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Biochemia

DETERMINATION OF ELASTASE AND ELASTASE INHIBITOR BY MEANS OF ORCEIN-ELASTIN

By

Ilona Banga

I[st] INSTITUTE OF PATHOLOGY AND EXPERIMENTAL CANCER RESEARCH, MEDICAL UNIVERSITY, BUDAPEST

(Received November 26, 1962)

A method has been developed for the assay of elastase and elastase inhibitor by means of suitably prepared orcein-elastin. The elastase inhibitor can be determined in the presence of elastase, too, if it is present in excess in the system.

The significance of the method is that it makes possible to determine the elastase inhibitor content of the pancreas. The pancreas of persons dead of arteriosclerosis contains no elastase, by the method described the excess "free" elastase inhibitor can be assayed quantitatively.

Although elastase is produced by the pancreas the enzyme could not be demonstrated in blood, presumably because of the presence of elastase inhibitor [1, 2, 3], which contains elastase in bound form. Any method suitable for assaying elastase activity [4, 5, 6, 7, 8, 9, 10] is theoretically suitable for the quantitative determination of the elastase inhibitor. Yet, experience has shown that the gravimetric method, employed at our Institute by Banga, Schuler and László [11], is the simplest and most precise of the assaying procedures. Both the serum and the pancreatic elastase inhibitor are substances of protein nature [3], and therefore the methods [8, 9, 10] which determine the quantity of dissolved elastin in the supernatant are complicated and not precise, as far as the assaying of the elastase inhibitor is concerned. Elastin stained with orcein has been used for elastase activity assay by Sachar, Winter, Sicher and Frankel [8], as well as by Scarselli [8a, 8b]. In the present study we used elastin stained with orcein to work out a more precise quantitative method for estimating both serum and pancreatic elastase inhibitor.

At our Institute we were not able to perform the elastase assay with orcein-elastin for years. As it turned out later, the cause of this was that the orcein preparation marketed by the *Light* Co. stained elastin very faintly so that large amounts of elastin had to go into solution to produce a colour that could be colorimetrized. This meant at the same time that large quantities of substrate and enzyme were required for the activity assay, which was obviously a disadvantage. After having tried out a number of preparations,

by the use of the orcein produced by *Merck*, Darmstadt (Orceinum $C_{28} H_{24}O_7N_2$ 7090) we obtained an orcein-elastin that proved to be most suitable for the elastase and elastase inhibitor tests.

Experimental

Preparation of orcein-elastin with different orcein preparations

SACHAR *et al.* [8], as well as SCARSELLI [8a, 8b] did not specify in their report the orcein preparation they had used. The orcein-elastin used by the *Worthington Biochemical Corporation* was prepared according to the method

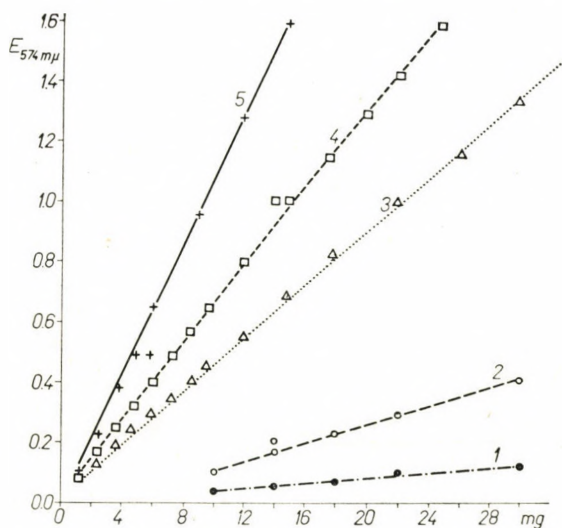

Fig. 1. Absorption curves of elastin stained with orcein produced by different firms
1. Orcein-elastin obtained from *Worthington Co.* *2.* Orcein *Light.* *3.* Orcein *Grübler.* *4.* Orcein *Gurr.* *5.* Orcein *Merck*
Abscissa: lysed orcein-elastin, mg. Ordinate: extinction

of SACHAR *et al* [8], and we have compared it with our own orcein preparations (Fig. 1, curve 1). For comparison we used orcein preparations from 4 firms, *Light*, *Gurr*, *Grübler* and *Merck*. In addition, we used freshly prepared resorcin-fuchsin for staining elastin [12]. This dye served at our Institute to determine the elastolysis in sections. The curve of this is not shown in Fig. 1, the data being identical with those shown by curve *3* in Fig. 1.

The method of staining according to SACHAR *et al.* [8] is as follows. Two g of elastin, isolated by PARTRIDGE's method [13] were stained for 24 hours with 0.5 g of dye, dissolved in 50 ml of 70 per cent alcohol containing 0.25 ml concentrated HCl. During the staining period the tubes were stirred

from time to time. After centrifugation the stained elastin was washed 7 times with 50 ml alcohol over 7 days and the washing fluid was removed by centrifugation in every case. After the last washing with alcohol the washing fluid was still coloured, but later it turned out that after so many washings no colour could be dissolved out from the preparation with water or buffer.

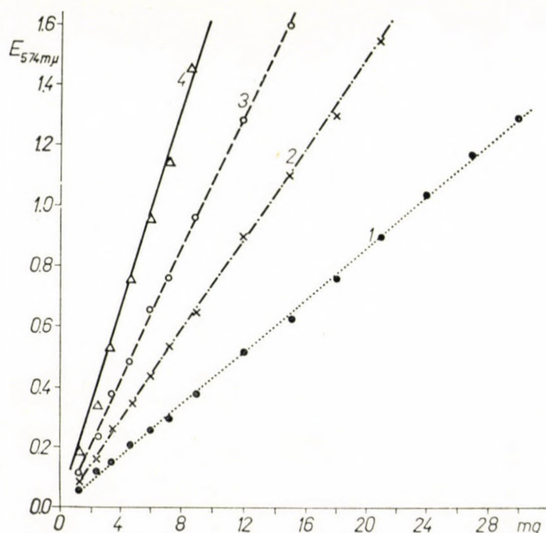

Fig. 2. Staining of elastin with different quantities of *Merck* orcein. Elastin: orcein ratio. *1.* 10 : 1. *2.* 10 : 2.5. *3.* 10 : 3.75. *4.* 10 : 5.
Abscissa: lysed orcein-elastin. Ordinate: extinction

The stained elastin after the 7th washing was washed twice with 100 ml acetone, dried in air and kept at 110° C until constant weight. The yield was 90 per cent.

Fig. 1 shows the curves of the 5 kinds of stained elastin. Known amounts of each sample were dissolved by means of elastase and the extinctions were plotted against the concentration of stained elastin. The curves indicate that the *Merck* and *Gurr* orceins stained elastin with suitable intensity so that small amounts (2 to 5 mg) of dissolved elastin could be determined, while from the *Worthington* elastin prepared according to Sachar *et al.* [8] and stained with *Light* and *Grübler* orcein, large amounts of elastin had to be lysed out to get an appreciable colorimetric extinction value. The elastin stained with *Merck* orcein appeared to be the most suitable preparation, so that this was used in our subsequent experiments. When studying the relative proportions of elastin and orcein, the more of the dye we used the more of it became bound and thus, with the lysis of the same amount of elastin, extinction increased with the increase of the amount of the dye. However, when

much of the dye is bound, the extinction curve is too steep and not proportionate to the amount of elastin lysed (Fig. 2). The best preparation was obtained when we used elastin and dye at a ratio of 10 : 1, with the same volume and with the same hydrochloric acid concentration; this corresponds to curve 2 of Fig. 2. Thus, the orcein-elastin used by us has been prepared in the following

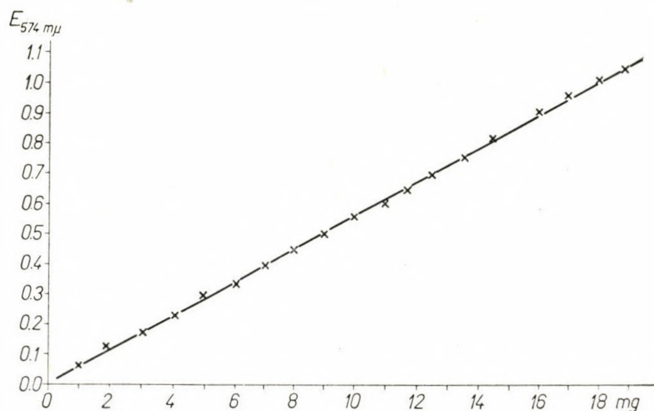

Fig. 3. Standard curve *1*. Abscissa: lysed orcein-elastin. Ordinata: extinction

way: Ten g of elastin were suspended in 100 ml of 70 per cent alcohol, in which 1 g of *Merck* orcein had been dissolved and to which 1.4 ml of concentrated HCl had been added. Staining and washing took place as described above.

Plotting of standard curves for elastase assay

In the next step we determined the optimal conditions for elastase assay and plotted the standard curves on that basis.

Tris buffer (pH 8.6, 0.08 *M*) was used; it was prepared by adding to 25 ml of 0.2 *M* tris (hydroxymethyl) aminomethane 12.5 ml of 0.1 *M* HCl and adjusting the volume to 62.5 ml with distilled water.

To plot standard curve *1*, 300 mg orcein-elastin were dissolved with 1 mg elastase in 50 ml pH 8.6 Tris buffer for 24 hours at 38°C. Of the pure solution, containing 6 mg of dissolved orcein-elastin per ml, volumes ranging from 0.1 ml to 6 ml were measured in and the volume was made up to 6 ml with buffer. The coloured solution was read against water in 1 cm cuvettes in a *Pulfrich* photometer, using a S_{57} filter. Plotting the extinction values against the lysed elastin, we obtained standard curve I., shown in Fig. 3.

When plotting standard curve *2*, we had started out from the observation that for the determination of the activities of elastase preparations of different purity we must know the amounts of elastin lysed by an elastase of 100 per

cent activity. Correspondingly, we accepted our purest preparation, containing 100 elastolytic units per mg, as having 100 per cent activity and plotted with it a concentration curve, representing dissolved orcein-elastin against elastolytic units (E. U.). This is standard curve *2*, shown in Fig. 4.

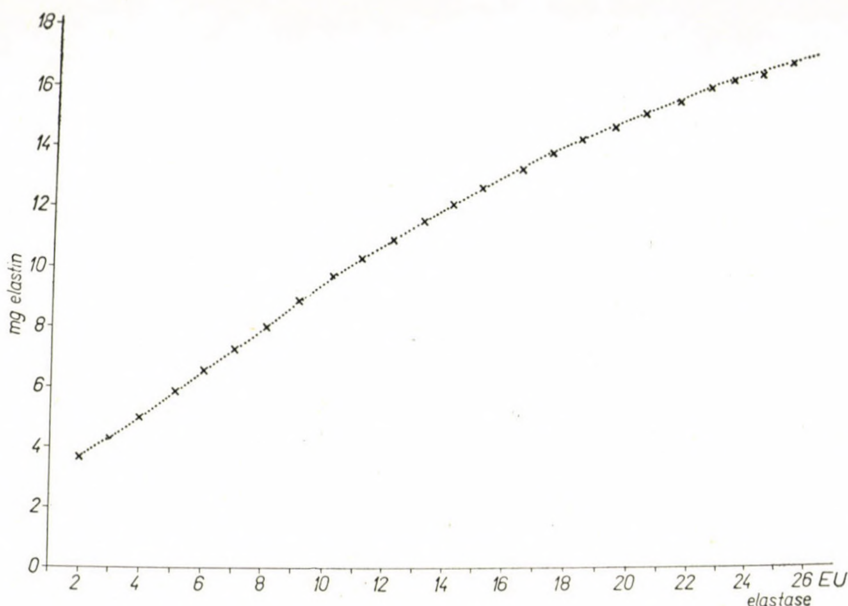

Fig. 4. Standard curve *2*
Lysed orcein-elastin (ordinate) plotted against elastase unit (E.U.) (abscissa)

The procedure of elastase assay and the calculation of E. U. was as follows. To each tube is added 30 mg orcein-elastin, weighed on analytical scales. To this are added x ml of elastase and (6—x) ml of the pH 8.6 Tris buffer. The blank is prepared without enzyme. The 0 minute blank prepared with enzyme gives namely no readable colour, just as the one without enzyme, but the use of a separate blank for each enzyme concentration retards the assay and reduces the reliability of determination. The tubes are incubated for 30 minutes in a water bath of 38° C under continuous stirring, and filtered. The filtrate is colorimetrized against the crystal-clear blank in 1 cm cuvettes in the Stupho-photometer with the S_{57} filter. From standard curve *1* (Fig. 3) the value for the elastin lysed by the given amount of the elastase preparation is read, and from standard curve *2* (Fig. 4) the E.U. corresponding to the dissolved elastin. The specific enzyme activity expresses the number of E.U. contained in 1 mg of the enzyme. Comparing the results thus obtained with those yielded by the other methods known from the literature, the procedure has proved to give the best reproducible data.

With the preparation of 100 specific activity (E. U./mg = 100) used by us, we plotted by the above method the time curve and the substrate concentration curve and compared them with those of the homogeneous elastoproteinase, obtained by chromatography on a Sephadex column, received

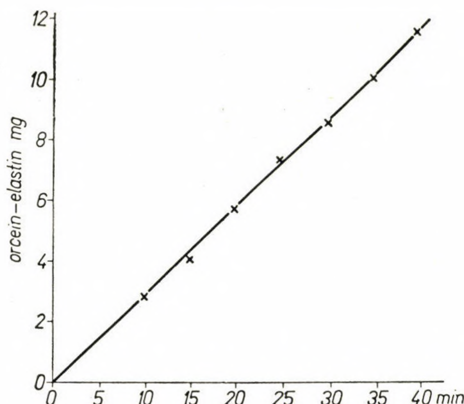

Fig. 5. Time curve of elastase (elastoproteinase) purified by Sephadex chromatography
Abscissa: time, minutes. Ordinate: lysed orcein-elastin
Amount of enzyme, 0.2 mg in every experiment

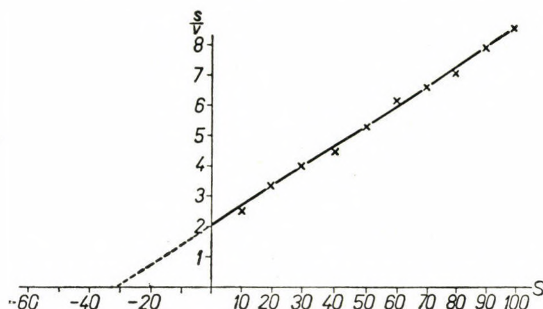

Fig. 6. Substrate concentration curve of elastase purified by Sephadex chromatography
Abscissa: orcein-elastin substrate (*s*). Ordinate: ratio of substrate (*s*) and reaction velocity (*v*)

from W. A. LOEVEN [14]. Both the time curve (Fig. 5) and the substrate concentration curve (Fig. 6) were of the same shape with the two enzymes. The elastase preparation of LOEVEN had a specific activity of from 50 to 60, as it was practically free from elastomucoproteinase, while the preparation used by us ($213/F_3$) contained 80 per cent elastase (or elasto-proteinase) and 10 per cent of each elastomucoproteinase and collagenmucoproteinase. On the basis of the substrate concentration curve $K_M = 30$, which means that in the case of 0.6 per cent orceinelastin the substrate is half saturated with the enzyme.

Determination of elastase inhibitor

The principle of the assay is that elastase of known activity is incubated with a given amount of serum or pancreas elastase inhibitor and the mixture is assayed for activity against orcein-elastin. The difference between the two activities gives the activity of the elastase inhibitor (E. I.). In the case of serum this is usually related to 1 ml of serum, and in the case of pancreas elastase inhibitor to 1 g of dry weight.

Thus, the assay of serum elastase inhibitor (E.I.) is carried out as follows. The preparation used for this purpose is usually one having a specific activity of 60. Of this a 2 mg/ml stock solution is made, in Tris buffer (pH 8.6). The following mixture is prepared,

0.5 ml elastase (60 elastolytic units E.U.),
0.25 ml serum,
0.25 ml Tris buffer pH 8.6.

The mixture is allowed to stand for 1 hour at 20° C. Then 0.4 ml of it (corresponding to 0.1 ml serum) is measured in. To ascertain the accuracy of measurement, we always set up as a control the elastase solution used. The E.U. obtained is subtracted from the 24 E.U. in 0.4 ml, and then we get the E.I. in 0.1 ml of serum. Ten times this gives the specific E.I. of 1 ml of serum.

Recently, the enzyme of proteolytic activity has been termed elastoproteinase [10], or, after Hall [6], E_2; the one of mucolytic activity either elastomucase [10] or E_1 [6]. We [15] call the latter enzyme elastomucoproteinase.

Table I

	Elastase inhibitor (E. I.)	
	per ml	per g
Human serum	150—160	—
Rabbit serum	95—110	—
Rat serum	170—180	—
Pancreas of arteriosclerotic human	—	25—44

With the assay for pancreas inhibitor we use pancreatic extract instead of serum, made from acetonic pancreas powder, with $N/10$ pH 4.7 acetate buffer. To 1 g of pancreas powder 5 ml buffer is added, and after mixing for 15 minutes it is centrifuged. The supernatant is neutralized and filtered. Of this extract 1 and 0.5 ml are added to 0.5 ml = 60 E.U. solution and the

volume is adjusted to 3 ml. One ml of this solution is used for assaying activity, that should yield 20 units, if there is no elastase inhibitor present. The difference between 20 and the result gives the amount of elastase inhibitor in the pancreatic extract tested. This amount is computed for 1 g of pancreas and is given as elastase inhibitor/g pancreas, designated E.I./g pancreas.

Table I shows the elastase inhibitor values obtained by the orcein-elastin method in human, rabbit and rat sera, as well as the inhibitor content of human arteriosclerotic pancreases. In pancreases which contain demonstrable amounts of elastase, the elastase inhibitor cannot be determined.

Discussion

In our earlier determinations of serum elastase inhibitor [11] no correlation could be established between elastase units and elastase inhibitor units. All we could establish were the relative changes of serum elastase inhibitor values in rabbits rendered hypercholesterolaemic by ammonium hydroxide treatment. Since then, we have succeeded in producing a standard elastase preparation considered to have a specific activity of 100 (E.U./mg = 100). Furthermore, we have worked out a method making it possible to express in units the amount of elastase inhibitor contained in serum. The elastase inhibitor unit (E.I.) is closely correlated with the elastase unit, insofar as one elastase inhibitor unit is identical with the amount of inhibitor capable of neutralizing the activity of one unit of elastase. This method also allows the quantitative determination of the amount of elastase inhibitor in the human pancreas. In earlier experiments [16], we found that the elastase of pancreatic extracts could be activated with acid, by dialysis and by ammonium sulphate precipitation. We then concluded that elastase was present in the pancreas in bound form, like trypsin, and could be freed from that linkage by certain procedures. Later, GRANT and ROBBINS [7] described the existence of proelastase in the pig pancreas. We consider the proelastase to be elastase bound by the elastase inhibitor. If no elastase activity is demonstrable in the pancreas after activation, that means that the inhibitor is present in larger amounts than is elastase. Thus far, there has been no method that could demonstrate this free or excess inhibitor. The method described above makes it possible to demonstrate and express in units also this free inhibitor. The presence of free inhibitor is presumably an important factor in arteriosclerosis. We had namely shown in earlier work [17] that the pancreas from subjects with severe arteriosclerosis contained no elastase, in contrast to the pancreas of healthy, young persons killed in accidents, that contained an average of 200 E.U./g of elastase. By means of this method it is now possible to determine quantitatively the amount of elastase inhibitor contained in the pancreas of arteriosclerotics, as well as

that in the pancreatic juice, *in vivo*. Such studies may eventually clarify the correlation of the elastase and elastase inhibitor contents of the pancreas and the severity of arteriosclerosis.

Acknowledgements

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Ilona BANGA
Orvostudományi Egyetem I. sz. Kórbonctani és Kísérleti Rákkutató Intézete, Budapest

PROTEIN SYNTHESIS IN THE SEMINAL VESICLE OF THE RAT

1. RAPIDLY LABELLED PROTEIN IN THE RNA FRACTION

By

S. MÁNYAI

INSTITUTE OF MEDICAL CHEMISTRY, MEDICAL UNIVERSITY, BUDAPEST

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A considerable amount of protein-like material is extractable from the $HClO_4$ precipitate of the seminal vesicles of the rat by boiling the precipitate with 10 per cent NaCl solution. Following the administration *in vivo* of glycine-1-^{14}C, or a previous incubation of the mince of vesicles in the presence of labelled amino acid, a protein-like material becomes intensively labelled.

The highly labelled substance has been found to belong to the components of the non-dialysable fraction. This fraction can be assayed by the biuret reaction or with Folin's reagent, and can be separated completely from the RNA by chromatography on a DEAE cellulose column. The HCl hydrolysate of this fraction consists of a mixture of amino acids in which glycine and serine proved to be radioactive in the presence of labelled glycine, both *in vivo* and *in vitro*. Glycine was incorporated into the polypeptide chain by peptide linkages.

The rapidly synthesized protein fraction which is extracted together with, but may be separated from, RNA, is characteristic of the seminal vesicles and can be found in this organ of rats, mice and guinea pigs.

It is known that the function of the male accessory organs of reproduction is controlled by the testosterone production of the testicles. Removal of the latter or a deficiency in their function is followed by an atrophy of the secondary sex glands. A number of studies have been devoted to the changes accompanying the involution of these organs, in their gross [42, 1, 6, 27, 66, 28, 30, 62, 34], microscopical [9, 14, 8, 63, 47, 29] and histochemical [15, 57, 49, 17] aspects, and concerning the profound alterations in chemical composition and metabolism [64, 55, 58, 38, 3, 65, 20, 61, 39, 52, 53, 18, 48, 22, 11, 32, 56, 4, 45, 5, 2, 59, 19]. Symptoms of regression may be reversed by testosterone administered parenterally; the involuted prostates and seminal vesicles regain their original size, metabolism is normalized, the secretory functions are restored.

According to experiments performed with labelled amino acids in castrated animals treated with testosterone, the hormone markedly enhances the incorporation of radioactive amino acids into the proteins of the prostate, seminal vesicles, and the perineal muscles [35, 10, 51, 12]. The same effect of testosterone in the same organ was demonstrated also *in vitro* [36, 37, 67, 21, 43].

The incorporation of labelled amino acids into the proteins of the accessory sex organs of male rats and the effect of androgens on this phenomenon has been studied in the present experiments. It has been observed that unlike

from other organs of the animal, considerable amounts of a substance of protein character could be extracted together with the RNA fraction of the seminal vesicles. It could be demonstrated by using labelled amino acids that this protein-like material was produced in a highly labelled state both *in vivo* and *in vitro*. Castration of the animals significantly depressed the incorporation of amino acids into this protein fraction. Administration of testosterone following orchiectomy restored the incorporation to its original level. The separation of the rapidly synthesized protein fraction from the RNA of the seminal vesicles is summarized in the following, the effects of castration and testosterone treatment will be discussed in a future paper. The preliminary results of the experiments have been reported previously [43, 44].

Methods

In the experiments male rats from the inbred strain of the Institute of Nutrition, Budapest, and the National Fodor Sanatorium were used. They were fed a normal laboratory diet.

The purity of the applied radioactive glycine (glycine-1-^{14}C having a specific radioactivity of 1.125 mc per mmol, produced by the Institute for Isotopes, Budapest) was controlled by means of paper chromatography and paper electrophoresis, and was found to be free of any contaminating ^{14}C-byproducts. Radioactivity measurements of samples were usually carried out in the dry state on aluminium planchets of 2.3 cm^2 surface area, using a thin mica end-window G.M. tube (window thickness, 1.8 mg per cm^2) or in some cases in a flow-counter flushed with a mixture of argon and amyl alcohol. Values corresponding for 1 μc ^{14}C-activity were of $1.7 \cdot 10^5$ counts per minutes (c.p.m.) (end window tube) and $1 \cdot 10^6$ c.p.m. (flow-counter), respectively. Results were corrected for self-absorption in all cases, when the dried samples contained more substance than 1 mg of dry material.

The seminal vesicles labelled *in vivo* or *in vitro* were processed according to the partly modified method of HOAGLAND *et al.* [31]. The organs were homogenized in water, proteins and nucleic acids were precipitated subsequently with 0.5 *N* $HClO_4$ (final concentration), at 0° C. The precipitates were thoroughly washed and centrifuged repeatedly. At least 5 volumes of washing-fluids were used and the following washings were carried out:

0.4 *N* $HClO_4$, 0° C, five times;

96 per cent ethanol, 0° C, once;

ethanol-ether (3 : 1) mixture, 65° C, 5 min., once;

ether, twice. The precipitate then was dried by mild heat. The ether dried powder was extracted with 5—10 volumes of a 10 per cent NaCl solution at 100° C for 30 mins. and centrifuged (in some cases the extraction procedure was repeated once or twice). The supernatant of this saline extract of the original precipitate (APHSE)* was filtered and after mixing with ethanol in a final concentration of 75 per cent stored overnight at —14° C.

The residue of the NaCl extraction was further extracted by 5—10 volumes of a 0.4 *N* $HClO_4$ solution at 90° C for 15 mins. (repeated in some cases); abbreviation APHPE refers to this extract.

The residue of the $HClO_4$ treatment was washed with 60, respectively 96 per cent ethanol, then with ether, and dried. The dried powder contained the "residual proteins".

Schleicher—Schüll's paper No. 2043b was used for paper electrophoresis and descending paper chromatography. The composition of the applied buffer solutions and solvents is described in the experimental part of this paper.

Sephadex gel-columns (*Pharmacia*, Uppsala) G-25, respectively G-50 (medium), were used for the gel-filtration technique. Separation of proteins and RNA was performed on a column

* The following abbreviations are used throughout the text,
APHSE: *a*cid *p*recipitate — *h*ot *s*aline *e*xtract
APHPE: *a*cid *p*recipitate — *h*ot *p*erchlorate *e*xtract
RNA: ribonucleic acid

of DEAE cellulose (*Whatman* DE 50). Samples to be separated were dissolved in 0.01 *M* borate buffer, pH 8.2, and eluted in the same buffer from the column with the aid of a NaCl gradient. The volume of the eluted fractions ranged from 2.6 to 2.9 ml. Protein content was determined according to LOWRY *et al.* [40]; u.v. absorption was mesured spectrophotometrically (*Beckman* Model DU).

Ribose estimations were performed according to MEJBAUM [46], the amount of RNA was calculated on the basis of the ribose content.

The radioactive assay of glycine and serine in the isolated protein was carried out as follows. 7.9 mg of protein in 8 ml of 6 *N* HCl were hydrolyzed *in vacuo* for 24 hrs., at 105° C, in sealed tubes. The hydrolysate was evaporated to dryness over solid KOH at reduced pressure, and, in order to remove the last traces of HCl, was redissolved several times and evaporated to dryness again. The hydrolysate obtained this way was subjected to chromatography according to the method of MOORE, SPACKMAN and STEIN [50], on Amberlite IR 120 cation exchanger resin column. The amino acid content of each fraction of 1.7 ml volume was determined by the method of SMITH and AGIZA [60]. After a tenfold dilution with water of the same fractions, dry samples were prepared and examined for ^{14}C-activity in the flow-counter.

Experimental results

(A) *Incorporation of glycine-1-^{14}C, administered in vivo, into the different protein fractions of the seminal vesicles of the rat.*

Six male rats of 230 g average weight were injected 100 μc glycine-1-^{14}C per kg body weight, intraperitoneally. The animals were killed 2 hours later by lighting gas, the $HClO_4$-homogenates of the seminal vesicles of each animal was three times extracted with hot 10 per cent NaCl and then with hot 0.4 *N* $HClO_4$. The protein content of APHSE (precipitated with 75 per cent ethanol), and APHPE, further the amount of residual proteins and the radioactivity of all the fractions is summarized in Table I. As seen, the ethanol precipitate of the first APHSE contained 3.2 per cent of the total proteins and 23.7 per cent of the

Table I

Incorporation of glycine-1-^{14}C into the different protein fractions of the seminal vesicles of the rat in vivo

	Total protein mg	Per cent	Total incorporated ^{14}C-activity, c.p.m.	Per cent	C.p.m. per mg protein
1. APHSE	1.36 ± 0.12	3.2	366 ± 37	23.7	279 ± 63
2. APHSE	0.26 ± 0.06	0.6	70 ± 50	4.4	255 ± 36
3. APHSE	0.15 ± 0.04	0.3	8 ± 10	0.5	
APHPE	1.26 ± 0.16	2.9	303 ± 99	18.8	242 ± 74
Residual protein	40.3 ± 3.2	93.0	863 ± 81	52.6	21 ± 4

total incorporated radioactivity. The amount of proteins obtained by the two subsequent hot NaCl extractions was 0.6 and 0.35 per cent, respectively, with radioactivity values of 4.4 and 0.5 per cent respectively. By means of the hot $HClO_4$ treatment — following the hot NaCl extractions — it became possible to remove an additional portion of material from the precipitate including

components that could be determined both with Folin's reagent and the biuret reaction, containing 18.8 per cent of the total bound radioactive amino acid. The remaining precipitate, the so-called residual protein fraction, amounting to 93 per cent of the total protein content, included 52.6 per cent of the total incorporated counts. Assuming on grounds of evidence to be discussed

Table II

Incorporation of glycine-1-^{14}C into the different protein fractions of the seminal vesicles of the rat in vitro

	Total protein mg	Per cent	Total incorporated ^{14}C-activity, c.p.m.	Per cent	C.p.m. per mg protein
(A) 1. APHSE	1.30	2.19	11430	37.1	8800
2. APHSE	0.22	0.37	1130	3.7	5130
3. APHSE	0.04	0.06	118	0.4	3360
1. APHPE	3.76	6.4	5320	17.3	1414
2. APHPE	3.00	5.0	2895	9.4	965
Residual protein	51.00	86.0	9900	32.1	194
(B) 1. APHSE	1.60	1.85	12190	39.4	7610
2. APHSE	0.23	0.26	419	1.3	1500
1. APHPE	6.04	6.9	3300	10.7	550
2. APHPE	4.44	5.1	3820	12.4	860
Residual protein	74.5	85.9	11190	36.2	150
(C) 1. APHSE	1.17	2.3	7925	35.8	6780
2. APHSE	0.05	0.13	98	0.5	1500
1. APHPE	6.40	12.6	2750	12.4	430
2. APHPE	3.25	6.4	1760	7.9	541
Residual protein	40.0	78.6	9560	43.4	239

later that all the radioactivity found in the APHSE was incorporated into protein, it follows that its specific radioactivity was 12—13 times higher than of the residual protein fraction.

(B) *Incorporation in vitro of glycine-1-^{14}C into the different fractions of the seminal vesicles of the rat.*

The results of three experiments are summarized in Table II, in which the mince of seminal vesicles was incubated for one hour in Krebs-Henseleit-phosphate solution, containing glucose, casein hydrolysate and 50 μc glycine-1-^{14}C per one g of tissue, at 37° C, under aerobic conditions. After incubation the minces were washed with ice cold 0.15 *M* NaCl solution and, as described

in the methodical part (see above), homogenized and precipitated with $HClO_4$, and assayed.

The first APHSE contained 2 per cent of the total protein content, and 37 per cent of the total incorporated radioactive glycine. The specific

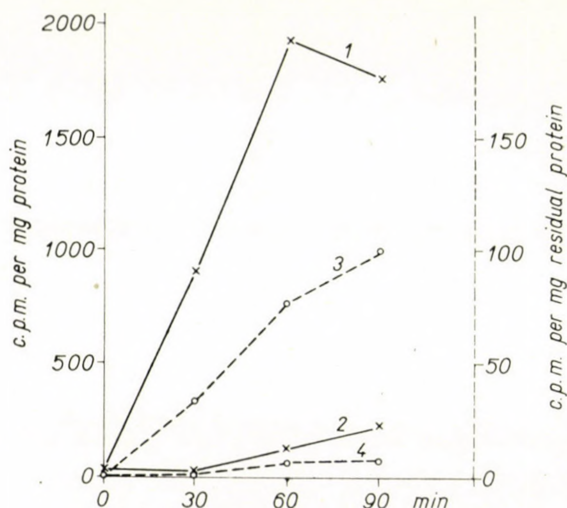


Fig. 1. Time curve of glycine-1-^{14}C incorporation into the different protein fractions of the seminal vesicles of the rat *in vitro*

Medium: 300 mg of minced vesicles + Krebs-Henseleit'-phosphate buffer solution, 3.0 ml; glucose 0.03 *M*; and glycine-^{14}C, 4 μc. Aerobic samples were flushed with a mixture of 5 per cent CO_2 and 95 per cent O_2, the anaerobic ones with 5 per cent CO_2 and 95 per cent N_2, and shaken (120 shakings per minute) at 37° C

1. APHSE, aerobic; *2.* APHSE, anaerobic; *3.* residual proteins, aerobic, *4.* residual proteins, anaerobic. For details see: methodical chapter

radioactivity of this protein fraction was 28—51 times higher than that of the residual proteins.

If the seminal vesicles were incubated *in vitro* in the presence of radioactive glycine, the label appeared in the APHSE fraction only under aerobic conditions. No amino acid incorporation could be observed anaerobically (Fig. 1). Aerobic amino acid incorporation into the above mentioned fraction proceeded linearly for about one hour; later the rate diminished. A decrease in the specific radioactivity of the protein often occurred after 90 or 120 minutes incubation.

(C) *Comparison of the amino acid incorporation into the different organs of the rat in vitro.*

In order to decide the organ-specific nature of the intensive labelling in the APHSE of the seminal vesicles of the rat in the presence of radioactive amino acids, the mince of the seminal vesicles, prostate, pancreas and liver

of the same animal was incubated under aerobic conditions in the presence of glucose and radioactive glycine. As seen in Fig. 2, the total amount of radioactivity extracted by hot 10 per cent NaCl from the $HClO_4$ homogenate of the seminal vesicles following one hour incubation, was 31 times more than the

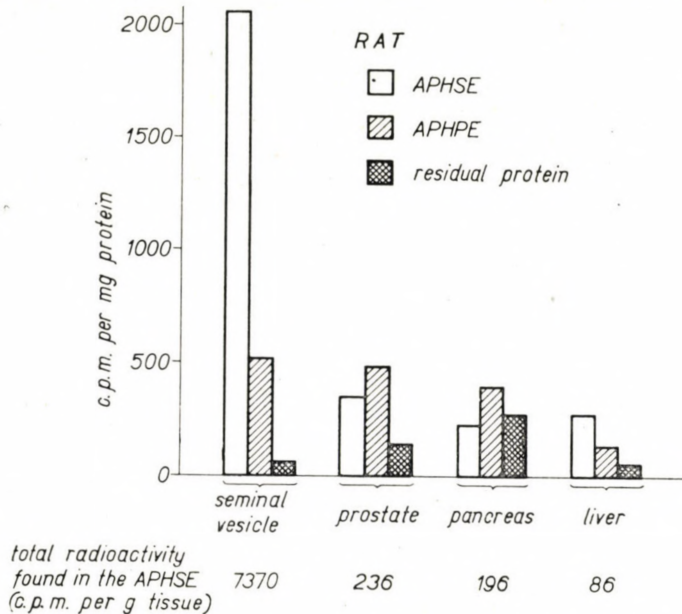


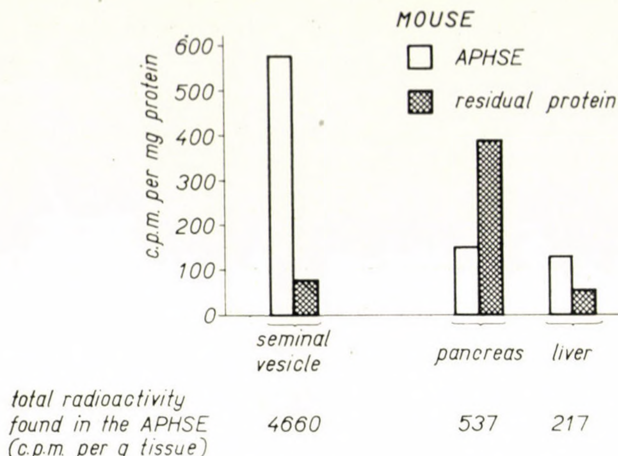
Fig. 2. Glycine-1-^{14}C incorporation into the different protein fractions of the seminal vesicles, prostate, pancreas and liver of the rat *in vitro*

Pooled organs of 4 rats (245 g each). Medium: 600 mg of minced seminal vesicles, prostate, pancreas or liver + Krebs—Henseleit'-phosphate buffer solution, 6.0 ml; glucose, 0.03 *M*; glycine-1-^{14}C, 8 μc. Gas-phase: 5 per cent CO_2 + 95 per cent O_2. Samples were shaken (120 shakings per minute) at 37° C for 60 min. For details see: methodical chapter

same value counted in a prostatic mince incubated with glycine of the same activity. The radioactivity of APHSE obtained from liver slices incubated under the same conditions contained only slightly more than 1/100 of the counts present in the APHSE of the seminal vesicles.

(D) *Comparison of the amino acid incorporation into various organs of different animal species in vitro.*

Amino acid incorporation in the mince of the seminal vesicles of other rodents was investigated in order to establish whether in species other than the rat the same intensive labelling of the APHSE fraction occurred. The results of comparative experiments concerning mice and guinea pigs are demonstrated in Fig. 3. The APHSE fraction of the seminal vesicles of these two species was highly labelled, the specific radioactivities were at least one order of magnitude higher than those of the other organs.



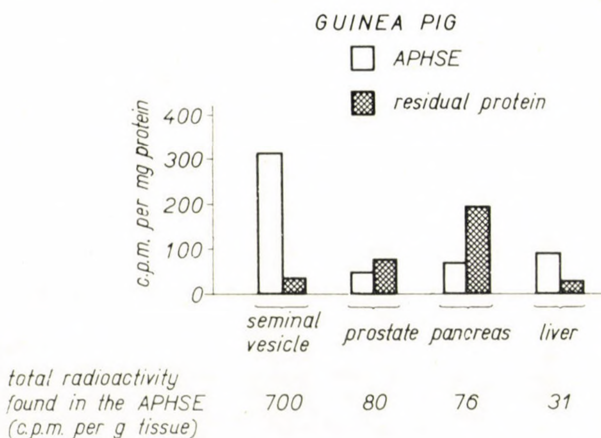


Fig. 3. Glycine-1-^{14}C incorporation into the different protein fractions of the seminal vesicles, prostate, pancreas and liver of mice and guinea pigs *in vitro*

Pooled organs of 21 mice (30 g each), and 3 guinea pigs (640 g each). Incubation mixtures and conditions were the same as in Fig. 2

(E) *On the chemical nature of the amino acid containing substance extracted by hot NaCl solution from the $HClO_4$ homogenate of the seminal vesicles of the rat.*

On removing all the low molecular, acid soluble compounds as well as the lipids from the $HClO_4$- or trichloroacetic acid homogenate, it is the nucleic acid (mainly RNA) content of tissues that is extractable from the residual ether dried powder by boiling it with a 10 per cent NaCl solution according to the procedures of DAVIDSON *et al.* [16], further HOAGLAND *et al.* [31]. On the other hand, the RNA obtained by these procedures is known to contain protein [41], and even RNA preparations purified several times are not free

of protein and/or peptide contamination [23, 28a]. Table III shows that in addition to RNA a considerable amount of protein (polypeptides) could be extracted with boiling NaCl from the $HClO_4$ precipitate of the seminal vesicles of mice, guinea pigs and rats. The amount of protein (polypeptides) could be

Table III

Protein and RNA content of the APHSE of different organs

	Protein	RNA	Protein/RNA
	mg per g wet weight		
RAT			
seminal vesicle (n=11)	2.56 (1.36—3.56)	1.93 (1.41—2.59)	1.33
prostate (n=3)	0.48 (0.39—0.64)	1.67 (1.55—1.73)	0.29
pancreas (n=3)	0.61 (0.49—0.84)	6.08 (5.20—6.74)	0.10
liver (n=3)	0.26 (0.23—0.30)	2.65 (2.33—3.15)	0.098
MOUSE			
seminal vesicle	8.60	1.60	5.38
pancreas	2.50	7.75	0.33
liver	1.10	2.72	0.41
GUINEA PIG			
seminal vesicle	3.54	0.61	5.80
prostate	2.15	0.51	4.20
pancreas	1.86	2.84	0.65
liver	0.54	1.34	0.40

(Numbers in brackets indicate the maximal deviations from the mean values)

measured by both the Folin—Ciocalteau-reagent or the biuret reaction. The two determinations yielded identical results.

The question arises whether the radioactive label found in the APHSE following the incubation of seminal vesicles in the presence of labelled amino acids, was bound to RNA or to protein, or was due to other components possibly present in the same fraction.

The labelled components may be precipitated in the presence of ethanol (75 per cent, final concentration). Reprecipitation from the aqueous solution of this primary precipitate may be accomplished by using ethanol in 60 per cent final concentration. The supernatant of the 60 per cent ethanol precipitate contained about 10 per cent of the protein (material reacting with Folin's

reagent), but at the same time only 0.5 per cent of the counts corresponding to the total radioactivity of the precipitate. The same precipitate is soluble in dilute salt solutions. If the protein concentration of the solution exceeded the value of 1 to 1.5 mg per ml, a temperature of 37° C was needed to achieve complete dissolution. Cooling of the solution resulted in the appearance of an opalescent gel under these conditions. No free, radioactive glycine could be detected in the solution of the dissolved precipitate. This was evidenced in two different ways.

(i) The precipitate of APHSE prepared from the vesicles labelled *in vitro* was reprecipitated twice with ethanol and then dissolved. The paper chromatographic assay in a butanol : acetic acid : water system [54] demonstrated that all the radioactivity remained at the start line.

(ii) After molecular sieving on a Sephadex G-25 as well as G-50 gel-column prewashed with 0.01 *M* borate buffer (pH 8.2), radioactivity was found only in those fractions which contained protein + RNA.

Glycine-1-^{14}C in tracer amounts was added to the material extracted from no-radioactive vesicles. It could be proved in this way that had labelled glycine been present in the free state in the above mentioned solutions, it would have been detected.

Dialysis of the APHSE prepared from the seminal vesicles without ethanol precipitation, against distilled water or 0.01 *M* borate buffer for 24 hours, led to the formation of a voluminous precipitate, containing 80 to 86 per cent of the regained radioactivity, protein and RNA. The precipitate was soluble in 0.3 *M* NaCl or at pH values more alkaline than 8.2, it could be dissolved in diluted salt solutions at 37° C. The solution stiffened to a gel on cooling. Part of the APHSE in the bag remained soluble during dialysis. The amount of protein, RNA and radioactivity amounted to 14 to 20 per cent of the total APHSE fraction, its specific radioactivity referred to 1 mg protein was usually lower than that of the protein fraction which had precipitated in the course of dialysis.

(F) *Separation of the protein and RNA components in the APHSE of the seminal vesicles.*

As it became evident that the radioactivity found in the APHSE of the seminal vesicles was not due to free glycine contamination possibly present in this fraction, it had to be determined whether the high label was to be attributed to the RNA, or was bound to RNA in the form of a peptide, or else, was incorporated into a rapidly synthesized protein fraction. The following experiments were performed to decide the issue.

1. The ethanol precipitate of the APHSE prepared from the seminal vesicles incubated with labelled glycine was hydrolyzed in 0.4 *N* $HClO_4$ at 100° C for various periods (30, 60, 180 min.). The $HClO_4$ was removed in the

form of $KClO_4$ following neutralization with KOH; the hydrolysate was subjected to paper electrophoresis in a barbital-acetate buffer solution ($\mu = 0.07$, pH 8.6) for 6 to 10 hours, the applied voltage gradient was 4 V per cm. In this case spots reacting with ninhydrin appeared on the paper in varying numbers (4 to 9), depending on the duration of hydrolysis. The spots reacting with ninhydrin were eluted and then hydrolyzed in 6 *N* HCl at 105° C for 24 hours. This procedure yielded a mixture of amino acids as revealed by paper chromatography.

2. The ethanol precipitate was hydrolyzed in 0.3 *N* KOH at 37° C for 20 hours, the hydrolysate was subjected to paper electrophoresis in a phosphate — borax buffer solution ($\mu = 0.05$, pH 8.4) [24], under the same conditions as described above. The results of this experiment are plotted on Fig. 4. The radioactivity was definitely separated from the nucleotides. Paper electrophoresis of the concentrated solution of the ethanol precipitate of the APHSE without previous hydrolysis was also attempted, the total amount of protein and radioactivity was found on the start line under these conditions as a consequence of the high viscosity of the system.

3. The distribution of the incorporated radioactivity following separation of the RNA from proteins by means of the phenol extraction procedure [25, 33], seemed to be of interest. In order to study this problem, the APHSE of the mince of vesicles incubated in the presence of labelled glycine for one hour, was precipitated by ethanol, the precipitate was redissolved in phosphate—borax buffer solution at pH 8.4 ($\mu = 0.05$). To this solution containing 0.3 mg of protein per ml water-saturated phenol was added at a volume ratio 1 : 1, and the mixture was homogenized for 10 minutes at room temperature (18° C) in a Potter—Elvehjem type all-glass homogenizer. Both the aqueous and phenol layers as well as the precipitate accumulated on the phase boundary were carefully separated and assayed for radioactivity. As demonstrated in Table IV, not more than 3.5 per cent of the total regained radioactivity were found in the RNA-containing aqueous phase, the rest was partitioned between the phenol phase and the precipitate floating on the surface of the latter.

4. According to these data, the radioactive label of the APHSE of the seminal vesicles most probably does not belong to the RNA, but is incorporated into proteins. Nevertheless, the possible presence of RNA-glycine in the APHSE of the vesicles had to be excluded. It should be noted that an extract has been prepared by WILSON [67] essentially by the same method as the APHSE characterized in the present paper, *i.e.* the $HClO_4$ precipitate of the mince of the seminal vesicles incubated in the presence of ^{14}C-labelled tyrosine and valine, was extracted by hot NaCl. The radioactive label of this fraction was considered to be RNA-tyrosine, respectively RNA-valine [67]. This circumstance underlined the necessity of further investigations concerning the character of the labelled substance in the APHSE fraction.

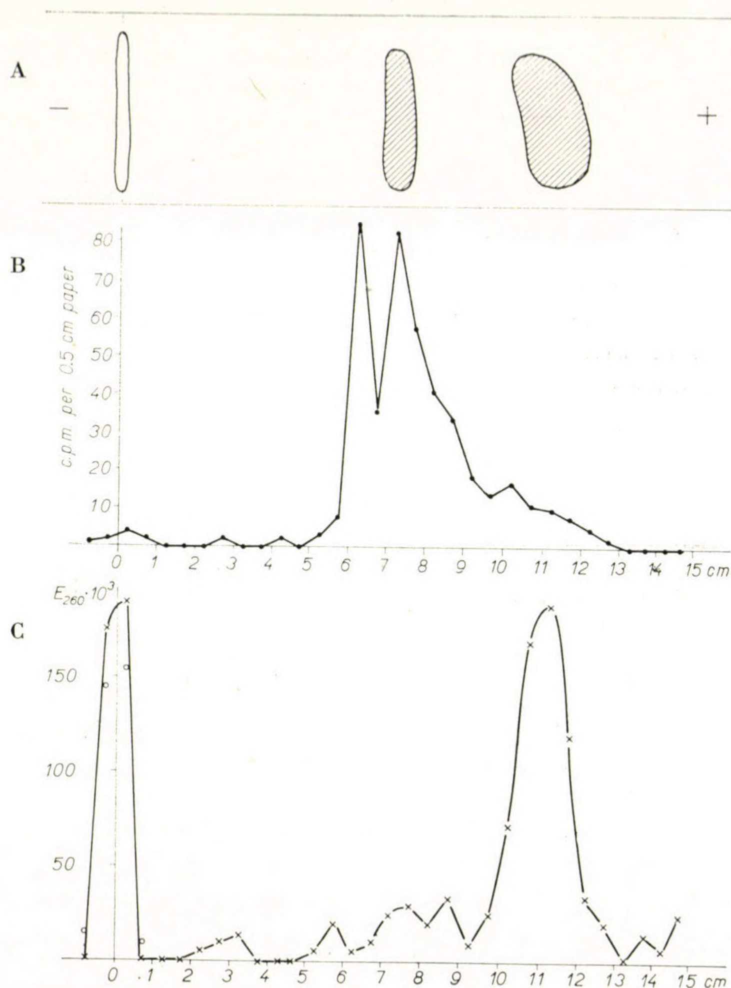


Fig. 4. Paper electrophoresis of the 0.3 *N* KOH hydrolysate of the APHSE isolated from seminal vesicles of the rat labelled with ^{14}C-glycine *in vitro*. Schleicher—Schüll' paper (2043*b*); phosphate-borax buffer solution ($\mu = 0.05$, pH 8.4); 4 V per cm voltage gradient
A : u. v. photo of the electropherogram; *B* : radioactive counts on the electropherogram (5×25 mm strips); *C* : u. v. absorption at 260 mμ of the 0.5 *N* NaOH eluate of the same strips

For this reason the ethanol precipitate of APHSE was hydrolyzed with 0.01 *N* NaOH for 20 minutes at room temperature, neutralized and reprecipitated by ethanol (60 per cent final concentration) at 0° C. Total radioactivity, protein and RNA content were determined in the aqueous solution of the precipitate. According to the data of Table V, the labelled substance in the APHSE of the seminal vesicles, in contrast to the RNA-amino acid complexes, is not labile in alkaline solutions. The radioactive label of the APHSE of the

Table IV

The effect of the phenol separation technique on the distribution of ^{14}C-activity in the ethanol precipitate of the APHSE of seminal vesicles

	C.p.m.	Regained ^{14}C-activity, per cent
Original, total ^{14}C-activity	1410	
^{14}C-activity following the phenol treatment in the		
aqueous phase	44	3.5
phenol phase	442	36
precipitate	744	60.5

Table V

The effect of 0.01 N NaOH hydrolysis on the specific radioactivity of the APHSE

Total c.p.m. before (B) and after (A) hydrolysis		Per cent	Change of the the protein content, per cent	Change of RNA content, per cent	C.p.m. per mg protein before (B) and after (A) hydrolysis		Per cent
B	A				B	A	
756*	688	− 9.0	−16.6	− 6.0	4200	4590	+ 9.2
708*	648	− 8.5	−14.3	− 7.0	4860	5400	+11.0
1060	1112	+ 5.0	0	−20.5	2070	2175	+ 5.0
7645	6400	−16.2	+ 4.5	+ 4.5	3450	2910	−15.5
7560	7920	+ 4.8	−15.5	−15.5	1420	1578	+10.8

Samples were incubated for 20 minutes in 0.01 *N* NaOH at 27° C, and subsequently precipitated by 60 per cent ethanol at 0° C. Determinations were carried out from the aqueous solution of the precipitate.

Samples marked by * were incubated in the aqueous solution for 20 minutes, and reprecipitated by 60 per cent ethanol as well.

prostate, pancreas, and liver, which was anyhow considerably lower than that of vesicles, decreased to 10 per cent of the original values by alkaline treatment. There was no considerable loss of radioactive counts observable in the ethanol precipitate APHSE of the seminal vesicles even when the 0.01 *N* NaOH hydrolysis had been continued for one hour at room temperature. The effect of prolonged hydrolysis was just the opposite; the protein content of the precipitates was slightly decreasing and the specific activities referred to 1 mg protein were showing a small increase. The results were the same if the radioactivities were referred to RNA instead of protein.

5. The following experiment was carried out to decide whether the labelled amino acids incorporated into the proteins of the APHSE of the seminal

vesicles, were merely attached to the ends, or were built in into the main internal bulk, of the polypeptide chain.

The ethanol precipitate of APHSE prepared from the mince of the seminal vesicles incubated in the presence of ^{14}C-labelled glycine was dissolved in 0.3 *M* NaCl solution. Five mg of $NaHCO_3$ and 2 ml of 5 per cent dinitrofluorobenzene (solution in ethanol) were added to the solution containing 1 mg of protein, and the mixture was shaken for 2 hours at room temperature and then acidified with 0.1 ml concentrated HCl. The easily sedimentating precipitate was centrifuged; no radioactivity was detectable in the supernatant, supporting once more the fact that it did not contain free glycine. The centrifuged DNP-protein was washed with ether several times, the total amount was subjected to hydrolysis in the presence of 2 ml 6 *N* HCl, at 110° C for 18 hours, in a sealed tube. The hydrolysate was diluted with water and repeatedly shaken out with ether. The separated aqueous phase was washed with ether. Total radioactivity was counted in both the aqueous phase and the combined ether extracts. The total activity in the ether extract amounted to 6 c.p.m., in the aqueous phase to 1470 c.p.m. From these results it seems most likely that the radioactivity counted in the proteins of the APHSE, found its origin in amino acids incorporated by peptide linkages in the internal part of the polypeptide chain, especially considering the easy decomposition of DNP-glycine.

6. It was essential to find a mild procedure by which the rapidly labelled protein fraction present in the APHSE of the seminal vesicles could be separated from the RNA, to allow a detailed study of the properties, the mechanism of synthesis and the eventual physiological role of the protein. Chromatography on DEAE cellulose columns was chosen for this purpose. In the experiment plotted on Fig. 5 the precipitate (reprecipitated twice by ethanol) of the APHSE prepared from vesicles previously labelled with radioactive glycine was chromatographed in an 0.01 *M* borate buffer solution, at pH 8.2, using a NaCl gradient. The proteins separated well from RNA. The counting results which had to be corrected for self-absorption due to the high NaCl concentrations in the various fractions, showed the absence of labelled components in the RNA. At the same time, the maximum value of radioactive counts in the protein-containing fractions and that of the protein peak were not completely overlapping. This phenomenon was observed consistently in a number of experiments and led to the conclusion that the APHSE of the seminal vesicles consists besides its RNA content of not a single but of several protein fractions which are synthesized at different rates. Separation of these fractions is in progress.

7. In order to prove that the radioactive label separated from RNA on the DEAE cellulose column was bound to proteins of high molecular weight and does not occur in any other compound eluted togheter with the proteins, the following experiments were undertaken.

All the fractions obtained by DEAE cellulose column chromatography containing radioactivity and proteins were pooled, and

(*a*) sieved on Sephadex G-50 gel-column. The protein content and radioactive counts in the gel-filtered fractions are presented in Fig. 6. The distribution of proteins and radioactive counts was overlapping in each fractions.

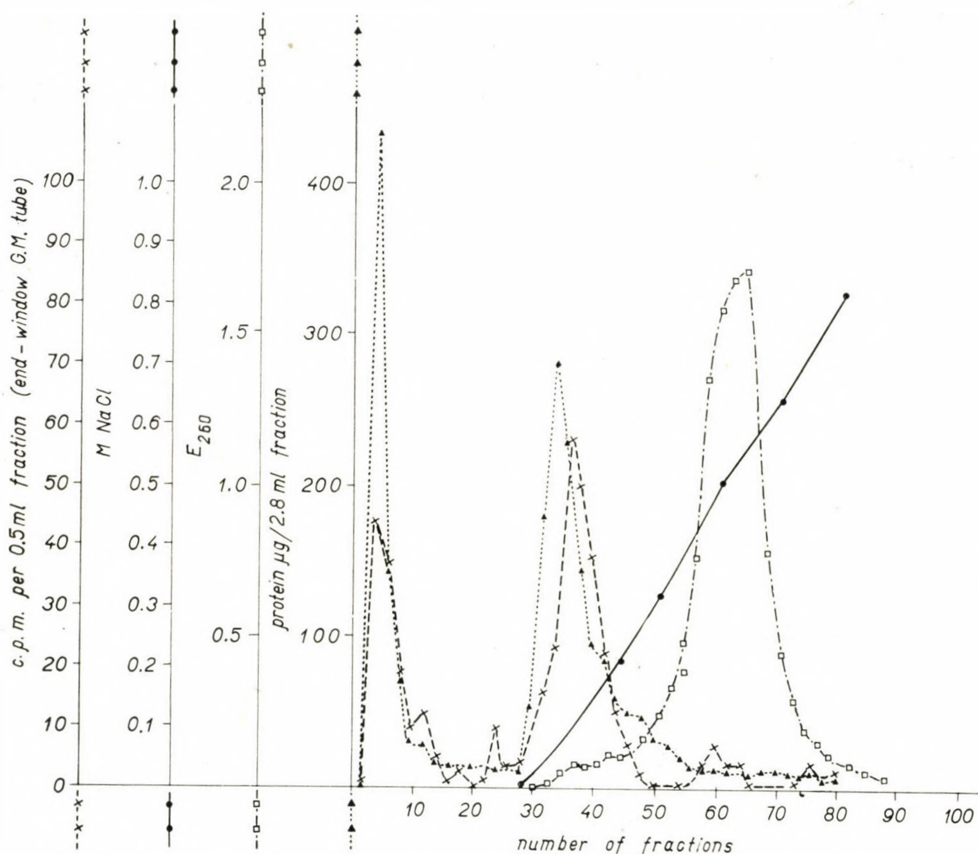


Fig. 5. DEAE cellulose column chromatography of the APHSE isolated from the *in vitro* ^{14}C-glycine labelled seminal vesicles of the rat
For experimental details: see text

(*b*) treated with an identical volume of water-saturated phenol, according to the procedure described under F/3. The distribution of label between the water and phenol phase was counted; 97 per cent of the radioactivity was found in the phenol phase.

(*c*) hydrolyzed with 6 *N* HCl, at 110° C, for 25 hours. The individual amino acids were separated from the hydrolysate according to MOORE, SPACKMAN

and STEIN [50] on Amberlite IR 120 cation exchanger resin. Radioactivity was not detectable in any other amino acid except glycine and serine. The radioactivity of humin formed during the hydrolysis was not significant. Comparing the specific radioactivities of glycine and serine, the ratio of spe-

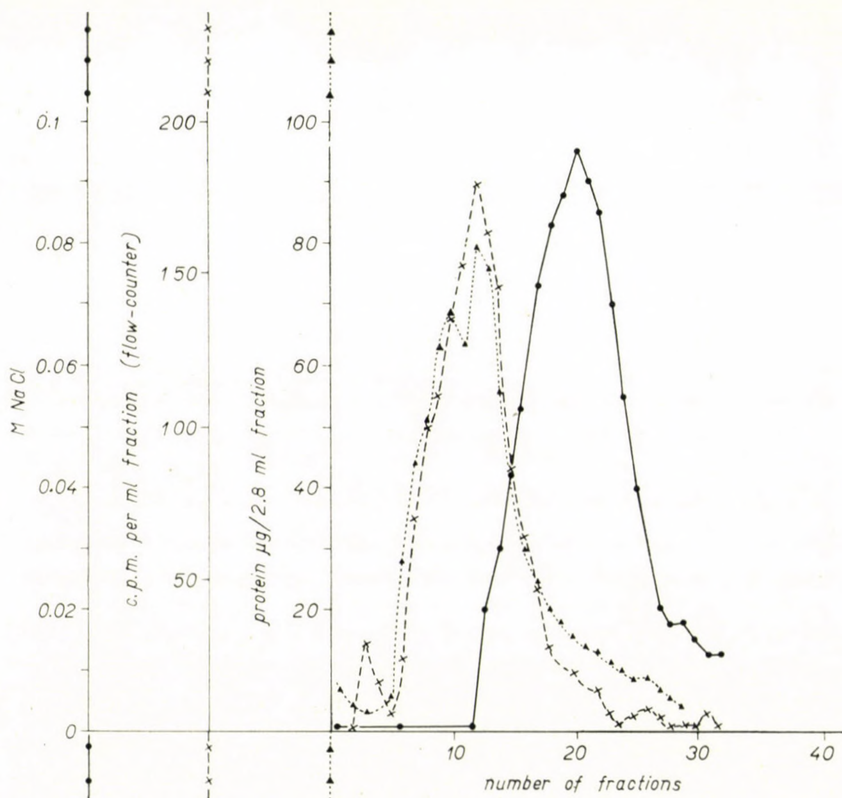


Fig. 6. Distribution of protein and radioactivity on rechromatographing the ^{14}C-labelled APHSE (isolated by DEAE cellulose column chromatography) on a Sephadex G-50 column For experimental details: see text

cific radioactivity of glycine to the specific radioactivity of serine was nearly 2. 89.2 per cent of the total ^{14}C-activity incorporated into the protein was present in glycine, the corresponding value for serine was 5.6 per cent.

All this seems to furnish sufficient evidence to establish that the radioactive label separated from RNA on the DEAE cellulose column was bound to high molecular weight proteins.

Discussion

The seminal vesicles of the rat, mouse and guinea pig have been found to contain a particular protein fraction characterized by a high radioactive label following the labeling of the seminal vesicles in the presence of radioactive amino acids, both *in vivo* and *in vitro*. The specific radioactivities (counts per minute per mg of protein) were 12 to 13 times higher than those of the so-called residual proteins consisting mainly of the structural proteins under conditions *in vivo*. The same ratio amounted to 28—51 in experiments *in vitro*.

A further characteristic feature of the highly labelled protein fraction of the seminal vesicles has been revealed by the fact that it is extracted togheter with the ribonucleic acids of the cell. Extracts prepared with hot 10 per cent NaCl solution from liver, prostate and pancreas homogenates contain, in addition to RNA measurable quantities of protein-like substances (substances reacting with Folin's reagent) but the concentration of this protein is nearly one order of magnitude higher in the seminal vesicles than in the other organs.

It is a moot point whether it is justified to use the term protein for the hot NaCl extract of the tissue homogenate precipitated with $HClO_4$. The analogy to consider the ^{14}C-labelled substance appearing togheter with the extracted RNA to be an RNA-amino acid complex [67] is certainly tempting, nevertheless the experimental results have clearly contradicted this assumption, since the ^{14}C-activity can be separated from the RNA with certainty, and is bound within the polypeptide chain of the protein.

The substance in question is most probably a degraded and denaturated product, but unquestionably of a high molecular weight and of polypeptide character; it is precipitated by 60 per cent ethanol (final concentration), or in the course of dialysis; it can be assayed by the biuret reaction and reacts with Folin—Ciocalteau's reagent; its hydrolysis in 6 *N* HCl results in the formation of an amino acid mixture. Proline and hydroxyproline are represented in the hydrolysate in considerable amounts, presumably as constituents of the simultaneously extracted collagen.

According to some further experiments, it is possible to obtain an extract from the $HClO_4$ precipitate of the secretion of the seminal vesicles by boiling with 10 per cent NaCl solution. This extract is rich in polypeptides and resembles the one obtained in a similar way from the gland.

Further experiments are in progress to clarify the possible physiological and functional relationships of the RNA and protein extracted from the seminal vesicles. The problem remains whether the protein in question is a true nucleoprotein, which may serve as a precursor in the synthesis of secretory proteins, or it becomes attached to the RNA owing to the special chemical structure of secretory proteins just during the chemical extraction of the cells.

Acknowledgements

The author wishes to express his indebtedness to Prof. F. B. STRAUB for his interest and critical remarks in the course of the present study. Thanks are due to Dr. L. BENEY for the quantitative column chromatography of amino acids, and to Mrs. M. PATAY and Mrs. K. OROSZ for skilful and conscientious assistance.

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Sándor Mányai

Orvostudományi Egyetem Orvosi Vegytani Intézete, Budapest

STUDIES ON THE SULFHYDRYL GROUPS OF LDH FROM SKELETAL MUSCLE

By

GY. JÉCSAI and P. ELŐDI

BIOCHEMICAL INSTITUTE OF THE HUNGARIAN ACADEMY OF SCIENCES, BUDAPEST

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As determined by *p*-chloromercuribenzoate (PCMB) titration, the lactate dehydrogenase (LDH) prepared by our method from pig skeletal muscle contains 19 to 20 sulfhydryl groups.

In the native state, in neutral medium the SH groups of skeletal LDH do not react with PCMB. They are demonstrable only at alkaline reaction, or after denaturation of the protein (treatment with urea, detergent). As indicated by viscosity data, the alkaline medium changes the tertiary structure of the protein.

In the lactate-DPN reaction, at pH 10.0, the blocking of 20 SH groups inhibits the enzyme activity to about 50 per cent.

Thus, in the pig's skeletal muscle LDH the role of SH groups in enzymatic activity is questionable, whereas in the case of the LDH isolated from the heart muscle of the pig the SH groups are essential for enzymatic activity.

As regards molecular form, several kinds of lactate dehydrogenase occur in the tissues of vertebrates. According to earlier reports [1], five isozymes can be demonstrated by gel electrophoresis in different tissues *(e. g.* liver, plasma, *etc.).* They can be distinguished on the basis of mobility, activity measured with coenzyme analogues, pyruvate inhibition, etc. It has, however, been shown recently that only two kinds of LDH* exist, heart muscle and skeletal muscle LDH. [2.] Of the five isozymes separated by gel electrophoresis 1 is heart muscle LDH, another is skeletal muscle LDH, whereas the remaining three appear to be hybrid molecules built up from the basic units of the two types.

Pig heart muscle LDH prepared in crystalline form by STRAUB's method contains 14 SH groups computed for a molecular weight of 127 000 g. From the point of view of enzymatic activity two SH groups are essential, because blocking of these two with PCMB leads to a cessation of activity [3].

In the present work we studied the role in enzymatic activity played by the SH groups of pig skeletal muscle LDH, and its relation to the protein structure.

* The abbreviations used in this paper are:
LDH = lactate dehydrogenase
DPNH/DPN = (reduced) diphosphopyridine nucleotide
PCMB = *p*-chloromercuribenzoate
SDS = sodium dodecylsulphate

Materials and methods

LDH was prepared from pig skeletal muscle by the method described earlier [4] and was used after three recrystallizations.

DPNH, DPN were preparations of 70 per cent purity *(Reanal*, Budapest).

DL-Na-lactate was produced by *Riedel de Haen A. G.*

SDS was a commercial Na-dodecylsulphate preparation used after two recrystallisations from alcohol.

PCMB was a preparation of 92 per cent purity *(Light)*.

The other substances used were of reagent quality.

The activity assays and the measurements of absorption were carried out by means of a *Hilger Uvispek* or *Unicam* SP—500 spectrophotometer.

Optical rotation was measured at 20 C° by means of a precision ring polarimeter manufactured by Schmidt and Haensch, provided by an ultrathermostat.

Internal viscosity was measured by an Ostwald viscosimeter (water outflow time about 100 seconds), at 20 C°.

Procedures

The concentration of LDH was determined spectrophotometrically [4].

LDH activity was assayed at room temperature (20 to 25 C°), in the following reaction mixture:

DPNH-pyruvate reaction:

$1.3 \cdot 10^{-1}$ μmol DPNH/ml
0.33 μmol Na-pyruvate/ml
$1.48 \cdot 10^{-6}$ μmol LDH/ml (computed for 135 000 g molecular weight)
in 0.1 *M* phosphate buffer pH 7.5

DPN-lactate reaction:

$1.63 \cdot 10^{-1}$ μmol DPN/ml
18.5 μmol Na-lactate/ml
$7.4 \cdot 10^{-6}$ μmμmol LDH/ml
in 0.1 *M* glycine buffer, pH 10.0.

The free SH groups of the protein were determined by Boyer's method [5].

Every value was computed for a molecular weight of 135 000 [6].

Results

After 60 minutes of preincubation with 6 M urea, the skeletal muscle LDH contains 20 to 22 sulfhydryl groups per molecule.

We have determined the number of reactive sulfhydryl groups in the native LDH at pH 7.5 and 10.0, at which the enzyme reaction is studied. The determinations were made at constant PCMB and variable protein concentrations (10 to 200 μg/ml).

Combination with PCMB at pH 7.5

No S—Hg linkage could be demonstrated after incubation at room temperature for 60 minutes in 0.1 M phosphate or 0.1 M tris buffer (Fig. 1).

Evaluation was rendered difficult by the appearance of opalescence in the incubation mixture after several hours of incubation with 50 to 100 μg/ml protein, and in 10 to 20 minutes in the case of 250 to 300 μg/ml LDH concentration.

Combination with PCMB at pH 10.0

S—Hg formation could be demonstrated at pH 10.0. In 0.1 M glycine buffer, depending on the duration of incubation, the SH groups of LDH combined with PCMB (Fig. 1).

As shown in Fig. 1, at alkaline reaction about 19 SH groups were bound in 40 minutes. No further S—Hg linkages were formed on prolonged incubation.

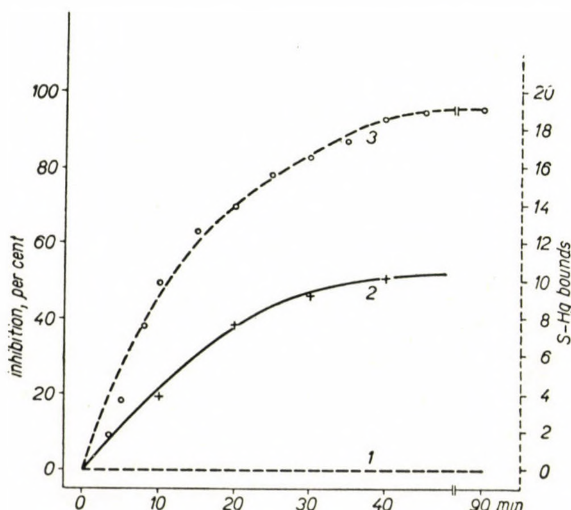

Fig. 1. Correlation between formation of the S—Hg linkage and inhibition
Activity was assayed in the direction of the DPN^+ + lactate → DPNH + pyruvate reaction, at pH 10.0
The number of S—Hg linkages was determined on the basis of the extinction increment measured at 250 mμ:

$$\Delta E_{250/\text{mol LDH}} \cdot \text{S—Hg} = 6.5 \cdot 10^3$$

In 10 minutes eight, in 30 minutes of incubation about sixteen S—Hg linkages were formed, while for the formation of the further three or four S—Hg linkages further 20 minutes of incubation were required.

It has been investigated how incubation with PCMB influences enzymatic activity at alkaline reaction (pH 10.0), at which the SH groups can be bound. The effect of 25 eq PCMB on the enzymatic activity of LDH is shown in Fig. 1. Incubation took place at room temperature, in 0.1 *M* glycine buffer. At the indicated points of time the incubation mixtures were diluted with 0.1 *M* glycine buffer (pH 10.0) for activity assay. LDH activity was measured in the direction of the DPN^+ + lactate → DPNH + pyruvate reaction.

Blocking of the SH groups was almost complete in 35 to 40 minutes, but this resulted in 50 per cent inhibition only.

In Fig. 2 the inhibitor effect of PCMB is plotted against the pH. The incubation mixtures containing $1.12 \cdot 10^{-3}$ μmol/ml LDH and $1.65 \cdot 10^{-2}$ μmol/ml PCMB were incubated between pH 7.5 and 9.1 in 0.1 *M* tris buffer, between pH 9.7 and 10.0 in 0.1 *M* glycine buffer, for various lengths of time.

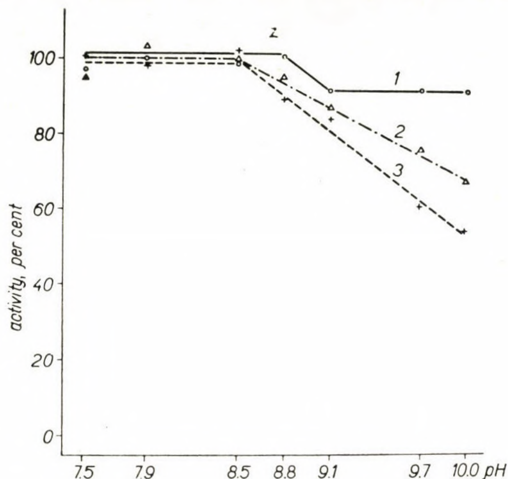

Fig. 2. Inhibitory effect of PCMB *vs.* pH
Activity was assayed in the direction of DPNH formation. Incubation was carried out at room temperature

The mixtures were diluted with pH 10.0 0.1 *M* glycine buffer and enzymatic activity (DPN reduction) was measured.

In neutral medium, up to pH 8.5, the activity of the preincubated protein is not different from that of the control protein solution not containing PCMB. At more alkaline reactions the inhibition in response to the PCMB effect increased with the increase of the pH. Inhibition was influenced by the duration of incubation. In response to incubation at alkaline reaction, in the absence of PCMB activity slightly decreased, by about 20 per cent in 60 minutes.

To determine whether PCMB, which combined with LDH in alkaline medium only, inhibited the DPNH + pyruvate → DPN^+ + lactate reaction measurably at pH 7.5, we had to acidify to pH 7.5 the alkaline solution containing the LDH—PCMB complex. In more concentrate solution (about 200 μg/ml LDH) neutralization led to the precipitation of LDH even at 0°C. The same happened in the absence of PCMB. For this reason neutralisation was carried out in a 5 μg/ml solution. Under such conditions the activity of the enzyme (DPNH oxidation) was identical with that of the control containing no PCMB and incubated at pH 7.5. However, owing to the low protein concentration it could not be determined in this case whether the Hg-compound was still linked to the protein.

At the above mentioned two pH-s the optical rotation values of LDH were measured after incubation in the presence of 20 eq of PCMB at 20 C°. The values obtained were $[\alpha]^{20}_{546} = -41.3° \pm 0.4$ in the case of 0 to 25 minute incubation at pH 7.5, and $-44.9° \pm 0.2$ in the case of incubation for 0 to 25 minutes at pH 9.8, which agreed within the limits of error with the optical rotation shown by untreated LDH ($42.6° \pm 3.0$). At the same time, the intrinsic viscosity of the protein at pH 7.5 was $[\eta] = 4.8$, at pH 10.0 $[\eta] = 11.5$.

In a previous paper [7] dealing with the effect of Na-dodecylsulphate on LDH, we have shown that the native structure of the enzyme is broken up in the presence of the detergent. We investigated whether in response to SDS at neutral pH the SH groups of LDH could be bound. Under conditions identical with those outlined above, in 60 minutes, at room temperature, in the presence of 200 to 2000 moles of SDS per mole of LDH, 17 to 19 moles of PCMB were bound, practically as many as at pH 10.0, without any other pretreatment.

Discussion

Kaplan and Ciotti [8] on the basis of studies made with coenzyme analogues classified human LDH's into two main groups; to group 1 belong the LDH's isolated from skeletal muscle, liver, spleen and connective tissue, to group 2 those isolated from heart muscle, kidney, pancreas, stroma and erythrocytes. The LDH isolated from lung tissue is intermediate between the two groups.

On the basis of the reaction velocity measured with coenzyme analogues, the difference between bovine heart LDH and bovine skeletal muscle LDH is greater than the difference between chick heart LDH and bovine heart LDH. According to our investigations there is a structural difference between heart muscle LDH and skeletal muscle LDH of the pig, manifested in the reactivity of the SH groups.

Two SH groups of the heart muscle LDH are essential from the point of view of activity, since binding of these two SH groups by PCMB makes activity to cease [3]. On the other hand, the SH groups of skeletal muscle LDH combine with PCMB at alkaline reaction only. However, as indicated by the data in Fig. 1, if all the SH groups are bound by PCMB, enzmyatic activity is decreased by about 50 per cent only. At neutral reaction no formation of linkage with PCMB is demonstrable by the Boyer titration method and the presence of the SH reagent does not influence enzymatic activity, either.

It is known that the SH groups of the different enzymes do not react with the same speed with specific heavy metal reagents. In certain cases the SH groups are demonstrable only after the native protein structure has been

deranged. "Masked" SH groups have been found in urease [9], D-glyceraldehyde-3-phosphate dehydrogenase [10], phosphorylase [11], aldolase [12], *etc*. According to SZABOLCSI *et al.* [13] in the case of aldolase the binding of the masked SH groups leads to a diminution of activity, yet they play no direct role in catalytic activity. On the other hand, they play an important role in the maintenance of the native protein structure.

At neutral reaction the SH groups of pig skeletal muscle LDH can be considered to be masked, being titratable only after treatment with urea or detergent.

At pH 10.0 every SH group of the skeletal muscle LDH is reactive. This may be due to a structural change caused by the alkaline reaction. The alkaline medium does not seem to damage the secondary structure of the protein, because no change in optical rotation is demonstrable. In contrast, the intrinsic viscosity of the protein is more than double of that measured at neutral reaction. Thus, the fact that the SH groups become reactive may be due to changes in the tertiary structure.

According to data in the literature, pig heart muscle LDH is a typical "sulfhydryl" enzyme [3], whereas the SH groups of pig skeletal muscle LDH seem to play no role in the activity demonstrable by PCMB treatment. The decrease of activity in response to PCMB at alkaline reaction, like that found with aldolase [13], is presumably a secondary effect, apparently a result of some change taking place in the tertiary structure.

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György JÉCSAI, Pál ELŐDI
Magyar Tudományos Akadémia Biokémiai Kutató Intézete, Budapest

A STUDY OF PURIFICATION AND PROPERTIES OF BACILLUS SUBTILIS EXO-PENICILLINASE

By

M. Ishimoto

INSTITUTE OF MEDICAL CHEMISTRY, MEDICAL UNIVERSITY, BUDAPEST

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Exo-penicillinase of *B. subtilis* was purified by means of chromatograhy on carboxymethyl cellulose. The pH optimum and resistance to inactivation by iodine revealed more similarity to *B. cereus* exo-penicillinase than to *B. subtilis* cell-bound penicillinase. Antiserum prepared against the enzyme precipitated not only the same enzyme but also the *B. cereus* exo-penicillinase. The results of absorption tests showed that the same antibody reacted with both enzymes.

Penicillinase is an enzyme widely distributed in microorganisms; it has been studied in the field of enzymology as well as its applications [1]. While penicillinase from *B. cereus* was purified [2] and studied extensively, only a few reports appeared about the enzyme *B. subtilis*. In the present paper a preparation of *B. subtilis* exo-penicillinase is described, the specific activity of which is equivalent to that of the purest preparation of *B. cereus*. It is different from the enzyme prepapered by Kushner [3] from another strain of *B. subtilis*.

Materials and methods

Culture. B. subtilis T98, isolated by V. Csányi in this laboratory was used. The culture medium was similar to that used for growing *B. cereus* [4], except that "Lactamin" *(Phylaxia*, Budapest) was used instead of casaminic acid; 0.25 g of yeast extract *(Difco)* and 1.5 g of glucose were added for 1000 ml of the medium. An inoculating culture was grown overnight at 37° C, then transferred to 10 volumes of the same medium, and cultivation was made at 35° C with aeration for 24 hours. The medium contained 2500—5000 units/ml of penicillinase.

Penicillinase was measured according to Csányi [5] and expressed in enzyme units; 1 unit of penicillinase being the amount of enzyme which hydrolyzes 1 μmol of penicillin in 60 min. at 30° C [6]. Protein was determined by the method of Lowry *et al.* [7]. The assay method for non-volatile organic substances was described by Johnson [8].

Exo-penicillinase of *B. cereus* was partially purified by chromatography on diatemaceous earth according to Citri [9]. Cell-bound penicillinase was the crude extract obtained from *B. subtilis* by crushing the cells in a thick suspension with sonic vibration (20 kc, 60 W, 6 min.).

Preparation of antiserum. B. subtilis purified exo-penicillinase preparation ($1.8 \cdot 10^6$ units/mg N) mixed with Freund's adjuvant was injected into the subscapular region of rabbits (about 400 000 units/kg). After 5 weeks, 5 injections of 150 000 units of the enzyme adsorbed on aluminium hydroxide were administered to the animals successively every third day. The preparation to be injected was made as follows, 1 ml of 1 per cent alum solution was added to 15 ml enzyme solution (400 000 units in 0.005 *M* phosphate buffer, pH 7.0) and the precipitate was collected by centrifugation and suspended in 0.85 per cent sodium chloride. One week after the last injection, the rabbits were sacrificed and blood was collected in a vessel containing heparin. In some cases, if the titre of the antibody was low at that time, 5 more injections of

alum-precipitated penicillinase were administered before sacrificing the animals. 1 ml plasma had an activity to neutrilize 40—70 000 units of penicillinase.

The plasma was diluted three times with water and the pH adjusted to 7.6 by adding 1 *N* HCl. Antipenicillinase was precipitated with an equal volume of ethanol in cold, collected by centrifugation, dissolved in 0.85 per cent saline (0.4 of the original plasma volume) and then lyophilized. Before use, the lyophilized antipenicillinase was dissolved in distilled water, the volume of which was equal to the solution before lyophilization.

Antiserum against *B. cereus* exo-penicillinase was obtained from Dr. KRAMER. The lyophilized preparation contained 58 000 neutralizing units/ml dissolved in distilled water of a volume corresponding to the original serum.

Results and discussion

Purification of penicillinase. The supernatant obtained by centrifugation of a *B. subtilis* culture was employed as a starting material for purification. It contained more than 90 per cent of the activity of the whole culture. After adjusting the pH of the supernatant to 5 by the addition of acetic acid, it was passed through a column of diatomaceous earth (Celite 535), according to CITRI [9]. After washing the column with distilled water, the enzyme was eluted with 1 *M* sodium chloride at pH 8.5. Recovery of enzymic activity was 85—100 per cent in this step. The main part of the effluent containing penicillinase activity over 20 000 units/ml was collected and dialyzed against 20 volumes of 0.001 *M* phosphate buffer, pH 6.5, for 24 hours at 4° C, with one change of the external solution. Phosphate buffer was added to the dialyzed solution to adjust the phosphate concentration to 0.01 *M* and the pH to 5.5. The enzyme was adsorbed on a column of carboxymethyl cellulose pretreated

Table I

Effects of iodine on penicillinase activity

Conditions of incubation*	In absence of iodine		With iodine	
			3 min.	30 min.
Reaction condition with penicillin*	—	Iodine was added together**	—	—
Enzyme preparation	Penicillinase found (units)			
B. subtilis exo-penicillinase	260	220	40	10
B. subtilis cell-bound penicillinase	300	10	0	0
B. cereus exo-penicillinase	390	330	60	10

* The enzymes were incubated in 4 ml containing 0.25% gelatin with or without 0.0125 *N* iodine at 30° C and then 1 ml penicillin solution was added and the amount of iodine-consuming substance formed in 10 minutes was determined according to CSÁNYI [5] and expressed in units of penicillinase in 4 ml.

** The amount of iodine added was the same as in other experiments, 50 μ atoms.

with 0.01 *M* phosphate buffer, pH 5.5. The column was then washed successively with the same buffer and 0.01 *M* phosphate buffer, pH 7.6. 90 per cent of the penicillinase was adsorbed and retained by the column. Sodium chloride gradient elution was then carried out. The mixing chamber contained 0.01 *M* phosphate buffer, pH 7.6, at the start, to which 1 *M* sodium chloride was

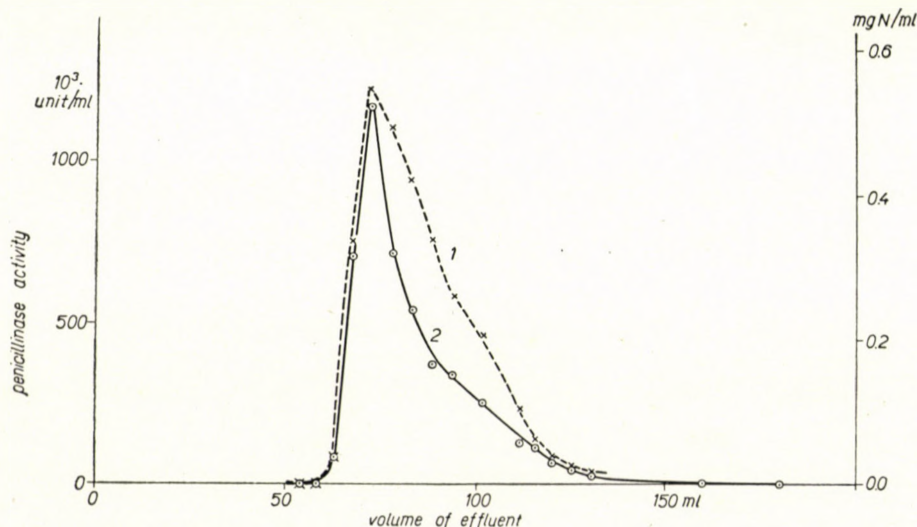

Fig. 1. Chromatogram of penicillinase on carboxymethyl cellulose column
24 000 000 units of penicillinase were adsorbed on a carboxymethyl cellulose column, 12 × 1.6 cm.
The capacity of the mixing chamber was 190 ml
1: Amount of protein (mgN/ml); 2: Penicillinase activity (units/ml)

added dropwise from a tank. One typical experiment is shown in Fig. 1 Though the enzyme as well as the proteins are eluted as a single peak, the specific activity of the fractions constantly decreases after the maximum. This indicates that some protein impurities had been eluted by the high salt concentration. The maximum specific activity was $2.1 \cdot 10^6$ units/mg N. The crystalline preparation from *B. cereus* has been reported by Pollock *et al.* [2] to have an activity of $1.53 \cdot 10^6$ units/mg N.

Properties of the B. subtilis penicillinase. The pH optimum of the enzyme is not sharp; maximum activity was found at pH 6.5, similarly to the exo-penicillinase of *B. cereus* which has the optimum at pH 6.7.

An experiment concerning the effect of iodine (Table I) also showed the similarity of the two enzymes. The exo-penicillinase of *B. subtilis* was more stable against iodine than cell-bound penicillinase. A similar difference has been described between the penicillinase and the cell-bound enzyme of *B. cereus* [10].

Immunological study. The antiserum precipitated the exo-penicillinase and inhibited enzymatic activity. The degree of inhibition, however, was somewhat different with the different preparations. On the other hand, the antiserum scarcely inhibited the cell-bound enzyme from the same organism and formed no precipitate at a dilution of 1 : 10 with this or with the culture medium from an uninduced inducible strain of *B. subtilis* T215, isolated in this laboratory. It was shown by the agar diffusion method [11] with partially purified enzyme (dialyzed eluate from Celite adsoraption) that it contined at least 3 different antibodies. As the purified preparation of the penicillinase

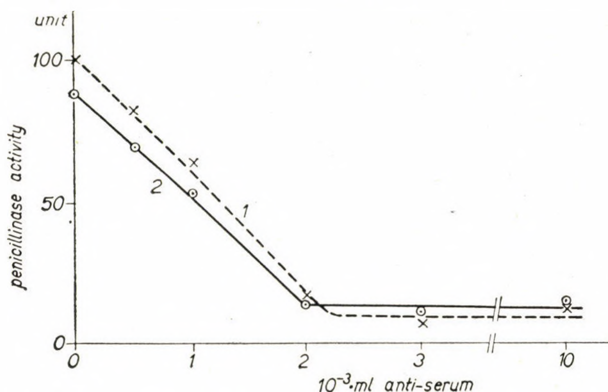

Fig. 2. Titration curve of *B. subtilis* and *B. cereus* penicillinase with anti-penicillinase against *B. subtilis* penicillinase

The enzyme and anti-penicillinase were mixed in the presence of 0.85% NaCl and 0.25% gelatin, and after 30 minutes incubation the penicillinase activity of the mixture was estimated 1: *B. cereus* penicillinase; 2: *B. subtilis* penicillinase

gave a precipitate with only one, the other two antibodies should be against concomitant proteins in the preparation.

Tests for comparison using *B. cereus* exo-penicillinase revealed that it was also precipitated by *B. subtilis* penicillinase-antiserum. The titration curve of both enzymes is indicated in Fig. 2. The serum neutralized an almost equal number of units of them. The results of the precipitation reaction indicate that the enzymic activity remaimed in the supernatant when both enzymes were present at the same time, even if either of them alone could almost completely be precipitated by the antibody. These results suggest that the same antibody reacted equally with either of the penicillinases. The results of double diffusion in agar [12] also showed that the penicillinase from *B. cereus* and that from *B. subtilis* are identical as antigen, they form a continuous precipitation band, and no crossing lines.

Antiserum against *B. cereus* exo-penicillinase neutralized also both of the penicillinases.

KUSHNER [3] studied purification as well as the immunological properties of penicillinase from a strain of *B. subtilis*. According to his results, the enzyme was not adsorbed on glass and did not react with the antiserum against *B. cereus* exo-penicillinase while the antiserum against the *B. subtilis* did not combine with *B. cereus* exo-penicillinase.

The discrepancy between our results and those of KUSHNER was probably due to the difference of the strains. According to KUSHNER, his enzyme was

Table II

Precipitation of penicillinase of B. subtilis and B. cereus by anti-penicillinase against B. subtilis penicillinase

B. subtilis exo-penicillinase (1940 units) and/or B. cereus exo-penicillinase (2000 units) were mixed with anti-B. subtilis exo-penicillinase (1400 neutralizing units in amounts corresponding to 0.04 ml of the original plasma).

The precipitation reaction was carried out at 0° C in the presence of 0.45% gelatin and 0.85% NaCl in 2 ml. After 2 hours any precipitate formed was centrifuged down, washed with 0.3 ml of 0.85% NaCl and used for the determination of non-volatile organic substances [8]. The supernatant was used for determination of penicillinase activity.

B. subtilis penicillinase	*B. cereus* penicillinase	Antiserum	Penicillinase in supernatant units	Non-volatile organic substance in precipitate, mg
+	+	+	2200	0.106
—	+	+	180*	0.162
+	—	+	60*	0.205
+	+	—	4380	—
—	+	—	1980	—
+	—	—	2190	—
—	—	+	—	0.045

* The supernatants did not neutralize either the penicillinase activity of *B. subtilis*, or that of *B. cereus*.

not an exo-enzyme and the strains were inducible ones. We employed a strain which produced penicillinase constitutively and the larger part was released into the external medium, although the ratio of the enzyme in cell and in medium changed, depending on the age of the cells. There is no reason to suppose that the enzymes were in any way identical.

The results stated above indicate the close similarity or identity of *B. subtilis* and *B. cereus* exo-penicillinases, at least as far as enzymic and immunological properties are concerned.

Acknowledgement

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Makoto Ishimoto
Orvostudományi Egyetem Orvosi Vegytani Intézete, Budapest

ENZYMIC FORMATION OF THE DISULFIDE BRIDGES OF RIBONUCLEASE

By

P. Venetianer and F. B. Straub

INSTITUTE OF MEDICAL CHEMISTRY, MEDICAL UNIVERSITY, BUDAPEST
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An enzyme was found in the pancreas of several animal species which is able to catalyze the reactivation of reduced bovine pancreatic ribonuclease.

The enzyme was partially purified from chicken and pig pancreas.

A heat-stable factor was essential to the activity of the enzyme. This substance could be replaced by dehydroascorbic acid.

The possible significance of these results in the problem of protein biosynthesis is discussed.

Introduction

Anfinsen and Haber [1], and White [2] described the reversibility of the reduction of ribonuclease. These authors reduced the disulfide groups of bovine pancreatic RNase*, and after removing the reducing agent reoxidized them with atmospheric oxygen. The reoxidized enzyme proved to be identical with the original, both in respect to physico-chemical properties, and enzyme activity. Consequently the reformation of the disulfide bridges was a specific and not a random process. Since then analogous reactivation of several other reduced proteins has been observed [3—8].

The significance of these results is obvious, considering the probable identity of the reduced protein and the polypeptide chain which is formed on the ribosomes during protein biosynthesis. Accordingly such reoxidation experiments may serve as *in vitro* modells in studying the final steps of the biosynthesis — *i.e.* the formation of the properly folded protein molecule. Indeed, it is generally believed on the basis of these experimental facts, that no new information is needed for this final step, the tertiary structure being perfectly determined by the primary, *i.e.* the correct pairing of cysteine residues is coded in the amino acid sequence itself. However, the rate of reactivation is too slow to account for the *in vivo* observed biosynthesis, even under optimal conditions. Moreover it must be kept in mind that the optimal conditions (pH 8.2 24° C) [9] are not physiological.

At the start of this work we assumed therefore, that ribonuclease-synthesizing tissues may contain an enzyme, or enzyme system which catalyzes the final step of the biosynthesis, *i.e.* the reactivation of reduced RNase. In this paper we describe the existence of this enzyme in the pancreas, its partial

* RNase: ribonuclease; tris: trishydroxymethylaminomethane; DHA: dehydroascorbic acid; EDTA: ethylenediaminotetraacetic acid; GSH, GSSG: reduced and oxidized glutathione; NAD: nicotinamide adeninedinucleotide (DPN); NADP nicotinanideadenine dinucleotide phosphate (TPN).

purification, and some properties of the reaction. A preliminary report on this subject was already published [10]. Recently GOLDBERGER *et al.* [11] reported a rat liver system capable of accelerating the reactivation of reduced ribonuclease. Considering the similarities we believe that this system and the pancreatic enzyme described by us are identical.

Materials and methods

"*Reanal*" bovine pancreatic RNase was purified by chromatography on IRC—50 column. The protein peak corresponding to RNase A was lyophilized, Purified RNase was reduced with 2-mercaptoethanol in 8 M urea according to the method of ANFINSEN and HABER [1]. The reagents were removed by means of a Sephadex G—25 column, with 0.1 N acetic acid as eluent. Reduced RNase could be stored for several weeks in the acid solution without considerable reactivation. It was neutralized immediately before use.

RNase activity was assayed by the method of SCHUCHER and HOKIN [12] with one modification. The incubation time was 10 min. instead of 30 min. In the calibration of the assay method the concentration of the standard enzyme solution was determined by measuring the optical density at 275 mμ. At this wavelength, the molar extinction coefficient of the native as well as the reduced RNase is 9200 [13].

SH groups were determined by the method of BOYER [14], protein concentration was measured by the method of LOWRY *et al.* [15].

In the assay of the reactivating enzyme the following procedure was employed. The enzyme preparation to be tested, was shaken at 37° C in pH 7.5 tris-HCl buffer with eventual additions as noted in the figures. After 5 min. preincubation, the reaction was started with the addition of reduced RNase. At intervals 0.1 ml aliquots were taken out from the reaction mixtures and pipetted into 1 ml 0.25 N H_2SO_4. Before RNase assay these solutions were neutralized with 1 N NaOH and appropriately diluted with 0.04 M pH 7.5 phosphate buffer. In work with pancreatic homogenates or crude extracts this sulfuric acid treatment was essential in order to release the particle-bound RNase. When purified enzyme preparations were tested, the 0.1 ml aliquots were pipetted into cold 0.04 M phosphate buffer and immediately assayed for RNase activity.

Dehydroascorbic acid was always freshly prepared by oxidizing ascorbic acid in dilute solution with bromine and removing excess bromine with a current of air. Crystalline DHA was prepared according to KENYON and MUNRO [16].

Results

1. *Experiments with pigeon pancreas*

Pigeon pancreas was homogenized in 0.25 M sucrose. After removal of cell debris and the nuclear fraction with centrifugation (1000 g, 10 min.), the supernatant was incubated with reduced RNase. As shown on Fig. 1 the homogenate greatly accelerates the reactivation of RNase. This effect disappears after heating the homogenate for 5 min. at 100° C.

The extract of an acetone-dry powder of pigeon pancreas exhibited similar catalytic effect, indicating that the activity was not necessarily bound to intact structures.

2. *Experiments with chicken pancreas*

Owing to the fact that pigeon pancreas was not available in sufficiently large quantities to purify the catalytic factor, we tried other species. Attempts to demonstrate the activity in extracts of beef and pig pancreas powder were

unsuccessful. However, a slight activity was observed in chicken pancreas. The addition of a boiled (*i.e.* in itself inactive) pigeon pancreatic extract enhanced the rate of reactivation of reduced RNase. The boiled extract could be replaced by yeast extract. These data, summarized in Table I suggest that a heat-stable factor is the necessary component of the reactivation system. The concentration of this factor(s) is rate limiting in chicken pancreas.

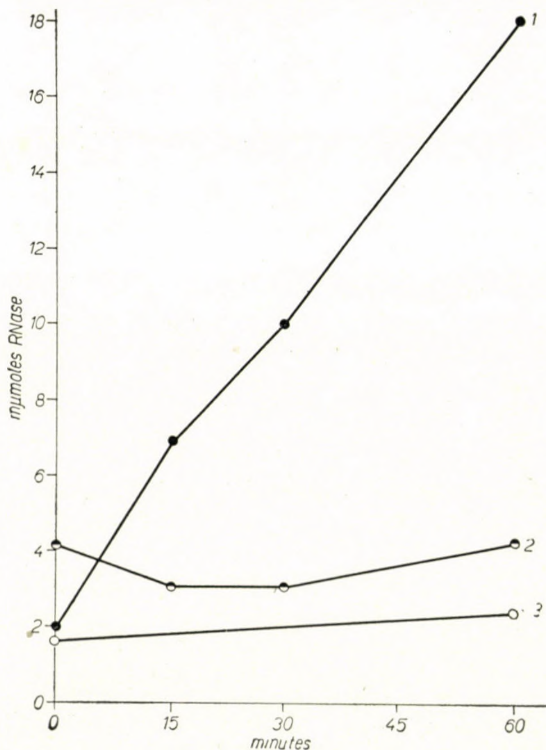

Fig. 1. The effect of pigeon pancreas homogenate on the reactivation of reduced RNase Reaction mixtures: 0.03 *M* pH 7.5 tris-HCl; 23 mμmoles reduced RNase; plus additions. Final volume: 3.2 ml
Curve *1*: 0.5 ml 20% pigeon pancreas homogenate. *2* : 0.5 ml boiled (5 min 100° C) homogenate, *3* : no addition (control)

3. *Purification of the reactivating enzyme from chicken pancreas*

When testing the purification procedure, a heat-stable factor *(Difco* lyophilized yeast extract) was always added to the assay mixtures. The unit of activity was defined arbitrarily as follows: one unit of enzyme is that which reactivates 1 millimicromole (13.7 microgram) reduced bovine pancreatic RNase in 1 min., based on the initial velocity. The exact quantitative evalu-

ation of the purification procedure was not possible because the conditions were not always optimal, and the crude extracts contained inhibitory substances (see later).

Step 1. **The pancreas of 6 weeks-old chickens were homogenized in Waring blendor in 10 vol. twice destilled —10° C cold acetone. After centrifugation, the homogenization was repeated, the homogenate filtered, and the cake dried *in vacuo.* This powder could be stored for several months without loss of activity.**

Table I

The effect of the heat-stable factor(s)

Incubation mixture: 0.1 *M* pH 7.5 tris-HCl; 10 mμ moles reduced RNase; + additions as noted in the table. Final volume: 0.5 ml. Pancreatic extracts: acetone-dried pancreas powder was extracted with 10 volumes of buffer as described in the text.

Boiled extract: pigeon pancreatic extract was heated at 100° C for 5 min.

Yeast extract: 2 mg *Difco* powdered yeast extract.

Exp.	Additions	Reactivation rate mμ mole RNase/min.
1	No addition (control)	0.003
	0.1 ml pigeon extract + 0.05 ml boiled extract	0.051
	0.1 ml pigeon extract	0.033
	0.05 ml boiled extract	0.003
2	No addition	0
	0.01 ml chicken extract	0.041
	0.01 ml chicken extract + 0.05 ml boiled extract	0.056
3	No addition	0.002
	0.1 ml chicken extract + 0.05 ml boiled extract	0.039
	0.1 ml chicken extract + 0.05 ml boiled dialyzed extract	0.011
	0.1 ml chicken extract + 2 mg yeast extract	0.056

Step 2. The dry powder was extracted with 0.05 *M* pH 7.5 tris-HCl buffer for 30 min. in the cold room. The buffer contained 0.1 mg/ml diisopropylfluorophosphate to inhibit proteolytic enzymes. The extract was centrifuged with 20 000 *g* for 10 min. and the insoluble residue was discarded. All subsequent operations were carried out in the cold.

Step 3. Solid ammonium sulfate was added slowly to the supernatant to 54 per cent saturation. It was centrifuged after standing overnight, the precipitate was discarded and ammonium sulfate was added to the supernatant to 75 per cent saturation. After centrifuging the supernatant was discarded and the precipitate was dissolved in a minimal amount of the extracting buffer. This solution was dialyzed against 0.01 *M* pH 7.5 tris-HCl buffer.

Step 4. The dialyzed solution was submitted to chromatography on DEAE-cellulose with a gradient elution technique. (Gradient from 0.01 *M* pH 7.5 tris-HCl to 0.7 *M* NaCl.) The active fractions were pooled and dialyzed

against saturated ammonium sulfate. The precipitated protein was centrifuged, dissolved in a minimal amount of tris buffer and dialyzed.

The results of some purification experiments are summarized in Table II. It is remarkable that in some cases the crude extract was completely inactive, activity appeared only after the ammonium sulfate fractionation. Probably the crude extract contained inhibitory substances which had to be removed. This observation led us to try a similar purification from pig pancreas which appeared to be inactive in earlier experiments.

Table II

Purification of the reactivating enzyme from chicken pancreas

Exp.	Specific activity: mμmole RNase/min./mg protein		
	Crude extract	After $(NH_4)_2SO_4$ fractionation	After DEAE chromatography
1	0.009	0.041	0.352
2	0	0.089	0.390
3	0	0.095	0.476

4. *Purification of the enzyme from pig pancreas*

After removal of the fat and connective tissue, pig pancreas was chopped up with scissors and homogenized in a Waring blendor for 2 min. in five volumes of 0.025 *M* pH 7.5 phosphate buffer which contained EDTA (0.002 *M*) and diisopropyl-fluorophosphate (0.1 mg/ml). This, and all subsequent operations were carried out in the cold. The homogenate was centrifuged for 30 min. with 1000 *g*, the supernatant was decanted and filtered through glass wool to remove fat. Ammonium sulfate was added slowly to 54 per cent saturation. After standing overnight the precipitate was removed by centrifugation. The clear pink supernatant was already active. Ammonium sulfate was added slowly to 70 per cent saturation. The solution was centrifuged, the supernatant discarded and the precipitate dissolved in 0.01 *M* pH 7.2 tris-HCl buffer. This solution was dialyzed against the same buffer overnight. The white insoluble material which precipitated during dialysis was removed by centrifugation. The activity remained in the supernatant. The next step was the DEAE chromatography, with a gradient from 0.01 *M* pH 7.2 tris-HCl to 0.6 *M* NaCl in the same buffer. The active fractions were concentrated as described above. The solution of the enzyme at this stage was light yellow, and always contained slight amounts of nucleic acid besides protein. The data of the purification procedure are summarized in Table III.

Table III

Purification of the reactivating enzyme from pig pancreas

Exp.	Specific activity: mμmole RNase/min./mg protein		
	Crude extract	After $(NH_4)_2SO_4$ fractionation	After DEAE chromatography
1	0	0.60	2.28
2	0	0.81	4.35

5. *Experiments with the partially purified enzyme*

The reactivating enzyme at this stage of purification appeared to be suitable to study the conditions and requirements of the reaction. Fig. 2 shows the dependence of the reaction rate on the concentration of the sub-

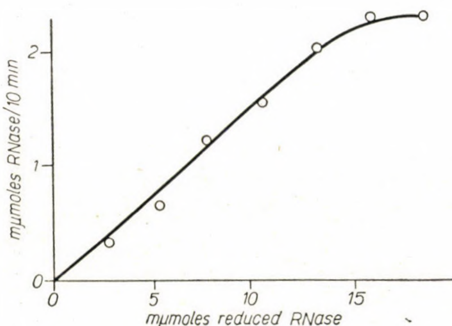

Fig. 2. Effect of the concentration of reduced RNase on the rate of reactivation. The amount of RNase reactivated in the first 10 minutes is plotted against the initial concentration of reduced RNase

Incubation mixtures: 0.05 *M* pH 7.5 tris-HCl; 4 mg *Difco* yeast extract; 1.65 mg purified chicken reactivating enzyme; plus varying amounts of reduced RNase. Final volume: 1 ml

strate, *i.e.* the reduced RNase. This increased velocity with higher concentrations is in sharp contrast with the non-enzymic reactivation [9] where an inverse relationship was observed at similar substrate concentrations.

In contrast to the marked pH dependence of the non-enzymic reoxidation, the enzyme reaction is only slightly influenced by alteration of the pH in the range between 7—8.5. This is in agreement with the results of GOLDBERGER *et al.* [11].

The kinetics of reactivation were compared at 25° C and 37° C. Again in contrast to the non-enzymic reaction [9], the rate was higher at 37° C.

Table IV summarizes data on the effects of various additional compounds on the reactivation. The SH compounds tested are all strongly inhibitory at

higher concentration, whereas at lower values the effect can be slightly stimulatory. Urea and Cu^{++} are strong inhibitors. The marked activating effect of EDTA or cyanide might be explained by assuming that these compounds act by removing the inhibitory heavy metal ions present in the reagents employed. Therefore in later experiments EDTA was always added to the reaction mixtures.

Table IV

Inhibition and activation of the enzymic reactivation

Incubation mixtures in all these experiments contained: 0.1 *M* pH 7.5 tris-HCl buffer; 8—14 mμ moles reduced RNase; 2 mg *Difco* yeast extract; 1—2 mg purified activating enzyme; + additions as noted in the table. Final volume 0.6 ml. Activities are expressed in the percentage of the control values.

Additions	Activity %
No addition (control)	100
5. 10^{-3} *M* cystein	20
5. 10^{-3} *M* GSH	4
5. 10^{-3} *M* 2-mercaptoethanol	20
10^{-4} *M* ,,	107
5. 10^{-3} *M* thioglycolic acid	3
10^{-3} *M* Cu^{++}	0
1.3 *M* urea	21
8. 10^{-4} *M* NaCN	120
8. 10^{-3} *M* NaCN	147
8. 10^{-3} *M* EDTA	155

The reactivating enzyme could be stored even in very dilute solutions in the cold for several weeks without loss of activity. It is sensitive to acid and heat treatment. 5 min. heating at 65° C decreases the activity by 43 per cent. Precipitation at pH 4.8 results in an irreversible decrease in activity. If the enzyme was treated with *p*-chloromercuribenzoate, after removal of the reagent with Sephadex G—25 no decrease of activity was observed. This result suggests that SH groups do not play any role in the activity of the enzyme.

6. *The heat-stable factor*

Our earlier experiments with crude pancreatic extracts indicated — in agreement with the results of GOLDBERGER *et al.* [11], — thet the heat-stable non-protein factor required in the reactivation process can be removed only

partially by dialysis. Partially purified preparations of the reactivating enzyme were sometimes completely inactive without the addition of yeast or boiled pancreatic extract; other preparations did not require the addition of the heat-stable cofactor. Probably uncontrolled variations in the purification procedure resulted in the loss of bound cofactor in the former case.

Fig. 3 shows the effect of cofactor *(Difco* yeast extract) concentration on the reactivation catalyzed by a cofactor-dependent enzyme preparation. The initial velocity was proportional to the concentration. However, the

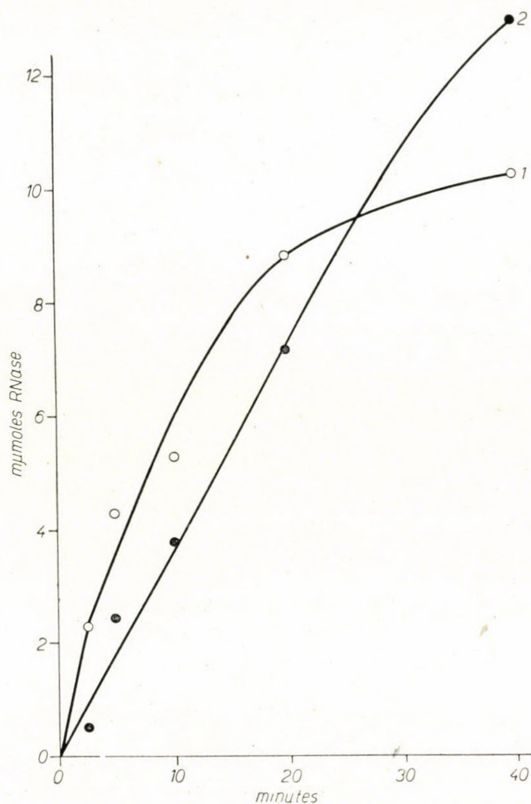

Fig. 3. Effect of the concentration of the yeast extract. Incubation mixtures: 0.1 *M* pH 7.5 tris-HCl; $5 \cdot 10^{-3}$ *M* EDTA; 50 mμmoles reduced RNase; 0.05 mg purified pig enzyme. Final volume: 1 ml. Curve *1 :* 20 mg *Difco* yeast extract, *2 :* 6.6 mg *Difco* yeast extract

maximal extent of reactivation was always higher with lower cofactor concentration. We tried to replace yeast extract with NAD, NADP, GSSG, but these attempts were unsuccessful. GOLDBERGER *et al.* [11] tested a number of other substances but none of them proved to be active. In view of the known hydro-

gen acceptor role of dehydroascorbic acid in plant systems, we tried this substance as cofactor. It was found, that crystalline DHA as well as DHA prepared by the bromine oxidation of ascorbic acid could replace yeast extract in our system. Ascorbic acid was also active but only after a lag period. This is not surprising, owing to the easy oxidizability of ascorbic acid by atmospheric oxygen (Fig. 4).

The kinetics of the reactivation process were therefore studied with DHA. Fig. 5 shows the variation of the kinetics with different DHA concentrations

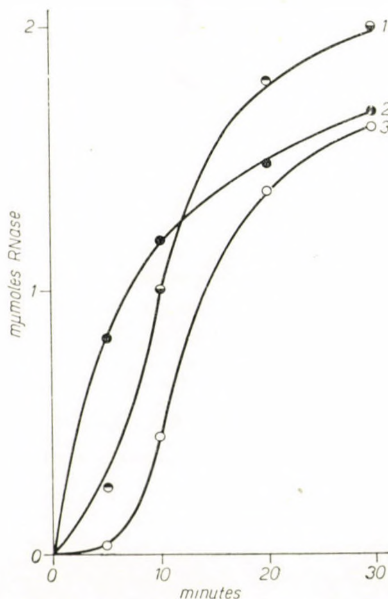

Fig. 4. Replacement of the yeast extract with ascorbic acid and DHA. Incubation mixtures: 0.1 *M* pH 7.5 tris-HCl; $3 \cdot 10^{-3}$ *M* EDTA; 12 mμmoles reduced RNase; 0.12 mg purified pig enzyme. Final vol: 0.8 ml. Curve *1* : 0.05 ml boiled yeast extract, *2* : $2 \cdot 10^{-3}$ *M* DHA, *3* : $6 \cdot 10^{-3}$ *M* ascorbic acid

using a cofactor-dependent enzyme. The shapes of the curves are very similar to those of Fig. 3, where yeast extract was used as cofactor. Initial velocity is proportional to the DHA concentration, but the maximal extent of reactivation is achieved with low DHA level. The kinetics are even more complicated in the case of cofactor-independent enzyme preparations. Using a concentrated enzyme solution, the effect of DHA is slightly inhibitory even at low concentration level. However, at lower enzyme concentrations DHA acts as a stimulant, its concentration being directly proportional to the initial rate and inversely proportional to the extent of reactivation (Fig. 6).

Discussion

The experiments described above, demonstrate the presence in the pancreas of several animal species of an enzyme which greatly accelerates the reactivation of reduced RNase. The enzymic nature of the catalysis is supported by the facts that: (i) Activity is bound to a heat-labile, non-dialyzable

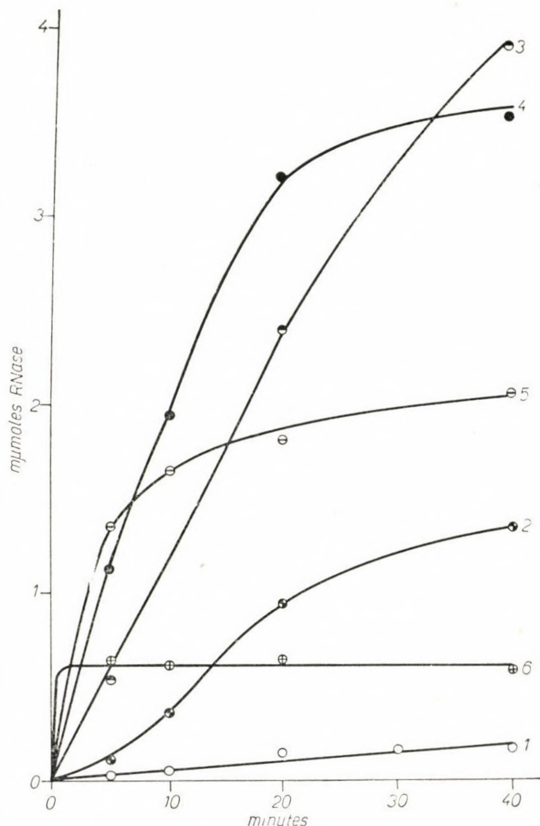

Fig. 5. Effect of DHA concentration. Reaction mixtures: 0.1 M pH 7.5 tris-HCl; $3 \cdot 10^{-3}$ M EDTA; 18 mμmoles reduced RNase; 0.05 mg purified pig enzyme. Final volume: 0.7 ml. DHA was added in the following concentrations: Curve *1* : 0 (control), *2* : $2.1 \cdot 10^{-4}$ M, *3* : $7.1 \cdot 10^{-4}$ M, *4* : $1.7 \cdot 10^{-3}$ M, *5* : $3.4 \cdot 10^{-3}$ M, *6.* $1.7 \cdot 10^{-2}$ M

factor which gives a well-defined protein peak in DEAE chromatography. (ii) The optimal pH, temperature and substrate concentration values are different from the optimal conditions of the spontaneous reactivation.

Under optimal conditions maximal reactivation may be achieved enzymically within 10 min., while no reactivation occurs in the non-enzymic con-

trols. Our best preparations catalyzed the reactivation of RNase at a rate of 260 μg/minute/mg enzyme.

Several enzymes are known which catalyze this type of reaction, *i.e.* the —SH → —S—S— redox process, however most of these, for example glutathion reductase or cystine reductase [18—20] are specific for the low molec-

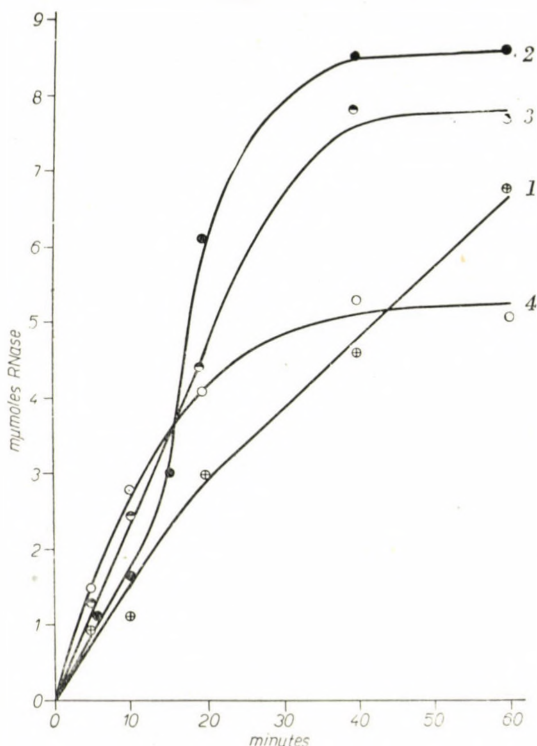

Fig. 6. Effect of DHA concentration. Reaction mixtures: 0.1 *M* pH 7.5 tris-HCl; 3.10^{-3} *M* EDTA; 12 mμmoles reduced RNase: 0.14 mg purified pig enzyme. Final volume 0.7 ml. DHA was added in the following concentrations: Curve *1* : 0 (control), *2* : $5 \cdot 10^{-4}$ *M*, *3* : 10^{-3} *M*, *4* : $4 \cdot 10^{-3}$ *M*

ular weight substrates. Recently some enzymes have been described in yeast [21], or in plant seeds [22], which specifically reduce protein disulfides. Tomizawa [23] purified an enzyme which specifically catalyzes the reduction of insulin with GSH. Katzen *et al.* [24] demonstrated the reversibility of the transhydrogenation catalyzed by this enzyme. They discussed the possible significance of this fact in protein biosynthesis. The RNase rectivating enzyme appears to be similar in function to this enzyme. However in our case DHA serves as hydrogen acceptor in the transhydrogenation whereas GSSG is inactive. The question whether DHA is the physiological hydrogen acceptor,

or only replaces the unknown cofactor, cannot be resolved as yet. A suitable proof would be the demonstration of protein-bound DHA in purified enzyme preparations which do not require added cofactor. Attempts to do this, have been as yet unsuccessful.

To draw conclusions concerning the mechanism of the catalyzed reaction from the kinetic data, would be premature at this stage of the work. The assay method employed — the determination of RNase activity — can measure only the end-product of a complex process. The question arises: which of the four disulfide bonds is or are formed enzymically. On the basis of the kinetics of the non-enzymic reactivation, ANFINSEN *et al.* [13] assume that in the first stage of the reaction disulfide bonds are formed randomly and the resumption of the correct „native" structure takes place by way of interchange reactions. On the basis of the kinetics of enzymic reactivation reported here, we have attempted to formulate a working hypothesis, compatible with the facts. According to this hypothesis the role of the enzyme would be to connect two specified cystein residues of the reduced RNase chain and oxidize them with the aid of DHA. The new bond facilitates the *correct* pairing of the other six sulfhydryls which takes place rapidly and non-enzymically in the presence of DHA. Without enzyme, or in the presence of excess DHA, a rapid aspecific oxidation occurs, measured by the disappearance of SH groups, but this process is not accompanied by reactivation because the incorrect randomly oxidized structure is formed. Conversely, in the presence of enzyme at low DHA level the correct structure is formed immediately, without interchange.

Finally one may ask what the significance of these results with respect to the problem of protein biosynthesis may be. If the biological function of the enzyme is really to catalyze the specific formation of disulfide bridges as the final step of the biosynthesis, then in contrast to the general speculation, the formation of the tertiary structure does require additional information. This information is not encoded directly in nucleotide triplets but in a specific enzyme protein which catalyzes the formation of one of several possible isomers.

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Pál Venetianer, Ferenc Brunó Straub
Orvostudományi Egyetem Orvosi Vegytani Intézete, Budapest

Physiologia

STUDIES OF THE DOUBLE INNERVATION IN THE REGULATION OF ADDUCTOR MUSCLE TONE IN THE CLAM ANODONTA CYGNEA L.

By

J. SALÁNKI and E. LÁBOS

INSTITUTE OF PHYSIOLOGY, MEDICAL UNIVERSITY, DEBRECEN

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The responses given by the posterior adductor muscle consisting of smooth muscle fibres in the clam *(Anodonta cygnea L.)* to the electrical stimulation of the nerve (cerebrovisceral connectivum) running to it and the action potentials obtainable from the isolated cerebro-visceral connectivum in response to single impulses have been investigated.

On changing the stimulation parameters (voltage, frequency, duration of impulse, time of stimulation) significant changes were observed in the muscle responses. With the other parameters unchanged, low voltage (under 1 or 2 V) evoked only contraction, higher voltage (over 2 to 5 V) evoked contraction following by significant relaxation or caused relaxation of the adductor muscle in the state of permanent tonic contraction. Too low and too high frequencies (under 3 to 5/sec. and over 20 to 60/sec.), too short and too long impulse widths (under 3 msec and over 80 msec), and short stimulation (one lasting less than 2 to 4 sec) produced the same effect as low voltage. Effects similar to that produced by high voltage were brought about by stimulations with 5 to 20/sec, series of 4 to 100 msec impulses and stimulation lasting longer than 20 sec.

The action potential recorded from the isolated cerebro-visceral connectivum in response to stimulation with single impulses has several, clearly distinct components, of these the two principal ones could be separated by changing the parameters of stimulation.

It is assumed that the adductor muscle has a double, antagonistic innervation, special innervation serving not only for contraction, but also for the relaxation of the muscle in the tonic state. Correspondingly, relaxation of the muscle in the tonic state is not a passive process, but appears to result from an active nervous effect.

In lower species, including the molluscs, an important functional role is played by the smooth muscles, which are capable of long-lasting tonic contraction with very low energy consumption. In clams the tonic contraction of the adductor muscles ensures that the shells be closed tightly for hours. However, the adductor muscles of the clam can maintain not only tonic contraction, but are also capable of performing fast, phasic contraction, followed immediately by slow relaxation. Many aspects of the regulation of tone are unclear, and therefore the views concerning these two kinds of activity are not uniform.

The first observations on the control of tone of the adductor muscle in clam were made by PAVLOV [6], who found that in response to the stimulation of the cerebrovisceral connectivum (CVC) or the postganglionic nerves supplying the posterior adductor muscle, contration, then relax-

ation of the adductor muscle resulted. He observed that the effect of CVC stimulation depended greatly on the parameters of stimulation, concluding that special inhibitory fibres would be responsible for the decrease of the adductor muscle tone. According to PAVLOV, these inhibitory fibres are different from the tone-increasing fibres, thus the adductor muscles would have a double innervation. FLETCHER [1] found that direct current caused merely contraction and a minimal relaxation, alternating current excessive relaxation in the case of direct stimulation of the retractor muscle of *Mytilus*. LOWY [4, 5] recorded action potentials from the adductor muscle of *Mytilus edulis* and *Pecten maximus* and found that relaxation was accompanied by electrical activity. This suggests that relaxation is an active process, and confirms PAVLOV's view.

ZHUKOV *et al.* [10] as well as ZHIRMUNSKAYA [9] disagree with PAVLOV and claim that the cause of the difference in response to CVC stimulation (relaxation, then contraction) is not a difference in the functional character and behaviour of the nerve elements, but a change in the condition of the adductor muscle. They think that an inhibitory mechanism of the Vedensky type may be involved, *i.e.* the different state of the same neuromuscular structures would be responsible for the difference in the response, so that there was no need to assume the presence of a double innervation (tone-increasing and tone-lowering).

The aim of our investigations was to study the dependence of the muscle responses to CVC stimulation with different parameters reproducing and partly supplementing by up-to-date methods the Pavlovian experiments, and to obtain information as to the fibre composition of CVC by electrophysiological techniques. On the basis of the results obtained we have endeavoured to clear the problem of adductor tone regulation and to establish whether they support the view put forward by PAVLOV or that suggested by ZHUKOV.

Methods

Adult specimens of *Anodonta cygnea L.*, kept for several days in running tap water prior to the experiment, were used. The middle part of the valve was removed, the connecting ligament was cut in the midline, and thus the movements of the posterior half of the shell were separated from those of the anterior half. By cutting the mantle, the 2 to 3 cm long part of the CVC lying between the cerebral ganglia and the retractor pedis muscle, was exposed. In some cases the connexion between the CVC and cerebral ganglia was interrupted, to eliminate eventual interferences by the latter. The intact valve was fixed in a groove, and the shell that remained free was attached to a writing lever. The movements of the posterior half of the shell were recorded kymographically. The lever moved upward in the case of conttaction (Fig. 1). The time signal was presented every minute. A square wave impulse generator was used for stimulation. The parameters examined changed between 0 and 20 V, 0.2 and 120 msec, 0.5 and 200 impulses/sec, duration of stimulation from 1 and 64 sec. The stimulating electrodes were bent silver wires 250 μ in diameter, at a distance of 2 mm from each other, that stimulated both bundles of the CVC. To avoid exsiccation of the nerve, non-hygroscopic paraffin oil was layered over, the CVC was pulled into that. The electrodes were placed between

the anterior adductor and the retractor pedis muscles, about 5 mm from the latter. The studies began 15 to 20 minutes after having made the necessary preparations. The duration of an examination varied from 1 1/2 to 2 hours. At one time only one parameter was changed.

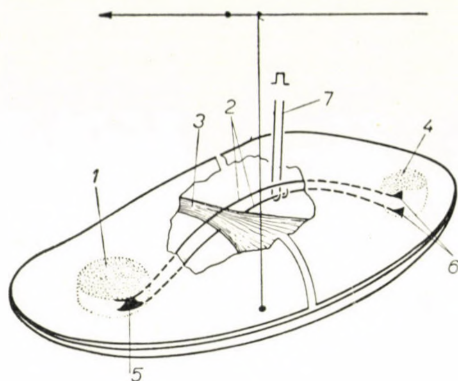

Fig. 1. 1. Posterior adductor muscle. *2.* Cerebrovisceral connectivum. *3.* Retractor pedis muscle. *4.* Adductor anterior muscle. *5.* Visceral ganglion. *6.* Cerebral ganglion. *7.* Stimulating electrodes

The electrophysiological studies were made on isolated CVC. It was prepared out in its entire length, the ganglia were removed, then the nerve was placed into the special electrode holder apparatus, under a layer of paraffin. Single pulses from the square wave generator were applied and the action potentials were led off unipolarly.

The studied properties of the muscle response were, *a)* size of contraction; *b)* time of onset, chronological course and size of relaxation; *c)* the relation of contraction and relaxation. These properties are shown diagrammatically in Fig. 2, where t_0 is the initial tone level, c the size of contraction in mm, T the time between onset of stimulation and the measurement of the size of relaxation (this was usually made at maximal relaxation, and r_T the size of relaxation in mm at T. The relative sizes of contraction and relaxation are indicated by the quotient c/r_T. Maximum relaxation is signified by the letter r.

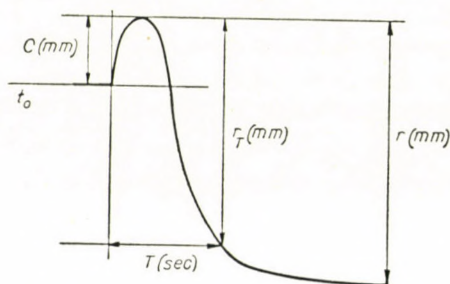

Fig. 2. Explanation in text

In the case of the action potentials their shape and size were analysed.

In every series the number of experiments was more than 15. The results obtained were in all cases entirely corresponding.

Results

After preparation, the posterior adductor muscle was in the state of permanent contraction in most cases. The muscle in that state could be induced to relax

by the repeated application of 10 to 20 V, 4 msec, 8/sec stimuli for 20 to 60 sec. Later, these values were considered to be the basic ones, when we were changing one or another of the parameters. The relaxed muscle could be induced to contract by a mechanical stimulation of the mantle. In order to be able to study the changes in contraction and relaxation, the muscle had to be brought into the condition of moderate relaxation, attained by the application of the relaxing stimulus, or by mechanically stimulating the mantle.

The muscle responses to the electrical stimulation of the CVC did not differ from one another, irrespective of the fact whether or not the CVC was connected with the cerebral ganglia.

The muscle responses to CVC stimulation differed in dependence on the parameters. The response was usually composed of contraction and relaxation. Depending on the parameters of stimulatión, the sizes of contraction and relaxation changed in both absolute and relative values.

When certain parameters were applied, only contraction could be observed, without subsequent relaxation.

A) *Dependence of muscle responses on the voltage of stimulation*

When the CVC was stimulated at different voltages, with the other parameters unchanged, the contractions differed in size. The results of such an experiment can be seen in Fig. 3. Voltage was changed from 1 to 12 V, with the other parameters 4 msec, 8/sec, and the duration of stimulation 60 sec. With increasing voltage the size of contractions increased at first, reaching a maximum at 12 V. A further increase of voltage did not increase the size of the contractions. However, the maximum contraction obtained did not mean that the muscle was absolutely contracted; greater contractions could be induced by the mechanical stimulation of the mantle (Fig. 3, m).

Analysis of the relaxations following contractions showed that at low voltage stimulation contraction dominated and this was followed by slight or no relaxation. On increasing the voltage, relaxation became more and more marked and its velocity, too, increased. In the case illustrated, relaxation reached the size of the contraction between 4 and 10 V, at a time the muscle returned to the pre-stimulation tone level. When voltage was further increased, the size of relaxation exceeded that of contraction and the muscle became set at a tone level lower than the initial. It is noteworthy that at 12 V contraction reached a maximum that could not be increased by increasing voltage, whereas the subsequent relaxations continued to increase, reaching the maximum at 20 V (Fig. 7/A).

When we examine that point of time at which relaxation begins, we find that it is not in correlation with the cessation of stimulation. If adequate

voltage and other parameters are used, relaxation begins even when stimulation is not stopped at 60 sec. However, on applying low voltage again, or stimulating the mantle mechanically, muscle tone is increased and the muscle remains in lasting contraction (Fig. 3, m).

Thus, there is a voltage under wich contraction is not followed by relaxation, provided the other parameters are adequate. If with unchanged other

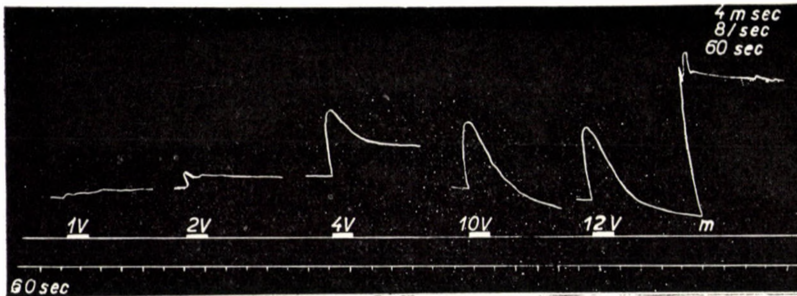

Fig. 3. Posterior adductor muscle response. *vs.* voltage. m: mechanical stimulation of posterior margin of the mantle
Constant parameters: 4 msec, 8/sec, 60 sec

parameters the voltage is increased, the tone-lowering effect of the stimulus comes to the fore. When voltage is increased, at frist no relaxation follows contraction, later relaxation also occurs, although weaker than the contraction, and, finally, when voltage is further increased, the size of relaxation exceeds that of contraction. The dependence of c/r on voltage is shown in Fig. 7/A, drawn from a typical experiment.

In our experiments the lowest voltage releasing a muscle response, varied from 0.5 to 2 V, depending on the other parameters. The voltage not yet followed by a decrease of tone also varied from case to case, averaging 1 to 3 V. The minimum voltage producing relaxation was 3 to 5 V. The absolute size of these limiting values was variable, but clearly defineable in every case.

As to the correlation between the course of contraction and voltage, it has been found that in the case of high voltages the time of onset of the peak fell near to the time of beginning stimulation. There was a definite correlation between the velocity of relaxation and the voltage. The higher the voltage, the steeper the relaxation, and, correspondingly, the shorter the time during which in the course of relaxation the muscle returns to the initial tone level.

Thus, with high voltage the conditions for the development of permanent tonic contraction are less favourable and the tonic contraction is short.

B) *Dependence of muscle responses on the frequency of stimulation*

The effect of different frequencies has been studied at the parameters of 20 V, 4 msec, 60 sec. The frequencies tested varied from 0.5 to 200 impulses/sec. We have found that like at different voltages, at different frequencies, too, contractions and relaxations of different magnitude could be produced. Such a series is shown in Fig. 4. The greatest contraction resulted from stim-

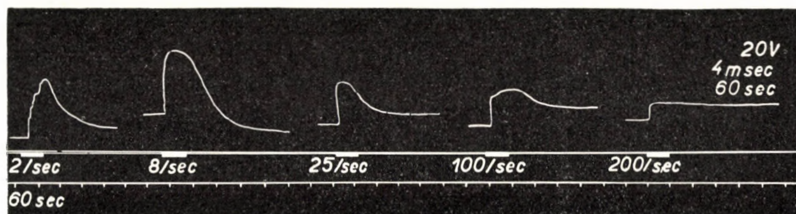

Fig. 4. Muscle response. *vs.* frequency
Constant parameters: 20 V, 4 msec, 60 sec

ulation at 8/sec. Frequencies higher or lower than that produced contractions of smaller size. The size of relaxation following contraction was also the greatest with a frequency of stimulation of 8/sec. Then the muscle relaxed below the initial tone level. With the increase of frequency the contractions of decreasing size were followed by relaxations of smaller and smaller size, and finally the latter disappeared almost completely. Thus, higher frequencies are less favourable for the decrease of tone following contraction, than for contraction itself. The relaxations following stimulation at 2/sec suggested that lower frequencies are again less favourable from the point of view of tone decrease. As it is visible in Fig. 7/B, the relation between relaxation and contraction showed an excessive value in the case of stimulation at 7/sec, in favour of relaxation. In that experiment the dependence of the c/r ratio on frequency was examined with the parameters of 10 V, 4 msec and 20 sec.

Thus, there exists an optimum frequency for both contraction and lowering of tone. Deviating from the optimum frequency is, however, more detrimental for relaxation than for contraction. For this reason under or over at certain frequency the c/r ratio increases to above 1 (Fig. 7/B).

C) *Dependence of muscle response on the duration of the impulses*

In this series the constant parameters were 20 V, 8/sec and 20 sec. The impulse lasted from 0.2 to 120 msec. The muscle responses obtained are shown in Fig. 5. The size of the contraction, the velocity and size of relaxation were correlated with the duration of the impulses. Notably, the increase of the

latter was followed by an increase in the size of contraction. When the duration of the impulses was increased to about 10 msec, relaxation became faster and faster, to reach a high value within the same length of time, and the interval between its onset and the onset of stimulation became shorter.

As to the behaviour of the c/r ratio, contraction was found to dominate with the impulses of short duration, *i.e.* c/r was higher than 1; at 3 and 4 msec

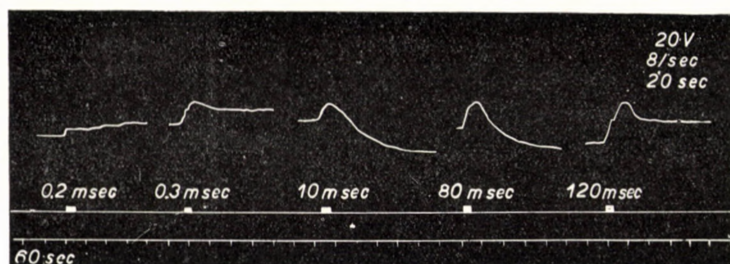

Fig. 5. Muscle response. *vs.* duration of impulses
Constant parameters: 20 V, 8/sec, 20 sec

c/r was 1, then it fell below 1; this was correlated with a significant increase of the relaxation. Over 5 msec the size of relaxation decreased again and over 100 msec the value of c/r was again higher than 1. (Fig 7/C)

Thus, there is a certain impulse width domain favourable from the point of view of the cessation of tonic contraction, *i.e.* for the dominance of relaxation, whereas impulses shorter or longer in duration produce tonic contraction.

D) *Dependence of the muscle response on the duration of stimulation*

The effect of changes in the duration of stimulation were examined at the parameters of 20 V, 4 msec and 8/sec. The duration of stimulation was 5, 10, and 60 sec.

The results shown in Fig. 6 indicate that while there was hardly any difference in the size of contractions, relaxation was significantly reduced with short stimulations, though the other parameters were favourable for relaxation. So with stimulation lasting 5 sec. the tone decreased to about the initial level, with 10 sec stimulation it fell below the initial level, and in response to 60 sec. stimulation excessive relaxation was observed. There were differences also in the velocity of relaxation. Relaxation was quicker in response to protracted stimulation. On short stimulation relaxation started immediately after stimulation has been stopped, and in the case of the 60 sec. stimulation it followed by 35 to 90 sec. the onset of stimulation. If stimulation had lasted

longer than 60 seconds, relaxation began at from 35 to 90 sec, *i.e.* not only after stimulation had been discontinued, but even during it. When stimulation had been discontinued, the speed of relaxation increased.

Fig. 7/D shows, on the basis of a typical experiment, the effect of the duration of stimulation on the c/r ratio. As it is visible, with the increase in the duration of stimulation the muscle response shifted more and more in the direction of relaxation.

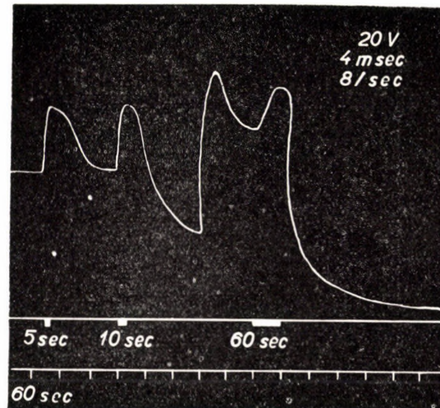

Fig. 6. Muscle response. *vs.* duration of stimulation
Constant parameters: 20 V, 4 msec, 8/sec

E) *Electrophysiological studies on CVC*

On stimulating the isolated CVC with single square impulses of different parameters, the action potentials changed depending on the parameters employed. Changes in voltage and duration of the impulse manifested themselves equally in the size and shape of the action potential. In these experiments we used the same voltage and msec ranges which had been found to cause the c/r value to fall below 1 in experiments *in situ*. In this sense we examined the action potentials produced by 1 to 10 V and 1 to 4 msec. The characteristic results are illustrated in Fig. 8, showing the action potentials in response to stimulation with single pulses of 3 msec duration, at 2, 4 and 10 V. The recording electrode lay 20 mm from the stimulating electrode. With 2 V stimulation the action potential was just one single wave, in the case of a 4 V stimulus another wave also appeared, and in response to 10 V the latter became marked and partly merged with the first wave. The size of the action potential increased with the increase of voltage and finally reached about 3 mV. It is also visible that the first wave of the action potential did not remain uniform when voltage was increased, but was broken up into at least 3 components.

The shape of the action potential suggested that the fibre composition of the CVC is not homogeneous, but falls into at least two groups from the point of view of conduction velocity. The two fibre systems can clearly be distinguished from the point of view of the stimulation threshold. In general, it was between 2 and 4 V that the second component of the action potential appeared in our experiments. If we increased the msec value, voltage had to

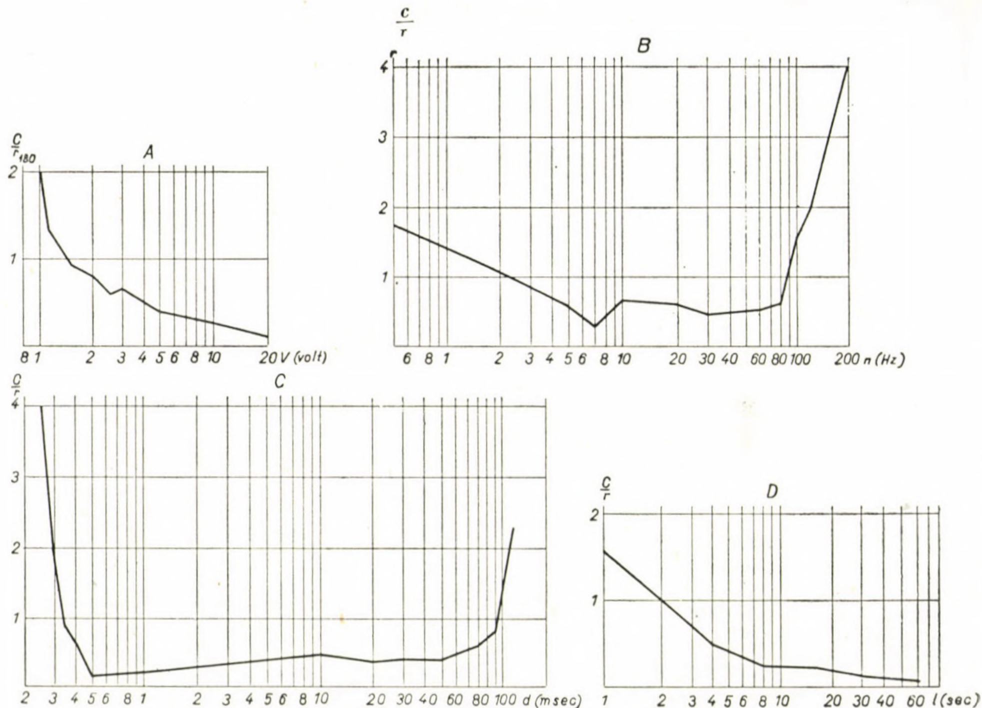

Fig. 7. Dependence of the contraction/relaxation ratio on the parameters of stimulation *A)* Voltage *vs* c/r_T. Constant parameters: 4 msec, 8 Hz, 20 sec. $T = 180$ sec. *B)* Frequency *vs.* c/r. Other parameters: 10 V, 4 msec, 20 sec. *C)* Width of impulse *vs.* c/r. Other parameters: 20 V, 8 Hz, 20 sec. *D)* Duration of stimulation *vs.* c/r. Other parameters: 20 V, 4 msec, 8 Hz

be lowered so that only the first component of the action potential should appear and, reversed, the decrease in msec value of the stimulus had to be compensated for by an increase of voltage, if we wanted the second component to appear.

Discussion

The results obtained by stimulating the CVC at different parameters indicate that the quality of the muscle response depends decisively on the quality of stimulation. Low voltage causes merely contraction, higher voltages produce relaxation following the contraction. Studies of the other parameters

have revealed that certain frequencies, duration of impulses and time of stimulation are followed by contraction alone, while others are followed by both contraction and relaxation. By changing the parameters the measure of relaxation, too, could be influenced: in certain cases the relaxation following contraction reached merely the initial tone level, in others it fell considerably below it.

The fact that a certain increase of both voltage and duration of impulses brought about an increase in the size of contraction, may be explained by an increase in the number of fibres excited. And the fact the relaxation results only beyond certain parameter values induces one to think that the decrease of tone is

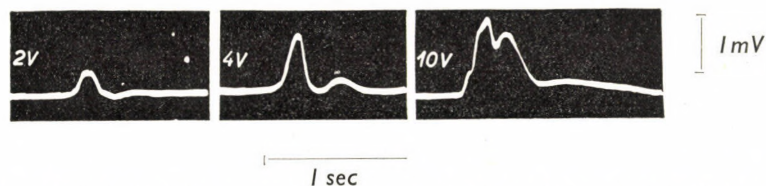

Fig. 8. Action potential of the CVC in response to stimulation with single pulses, at 2, 4 and 10 V The impulse lasted 3 msec in every case

caused by the stimulation of some fibres of higher threshold. The increase in the size of relaxation on increasing voltage and the duration of the impulse can be explained likewise by an increase in the number of excited tone-decreasing fibres. The fact that short stimulation with protracted high voltage and high duration stimuli produces merely contraction, without relaxation, suggests that on stimulating the fibres inducing a decrease of tone some relaxant substance is liberated, the release of which in effective amounts requires time. This contention is supported by the observation that under the effect of stimulation with suitable parameters relaxation begins not on stopping the stimulation, but already during it, and may reach a maximum in the course of stimulation, although the muscle is not exhausted since it can be caused to contract by other methods.

The view concerning the existence of fibres different in physiological function and threshold is supported by our electrophysiological studies. The action potentials recorded in response to stimulation with single pulses suggested the presence of two main fibre groups. The results obtained after the parameters have been changed indicate that there undoubtedly exists a threshold of stimulation below which only the first group of fibres is excited, and that threshold is practically the same below which we obtained solely contraction in the experiments *in situ*. At parameters which produced both contraction and relaxation in the experiments *in situ*, the second wave of the action potential of the CVC became marked. This suggests a correlation between the process of relaxation and the second wave of the action potential. For this reason we think that it is the excitation of the second fibre group giving the second wave of the action potential that gives rise to relaxation.

Consequently, relaxation is not a passive phenomenon, but a result of an active nervous process, presumably causing the release of a relaxant substance and leading thereby to the relaxation of muscle in tonic contraction.

The fact that a lowering of frequency below a certain limit is favourable for tonic contraction may be explained by assuming that the number of pulses per unit of time does not suffice to cause the release of the above mentioned relaxant in effective concentrations. The induction of tonic contraction by a significant increase in the impulse duration may be explained by assuming that as a result of the impulse duration the single pulses coming one after the other fall into the refractory period and cannot sufficiently excite the relaxant fibres of higher threshold.

Thus, we interpret our results as supporting the Pavlovian view of tone regulation and we ascribe the differences in response to a double innervation, and not to an excessive lability of the muscle fibres. The double innervation, as we imagine it, differs from that described by PUMPHREY [7] as occurring in *Mya arenaria* where one system is giving fast potentials, ensuring phasic activity, the other one shows long discharge-duration, serving to produce tone. HOYLE and LOWY [2] interpreted in a similar manner the double innervation of the retractor pedis muscle of *Mytilus edulis* byssus. Our interpretation of the concept of double innervation is similar to that put forward by WINTON [8] and FLETCHER [1] who described the tone-increasing effect of direct current and the tonic contraction-abolishing effect of alternating current in the retractor pedis muscle of the clam. Adding to this the double innervation described by PUMPHREY [7], as well as HOYLE and LOWY [2], we may speak essentially about a triple innervation. In other words, there would be separate innervations for *a)* inducing phasic contraction, *b)* for inducing tonic contraction; and *c)* for inducing relaxation abolishing the tonic state.

In our studies we, too, observed the phenomenon described by ZHIRMUNSKAYA [9] that the muscles of different specimens respond differently to the same parameters. This we think may be explained, among others, by the decrement in the conduction of the CVC [3]. Depending namely on the size of the animals and other conditions the stimulating electrodes are at different distances from the muscle, which because of the decrement conduction decisively influence the size of the impulse reaching the muscle. This explains why even the same animal may give different responses to stimulation with the same parameters, if the distance between the muscle and the electrodes is changed. When the electrodes are farther away from the muscle, the voltage of stimulation must be increased. This is supported also by the fact that we get identical responses to stimulation applied at the same parameters to the same site, under otherwise identical conditions.

In the course of our studies of the dependence of the muscle response on the parameters of stimulation we have found that there are some further fac-

tors that influence the size, velocity and relation to one another of tone increase and tone decrease. Such factors have proved to be the tone at the onset of stimulation, the direction of the change in tone, and its size at the moment stimulation is begun, previous stimuli and the interval between them and the onset of stimulation, as well as the actual state of the animal's motor activity. It is most difficult to keep all these factors constant and we could not achieve it perfectly in our experiments. However, the dependence of the properties of the muscle response on the parameters of stimulation is most conscipucious and unequivocal, and the electrophysiological evidence obtained is also in close agreement with the conclusions drawn from it. Further investigations are in progress to elucidate the mode by which relaxation develops and to obtain information as to the quality of the supposed relaxant factor playing a role in that process.

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János SALÁNKI, Elemér LÁBOS
MTA Biológiai Kutatóintézete, Tihany

EFFECT OF HYPOTHALAMIC AND BRAIN STEM STRUCTURE STIMULATION ON PITUITARY-ADRENOCORTICAL FUNCTION

By

E. ENDRŐCZI and K. LISSÁK

INSTITUTE OF PHYSIOLOGY, MEDICAL UNIVERSITY, PÉCS

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In cats with chronic implanted deep electrodes different structures of the diencephalon and mesencephalon have been stimulated and the corticosteroid content of adrenal venous blood has been studied. It has been found that stimulation of the basal septum, antero-lateral hypothalamus and lateral hypothalamus inhibits adrenocortical secretion. No significant change resulted from the stimulation of the supraoptic and paraventricular nuclei. Secretion increased markedly in response to the stimulation of the posterior tuber, premamillary region, mesencephalic reticular formation, and ventral tegmentum. Stimulation of the dorsal tegmentum at the level of the superior colliculus caused inhibition. The structural connexions involved in the central nervous control of pituitary-adrenocortical function are discussed.

In recent years numerous data have been published indicating that the diencephalon, mesencephalon and rhinencephalon play a role in the control of pituitary-adrenocortical function. In electrocoagulation and ablation experiments it has been demonstrated that the absence of certain structures significantly increases ACTH secretion, while stimulation with chronic implanted deep electrodes supplied evidence indicating that certain central nervous structures may not only activate, but also inhibit the ACTH-adrenocortical activity [1, 2, 3, 4, 5, 6]. It is not yet known through which structures the central nervous system activates or inhibits pituitary-adrenocortical function. In the present work we have undertaken to analyze the role played by different diencephalic and mesencephalic structures in the regulation of pituitary-adrenocortical activity, by means of stimulation with chronic implanted deep electrodes.

Methods

A total of 56 cats of either sex, weighing 1.6 to 2.3 kg, were used. The chronic deep electrodes were implanted under pentobarbital anaesthesia (30 mg per Kg body weight, intraperitoneally). Bipolar enamel insulated nickel-chromium electrodes, 0.2 mm in diameter were implanted [7]. The electrodes were held in place with slowly polymerizing acrylate (Protenit, *Magyar Kémiai Művek*, Budapest), the outgoing electrodes were soldered to a micro-valve socket. A square pulse generator with adjustable intensity, duration and frequency of stimulation, was used. Stimulation was begun 2 weeks after the electrodes had been implanted, to allow time for the wounds to heal completely. The controls were animals with implanted electrodes; they were not stimulated.

Pituitary-adrenocortical function was estimated by determining the corticosteroid content of adrenal venous blood. At 45 minutes following stimulation the animals were anaesthetized with intraperitoneally administered pentobarbital, a glass-tipped polyvinyl cannula

was inserted into the left lumboadrenal vein, and after giving 400 units/kg body weight of heparin intravenously, adrenal venous blood was collected for 60 minutes, then the adrenals were removed and weighed with mg accuracy on torsion scales. The brain was then perfused through the carotid with 40 per cent formalin (40 to 50 ml), then removed for histological examination. The localization of the electrodes was determined in serial frozen sections.

The methods of extracting corticosteroids from adrenal venous blood, separation by paper chromatography, and photometric determination with tetrazolium blue have been described previously [8].

Results

Effect of septum, preoptic region and antero-lateral hypothalamus stimulation

There were 14 experimental animals and 4 controls in this group. Stimulation lasted 15 minutes in every case, followed 45 minutes later by the collection of adrenal venous blood. The parameters of stimulation were 0.75

Table I

Effect of stimulation of septum, preoptic region, and antero-lateral hypothalamus

	Corticosteroids, μg/kg/hour			Site of electrodes
	Cpd F	B	Total	
Control group				
1	27.0	21.0	48.0	Basal septum
2	24.5	17.0	41.5	Basal septum
3	24.0	18.5	42.5	Preoptic region
4	22.0	22.0	44.0	Preoptic region
			44.0 ± 3.1	
Experimental group				
1	12.0	12.5	24.5	Basal septum
2	9.5	14.0	23.5	Basal septum
3	14.0	11.0	25.0	Basal septum
4	11.5	9.0	20.5	Basal septum
5	15.5	12.0	27.5	Preoptic region
6	8.5	12,0	20.5	Preoptic region
7	10.0	8.5	18.5	Lateral hypothalamus
8	18.0	11.0	29.0	Lateral hypothalamus
9	14.5	12.0	26.5	Lateral hypothalamus
10	15.0	12.5	27.5	Lateral hypothalamus
11	13.0	13.0	26.0	Lateral hypothalamus
			24.5 ± 3.5	
12	26.0	23.0	49.0	Descending fornices
13	32.0	27.0	59.0	Descending fornices
14	29.0	21.0	50.0	Descending fornices
			52.0 ± 5.5	

to 1.5 V, 0.5 msec and 30 c/sec. During stimulation the animals showed orientation reaction and increased attention to environmental effects. No emotional reaction (rage, escape, its vegetative signs), or incoordinated motor activity could be observed.

Pituitary-adrenocortical activity was diminished by the stimulation of the septum, preoptic region and antero-lateral hypothalamus alike. The

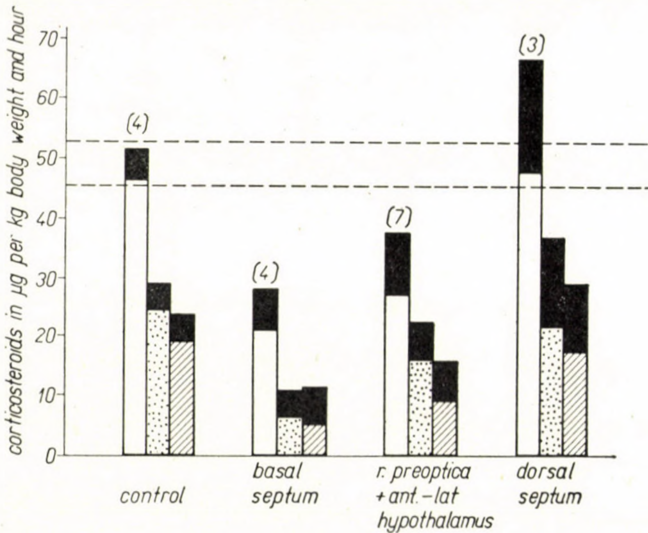

Fig. 1. Stimulation of septum and anterior hypothalamus and corticosteroid secretion. The limit between the broken lines shows the normal range. White column: total corticosteroid content. Dotted column: hydrocortisone secretion. Shaded column: corticosterone secretion. The figures above the columns show the number of cases, the black layer shows the range

decrease was most marked following stimulation of the basal septum and lateral preoptic region. Stimulation of the area of the dorsal septum, thus that of the descending fornix, caused a small decrease of corticosteroid secretion, and in 2 cases a definite increase was observed. In these experiments the anterior commissure was not stimulated by itself, but stimulation with the electrodes in the immediate vicinity of that structure caused a marked decrease similar to that observable in response to stimulation of the basal septum, (Table I, Fig. 1).

Effect of medial hypothalamic stimulation

This group contained 8 experimental and 5 control animals. Anatomically, the electrodes were placed from the plane of the optic chiasma to that of the premamillary region. The parameters of stimulation were 0.75 to 2.0 V, 0.5 msec

and 30 c/sec. No significant change in corticosteroid secretion could be observed following stimulation for 15 minutes of the supraoptic nucleus, the ventral paraventricular nucleus or the ventromedial nucleus. A marked activation followed the stimulation of the posterior tuber cinereum, medial eminence and premammillary region. Stimulation of the lateral hypothalamus was

Table II

Effect of stimulation of medial part of hypothalamus

	Corticosteroids, μg/kg/hour			Site of electrodes
	Cpd F	B	Total	
Control group				
1	28.0	22.0	50.0	Supraoptic nucleus
2	24.5	21.0	45.5	Supraoptic nucleus
3	23.0	22.0	45.0	Paraventricular nucleus
4	23.0	23.0	46.0	Paraventricular nucleus
5	21.0	23.0	44.0	Posterior tuber
			46.0 ± 2.4	
Experimental group				
1	26.5	23.0	49.5	Supraoptic nucleus
2	24.0	22.0	46.0	Supraoptic nucleus
3	31.0	26.0	56.0	Paraventricular nucleus
4	25.0	22.0	47.0	Paraventricular nucleus
			49.5 ± 4.6	
5	47.0	36.0	83.0	Posterior tuber
6	54.0	44.0	98.0	Median eminence
7	22.0	24.0	46.0	Ventromedial nucleus
8	19.0	14.0	33.0	Lateral hypothalamus

followed by a moderate decrease in corticosteroid secretion. The animals' behaviour varied during stimulation. In most cases the pattern was characterized by emotional reactions, such as rage, vocalization and escape. Salivation and urination were often observed. It was in this group that incoordinated motor activity characterized by contralateral head-bending or walking occurred most often. Salivation and urination also occurred. These changes in behaviour normalized within a few seconds after stimulation had ceased. In an animal with the electrode in the paraventricular nucleus, epileptic seizure developed during stimulation. This animal showed a somewhat higher corticosteroid level than the control value (Table II, Fig. 2).

Effect of stimulation of the posterior hypothalamus and mesencephalic reticular formation

This group contained 23 experimental animals and 4 controls. The conditions of stimulation were the same as in the former groups. Adrenocortical activity was unequivocally increased by the stimulation of the posterior hypothalamus and mammillary body; a similar response was observed on stimulation

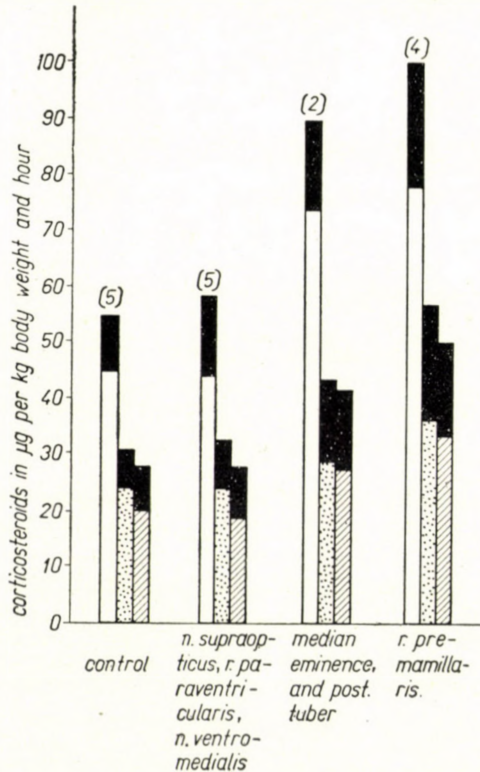

Fig. 2. Adrenocortical hormone production under stimulation of supraoptic nucleus, paraventricular nucleus, tuber and premammillary region. Signs as in Fig. 1

of the tegmento-mammillary tracts. Stimulation of the lateral posterior hypothalamus produced no substantial change in ACTH secretion. Corticosteroid secretion definitely increased in response to the stimulation of the mesencephalic reticular formation, at the level of the superior colliculus, as well as rostrally and ventrally from that plane. Stimulation of the dorsal tegmentum resulted in a decrease of adrenocortical secretion. Whereas the ACTH secretion was most increased on stimulation of the ventral tegmentum, stimulation with the electrodes in the pretectal area inhibited corticosteroid secretion. The change

Table III

Effect of stimulation of posterior hypothalamus, mesencephalic reticular formation

	Corticosteroids, μg/kg/hour			Site of electrodes
	Cpd F	B	Total	
Control group				
1	28.0	28.0	56.0	Mammillary body
2	34.0	24.0	58.0	Subthalamic region
3	27.0	29.0	56.0	Reticular formation
4	25.0	23.0	48.0	Reticular formation
			54.0 ± 3.6	
Experimental group				
5	43.0	36.0	79.0	Subthalamic region
6	52.0	36.0	88.0	Subthalamic region
7	54.0	36.0	92.0	Premammillary region
8	58.0	38.0	96.0	Mammillary body
9	62.0	33.0	95.0	Interpeduncular tract
10	62.0	43.0	105.0	Tegmentomammillary tract
11	54.0	42.0	96.0	Reticular formation
12	47.0	40.0	87.0	Reticular formation
13	58.0	34.0	92.0	Reticular formation
14	67.0	20.0	87.0	Reticular formation
15	57.0	23.0	80.0	Reticular formation
16	48.0	42.0	90.0	Central gray matter
17	52.0	26.0	78.0	Central gray matter
			90.0 ± 8.0	
18	54.0	32.0	86.0	Ventral tegmentum*
19	56.0	28.0	84.0	Ventral tegmentum
20	44.0	36.0	80.0	Ventral tegmentum
21	65.0	26.0	91.0	Ventral tegmentum
			85.0 ± 4.6	
22	26.0	11.0	37.0	Pretectal area
23	23.0	15.0	38.0	Pretectal area
24	19.0	17.0	36.0	Dorsal tegmentum
25	25.0	10.0	35.0	Dorsal tegmentum
26	17.0	14.0	31.0	Dorsal tegmentum
27	24.0	11.0	33.0	Dorsal tegmentum
			35.0 ± 2.6	

* At the level of the superior colliculus.

in behaviour evoked by the stimulation of the posterior hypothalamus was characterized by an escape reaction, accompanied in some cases by vegetative signs, such as increased respiration, dilatation of the pupils. Stimulation of the tegmentum with the specified parameters gave rise to an increased orientation reaction, accompanied by vocalization in single cases (Table III, Fig. 3, Fig. 4).

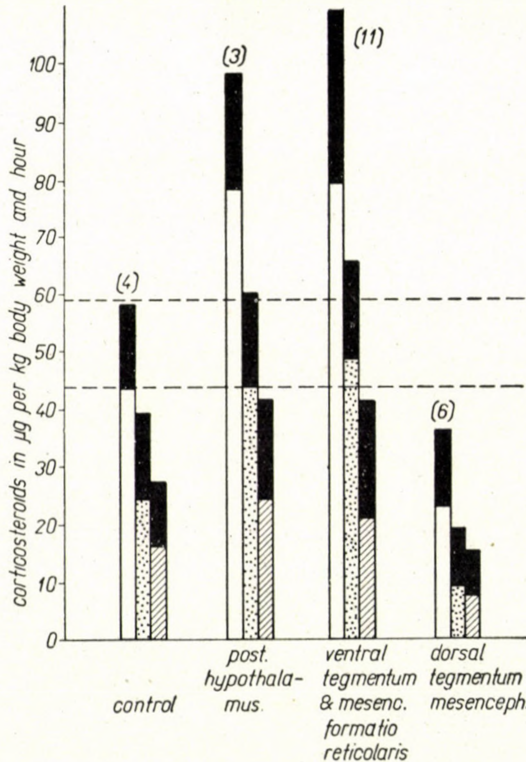

Fig. 3. Adrenocortical hormone production under stimulation of posterior hypothalamus, mesencephalic reticular formation and dorsal tegmentum. Signs as in Fig. 1 and Fig. 2

Discussion

The role of forebrain structures in the inhibition of pituitary-adrenocortical activity has been observed previously [1, 2, 4, 6]. In earlier investigations we found that stimulation of the archicortex inhibited the increase of ACTH secretion in response to stress, without knowing anything definite about the efferent mechanism of inhibition [3]. The present data indicate that the septum, antero-lateral hypothalamus and the dorsal tegmentum form a homogeneous structural mechanism in the inhibition of pituitary-adrenocortical

activity. Neurophysiological and morphological data suggest that most of the connexions between the rhinencephalon and brain stem go through the septum, and that some of the principal afferent and efferent pathways run across the hypothalamus. Such a connexion is formed first of all by the medial forebrain bundle which, as we have reported recently, plays an inhibitory role in the

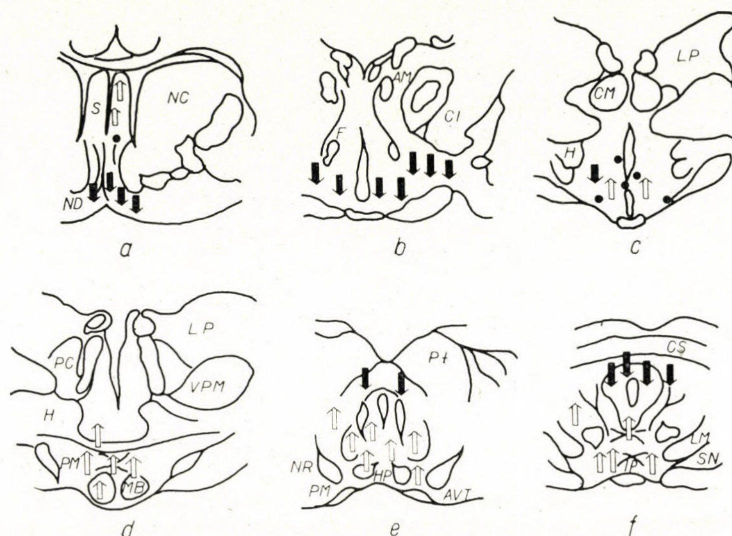

Fig. 4. Direction of corticosteroid secretion in response to stimulation of the diencephalon and mesencephalon. Location of the electrodes. The white arrow pointing upward indicates an increase, the downward pointing black one a decrease of corticosteroid production. Black dot: no change in corticosteroid production

a : section at the level of the septum; *b* : at the optic chiasma, *c* : at medial eminence, *d* : at mammillary body, *e* : at interpeduncular tract, *f* : at superior collicle

control of pituitary-adrenocortical activity, as well as in the organization of behavioural processes and in the formation of "instinctive"-"motivation" activities.

The present investigations did not elucidate the origin of the inhibitory structures of the septum and lateral hypothalamus. Considering that inhibition was most intensive if evoked from the basal septum and anterolateral hypothalamus, while stimulation of the fornix fibres running under the splenium corporis callosi elicited a slight response, and even an increase of the corticostreoid level could be observed, one might conclude that in this phenomenon certain forebrain structures independent of the arhicortex may play an inhibitory role. The medial forebrain bundle arises from the medial groups of nuclei of the subcallosal gyrus, but many fibres join it in the preoptic and basal septal areas from the terminal stria, fornix, then, more caudally, from the tegmento-subthalamic and periventricular pathways. The medial forebrain

bundle establishes a mutual connexion between basal septum and tegmentum, with some fibres forming junctions in the multipolar cells of the posterior hypothalamic nucleus. Other fibres terminate in the nuclei of the tegmentum and create connexions with the forebrain structures caudally, to the plane of the pons [9, 10, 11, 12].

Stimulation of the medial hypothalamus gave rise to extreme behavioural changes, but the pituitary-adrenocortical system showed activation exclusively in response to the stimulation of the tuberal and premammillary regions. The increased ACTH secretion observed following the stimulation of the tuber cinereum, medial eminence and premammillary area confirmed our earlier observations in this respect [3, 22]. Stimulation of the supraoptic nucleus had no effect on secretion, indicating that direct stimulation of the neurosecretory mechanism does not result in such a neurohormonal change in consequence of which ACTH secretion would increase. This is at variance with the hypothesis put forward in the literature that the neurosecretory system might play a role in the secretion of some specific ACTH-relasing factor, though in the appraisal of the problem an electrical non-stimulability of the cells of such type may be suggested [13, 14, 15, 16]. The negative evidence obtained in connexion with the stimulation of the neurosecretory system is in agreement with similar data obtained earlier by us and others [3, 6, 17, 18].

The emotional changes observed in response to stimulation of the medial hypothalamus were not accompanied by significant changes in pituitary-adrenocortical activity. Although in recent years evidence has been accumulating to suggest that emotional behaviour and stress might be organized through the same central nervous structures, the present data tend to indicate that there may be a discreet central nervous control of pituitary-adrenocortical activity. Stimulation of the medial areas of the hypothalamus is known to cause endogenous adrenalin and noradrenalin hypersecretion which in connexion with the present observations indicates that endogenous adrenalin secretion plays no significant role in the activation of pituitary-adrenocortical function [19, 20]. The results of experiments with medial hypothalamic stimulation have shown that the tuber cinereum and the premammillary area contain numerous elements playing a role in ACTH secretion. At the same time, before saying that other structures may be inactive from this point of view, it is to be suggested that when the paraventricular, ventral-or dorsomedial groups of nuclei are being stimulated, structures both activating and inhibiting ACTH secretion are excited. Stimulation of such a nature may, of course, have no endocrinological effect; this is supported by the fact that the activating pathways running in the medial and caudal hypothalamic area from the forebrain to the tegmentum, and those coming from the tegmentum to the hypothalamus, are interwoven and cannot be distinguished by electrical stimulation.

Stimulation of the posterior hypothalamus, corpus mammillare and the tegmento-mammillary tract causes a significant increase in the corticosteroid level of adrenal venous blood. Recent investigations have suggested an eventual role of the tegmento-mammillary tract in the control of adenohypophyseal hormone production and secretion; destruction of these fibres was observed to inhibit ovulation and reflex hormone secretion by the testis [21, 22]. The present observations indicate that the structure plays an important part in the control of ACTH secretion.

Stimulation of the mesencephalic rostral reticular formation, ventral tegmentum and habenulo-peduncular tract increases adrenocortical secretion. In response to stimulation of the pretectal area and dorsal tegmentum, ACTH secretion decreased. It was reported earlier that the mesencephalic reticular formation had a role in the activation of the pituitary-adrenocortical system, whereas experimental lesions indicated that not only activating, but also inhibitory representation could be found in the mesencephalic area [3]. The effect of the inhibitory zone running in the area of the dorsal tegmentum is less marked than what could be observed on stimulating forebrain and archicortical structures [3]. The question emerges whether these structures are involved in bringing about of the inhibitory effect evoked from the tegmentum. Degeneration experiments have shown that the projection coming from the dorsal tegmental nucleus gives off many fibres to the lateral hypothalamic zone and the anterolateral preoptic region, but supplies no direct connexions to the medial and caudal hypothalamic nuclei [10]. These morphological data suggest that inhibition elicited from the area of the tegmentum is the result of a multineuronal, eventually complex feed-back mechanism, involving an activation of forebrain structures.

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Elemér Endrőczi, Kálmán Lissák
Orvostudományi Egyetem Élettani Intézete, Pécs

CORRELATIONS BETWEEN AVOIDING CONDITIONED REFLEX ACTIVITY AND PITUITARY-ADRENOCORTICAL FUNCTION IN THE RAT

By

B. BOHUS, E. ENDRŐCZI and K. LISSÁK

INSTITUTE OF PHYSIOLOGY, MEDICAL UNIVERSITY, PÉCS

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It has been shown that the performance of the conditioned avoiding reflex in individual rats is more intensive in the animals showing higher resting corticosterone secretion, than in the rats with low corticosterone secretion.

There is ample evidence in the literature of the role of humoral factors in the control of higher nervous activity. The pituitary-adrenocortical system, as one of the central regulator of the processes of general adaptation, has a particularly important part to play. The experimental data published by LIDDEL and ANDERSON [1, 2], and particularly those reported by WOODBURY [3] have furnished proof of the influence of corticosteroids on higher nervous activity, first of all the polar derivatives. These data have been amply confirmed by observations made in human pathology [4, 5].

At the same time, our own observations made in studies concerning the alimentary conditioned reflex, as well as the spontaneous goal-directed behaviour have likewise disclosed several correlations between higher nervous function and pituitary-adrenocortical function [6, 7, 8].

The present paper deals with the correlations found in the rat in studies concerned with avoiding conditioned reflex activity and adreno- cortical secretion.

Methods

Albino rats of either sex, weighing 120 to 180 g, were used. The animals were fed on a standard diet and were allowed water *ad libitum.*

Method of studying the avoiding conditioned reflex

The avoiding conditioned reflex was elaborated in the box shown in Fig. 1. The conditioned stimulus was the ringing of a bell, the unconditioned one a 50 V electric shock, delivered by metal screen serving as the floor of the conditioning apparatus. The animal can escape from shock by jumping onto an about 20 cm high bench.

The reflex was elaborated as follows. The duration of the auditory stimulus was 10 sec; the electric shock was delivered during the last two seconds of the auditory stimulation, in the form ot two stimuli 0.5 sec in duration each. The interval between the single associations was 1 minute. Ten such trainings were carried out daily with every animal. The conditioned reflex performance, the intersignal reactions were recorded kymographically, and the duration of the atency period was measured.

Every animal was subjected to 150 associations, and throughout the experiment the reflex was reinforced by the unconditioned stimulus, if the animal failed to escape to the bench spontaneously on presenting the conditioned stimulus.

Method of studying pituitary-adrenocortical function

After the conditioned reflex studies had been completed, the animals were anaesthetized with 5 mg/100 g body weight of intraperitoneally injected pentobarbital, given heparin intra-

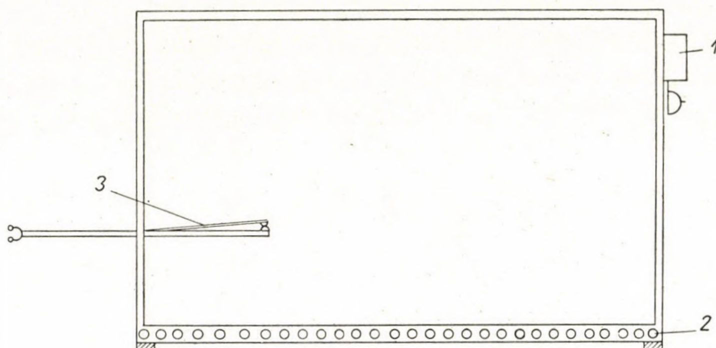

Fig. 1. Schematic representation of the box used for establishing avoiding conditioned reflex *1.* Bell for auditory stimulation. *2.* Floor of wire mesh, from which the animal is shocked. *3.* Bench for escaping

venously, and the blood flowing from the left adrenal was collected for 60 minutes by means of a glass cannula.

Corticosteroids were extracted with ether: ethylacetate (4 : 1), partitioned by paper chromatography and assayed by photometric analysis of the tetrazolium-positive areas by methods described previously [9, 10].

Results

Positive avoiding conditioned reflex was established after 5 to 15 trainings. Meanwhile, it could be observed that parallel with the advance of training the spontaneous intersignal activity, as well as the orienting and washing automatisms diminished.

After the reflex had been established, during subsequent training the avoiding conditioned reflex performance, *i.e.* the number of positive responses to the conditioned sound stimulus, varied from rat to rat. Evaluation of conditioned reflex activity, *i.e.* determining of the percentage of the total number of trainings when the animal performed the reflex without reinforcement, yielded from 62 to 97 per cent. At the same time in the course of training the animals always escaped to the bench in response to reinforcement after the reflex had been established, *i.e.* no absolutely negative response occurred.

An analysis of the duration of latency showed that every animal began the avoiding motor reaction in less than 1 sec after the presentation of the sound stimulus, while the reflex was performed completely in from 1 to 4

seconds, depending on the motor activity of the animal and on the distance from the bench.

According to the results of adrenal venous blood analysis made after completion of the conditioned reflex studies, the resting values of corticosterone secretion ranged from 8 to 30 μg/100 g body weight/hour, averaging 16 μg/g body weight/hour.

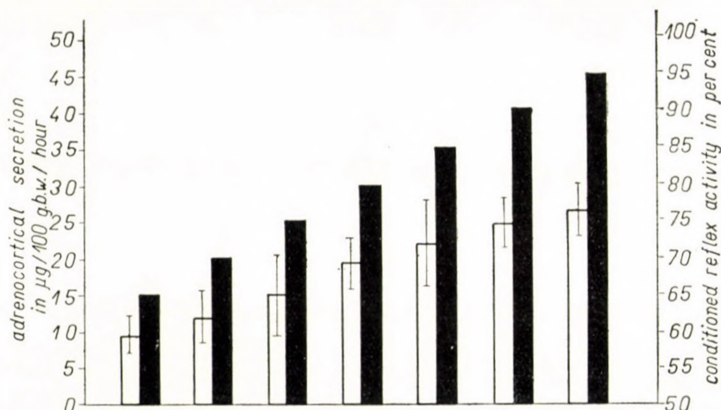

Fig. 2. Correlation between avoiding conditioned reflex activity and adrenocortical secretion White columns: Adrenocortical secretion. Black columns: Avoiding conditioned reflex activity

Individual evaluation of the results of the conditioned reflex studies and those of the pituitary-adrenocortical function tests showed a correlation to exist between reflex activity and corticosterone secretion. In the animals showing lower reflex performance, *i.e.* those needing reinforcement with the unconditioned stimulus in the major part of the training, the resting secretion, too, was low, whereas the animals displaying a high reflex activity had high secretion values. These correlations are illustrated in Fig. 2, showing the combined secretory values of the animals with reflex activity differences within 5 per cent.

Analysis of adrenal weight has shown that no significant correlation exists between the adrenal weights found in the individual animals and their reflex activity.

Discussion

Emotional factors are known to be capable of activating the adrenal cortex, as it can be demonstrated by the increase of 17—OHCS in peripheral blood or of the urinary excretion of corticosteroids [11, 12, 13, 14]. MASON [15] found in the monkey an increased 17—OHCS content of peripheral blood

during conditioned anxiety or defensive behaviour. Likewise, the plasma 17—OCHS level was shown to be increased in the dog during the conditioned reflex to painful stimulation [16].

In the present study we have shown that after the defensive conditioned reflex experiments the average resting corticosterone secretion value was 16 μg/100 g body weight/hour (from 8 to 30 μg/g body weight/hour). These values were not different from those found in normal animals. At individual evaluation, however, the animals showing corticosteroid secretion values in the higher part of the range had a high reflex activity, whereas the animals showing lower reflex activity had lower secretion values. This might suggest that in the animals showing a high secretion emotional motivation was increased, and this would go together with a high avoiding conditioned reflex activity. The observation that the animals showing increased emotional reaction require less reinforcement for the establishment of the conditioned reflex, seems to confirm this hypothesis [17].

At the same time, our own investigations and the data published in the literature are indicative of the pituitary-adrenocortical system having a role in the control of higher nervous function. WOODBURY observed that the polar corticosteroids, thus hydrocortisone and cortisone, increase the excitability of the central nervous system, whereas corticosterone is ineffective, or even decreases the effect of polar derivatives [3]. APPLEZWEIG and BAUDRY reported that the establishment of the avoiding reflex is inhibited in hypophysectomized animals [18]. We have shown in the dog that the ratio of hydrocortisone: corticosterone is higher in the animals with more intensive internal inhibition; in the animals with weaker inhibition hydrocortisone was found to increase the duration of internal inhibition [6]. At the same time, hydrocortisone increases aggressivity during lactation in female rats [7]. The above investigations, as well as alimentary reflex studies in the cat indicate that the activation of the pituitary-adrenocortical system influences higher nervous function through the polar corticosteroids, first of all by promoting the processes of internal inhibition [8].

The establishment, or the stability after learning, of the conditioned reflex processes is a significant function of discriminatory internal inhibition. The differences in reflex activity found in our experiments may be ascribed in part to differences in the processes of discriminatory internal inhibition. According to this, high secretion values will be found in the animals with more intensive internal inhibition, as it has been shown in our earlier investigations. However, attention is to be called to the fact that the earlier statements had related to higher animal species, in which hydrocortisone secretion was held responsible for the above phenomena. In contrast to this, the adrenal cortex of the rat produces exclusively corticosterone. In the lack of experimental data we can merely suggest that in this species corticosterone would have a role

different from that in higher species, or that the more polar derivatives excreted in small quantities and aldosterone, secreted in large amounts during anxiety in higher species [19, 20], may also play a role in the genesis of internal inhibitory processes.

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Béla Bohus, Elemér Endrőczi, Kálmán Lissák

Orvostudományi Egyetem Élettani Intézete, Pécs

FURTHER DATA CONCERNING THE SEX DIFFERENCES OF THE PITUITARY-ADRENAL SYSTEM IN THE RAT

By

B. Bohus, E. Endrőczi and K. Lissák

INSTITUTE OF PHYSIOLOGY, MEDICAL UNIVERSITY, PÉCS

(Received February 14, 1963)

In rats aged 27 to 29 days and 37 to 39 days there is no difference in the measure of compensatory adrenocortical hypertrophy between the male and the female animals, whereas in rats aged 52 to 56 days the measure of compensatory hypertrophy is much greater in the female.

Corticosterone production *in vitro* by the hypertrophic adrenals increases with the advance of age; at the age of 52 to 56 days females produce more corticosterone than males.

In response to prepubertal castration the compensatory adrenal hypertrophy following contralateral adrenalectomy on the 100th postnatal day significantly decreased in 107 days old female rats, whereas it was unchanged in male rats, as compared with sham-operated controls.

Oestrone treatment given prior to puberty increases the measure of compensatory hypertrophy in the adult male rat, while it leaves hypertrophy unchanged in the adult female. Prepubertal administration of testosterone produces no effect in either sex.

In response to oestrogen administration before sexual maturation, corticosterone secretion by the compensatory hypertrophied left adrenal increases in adult males and females alike, whereas it decreases in adult animals ovariectomized between the 22nd and 25th day of their lives.

The results indicate that the marked compensatory hypertrophy observed in the adult female rat develops in the period of sexual maturation, in response to oestrogens.

In previous investigation we have shown that the compensatory adrenal hypertophy developing after contralateral adrenalectomy was significantly more marked in the adult female rat than in the male. We have found that in the adult animal the difference in hypertrophy cannot be influenced either by gonadectomy, or by oestrogen or androgen treatment [1]. On the basis of these findings, as well as on grounds of the evidence published by other authors, first of all by Yates *et al.* [2], Kitay [3], Zarrow and Denison [4] and Hruza [5], we have suggested the possibility that the period of sexual maturation is of significance in the development of the sex difference in the pituitary-adrenal system of rats.

The present paper deals with pre-pubertal compensatory hypertrophy and the effect of pre-pubertal castration and sexual hormone treatment on the measure of compensatory hypertrophy in the adult animal.

Materials and methods

Male and female albino rats of the Institute's stock were used. Until the 28th postnatal day the animals had been kept together with their mother. After weaning the animals were fed on a standard diet, on milk for a short while, then water was allowed *ad libitum.*

The experiments were conducted in the following groups.

1. Right adrenalectomy through lumbar incision was performed at the age of 20 to 22, 30 to 32 and 45 to 49 days, and 7 days later the measure of the contralateral adrenal hypertrophy, as well as corticsterone production *in vitro* were determined, so that the adrenals from every male and female rat from the same litter were incubated together, separated according to sex.

2. The testes or ovaries were removed at the age of 22 to 25 days. Animals from the same litter served as the controls; these were subjected to sham operation. On the 100th postnatal day the right adrenal was removed, then 7 days later the compensatorily hypertrophied adrenal was assayed for adrenocortical secretion and the measure of hypertrophy was determined by weighing with 0.1 mg precision.

3. One group of 20-days old animals was treated with oestrone benzoate (Glandubolin, *Richter)*, 5 μg daily, another group was treated with testosterone propionate (Androfort, *Richter)*, 50 μg daily. The preparations were diluted in sunflower seed oil. In both groups treatment was continued until the vagina of the females had opened. Animals from the same litter served as the controls; these were treated with sunflower seed oil alone. In this group, too, right adrenalectomy was performed on the 100th postnatal day and the measure of compensatory hypertrophy and corticosterone output were determined 7 days later.

The experimental methods employed were as follows.

The right adrenal was removed through lumbar incision and was weighed with 0.1 mg precision. Likewise, ovariectomy was carried out through lumbar incision. The operations were performed under hexobarbital anaesthesia.

The animals of Group 1 were decapitated 7 days after right adrenalectomy, the hypertrophic left adrenals were removed without delay, freed from connective tissue, weighed, then kept in ice in a small dish slightly wetted with Krebs—Ringer's bicarbonate buffer until incubation. Incubation took place in Warburg vessels. The adrenals were cut up into fine slices with an ophthalmological scalpel, the slices were placed into 2 ml pH 7.4 Krebs—Ringer bicarbonate buffer containing 200 mg glucose per 100 ml, further ATP and nicoticacidamide, and were incubated for 90 minutes in 95 per cent O_2 and 5 per cent CO_2 atmosphere at 37° C, under continuous shaking.

After incubation the incubation fluid was decanted, the adrenal tissue slices in the Warburg vessels were washed with 2 ml Krebs—Ringer bicarbonate buffer. The incubation and washing fluids were pooled and the corticosteroids were extracted with 25 ml ether-ethylacetate, 4 to 1. After separation by chromatography [6], the corticosterone content was measured as described previously [7].

In Group 2 and Group 3 the animals were anaesthesized with 5 mg/100 g body weight of pentobarbital 7 days after right adrenalectomy, then, after the intravenous injection of heparin, the left adrenal venous blood was collected for 60 minutes by means of a glass cannula. The corticosterone was extracted and assayed as described [1].

The results were evaluated statistically by STUDENT's test.

Results

Compensatory adrenal hypertrophy in young female and male rats

In the rats aged 20 to 22 days there was no difference in the weight of the right adrenal between the two sexes (females: 5.0 $\pm$ 1.1 mg; males: 5.3 $\pm$ 1.4 mg). Seven days after right adrenalectomy the left adrenal was hypertrophied in both sexes; no difference in the measure of hypertrophy could be observed between the male and female animals.

Similar results have been obtained in 30 to 32 days old rats. There was no difference between males and females of that age either in the right adrenal weights, or in the weight of the hypertrophied left adrenals (Fig. 1).

Following the opening of the vagina which in our experimental groups took place around the 36th to 42nd postnatal days, in female rats aged 45 to

49 days the right adrenal weighed 10.2 ± 1.4 mg, whereas in the males of the same age it weighed 7.6 ± 1.4 mg; the difference was statistically significant ($p < 0.01$). Seven days after removal of the right adrenal the compensatory hypertrophy of the left adrenal was significantly more marked in the females (13.9 ± 2.0 mg), than in the males (9.7 ± 1.4 mg); the difference was significant ($p < 0.001$).

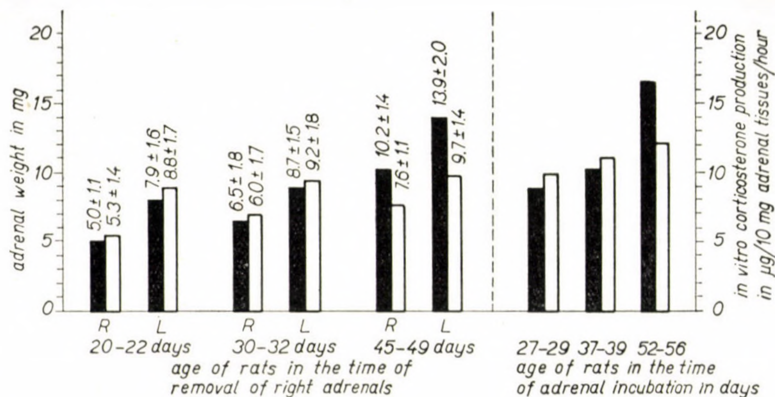

Fig. 1. Compensatory adrenal hypertrophy and in vitro corticoid production by the hypertrophic adrenals in young animals

Black columns: female rats. White columns: male rats. *R* : right adrenal weight. *L* : weight of the left adrenal with compensatory hypertrophy

In vitro corticosterone production by the hypertrophic adrenals of young animals

The corticosterone production *in vitro* by the hypertrophic adrenals increased parallel with the advance of age. While in the animals aged 27 to 29 days, corticosteroid production averaged 50 per cent of the adult value, in the animals 52 to 56 days of age production was comparable to that found by us earlier in adult rats (1.8 to 3.0 μg/10 mg adrenal tissue/hour). In the groups studied no difference in production was demonstrable between the two sexes until the 37th—39th postnatal days, while at the age of 52 to 56 days the females produced more corticosterone than the males (Fig. 1).

Effect of prepubertal castration on compensatory adrenal hypertrophy in adult animals

As determined on the 100th day, in female rats ovariectomized at the age of 22 to 25 days the weight of the right adrenal was not different from that of the controls from the same litter. Seven days after right adrenalectomy the hypertrophic left adrenal weighed 18.8 ± 2.6 mg/100 g body weight in the

ovariectomized females, while in the control females it weighed 23.6 ± 3.2 mg/100 g body weight; the difference was significant ($p < 0.001$ Fig. 2).

Male rats aged 22 to 25 days were castrated; at that time the tests had already descended in 90 per cent of the animals. Right adrenalectomy was performed on the 100th postnatal day, both in the castrated and the sham-

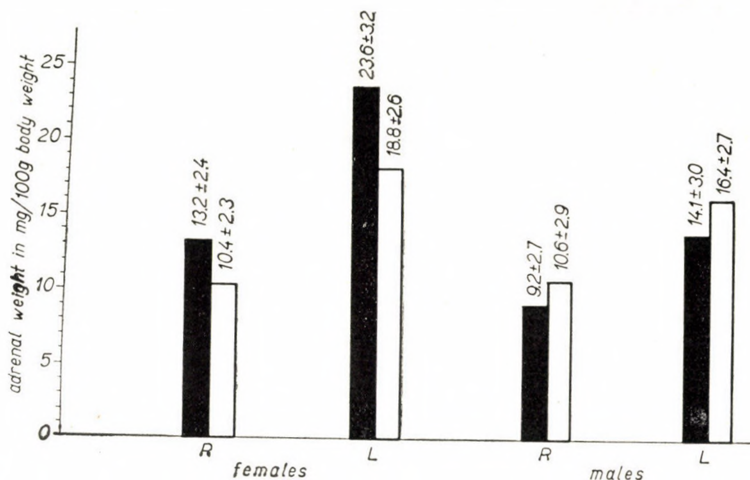

Fig. 2. Effect of prepubertal castration on compensatory adrenal hypertrophy in the adult rat. *R :* weight of right adrenal. *L :* weight of hypertrophic left adrenal. Black columns: sham-operated controls. White columns: castrated animals

operated control groups. There was no significant difference in relative adrenal weight between the two groups, nor in compensatory adrenal hypertrophy estimated 7 days later (Fig. 2), when the castrated animals weighed significantly more than the controls.

Effect of prepubertal oestrogen and androgen treatment on compensatory adrenal hypertrophy in adult male and female rats

Female rats were treated with 50 μg of testosterone propionate daily from the 20th postnatal day till the opening of the vagina. In response to this treament the vagina opened on the 30th to 31st day, while in the controls from the same litter it opened only after 37 to 40 days. There was no significant difference in relative adrenal weight between the treated animals and the controls, as determined by right adrenalectomy performed on the 100th day. Likewise, the left adrenals removed 7 days later showed no significant difference in weight (Fig. 3).

Following treatment with 5 μg of oestrogen daily the vagina opened on the 29th—30th day. No difference in right or left adrenal weight was demon-

strable between the treated animals and the controls at 100 and 107 days, respectively (Fig. 3).

Male animals had been treated with oestrogen or testosterone until the vagina of the females from the same litter had opened.

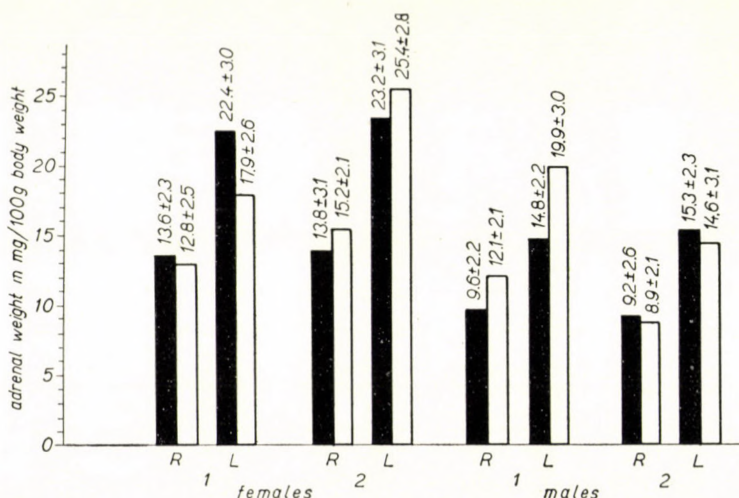

Fig. 3. Effect of prepubertal oestrogen and androgen treatment on compensatory adrenal hypertrophy in adult male and female rats

R : weight of right adrenal. *L* : weight of left, hypertrophic adrenal. Black columns: control rats. White columns: treated rats. Females: *1*. Testosterone propionate treatment, *2*. Oestron benzoate treatment. Males: *1*. Oestron benzoate treatment, *2*. Testosterone treatment

In the oestrogen-treated animals the right adrenal did not weigh significantly more on the 100th postnatal day than in the control ($p < 0.1$). Seven days later, however, a significant difference in left adrenal weight was found between the oestrogen-treated male rats and the control males ($p < 0.001$), with values for the treated animals 19.9 ± 3.0 mg/100 g body weight, and for the controls, 14.8 ± 2.2 mg. At the same time, no difference was found in testicular weight between the two groups.

No difference in adrenal weight between the males treated with 50 μg of testosterone daily and the control rats was detected either on the 100th, or on the 107th postnatal day (right adrenalectomy and left adrenalectomy, respectively) (Fig. 3).

Corticosterone secretion by the hypertrophied adrenals of adult male and female rats castrated, or treated with sexual hormone, prior to puberty

In castrated rats, as well as in rats treated with oestrogen or androgen, corticosterone secretion of the left, hypertrophic adrenal was examined 7 days after right adrenalectomy, *i.e.* on the 107th postnatal day, by analyzing the

left adrenal venous blood. As the controls, animals from the same litter were used. Analysis of all the control animals showed that the secretion values were identical in the male and female sham-operated and control-treated rats, so that, when comparing the results with those for the castrated, oestrogen- or androgen-treated rats, it was possible to group the controls according to sex.

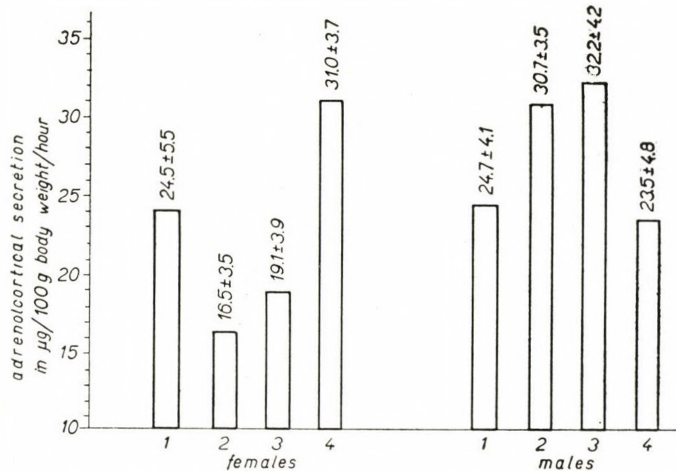

Fig. 4. Corticosterone secretion by the hypertrophied adrenals of adult rats castrated or sexual hormone-treated before sexual maturation

Females: *1.* Controls, *2.* Ovariectomized rats, *3.* Testosterone-treated rats, *4.* Oestron treated rats. Males: *1.* Controls, *2.* Castrated animals, *3.* Oestron treated animals, *4.* Testosterone treated animals

Corticosterone secretion was 24.0 ± 5.5 μg/100 g body weight/hour with the control females, and 24.7 ± 4.1 μg/100 g body weight/hour with the control males (Fig. 4).

After ovariectomy performed before sexual maturation corticosterone secretion was 16.5 ± 3.5 μg/100 g body weight/hour, less than in the normal female rat. The difference was significant statistically ($p < 0.001$).

Following prepubertal testosterone propionate treatment the left hypertrophic adrenal secreted 19.1 ± 3.9 μg/100 g body weight/hour corticosterone at the age of 107 days in the female rats. This value was not significantly different from the 24.0 ± 5.5 μg/100 g body weight/hour result obtained for the control female rats ($p > 0.01$).

The adult female rats treated with 5 μg of oestrone benzoate daily from the 20th till the 29th—30th postnatal days, corticosterone secretion was 31.0 ± 3.7 μg/100 g body weight/hour, significantly higher ($p < 0.001$) than that shown by the controls (Fig. 4).

In male rats castrated in the 22nd to 25th days of life, corticosterone secretion increased in adult age. In this group the secretion rate was 30.7 ± 3.5

μg/100 g body weight/hour, as compared with the 24.7 ± 4.1 μg/100 g body weight/hour value for the control males. The difference between the two values was significant ($p < 0.001$).

Likewise, prepubertal oestrone treatment resulted in a significant increase of secretion ($p < 0.001$). In the oestrone-treated males the value was 32.2 ± 4.2 μg/100 g body weight/hour, as compared with the control value of 24.7 ± 4.1 μg/100 g body weight/hour.

The adult males treated with testosterone during the same prepubertal period secreted 23.5 ± 4.8 μg/100 g body weight/hour, an amount not different from that secreted by the controls.

Discussion

We have shown previously [1] that beside the sex differences in the morphology and function of the pituitary-adrenal system, thus the higher adrenal weight [8], the higher corticosterone level [9, 11] and ACTH content [10] in peripheral blood, the more marked increase of the corticosterone level in response to exogenous ACTH and stress [9], the compensatory adrenal hypertrophy following unilateral adrenalectomy was also significantly greater in the female rat. Studies of the sexual fact orsresponsible for the sex differences have shown that in the adult rat the difference in adrenal hypertrophy between males and females is not influenced by gonadectomy, oestrogen or androgen treatment. On the basis of the experimental evidence obtained, and on grounds of the data reported by Yates *et al.* [2], Kitay [3], Zarrow and Denison [4] and Hruza [5] we have suggested the possibility that the sex differences in pituitary-adrenal activity, thus also in compensatory hypertrophy, would develop during the period of sexual maturation.

The present results have confirmed the validity of that hypothesis, since there was no difference in the measure of compensatory adrenal hypertrophy between the two sexes at the age of 27 to 29, as well as 37 to 39 days, whereas sex differences appeared at 52 to 56 days of age. No sex difference in basal adrenal weight was observable before 45 to 47 days, in agreement with the observation by Yates *et al.* that a sex difference in adrenal weight appears only after the 40th day, to become more and more marked subsequently [2].

As regards corticosterone production *in vitro*, the increase of secretion was identical in the males and females until the age of 37 to 39 days, while females aged 52 to 56 days showed already higher secretion than the males of the same age. Kitay [3] who found production *in vitro* to be higher in females, ascribed the difference to oestrogen secretion starting at the time of sexual maturation, because corticosterone production *in vitro* by the adrenal tissue from adult female animals cannot be increased by 17 β-oestradiol treatment, whereas such treatment enhances production following prepubertal ovariectomy.

Our castration experiments, as well as those involving oestrogen or androgen treatment, apparently confirmed the role of oestrogens, as the factor responsible for the difference in the measure of hypertropy. While in the femal ovariectomy performed on the 20th to 25th postnatal day significantly decreased the measure of compensatory hypertrophy, in the male orchidectomy performed at the same time had no influence on it. In the male animal prepubertal oestrogen treatment enhanced the hypertrophy as estimated at the age of 107 days, while testosterone treatment was ineffective in either sex.

The observation that no similar difference was demonstrable in the weight of the right adrenals removed on the 100th postnatal day, in other words the fact that basal adrenal weight had not changed under the effect of ovariectomy, or in males by oestrogen treatment raises a further problem which becomes especially conspicuous if the results for secretion are analyzed.

According to our observations, the corticosterone level of the venous blood coming from the compensatorily hypertrophied left adrenal is significantly increased if the animal had been treated with oestrogen before sexual maturation, whereas following ovariectomy a significant decrease in secretion occurs in the adult animal. These results indicate that the sexual-hormonal stimuli reaching the pituitary-adrenal system at the time of sexual maturation significantly influence secretion in the adult animal, in the presence of compensatory hypertrophy.

Activation of pituitary-adrenal function by oestrogens has been demonstrated by GEMZELL [12] on the basis of an increased plasma ACTH level, by BROLIN and HELLMANN with thymus involution, wihch does not take place after adrenalectomy [13]. The same has been shown for man by DÖRNER *et al.* [14], PETERSON *et al.* [15], WALLACE *et al.* [16, 17], among others. At the same time, VOGT and HOLZBAUER [18, 19] reported a decreased adrenal venous corticosterone level in the female rat after treatment with synthetic oestrogens and 17-β-oestradiol; this has been confirmed by TELEGDY *et al.* [6], who administered oestrone-benzoate.

Our observation of an increased output by the hypertrophic adrenals under the effect of oestrogen treatment before sexual maturation indicates that the mode of action is no identical with that of the secretory changes resulting in the normal female rat from direct oestrogen administration, partly because in the present case a late change was observed following oestrogen administration in young age, and partly because in our previous studies involving adult male and female rats there was no change in the measure of hypertrophy, yet adrenal secretion was increased in response to oestrogen treatment.

The development of compensatory adrenal hypertrophy, being a feedback mechanism, depends on the intactness of certain central nervous structures, notably on that of the medial eminence, as it has been shown in the dog by GANONG and HUME [20], as well as in the rat by BACHRACH and KORDON

[21], McCann *et al.* [22], Smelik *et al.* [23]. In the light of these data it may be suggested that the enhancement of this feed-back effect by oestrogen treatment would be due to an action of the oestrogens on the central nervous sites governing the feed-back meachanism, thus, on the medial eminence, tuber cinereum or on the mesencephalic reticular formation [24]. To lend support to this hypothesis, we may refer to the evidence reported by Barraclough [25] that a single dose of testosterone propionate administered on the 2nd to 5th postnatal day produced sterility in the female rat, by an action on hypothalamic centres.

The divergences in the results for the measure of compensatory hypertrophy and for the corticosterone output by the hypertrophic adrenals, for example the finding that in oestrogen-treated females there occurred no change in the measure of compensatory hypertrophy and at the same time secretion was increased, may be explained by our earlier observations, notably that the changes in secretion are not always followed by identical changes in adrenal weight [26].

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Béla Bohus, Elemér Endrőczi, Kálmán Lissák
Orvostudományi Egyetem Élettani Intézete, Pécs

EXTENT OF THE MOTOR CORTEX IN THE POSTERIOR SIGMOID GYRUS IN THE CAT

By

V. Golda, J. Petrěk and P. Lisoněk

LABORATORY OF HIGHER NERVOUS ACTIVITY AND DEPARTMENT OF ANATOMY, PALACKY UNIVERSITY, OLOMOUC, CZECHOSLOVAKIA

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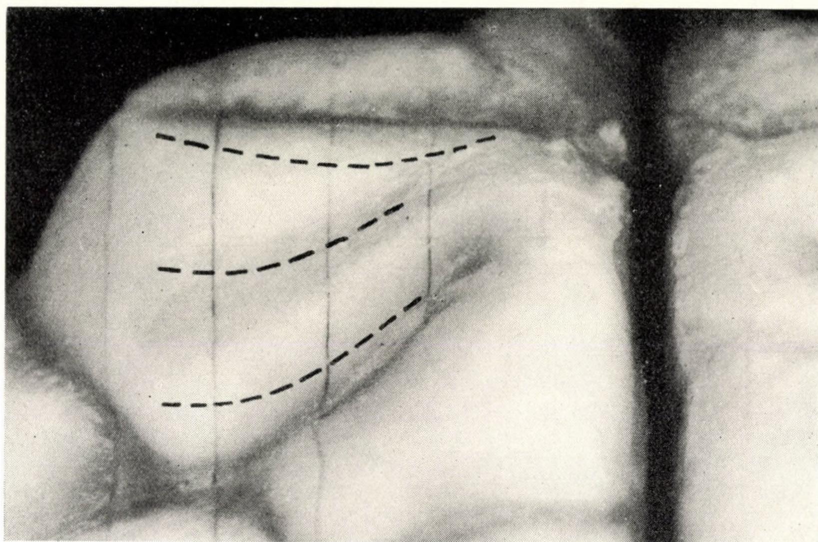
In agreement with the opinion of V. M. Svetukhina [12], in cat the borderline between the area of the motor and the sensory cortex in the region of the posterior sigmoid gyrus has been found to be formed by the postcruciate sulcus. From the results obtained it has been concluded that the different cytoarchitectonical regions of the posterior sigmoid gyrus correspond to the evoked potentials with different latencies of the positive phases. We found that during stimulation of the cutaneous and muscle branches of the radial nerve, in the area between the cruciate sulcus and the postcruciate sulcus the latency of the positive phase is considerably longer than in the region which is situated occipitally to the postcruciate sulcus. In some of the preparations a special form of evoked potential was observed at the site of the postcruciate sulcus.

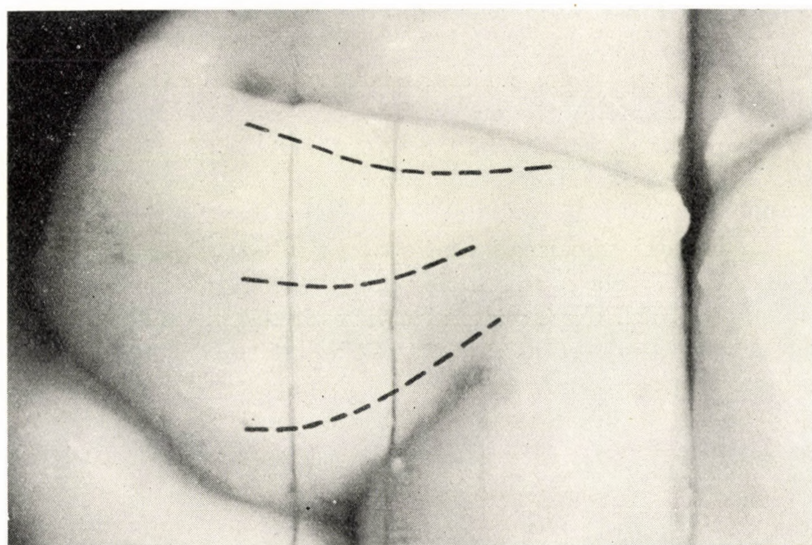
In the electrophysiological and neuroanatomical literature there is no uniform opinion regarding the occipital borderline of the motor cortex in the cat. In some of the electrophysiological studies it has been pointed out that the motor zone is represented by the gyrus sigmoideus anterior and the depth of the sulcus cruciatus [11, 5]. On the other hand, some morphological studies indicate that not only the area anterior to the sulcus cruciatus and the depth of this notch but also a considerable part of the gyrus sigmoideus posterior are involved [4, 1, 12]. These anatomical investigations are in conformity with the electrophysiological studies of J. P. Murphy and E. Gellhorn [10], and J. M. R. Delgado [2], who studied by electrical stimulation the extent of the cortical region in which the movements of the individual parts of the body are evoked.

On the basis of cytoarchitectonical studies, V. M. Svetukhina [12] is of the opinion that in the gyrus sigmoideus posterior of carnivores, the sulcus postcruciatus corresponds to the central notch of the primates. On verifying the cytoarchitectonical structure of the gyrus sigmoideus posterior, we have found the different cytoarchitectonical areas to account for evoked potentials of various nature.

Methods

The experiments were performed on 15 adult cats under intravenous chloralose (50 mg/kg) anaesthesia. After unilateral craniectomy, intratracheal and intravenous cannulas were introduced, and the exposed cutaneous and muscle branches of the radial nerve of the contralateral limb were placed in special activating electrodes preventing the preparation from drying and cooling during the experiment. Shortly before the recordings were taken,



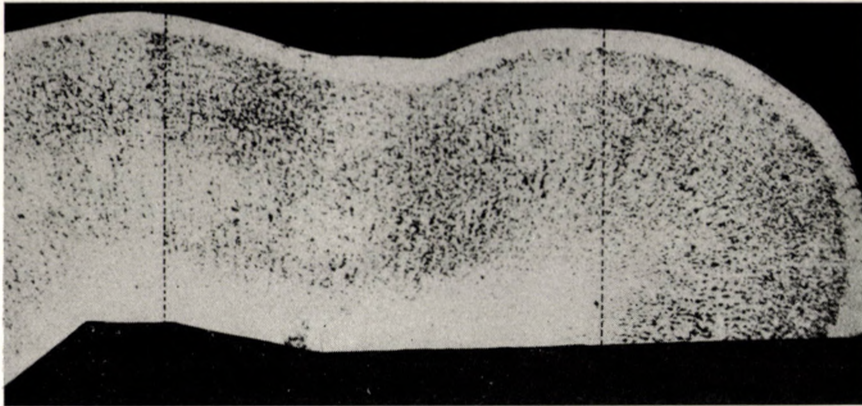


b

Fig. 1a, 1b. First line posterior to sulcus cruciatus — region of giant and large pyramidal cells (occipitally, only exceptionally, single Bec's cells. Second line posterior to sulcus cruciatus — boundary line of the cortex, frontally in the fifth layer large pyramids; occipitally, the fifth layer without any pyramidal cells. Third line posterior to sulcus cruciatus — an outline of the posterior boundary of the pyramid-free layer and the beginning of the fifth layer with pyramids present

durectomy was carried out. Artificial ventilation was provided and the animal was immobilized by intravenous Gallamine triethiodide. The temperature of the preparation was kept constant.

The stimuli were induced by means of rectangular oscillations of 0.3 to 0.9 msec. The intensity of the stimulating current varied from 0.02 to 0.5 mA. The stimuli were applied via an earthed transformer unit.



a

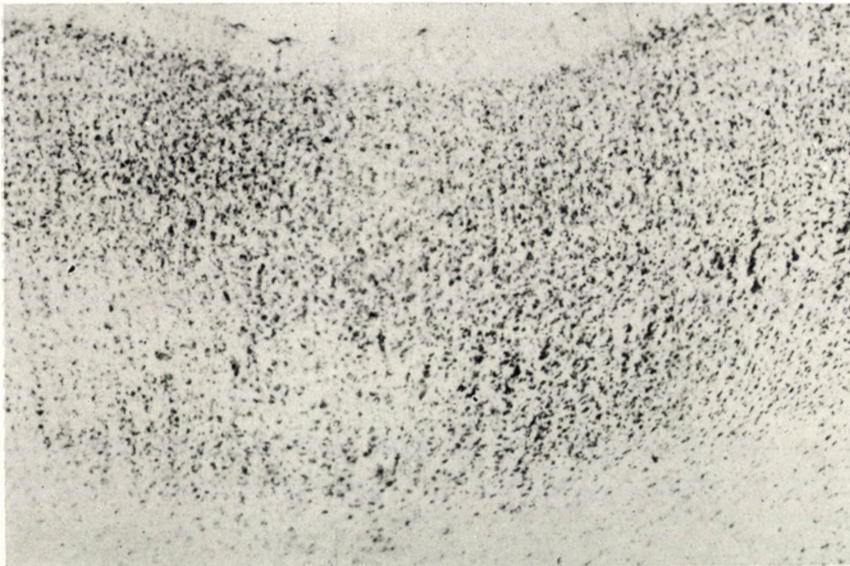


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Fig. 2a, 2b. On the right side of the microphotograph the upper lip of the sulcus cruciatus is to be seen. The concavity in the central part represents the sulcus postcruciatus (Fig. 2*a*). On the lower part of the figure there is a sector of the microphotograph surrounding the sulcus postcruciatus (Fig. 2*b*)

Recording of cortical electrical activity was carried out with the aid of silver electrodes. The evoked potentials were registered photographically from the screen of a 5-channel *Křižik* oscilloscope.

Spontaneous activity was followed up continuously with a 15-channel Alvar EEG apparatus.

During the experiment, the cortex was kept moist with warm Ringer's solution.

The cytoarchitectonical studies were carried out on 5 cats. The brain tissue was fixed intravitally with 100 per cent formalin which killed the animals during the experiment. The slices were stained with cresyl violet.

Results

The neuroanatomical findings have shown that, in the region of the gyrus sigmoideus, the sulcus postcruciatus is actually situated along the regional borderline between the motor and the sensory cortex in the cat. The enclosed microphotograph (Fig. 2) shows that the cortex which is situated between the sulcus cruciatus and the sulcus postcruciatus abounds in giant and large pyramidal cells whereas in the fifth layer of the cortex which is situated posterior to the sulcus postcruciatus the cells are practically missing.

It is noteworthy that from these cytoarchitectonically different regions of the postcentral gyrus, it was possible to record evoked potentials of different latency and form during electrical stimulation of the branches of the radial nerve (Fig. 3). The positive phases of the primary evoked potential in the area between the sulcus cruciatus and the sulcus postcruciatus. In some of the preparations the evoked potentials which were recorded directly from the region of the sulcus postcruciatus displayed two positive phases of low voltage in which the difference of the latency was about 5 msec. (Fig. 3, second channel). The latter phenomenon was observed only on stimulating the cutaneous branch of the nerve. In other respects there did not exist any differences between the muscle and the cutaneous branch.

Discussion

The evoked potentials of the so-called motor and sensory regions have been studied in detail by L. I. MALIS, K. H. PRIBRAM and L. KRUGER [9] in the monkey, E. OSWALDO-CRUZ and S. TSOULADZE [11] in the cat, and by S. LIBOUBAN [8] in the rat. MALIS *et al.* [9] demonstrated that the positive phase of the evoked potential in the region of the precentral gyrus has a latency of approximately 18 msec, whereas in the postcentral gyrus one of 13 msec, during the stimulation of both the cutaneous and the muscle branches of the ischiadic nerve. These findings were confirmed in cats under chloralose anesthesia by OSWALDO-CRUZ and TSOULADZE [11]. These authors started from the supposition that the anterior sigmoid gyrus in the cat corresponds to the motor region and the posterior sigmoid gyrus to the senzory zone. During electrical stimulation of the superficial radial nerve the positive phase of the evoked potential in the anterior sigmoid gyrus had a latency period of approximately 10 msec and the positive phase of the evoked potential in the posterior sigmoid gyrus one of approximately 5 msec. S. LIBOUBAN [8] observed a similar rate of latencies in rats under chloralose or pentothal anaesthesia during electrical stimulation of the limbs. He also found that, in the motor region, the latency of the positive phase of the evoked potential is markedly longer than n the somatosensory region.

According to data in the literature, in monkeys, cats and rats, during stimulation of the peripheral nerves the positive phase of of the evoked potential in the motor region is markedly longer than in the sensory region. We recorded such potentials of longer duration also from the area between the cruciate and postcruciate sulci. Considering the cytoarchitectony, in cat this

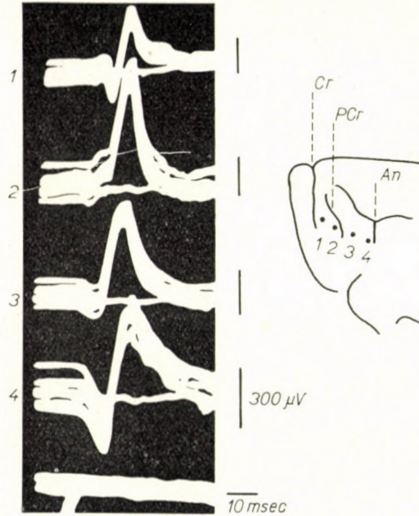

Fig. 3. Evoked potentials from cytoarchitectonically different cortical areas during stimulation of the n. radialis superficialis. To the left, the different latencies of the positive phase of evoked potentials (compare the first and fourth channels)
In the right, points Nos. 1, 2, 3 and 4 indicate the position of the recording electrodes. Abbrev.: Cr = sulcus cruciatus, PCr = sulcus postcruciatus, An = sulcus ansatus

area actually pertains to the motor region. Consequently, the boundaries of the motor and sensory cortex cannot be placed in the cruciate sulcus but are lying more occipitally, in the region of the posterior sigmoid gyrus, in the line which is approximately defined by the postcruciate sulcus.

There are two more questions to be answered, 1. how to explain the differences in the latencies, and 2. why the same dislocation of the evoked potentials is observed during stimulation of the cutaneous and the muscle branch.

Libouban [8] finds the answer to the first question in the differing thalamic relay. We, however, assume that the direct cortico-cortical connection has also to be taken into account [3].

The second question arises from the comparison of our results with those obtained by J. Hrbek [6] and J. Hrbek *et al.* [7]. These authors demonstrated on curarized unaesthesized preparations essential differences in the cortical projection of the muscular and cutaneous volleys. According to their findings,

the proprioception, contrarily to the exteroception (the skin sensation), is projected much more occipitally. This disproportion might be explained by the observation of J. HRBEK [6] and J. HRBEK *et al.* [7] that the proprioceptive analyzer, being the most sensitive anatomical and functional structure, is deeply affected by anaesthesia. Furthermore the authors are of the opinion that Mountcastle et al. did not succeed to prove the cortical projection of the Ia and Ib systems into the cortex because they studied anaesthesized preparations, in which we see the cortical projection only on the second and third group of the fibres of the afferent muscle. Naturally, this projection may differ from that of the Ia and the Ib group of the muscle afferents.

Acknowledgement

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V. GOLDA, J. PETRĚK, P. LISONĚK
Laboratory of Higher Nervous Activity, and Department of Anatomy, School of Medicine, Palacky University, Olmouc, Czechoslovakia

SUMMATION PHENOMENA IN THE AUTONOMIC NERVOUS SYSTEM

By

E. Bittman and N. Raiciulescu

D. DANIELOPOLU" INSTITUTE OF NORMAL AND PATHOLOGICAL PHYSIOLOGY, ACADEMY OF SCIENCES, BUCHAREST, RUMANIA

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Acute experiments have been performed in 16 dogs and 27 cats (curare or chloralose anaesthesia), to study the tensional phenomena occurring in simultaneous activation of the central vegetative projections which play a role in the control of arterial blood pressure. Thus, besides some tensional changes which take place at simultaneous activation of the cortical and hypothalamic vasomotor projections the relations between hypothalamus and midbrain reticular formation have been investigated, using as indicator the phasic changes of arterial blood pressure brought about by electric stimulation of those two nervous centres. Likewise, investigations were carried out concerning the effect of hypothalamus and midbrain reticular formation stimulation on the sinocarotic pressor reflex induced by bilateral occlusion of the common carotid arteries.

The results obtained showed that simultaneous stimulation of the pressor points of the motor cortex and the hypothalamus and anterior rhinencephalon in dogs and cats induces generally a phenomenon of spatial summation and rarely an effect of the occlusive type. When induced by tensional effects of various values, the summation phenomenon had a linear character, its correlation coefficient being calculated in terms of the nervous areas explored. Simultaneous stimulation of the hypothalamus and mesencephalic reticular formation usually brings about tensional effects of summation and occasionally occlusive or independent effects. Stimulation of these areas usually facilitates the sinocarotic pressor reflex, and, in rare cases, hinders or does not influence it.

The central nervous regulation of vasomotricity is subserved by a series of superposed nervous centres, starting with the bulbar centres, passing through the meso-diencephalic ones, and ending with the cerebral cortex. The manner in which these centres influence one another is not sufficiently known. Thus, although it is generally admitted that in man and mammals 2 or 3 zones the cortex act directly — from the functional point of view — on the nervous vegetative system, the exact nature of this relationship is not fully elucidated [17]. Furthermore, the interaction between the cortical vegetative projections concerning tensional effects has not been studied. On the other hand, although it is generally accepted that the action of the hypothalamus and the midbrain reticular formation upon blood pressure is of two kinds — phasic and tonic [15, 16, 18, 25, 26] — neither the connections between these areas nor their functional relationships are completely known. On account of the caudal-rostral hierarchy of the somatic functions which underlies the structure of the central nervous system, some authors believe that the vegetative effects caused by cortical stimulation are the expression of a corresponding structure in the nervous vegetative system. According to Benetato [2], the morphological and functional discrepancies between the mechanisms of somato-

visceral control — very conspicuous at the level of the medulla — diminish progressively the nearer we approach the cerebral cortex, blending in the cortex into a unitary system of coordination and adaptation of all functions.

In regard to the cortico-hypothalamic influences it was stated that bilateral ablation of the frontal cerebral pole alters arterial blood pressure, respiration and gastric motility, and reverts the effects of hypothalamic excitation. Likewise, frontal lobotomy induces in patients with essential hypertension a constant fall of arterial pressure. On the other hand, heating of the hypothalamus acts — via relay stations — upon the activity of spinal gamma motoneurones as well as on cerebral activity [13]. Thus, it seems that the thermoceptive structure of the hypothalamus are projected, as most of the peripheral receptors, on the activating relay stations sited in the brain stem. One knows, likewise, that the maintenance of thermal homeostasis has also considerable vasotomotor implications which are solved by the integrative activity of the hypothalamus. The bulbo-mesencephalic reticular system is involved in the maintenance of the tensional homeostasis by projections from the sino carotic baroreceptors and by the direct influence exerted by adrenaline [8, 11].

From the above data it seems that no investigations have been made of the tensional phenomena occurring in simultaneous activation of the central vegetative projections which play a role in the control of arterial blood pressure.

In the present study some of the tensional changes which take place in simultaneous activation of the cortical and hypothalamic vasomotor projections, and on the other hand the relations between hypothalamus and midbrain reticular formation have been investigated using as indicator the phasic changes of arterial blood pressure brought about by electric stimulation of the nervous centres.

The functional significance of tensional changes induced by simultaneous stimulation of the hypothalamus and mesencephalic reticular formation cannot be understood without exploration of the bulbar mechanism, that final common way by which all suprajacent activations are acting. This is why investigations were carried out in another series of experiments concerning the effect of hypothalamus and midbrain reticular formation stimulation on the sinocarotic reflex induced by bilateral occlusion of the common carotid [19]. In experiments carried out in dogs with isolated head, BENETATO et al. [4] demonstrated that adrenaline activation of the reticular formation increases both the sinocarotic reflex induced by clamping of the common carotid artery, and the tensional effects induced by stimulation of the central end of the vagus or the sciatic nerves.

Method

Acute experiments were performed in 16 dogs and 27 cats. Curare (1–3 mg/kg body weight in fractionated doses) was given to the animals in which the tensional effect of cortical stimulation was studied. Artificial breathing was performed and in most instances cervical

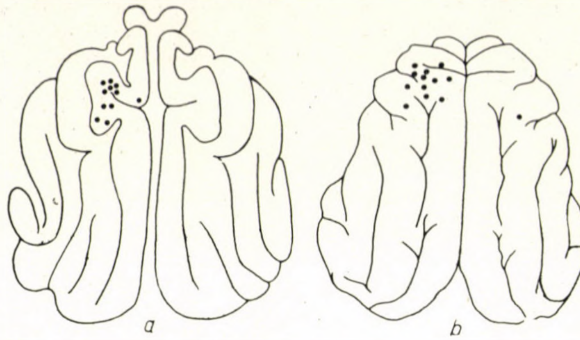

Fig. 1. Cortical points stimulated in dog (*A*) and cat (*B*)

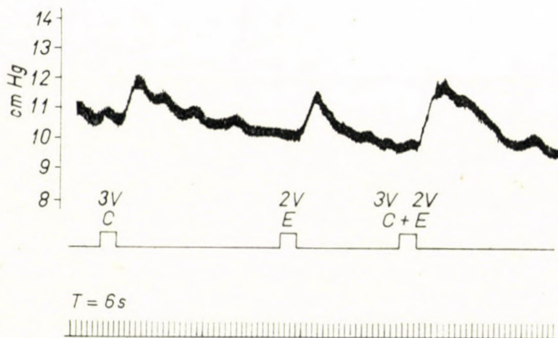

Fig. 2. Cat (chloralose + d-tubocurarine). C = stimulation of a point in the left sigmoid cortex, with 50 Hzm 3 V, sinusoidal current; E = stimulation of a point within the right mammillary body, with 50 Hz, 2 V sinusoidal current; $C + E$ = simultaneous stimulation of both points

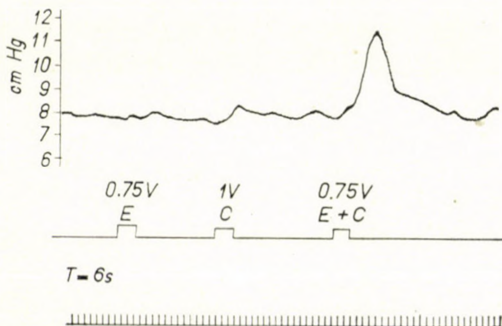

Fig. 3. Cat (Chloralose + d-tubocurarine). E = stimulation of a point in the parainfundibular region with 150 Hz, 0.75 V rectangular current; pulse duration: 0.5 mesc. C = stimulation of a point in the left sigmoid cortex with 50 Hz, 1 V, sinusoidal electric current. $E + C =$ = simultaneous stimulation of both points

bilateral vagotomy; occasionally chloralose was added (0.04 g/kg body weight) in order to prevent Traube—Hering waves, which may readily occur in curarized and vagotomized dogs. Chloralose anaesthesia (0.08 g/kg body weight intravenously) was used in animals in which the effect of stimulation of the hypothalamus and mesencephalic reticular formation was studied. Some animals were curarized in order to prevent motor effects which could derive from the stimulation of those structures. In some animals bilateral cervical vagotomy was performed. Arterial blood pressure was recorded from the femoral artery by means of a Ludwig manometer. For deep structures of the brain, silver electrodes 0.2—0.4 mm in diameter were used, insulated with epoxidic varnish excepting the last 1 mm. The electrodes were implanted stereotaxically, using for cats JASPER, AJMONE and MARSAN's Atlas [24] and for dogs our own method [5, 32]. Stimulation of the cerebral cortex was made by electrodes covered with a thin layer of cotton wool soaked in normal saline, the moisture of the cerebral cortex being maintained by drops of warm (38° C) saline.

Mono- and bipolar (1 mm distance between electrodes) stimulations were performed with electric monophasic rectangular impulses of 30—300 Hz, 0.05—0.4 mA, lasting 0.01—0.1 msec or with a 50 Hz., 1—6 V sinusoidal electric current. The electric stimulation was delivered during 15—20 sec in order to obtain clear tensional effects without their wearing out.

In the first series of experiments, the hypothalamic and cortical points were separately stimulated, then both areas simultaneously. In a second series of trials, the hypothalamus and the reticular formation were first stimulated separately, then simultaneously. In a third series of experiments, investigations were made of the effect of liminar and supraliminar stimulation of the hypothalamus and reticular formation upon the sinocarotic pressor reflex induced by bilateral occlusion of the common carotids over a 15—20 sec period.

After each experiment the nervous tissue surrounding the tip of the electrode was electrocoagulated (1—2 mA, 1—2 min) and the brain fixed in formalin. In all the cases gross and microscopic examination of the material was performed (haematoxilin-eosine and Nissl stainings), using the JASPER, AJMONE and MARSAN's Atlas [24] for cats and the atlases of ADRIANOV and MERING [1] and LIM, LIU and MOFFITT [28] for dogs.

Results

A) In the first series of experiments, 92 points were explored in the depth of the brain and 23 cortical points in the sygmoid gyrus (Fig. 1). Of the active subcortical points, 7 were explored in each of the following regions, anterior rhinencephalon, posterior and intermediary hypothalamus, and 6 in the anterior hypothalamus; their stimulation had a hypertensive effect.

The simultaneous stimulation of a cortical point which causes an arterial pressor effect and of a point sited in the depth of the brain which also causes hypertension, brings about in most cases a tensional effect larger than that of each separate effect, but smaller than the sum of the effects obtained by individual stimulation of the two points (Fig. 2). Occasionally, simultaneous stimulation produced a greater effect than the algebraic sum of the two components (Fig. 3), or an effect smaller than the value of one of the effects obtained by separate stimulation (Fig. 4).

In order to ascertain whether there is a relationship between the rise of the arterial pressure (S) when applying the two stimuli simultaneously and the rise of pressure after deep cerebral (E) and cortical (C) stimulation, the linear correlation equation with 3 variants was applied:

$$S = a \cdot E + b \cdot C + n$$

This formula disclosed a linear correlation between the tensional phenomena obtained. This correlation, expressed by the correlation coefficient (R)

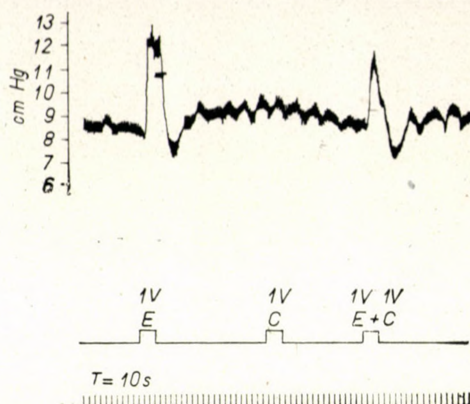

Fig. 4. Dog (chloralose + d-tubocurarine). E = stimulation of a point in the left mammillary body with 50 Hz, 1 V sinusoidal electric current. $E + C$ = simultaneous stimulation of both points

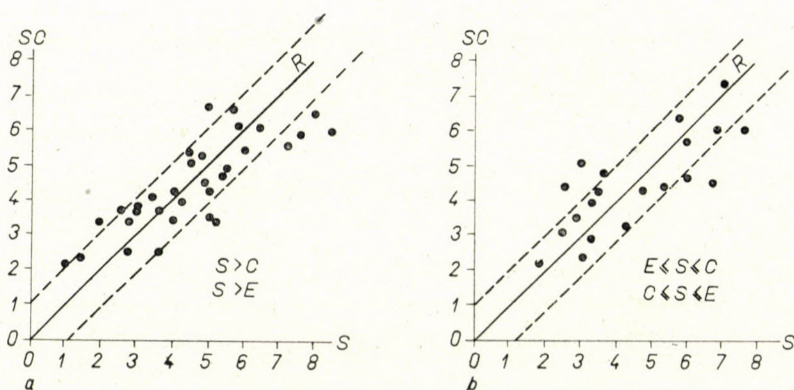

Fig. 5. Correlation between the summated effect found experimentally (abscissa) and the summated effect calculated according to the regression equation (ordinate)

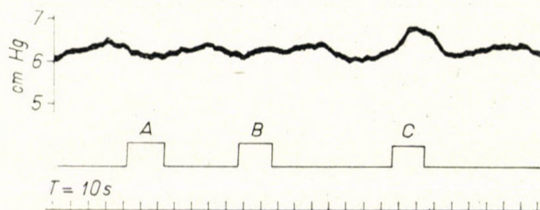

Fig. 6. Cat (chloralose). Bipolar electrodes. A = stimulation of left anterior hypothalamus, 30 Hz, 0.05 msec, 0.08 mA, 20 sec. B = stimulation of left mesencephalic reticular formation, 50 Hz, 0.15 msec, 0.1 mA, 20 sec. C = simultaneous stimulation of both points, with identical parameters

depends upon the various anatomical regions; it is closer in the hypothalamus-cortex correlations ($R = 0.67$) than in the rhinencephalon-cortex ones ($R = 0.49$). In regard to the hypothalamic areas, the correlation coefficient (R) had the following values:

anterior hypothalamus: $R = 0.92$,
intermediary hypothalamus: $R = 0.82$,
posterior hypothalamus: $R = 0.35$.

By applying the correlation calculation to the tensional rise induced by simultaneous delivery of both cortical and deep stimuli, one can establish — according to the size of the coefficient (E) or (C), — the importance of each factor in the genesis of the summated phenomenon. It is supposed that the figure (n) from formula (1) expresses the superposition of the excitation phenomena in a common subjacent area (reticular formation, bulbar vasomotor centers, etc.).

The values of summated arterial pressure obtained in all explored points were divided into 2 classes because the correlation between the tensional phenomena is less intense in some areas, and because the sum (S) corresponds in these areas to phenomena which may have a different physiological significance, exhibiting higher, or on the contrary, lower values than those of the component E and C elements: 1. (S) higher than (C) and higher than (E) (Fig. 5, A); 2. (S) with equal values to (C) and (E) or with intermediary values between (C) and (E) (Fig. 5, B).

B) In a second series of experiments, investigations were carried out concerning the effects of simultaneous stimulation of the mesencephalic reticular formation and the hypothalamus — by subliminar, liminar and supraliminar intensities — on the arterial blood pressure. Several types of tensional responses were found.

1. Simultaneous stimulation of the hypothalamus and reticular formation with subliminar intensities — which after separate stimulation of the two structures induces no alterations of the arterial blood pressure — results in a constant rise of the pressure (Fig. 6, 7).

When hypothalamus and reticular formation are stimulated with various supraliminar intensities, the tensional response to their simultaneous stimulation tends to be inversely proportional with the tensional effects induced by separate stimulation of both nervous structures, *i.e.* the smaller tensional effects are induced by separate stimulation of the hypothalamus and reticular formation, proportionally the greater is the tensional rise brought about by their simultaneous stimulation, and *vice versa* (Fig. 8).

When separate stimulation of the hypothalamus and reticular formation induces a hypertensive effect and a hypotensive effect, respectively, simultaneous stimulation results in the algebraic summation of both effects, one of them being more prominent (Fig. 9).

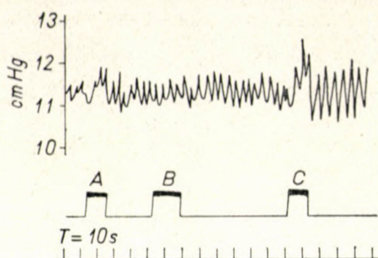

Fig. 7. Dog (chloralose + gallamine triethiodide). Monopolar electrodes. A = stimulation of right mesencephalic reticular formation, 50 Hz, 0.1 msec, 0.1 mA, 15 sec. B = stimulation of right intermediary hypothalamus, 100 Hz, 0.1 msec, 0.1 mA, 15 sec. C = simultaneous stimulation of both points with the same parameters

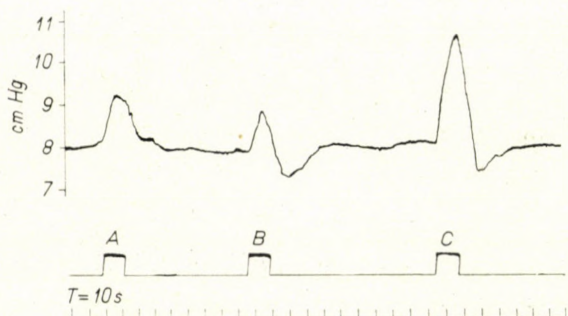

Fig. 8. Cat (chloralose + d-tubocurarine). Monopolar electrodes. A = stimulation of right intermediary hypothalamus, 100 Hz, 0.7 msec, 0.25 mA, 15 sec. B = stimulation of right mesencephalic reticular formation. 300 Hz, 0.15 msec, 0.1 mA, 15 sec. C = simultaneous stimulation of both points with the same parameters

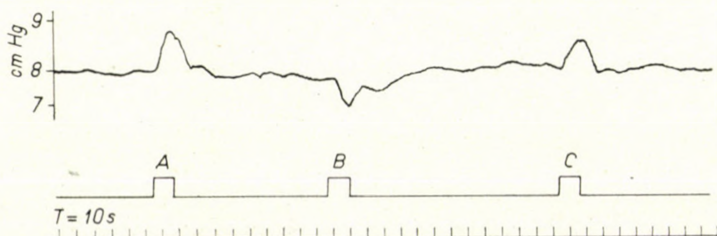

Fig. 9. Cat (chloralose + d-tubocurarine). Monopolar electrodes. A = stimulation of right intermediary hypothalamus. 100 Hz, 0.7 msec, 0.25 mA, 15 sec. B = stimulation of right mesencephalic reticular formation, 300 Hz, 0.03 msec, 0.1 mA, 15 sec. C = simultaneous stimulation of both points with the same parameters

In some cases, summation of reticular formation and hypothalamic stimulation exhibits particular features. For instance, stimulation of the hypothalamus occasionally induced a slight rise of arterial blood pressure, whereas stimulation of the reticular formation induced a consistent rise of arterial blood pressure followed by a hypotensive effect. The simultaneous stimulation of both nervous structures brought about a tensional rise equal to that induced by individual stimulation of the reticular formation, but this was not followed by a hypotensive effect. Likewise, in some instances, stimulation of the reticular formation had a hypotensive effect, and that of the hypothalamus a hypertensive one; during simultaneous stimulation one could well note the persistence of both effects without any alteration.

2. A simultaneous stimulation of the hypothalamus and midbrain reticular formation, occlusive phenomena may also take place. Thus, sometimes the hypertensive effect caused by stimulation of the reticular formation ceased completely during the simultaneous stimulation of the hypothalamus with liminar intensity (Fig. 10).

In other experiments, the hypothalamic hypertensive effect disappeared during simultaneous stimulation of a reticular point which induced a hypotensive effect when stimulated separately.

In other trials, simultaneous stimulation of both nervous areas resulted in an occlusive effect characterized by tensional responses approximately equal in magnitude with the responses to their separate stimulation.

C) In a third series of experiments, we studied the influence of liminar and supraliminar hypothalamic and reticular stimulation on the sinocarotic pressor reflex.

In most cases, concomitant liminar or supraliminar electric stimulation of the hypothalamus or reticular formation and bilateral occlusion of the common carotid arteries, brought about an increase of the sinocarotic pressor reflex (Figs. 11, 12, 13, 14).

In rare instances, electric stimulation of the hypothalamus or the reticular formation causing no liminar pressor effects, had no influence upon the sinocarotic pressor reflex or merely decreased its amplitude.

It appears that simultaneous stimulation of the hypothalamus and reticular formation may bring about a summation of their tensional effects. When investigating the influence of combined hypothalamic and reticular stimulation on the sinocarotid pressor reflex, this usually increased. Summation of tensional effects induced by concomitant stimulation of the hypothalamus and reticular formation does not, however, always implicate the increase of sinocarotic pressor reflex by separate stimulation of these centres, the phenomenon depending on the stimulation parameters. It must be mentioned that by concomitant stimulation of the hypothalamus and reticular formation during the sinocarotic pressor reflex, a summation of both tensional effects usually takes place.

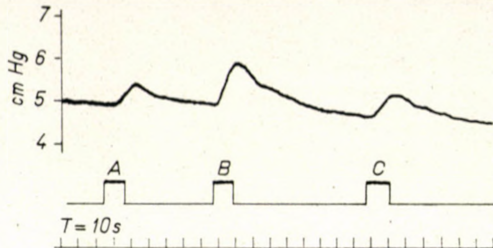

Fig. 10. Cat (chloralose + d-tubocurarine). Monopolar electrodes. *A* = stimulation of left intermediary hypothalamus. 100 Hz, 1 msec, 0.5 mA, 15 sec. *B* = stimulation of the left mesencephalic reticular formation. 300 Hz, 0.3 mesc., 0.3 mA, 15 sec. *C* = simultaneous stimulation of both points with the same parameters

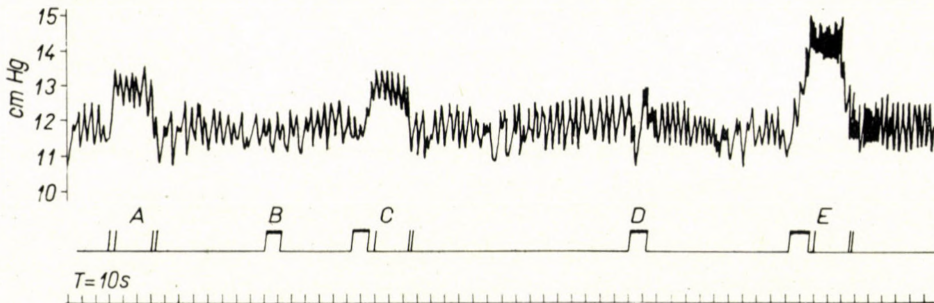

Fig. 11. Dog (chloralose). Monopolar electrodes. *A* = occlusion of common carotid arteries, 30 sec. *B* = stimulation of right intermediary hypothalamus. 100 Hz, 0.1 msec, 0.1 mA, 15 sec. *C* = separate stimulation of right intermediary hypothalamus (as in *B*), 15 sec, then together with occlusion of the common carotid arteries 30 sec. *D* = stimulation of right mesencephalic reticular formation, 60 Hz, 0.1 mA, 0.1 msec, 15 sec. *E* = separate stimulation of right mesencephalic reticular formation (as in *D*) 15 sec, then together with occlusion of the common carotid arteries, 30 sec

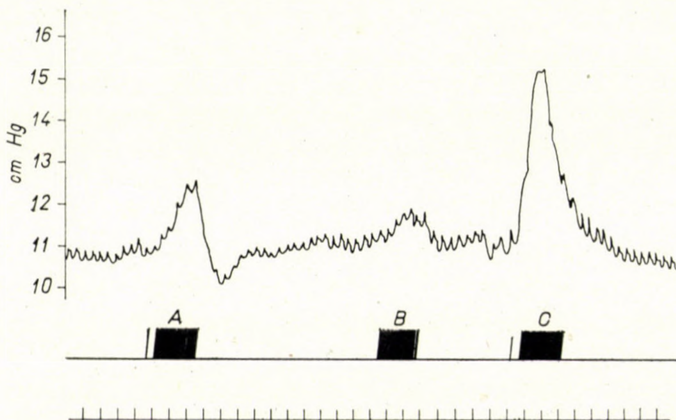

Fig. 12. Cat (chloralose). Bipolar electrodes. *A* = occlusion of common carotid arteries 20 sec. *B* = stimulation of left intermediary hypothalamus. 100 Hz, 0.03 msec, 0.27 mA, 20 sec. *C* = stimulation of left intermediary hypothalamus (as in *B*), together with occlusion of common carotid arteries 20 sec

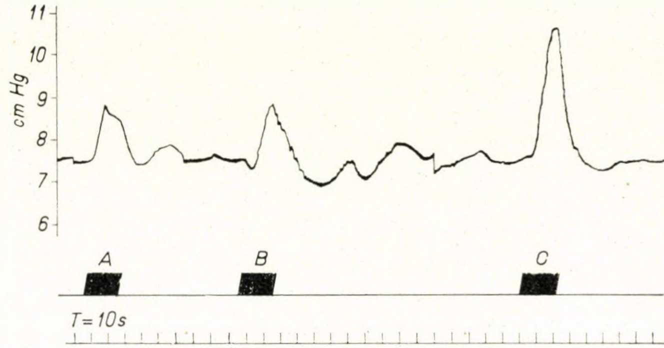

Fig. 13. Cat (chloralose + d-tubocurarine). Bipolar electrodes. *A* = stimulation of right mesencephalic reticular formation, 300 Hz, 0.3 msec, 0.3 mA, 20 sec. *B* = occlusion of common carotid arteries, 20 sec. *C* = stimulation of right mesencephalic reticular formation (as in *A*) together with occlusion of common carotid arteries, 20 sec

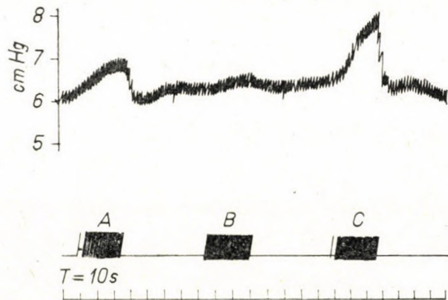

Fig. 14. Cat (chloralose + d-tubocurarine). Bipolar electrodes. *A* = occlusion of common carotid arteries, 30 sec. *B* = stimulation of right mesencephalic reticular formation, 70 Hz, 0.03 msec, 0.1 mA, 30 sec. *C* = stimulation of right mesencephalic reticular formation (as in *B*), together with occlusion of common carotid arteries, 30 sec

Gross and microscopic examination of the material showed that the points explored in the second and third series of experiments were grouped in the intermediary and anterior hypothalamus and in the nucleus reticularis pontis oralis, in the midbrain.

Discussion

In the terminology adopted by Sherrington for spinal reflexes, the above phenomena may be described as summation or occlusive phenomena. Due to the complexity of the structures stimulated in the present experiments, of which we ignore the elementary functional principles, the use of that terminology yields no information as to the fundamental mechanisms of the phenomenon. However, the investigations reported here have shown that simultaneous

stimulation of the central vasomotor projections leads to a reciprocal influence of the corresponding areas; at the same time, following stimulation of the upper structures in the neural axis a radiation of the excitation towards the subjacent structures takes place. The interpretation of these phenomena is difficult and it is a task of the future to demonstrate whether they have a physiological significance.

The tensional phenomena obtained are indicative of complex relations between the stimulated areas and raise the problem of the nature of the physiological mechanisms brought about by stimulation of the nervous areas under consideration.

a) Attention must be focussed first on the evidence that vasopressor points are sited throughout the entire hypothalamus. This finding is consistent with the view of other authors that the hypothalamus as a sympathetic station has functions which seem to conceal its parasympathetic activity. It may well be that the structures responsible for different reactional features which activate the sympathetic vasoconstrictive fibres would be disseminated throughout the hypothalamus, the specific activity depending not on the individual neurons but on the neural networks. Therefore most hypothalamic nuclei should not be considered as centres of specific function, the diversity of functions seeming to be relevant in the different combinations of firing neurons. The multiplicity of functions does not necessarily depend on the anatomical division; there may be comparatively few areas, but they have complex and close interconnections [27].

b) The occurrence of a pressor tensional effect after subliminar simultaneous stimulation of some points of the hypothalamus and midbrain reticular formation represents a very clear case of spatia lsummation. The bulbar mechanism by which the stimulation of the mentioned nervous areas acts was studied by simultaneous exploration of the sinocarotic pressor reflex. Under our experimental conditions, stimulation of the suprajacent nervous levels usually facilitates the pressor reflex of the lower effector station. It may well be that the hypothalamic stimulation which facilitates the sinocarotic bulbar reflex acts *via* the mesencephalic reticular formation, although the possibility of a direct action of bulbar level cannot be excluded. Bronk, Pitts, Larrabée [10] have demonstrated that hypothalamic activity may either increase or decrease the efficiency of the afferent impulses in the framework of the reflex regulation of the cardiovascular system; the works of Gellhorn [14] also support this view. Engelking and Willing [12] claimed that the state of the central sympathetic activity is dependent on the level of arterial blood pressure and that the presso-receptive afferences play a very important role. Likewise, Wilson *et al.* [37] state that the effect of hypothalamic stimulation is influenced by the hypotensive sinocarotic reflex induced at the same time; the depressive sinocarotic reflex abolishes the hypertensive effect of diencephalic stimulation.

Our present observations have demonstrated that hypothalamic stimulation alters the functional state of the bulbar level.

c) As seen above, the tensional pressor effect brought about by simultaneous stimulation of both nervous areas has the tendency of being inversely proportional with the individual tensional effects produced by the stimulation of each area. This fact suggests a more accurate superposition of the bulbar areas activated by the two suprajacent levels, as the intensity of stimulation increases.

Thus, as a greater number of descendent fibres is activated, their relative convergence towards the effector neurons increases.

d) Apart from the summation phenomenon, occasional occlusive phenomena were observed, the individual pressor effects brought about by stimulation of the hypothalamus and mesencephalic reticular formation being suppressed by simultaneous stimulation of the two areas. The occlusive phenomenon suggests that the bulbar area reached by the activated fibres does not merely represent a region of anatomical convergence, the messages of hypothalamic and mesencephalic projections being subjected here to an integrative control. On the other hand, some points of the hypothalamus and midbrain reticular formation may act as inhibitors at the bulbar level, decreasing the amplitude of the sinocarotic pressor reflex.

e) In the hypothalamus and mesencephalic reticular formation, points were found, the pressor effects of which do not influence one another, when simultaneousely stimulated. Likewise, there are points in both areas which do not influence the sinocarotic pressor reflex.

f) These findings suggest a differential activation of bulbar areas and prove the existence of subdivisions with precise functional significance of the bulbo-mesencephalic and bulbo-hypothalamic projections.

The study of the physiological responses induced in the mesencephalic reticular formation shows, in agreement with Hugelin and Bonvallet's findings [20], that the reticular formation does not increase the functional state at all the integrative levels. The reticular formation is organized functionally and does not seem to act without discrimination [33].

The various effects obtained in both areas could implicate, apart from the existence of functional subdivisions, that of positive and negative feedback effects, which are fired by stimulation and which have the role of reestablishing the initial level of activity.

g) The phasic effects obtained by artificial stimulation have a great physiological importance, as shown by the data demonstrating a tonic vasomotor activity at these integrative levels.

Thus, Sherrer [35] has shown that the stimulation of the hypothalamus may induce a phasic pressor response lasting longer than the stimulus, and Gellhorn [14] claimed the existence of a tonic activity of the hypothalamus.

This is also supplied by the great tensional fluctuations of thalamic animals as well as by the constant hypotension occurring after destruction of the hypothalamus or abolishment of experimental renal hypertension by hypothalamic damage [26, 34]. The tonic effect of the mesencephalic reticular formation upon the vasomotor nerves is well established [30]. As early as 1935, JAEGHER

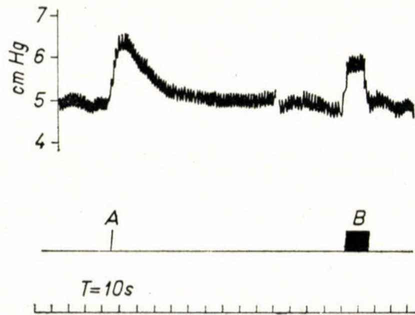

Fig. 15. Cat (chloralose + d-tubocurarine). $A = 200$ μg Ach are injected in the right intermediary hypothalamus. B = stimulation of the same point as in A with 200 Hz, 2 msec, 0.7 mA, 20 sec

and VAN BOGAERT [21, 22, 23] had shown that the electric stimulation of the cerebral penducle induces arterial hypertension even after removal of the hypothalamus. In view of the existence of vasomotor fibres which lead directly to the spinal cord, LINDGREEN [29] regards the mesencephalic reticular formation as the most caudal integrative vasomotor relay station. Likewise, it was shown that transection of the brain stem at the mesencephalic level induces a rise of arterial blood pressure [31].

A problem that has not been solved experimentally by electric stimulation of a nervous centre and especially a reticular area [9], is whether the effect obtained is due to the stimulation of the neurons of the respective area, or to that of by-passing fibres.

As regards the hypothalamus, van BOGAERT and JAEGHER [6, 7] attempted to dismiss this objection by chemical stimulation acting only the neurons. They injected ammonia, formaline and silver nitrate and obtained hypertensive effects from the posterior hypothalamus. BENETATO and MUNTEANU [3] showed that the electric stimulation of some hypothalamic nuclei releases an acetylcholine-like substance which is present in cerebral venous blood and, therefore, at least in some parts of that area, the synaptic transmission is cholinergic. Likewise, the hypothalamus is known to have a high cholinacetylase and cholinesterase content.

Acetylcholine does not activate the by-passing fibres of the region in which it is injected. Thus, an eventual suppressive effect induced by anti-

dromic impulses may also be avoided [36]. On the other hand, very small doses of acetylcholine prevent the propagation of the stimulation which cannot be inhibited by electric stimulation.

In spite of these advantages, stimulation of the nervous centres by local injections of acetylcholine cannot prevent a certain degree of injury to the nervous matter; furthermore, only large quantities of acetylcholine are effective in this respect and the fluid may propagate along the needle in the surrounding areas.

However, when acetylcholine injection induces a physiological effect similar to that produced by electric stimulation, it is undeniable that the respective area may be characterized as a nervous centre.

In the present investigations, identical tensional effects were induced by electric stimulation of the hypothalamus and the mesencephalic reticular formations as well as by their chemical stimulation with acetylcholine (Fig. 15).

Conclusions

1. Simultaneous stimulation of some pressor points of the motor cortex and the hypothalamus or anterior rhinencephalon, in dogs and cats, induces usually a phenomenon of spatial summation and rarely an effect of the occlusive type.

2. When the summation phenomenon is induced by tensional effects of various intensity, it had a linear character, its correlation coefficient being calculated in terms of the nervous areas explored.

3. Simultaneous stimulation of the hypothalamus and mesencephalic reticular formation usually brings about tensional effects of summation, and occasionally occlusive or independent effects.

4. Stimulation of these areas usually facilitates the sinocarotic pressor reflex and, in rare cases, hinders or even fails to influence it.

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E. Bittman, N. Raiciulescu
"D. Danielopolu" Institute of Normal and Pathological Physiology, Academy of Sciences, Bucharest, Rumania

Recensio

Methods in Experimental Medicine

Editor, A. G. B. Kovách. Volume VI

Publishing House of the Hungarian Academy of Sciences, Budapest 1962
1099 pages, 339 illustrations, many in colour, 5 appendices

The rapid advance of experimental medicine and the diversity of the methods employed have made it necessary to publish a series of volumes comprising in easily readable form the most important methods of examination applied in physiology, biochemistry, pathophysiology and pharmacology. The authors of the single chapters have been using the pertaining methods for many years, and so could add to the original description their own experience.

Volume VI, now published, begins with a chapter written by J. Knoll, on the methode of studying pharmacological effects on the nervous system. The effects of local anaesthetics, the drugs acting on the neuronmuscular junction, oblong medulla, autonomic ganglia, the parasympathetic and sympathetic stimulants and depressants, the spasmogenics and spasmolytics, as well as the analgesics, are described in detail. The chapter ends with a discussion on the methods used in psychopharmacology.

The next chapter is the most extensive and important one in this volume (pages 269 to 965). In it J. Szentágothai and O. Koref deal with the methods of experimental endocrinology. After an introductory part on experimental animal strains, feeding and microclimatic conditions, they discuss in detail the anatomy of the hypothalamic-pituitary system, the surgical methods used, the methods of morphological evaluation, as well as the quantitative estimation of the hormones of the anterior and intermediate lobes of the pituitary, and the procedures applied for studying the neurohypophysis and hypothalamus. The methods, both biological and biochemical, employed in studies on the thyroid (Takács—Mess), adrenals (Antal—Endrőczi—Erdélyi—Halász—Kovách—Monos), islets of Langerhans (Koref—Mess), the male (Mess) and female gonads and adnexa (Flerkó) are discussed in considerable detail. The methods of studying epiphyseal, parathyroid and thymic activity are also described in this chapter. The list of pertaining references is presented after every section. Having read this chapter, the reviewer feels that it ought to be translated to some world language as an independent monograph; it would be welcome by every endocrinological laboratory.

Chapter 3, written by G. Inke and M. Palkovits, deals with the anatomy of the circulatory system of experimental animals. The well-constructed diagrams and nicely executed drawings will be most instructive for research workers.

S. Mányai

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ACTA PHYSIOLOGICA

TOMUS XXIV — ВЫП. 1.

РЕЗЮМЕ

ОПРЕДЕЛЕНИЕ ЭЛАСТАЗЫ И ЭЛАСТАЗНОГО ИНГИБИТОРА ПРИ ПОМОЩИ ОРЦЕИН-ЭЛАСТИНА

И. БАНГА

Автором разработана методика определения эластазы и эластазного ингибитора при помощи препарированного орцеин-эластина. Эластазный ингибитор можно определить также в присутствии эластазы, поскольку ингибитор преобладает в системе по отношению к эластазе.

Метод автора имеет большое значение по той причине, что он предоставляет возможность для измерения содержания эластазного ингибитора в поджелудочной железе человека. Поджелудочная железа людей, умерших от артериосклероза, не содержит эластазу. При помощи описанного метода становится возможным количественное определение избыточного «свободного» эластазного ингибитора.

СИНТЕЗ БЕЛКОВ В СЕМЕННОМ ПУЗЫРЬКЕ КРЫС I. БЫСТРО МЕЧАЮЩИЕСЯ БЕЛКИ В ФРАКЦИИ РИБОНУКЛЕИНОВЫХ КИСЛОТ

Ш. МАНЬАИ

Из $HClO_4$-ого преципитата семенного пузырька крыс при кипячении в 10%-ном растворе поваренной соли вместе с рибонуклеиновыми кислотами можно экстрагировать также значительное количество вещества белковой природы, которое как **in vivo** так и **in vitro** интенсивно метится радиоактивными аминокислотами. Автор доказал, что большая активность ^{14}C наблюдается во фракции с большим молекулярным весом, которая не диализирует, дает положительную биуретовую реакцию и реагирует с реактивом Фолина. Эту фракцию можно отделить от рибонуклеиновой кислоты при помощи метода хроматографической адсорбции на колонке DEAE-целлюлозы. Хлористоводородный гидролизат этой фракции дает смесь аминокислот, в которой глицин и серин содержат атом ^{14}C. Радиоактивный глицин находится внутри полипептидной цепи этого белка в полипептидной связи.

Экстрагированная вместе с рибонуклеиновой кислотой, быстро синтетизирующаяся белковая фракция характерная для семенного пузырька, равным образом обнаруживается в семенном пузырьке крыс, мышей и морских свинок.

ИССЛЕДОВАНИЕ СУЛЬФГИДРИЛЬНЫХ ГРУПП ДЕГИДРОГЕНАЗЫ МОЛОЧНОЙ КИСЛОТЫ СКЕЛЕТНЫХ МЫШЦ

ДЬ. ЙЕЧАИ и П. ЭЛЁДИ

Авторы изолировали разработанным ими методом дегидрогеназу молочной кислоты из скелетной мышцы свиней, содержащую по результатам титрования с п-хлормеркурибензоатом 19—20 сульфгидрильных групп.

В нативном состоянии, в нейтральной среде, SH-группы дегидрогеназы молочной кислоты скелетной мышцы не являются реактивными, они не реагируют с п-хлормеркурибензоатом. Сульфгидрильные группы удалось выявить только в щелочной среде, или после

денатурации белков (мочевина, действие детергента). По величинам вязкости щелочная среда изменяет третичную структуру белков.

В реакции лактат-дифосфопиридиннуклеотид, при pH = 10,0, блоккирование 20 SH-групп задерживает ферментативную активность только прибл. на 50%.

Следовательно, по результатам исследований роль сульфгидрильных групп в ферментативной активности дегидрогеназы молочной кислоты, изолированной из *скелетной мышцы* свиней, еще не доказана, в противоположность дегидрогеназе молочной кислоты, изолированной из *сердечной мышцы* свиней, где для ферментативной активности необходимо присутствие SH-групп.

ОЧИСТКА И СВОЙСТВА ЭКЗОПЕНИЦИЛЛИНАЗЫ **Bacillus subtilis**

МАКОТО ИШИМОТО

Очистка экзопенициллиназы **Bacillus subtilis** проводилась методом хроматографии на карбоксиметил-целлюлозе. Оптимальный pH и сопротивление против инактивации йодом показали больше сходства с экзопенициллиназой **Bacillus cereus**, чем с пенициллиназой, связанной с клетками **Bacillus subtilis.** Противосыворотка, изготовленная с помощью экзопенициллиназы **Bacillus subtilis** вызывала осаждение как этого энзима, так и экзопенициллиназы **Bacillus cereus.** Результаты проб по всасыванию показали что одно и то же антитело действовало на оба энзима.

ЭНЗИМАТИЧЕСКОЕ ФОРМИРОВАНИЕ ДИСУЛЬФИДНЫХ МОСТИКОВ РИБОНУКЛЕАЗЫ

П. ВЕНЕЦИАНЕР, и Ф. Б. ШТРАУБ

Авторы показали, что в поджелудочной железе различных видов животных присутствует энзим, который способен катализировать реактивирование восстановленной рибонуклеазы панкреаса крупного рогатого скота.

Произведено частичное очищение данного энзима из поджелудочной железы цыплят и свиньи.

Для активности энзима необходимо и присутствие термостабильного фактора. Последнее вещество оказалось заменимым дегидроаскорбиновой кислотой.

В дискуссии обсуждено возможное значение полученных результатов в области синтеза белков.

ИССЛЕДОВАНИЕ ДВОЙНОЙ ИННЕРВАЦИИ В РЕГУЛИРОВАНИИ ТОНУСА СФИНКТЕРА У ПРУДОВОЙ БЕЗЗУБКИ (ANODONTA CYGNEA L.)

Я. ШАЛАНКИ и Э. ЛАБОШ

Авторы исследовали ответные реакции заднего сфинктера, состоящего из волокон гладких мышц, у прудовой беззубки (**Anodonta cygnea L.**), при электрическом раздражени приводящего нерва (**connectivum cerebro-visceralis**). Далее они исследовали акционные потенциалы, получаемые от изолированного **connectivum cerebro-visceralis**, при применении отдельных импульсов.

При изменении параметров раздражения (напряжение, частота, амплитуда импульсов, продолжительность раздражения) в ответных реакциях мышцы отмечались значительные отклонения. В случае неизменных прочих параметров низкое напряжение (ниже 1—2 в) вызывало только сокращение. Более высокое напряжение (выше 2—5 в) вызывало также значительное расслабление после начального сокращения, или расслабление сфинктера, находящегося в состоянии длительного тонического сокращения. Одинаковый с действием низкого напряжения эффект вызывала слишком низкая и чрезмерно высокая (ниже 3—5/сек или выше 20—60/сек) частота, слишком короткие или слишком длинные (ниже 3 мсек и выше 80 мсек) импульсы, далее применение короткого времени (ниже 2—4 сек) раздражения. Подобное эффекту высокого напряжения действие имело раз-

дражение с частотой 5—20 импульсов в сек, далее серия импульсов в 4—100 мсек, и применение времени раздражения выше 20 сек.

Акционный потенциал, отведенный от изолированного **connectivum cerebro-visceralis**, при раздражении отдельными импульсами, состоит из нескольких, хорошо дифференцируемых компонентов, два основных компонентов которых можно ясно обособлять друг от друга путем изменения параметров.

На основании проведенных опытов авторы придерживаются того мнения, что сфинктер обладает двойной, антагонистической иннервацией, причем как для сокращения сфинктера так и для расслабления находящейся в тоническом состоянии мышцы имеется специальная иннервация. В соответствии с этим расслабление сфинктера, находящегося в тоническом состоянии, не представляет собой пассивный процесс, а возникает в результате активного неврального воздействия.

ДЕЙСТВИЕ РАЗДРАЖЕНИЯ РАЗЛИЧНЫХ СТРУКТУР ГИПОТАЛАМУСА И СТВОЛА МОЗГА НА ФУНКЦИЮ СИСТЕМЫ ГИПОФИЗ-КОРА НАДПОЧЕЧНИКОВ

Э. ЭНДРЁЦИ и К. ЛИШШАК

В опытах на кошках после раздражения, при помощи хронически вправленных электродов, различных структур промежуточного и среднего мозга, исследовалось содержание кортикоидов в венозной крови надпочечников. Установлено, что раздражение **septum basalis**, антеро-латерального гипоталамуса и латерального гипоталамуса задерживает секреторную функцию коры надпочечников. При раздражении **nucleus supraopticus** и **n. paraventricularis** не отмечалось существенного изменения. Выраженное повышение секреции наблюдалось при раздражении **posterior tuber, regio premamillaris**, сетчатого образования среднего мозга и **tegmentum ventralis**. Торможение наблюдалось при раздражении **tegmentum dorsalis**, проведенном на уровне **colliculus superior**. Дается краткое обсуждение структурных связей, участвующих в центральной регуляции системы гипофиз-кора надпочечников.

ИССЛЕДОВАНИЕ СВЯЗИ МЕЖДУ АКТИВНОСТЬЮ УСЛОВНОГО ОБОРНОИТЕЛЬНОГО РЕФЛЕКСА И ФУНКЦИЕЙ СИСТЕМЫ ГИПОФИЗ-КОРА НАДПОЧЕЧНИКОВ

Б. БОХУШ, Э. ЭНДРЁЦИ и К. ЛИШШАК

При индивидуальном исследовании условного оборонительного рефлекса и функции коры надпочечников у крыс, можно наблюдать, что активность условного оборонительного рефлекса повышена у животных с более высокой активностью секреторной функции кортикостероидов и понижена у животных с низкой активностью секреторной функции кортикостероидов.

ДАЛЬНЕЙШИЕ ДАННЫЕ К ПОЛОВЫМ РАЗЛИЧИЯМ СИСТЕМЫ ГИПОФИЗ КОРА НАДПОЧЕЧНИКОВ У КРЫС

Б. БОХУШ, Э. ЭНДРЁЦИ и К. ЛИШШАК

Степень компенсаторной гипертрофии у 27—29 дневных и у 37—39 дневных крыс самцов и самок одинаковая, в то время как у 52—56 дневных животных степень компенсаторной гипертрофии самок значительно больше.

Синтез кортикоидов **in vitro** гипертрофированными надпочечниками постепенно повышается с возрастом, у 52—56 дневных самок продукция уже больше чем у самцов.

После кастрации, проведенной до пубертатного возраста, у 107 дневных крыс

самок степень компенсаторной гипертрофии надпочечников, наступающей после удаления контралатерального надпочечника на 100. день после рождения, значительно снижается, в то время как у самцов остается неизменной по сравнению с контрольными животными, у которых проводилась ложная операция.

Дача эстрон-бензоата до наступления половой зрелости вызывает у взрослых самцов повышение компенсаторной гипертрофии, в то время как у самок не наблюдается изменения. Введение тестостерона до пубертатного возраста остается без эффекта у обоих полов.

Под влиянием дачи эстрогена до наступления половой зрелости секреция кортикостерона компенсаторно гипертрофированными левыми надпочечниками у взрослых животных повышается как у самок, так и у самцов, в то время как после удаления яичников, проведенного на 22—25. день после рождения, у взрослых животных она уменьшается.

На основании результатов исследований, наблюдаемая у самок зрелых крыс большая компенсаторная гипертрофия наступает в период полового созревания под влиянием эстрогена.

РАЗГРАНИЧЕНИЕ МОТОРНОЙ КОРЫ ГОЛОВНОГО МОЗГА КОШКИ В ОБЛАСТИ GYRUS SIGMOIDEUS POSTERIOR

В. ГОЛДА, Ю. ПЕТРЕК, П. ЛИСОНЕК

В работе потверждают авторы мнение В. М. Светухины, что пределом между моторной и сенсорной корой головного мозга кошки в области **g. sigmoideus posterior** является **sulcus postcruciatus.** Сверх того устанавливают, что разным цитоархитектоническим областям в **g. sigmoideus posterior** соответствуют вызванные потенциалы с различным скрытым периодом положительной фазы. Эта положительная фаза вызванного потенциала имеет при стимуляции кожной и мышечной ветвей скрытый период в области между **s. cruciatus** и **s. postcruciatus** значительно длиннее чем в области, которая находится окципитально за **s. postcruciatus.** У некоторых препаратов авторы регистрировали в месте **s. postcruciatus** особую форму вызванного потенциала.

ЯВЛЕНИЕ СУММИРОВАНИЯ ДЕЙСТВИЯ В АВТОНОМНОЙ НЕРВНОЙ СИСТЕМЕ

Э. БИТТМАН и Н. РАЙЧУЛЕСКУ

Проводились острые эксперименты на 16 собаках и 27 кошках (анестезия курарили хлоралозом) в целях исследования кровяного давления, наблюдаемого при одновременной активации центральных вегетативных отростков, участвующих в регуляции артериального кровяного давления. Таким образом, кроме исследования некоторых изменений, имеющих место при одновременной активации корковых и гипоталамических вазомоторных отростков, изучалась также связь между гипоталамусом и сетчатым образованием среднего мозга. В качестве индикатора использовались фазовые изменения артериального кровяного давления, вызванные электрическим раздражением указанных двух нервных центров. Исследовался также эффект стимулирования гипоталамуса и сетчатого образования среднего мозга на синокаротидные прессорецепторные рефлексы, вызванные билатеральной окклюзией общей сонной артерии.

Результаты опытов показали, что одновременное стимулирование определенных прессорных точек двигательной коры, гипоталамуса или переднего обонятельного мозга у собак и кошек вызывает, как правило, явление пространственной суммации, и только в редких случаях эффект окклюзионного типа. Если явление суммации вызывалось действиями различной величины, то оно имело линейный характер, так как коэффициент корреляции был исчислен в отношении исследованных нервных центров. Одновременное раздражение гипоталамуса и сетчатой формации среднего мозга как правило вызывает суммированное действие на кровяное давление, и только изредка эффекты оклюзионного или самостоятельного типа. В общем, стимулирование этих областей имеет смягчающее действие на синокаротидный прессорный рефлекс, но в редких случаях, оно тормозит или не изменяет его.

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Biophysica

MOBILIZATION OF FLUIDS IN BIOLOGICAL OBJECTS BY MEANS OF TEMPERATURE GRADIENT

By

F. VETŐ

INSTITUTE OF BIOPHYSICS, MEDICAL UNIVERSITY, PÉCS, HUNGARY

(Received March 16, 1962)

Temperature gradients of 0.2—3.0° C/mm have given rise to significant differences in the water content of different vegetable tissues (apple, potato, *etc.*), so that, after the lapse of ¼ to 95 hours, the amount of water contained in the cooler part of the tissues was a few per cents higher than that in the warmer portion. It follows that — as in thermo-osmotic model experiments — a higher temperature is capable of driving water to spaces of lower temperature in biological systems. Thermal action may therefore be involved in the mobilization of cellular fluids, a problem of biological significance for the interpretation of "active" discharge of water by cells.

Introduction

The possibility that heat into which part of the energy inherent in the ingested food is converted, need not be regarded as a vaste product useless for the organism (as is still stated in textbooks of physiology [1]) but under adequate circumstances and in adequate structures it is able to perform work, as it had been suggested by ERNST [2, 3] in 1929 already. Experiments initiated by KOHNSTAMM in 1911 [4] and elaborated by ERNST and his associates [5—14] have shown that, if the temperature gradient was sufficient, warmer and more concentrated solutions (*i.e.* those with higher vapour pressure) were losing water through semi-permeable membranes to cooler and more dilute solutions, *i.e.* those with lower vapour pressure.

Heat may facilitate concentration work also by way of thermodiffusion, and capillary thermodiffusion [15], an aspect of the problem that is outside the scope of the present study.

Conditions favouring thermo-osmosis are given in the living organism, semi-permeable membranes as well as temperature gradients exist both intra- and intercellularly. A number of hitherto incomprehensible biological phenomena become clear if interpreted in terms of thermo-osmosis. It explains why cells are able to mobilize water against a drop of concentration, *e.g.* when pulsating vacuoles are filled [16—19]; how root pressure arises [20]; how the salivary gland is able to produce hypotonic secretion when pressure in the efferent duct is higher than in the artery [21]; how is regulated osmosis in fresh-water animals [22]; through what mechanism the cells are capable of "active" water discharge [23]. All these problems are clearly defined in the

works of DONHOFFER [24] and NETTER [25]. The concept of thermo-osmosis facilitates their solution.

We have failed to find data in the available literature concerning the question whether water in biological tissues can be mobilized by temperature gradients on the analogy of model experiments. It was only in GLASSER's work [26] that — at a time when our experiments were in progress — we found some reference to thermo-osmosis having been observed in certain vegetable substances. As regards the theoretical part of the problem, the thermodynamics of irreversible processes, the works of DENBIGH [27], DE GROOT [28] and PRIGOGINE [29] supply information on the energetistic intricacies of such processes.

Material and method

Easily procurable storing organs of plants were used. Vegetable was preferred to animal tissue because biological conditions are simpler in plants; osmotically, the cells of plants are nearer to the model than those of animals. We carried out three groups of experiments

1. on slices of plant tissue,
2. on models (controls),
3. on unimpaired organs of plants.

ad 1. The slices were cooled on one and heated on the other side. This done, changes in water content were measured. Disks measuring from 1 to 3 cm in thickness were cut from specimens of different fruits (apple, potato, carrot, kohlrabi, pumpkin, cucumber and onion).

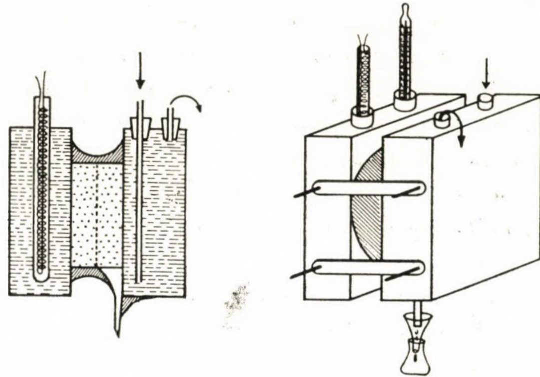

Fig. 1. For explanation see text

After measuring their weight, we placed the disks between two nickel-plated copper vessels with smoothly polished surface, being careful not to leave air between the cut surface and the vessel wall. The disks were then sealed hermetically by means of glazier's putty. Seeing that fluid was accumulating between the plant and the vessel on the cooled side, we mounted in later experiments a small glass funnel on this side likewise by means of putty (Fig. 1). Tap water (15 to 20° C) was then caused to flow through one of the vessels by way of cooling, while an electric heater was placed in the water contained in the second vessel, so that its temperature rose to approximately 40° C. The fluid trickling from the cool side was so collected as to avoid evaporation as far as possible. After an interval that varied from ¼ to 95 hours, the whole device was dismounted and the disks were cut in two, along a plane parallel to the original section. They were then weighed, minced, dried at 110° C until constant weight

and then the percentual water content of both the cooled and the heated portion was determined, the quantity and the freezing-point depression of the escaped fluid were determined. The original water content of the tissue was measured in another, untreated slice.

ad 2. The model experiments were done in a similar way. 2 to 8 per cent aqueous solution of agar-agar gel or agar-agar gel made with a 3.5 per cent solution of cane sugar was used instead of plant. The warm sol was poured into metal or plexiglass rings, left to solidify, and the contrivance was mounted in the usual way. At the end of the experiment the disks were cut in two, and the water content was determined on both the cooled and the heated side. In another group of experiments each of the plexiglass rings was filled with three superposed disks of synthetic sponge saturated with 3.5 per cent saccharose solution. At the end of the experiment, the solution was squeezed out of the disks, and the concentration change was determined by means of a refractometer.

ad 3. Unimpared whole tubers of potato were used in these experiments. We wrapped them in thin rubber pouches so as to prevent uptake of water, and placed the tubers on a

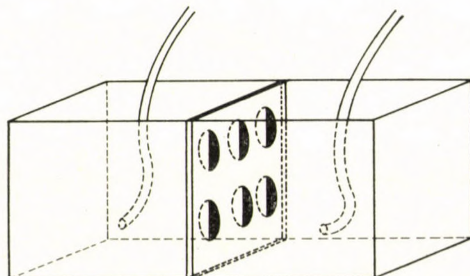

Fig. 2. For explanation see text

plexiglass plate provided with adequate apertures. After fixing the tubers with putty, the plate was so placed in a glass container as to divide the latter into two compartments (Fig. 2). The plate was fixed in the container likewise by means of putty, whereafter one side of the tubers was heated, the other cooled by flowing water (40°C and 17°C, respectively). Six tubers of potato were used in each experiment. The mean water content of the warmest and that of the coolest parts was determined at intervals ranging from 4 to 95 hours.

The results were evaluated statistically by means of the "*t*" test; differences were regarded significant if $p < 0.05$.

Results

1*a)* Altogether 67 experiments were made with slices of apple. The detailed results are shown in Table I. The quantity of water was invariably more in the cooled parts. The difference in the water content of the two parts ranged from 0.2 to 20.9 per cent, with a mean of 4.7 per cent. The water that invariably escaped on the cooler side has been neglected in these figures. The amount of the escaped water varied from 0.1 to 8.0 ml, with a mean value of 1.9 ml. The values of the freezing-point depression of this fluid were significantly different according to the direction in which the slices had been cut. The value was 0.72° C for juice trickling out of slices sectioned in a plane parallel, and 0.38° C for that from slices sectioned in a plane perpendicular, to that of the apple core, and 1.54° C for that obtained by squeezing the apple.

We furthermore ascertained whether there existed a correlation between the temperature gradient and the difference in water contents.

Table I

No.	Duration of experiment, hrs,	Temp. grad. C°/mm	Amount of fluid escaped, ml	Percentage of water content on cooled side		Water content on heated side	Percentage difference in water contents
				without	with*		
				escaped fluid			
1.	22	3.0	2.8	89.0	91.4	81.5	7.5
2.	23	1.0	0.1	90.8	90.9	86.9	3.9
3.	17	1.0	0.1	91.0	91.1	85.2	5.8
4.	5	3.0	1.0	92.2	92.8	88.8	3.4
5.	21	1.5	?	92.5	?	85.8	6.7
6.	24	1.0	?	91.8	?	84.6	7.2
7.	16	0.7	3.2	88.7	90.5	84.0	4.7
8.	16	2.0	1.0	90.5	91.0	85.4	5.1
9.	16	2.0	3.0	91.8	92.6	84.0	7.8
10.	18	2.0	8.0	90.5	94.3	84.3	6.2
11.	18	1.0	0.1	91.5	91.6	87.0	4.5
12.	18	1.0	5.0	91.5	92.9	87.5	4.0
13.	18	2.0	2.5	89.6	90.9	85.7	3.9
14.	18	1.0	2.5	90.6	91.7	89.2	1.4
15.	18	1.0	1.0	91.0	91.5	88.6	2.4
16.	21	1.0	1.6	91.1	94.0	87.4	3.7
17.	21	1.0	0.6	92.4	92.7	88.5	3.9
18.	22	1.0	6.5	89.0	91.2	80.4	8.6
19.	22	1.0	2.1	89.6	91.3	84.3	5.3
20.	22	0.5	2.8	90.3	91.2	89.0	1.3
21.	23	1.0	1.1	92.2	92.4	87.2	5.0
22.	23	1.0	1.2	92.6	92.8	90.3	2.3
23.	23	1.0	3.6	89.7	91.7	88.7	1.0
24.	19	1.0	2.0	92.0	92.6	88.7	3.3
25.	19	1.0	1.8	91.2	91.7	87.7	3.5
26.	19	0.9	1.6	90.3	90.9	83.7	6.6
27.	22	0.8	1.8	90.7	91.0	88.7	2.0
28.	22	0.6	1.6	92.8	92.9	90.0	2.8
29.	22	0.6	0.6	90.8	91.1	88.3	2.5
30.	26	1.0	3.5	90.4	92.0	88.2	2.2
31.	26	0.9	5.5	90.8	92.6	87.8	3.0
32.	26	0.8	1.1	92.0	94.6	89.4	2.6
33.	22	1.0	1.8	93.8	94.4	90.0	3.8
34.	22	0.7	2.0	91.5	92.1	88.0	3.5
35.	22	0.7	0.4	92.1	92.2	91.9	0.2
36.	21	1.0	0.8	92.3	92.5	88.9	3.4

No.	Duration of experiment, hrs.	Temp. grad. C°/mm	Amount of fluid escaped ml	Percentage of water content on cooled side		Water content on heated side	Percentage difference in water contents
				without escaped fluid	with* escaped fluid		
37.	21	0.8	1.1	92.0	92.3	87.8	4.2
38.	21	0.8	0.8	92.6	92.7	90.0	2.6
39.	18	1.0	3.7	91.9	92.7	89.5	2.4
40.	18	0.7	0.8	91.3	91.4	87.3	4.0
41.	18	0.7	2.1	92.7	93.1	89.0	3.7
42.	19	1.0	0.8	91.7	91.9	89.6	2.1
43.	19	0.7	0.6	92.0	92.1	90.2	1.8
44.	19	0.6	0.3	92.0	92.2	90.4	1.6
45.	23	0.8	4.2	91.0	92.2	88.0	3.0
46.	23	0.8	2.0	92.6	93.4	88.4	4.2
47.	23	0.7	1.4	92.6	92.8	88.7	3.9
48.	23	1.0	1.2	91.6	92.0	85.7	5.9
49.	23	0.9	3.8	90.6	92.6	87.9	2.7
50.	23	0.9	0.2	90.6	90.8	88.6	2.0
51.	22	1.3	4.1	91.4	92.5	87.1	4.3
52.	22	0.9	1.8	90.0	90.6	85.0	5.0
53.	22	0.8	1.4	92.2	92.5	88.6	3.6
54.	69	0.2	0.6	89.3	89.7	81.2	8.1
55.	69	0.2	2.8	86.9	89.0	79.3	7.6
56.	69	0.2	0.4	90.5	91.0	82.7	7.8
57.	43	0.2	3.7	87.4	91.5	77.9	9.5
58.	43	0.3	3.4	86.1	88.2	80.1	6.0
59.	43	0.3	1.4	95.0	95.5	74.1	20.9
60.	47	0.2	0.1	91.9	91.9	79.3	12.6
61.	47	0.3	1.4	90.3	91.2	83.6	6.7
62.	41	0.3	1.9	87.7	89.1	84.5	3.2
63.	41	0.2	1.3	91.7	92.4	84.9	6.8
64.	41	0.3	0.5	88.7	88.9	84.5	4.2
65.	43	0.3	0.5	89.5	89.7	83.7	5.8
66.	43	0.2	0.4	89.6	89.8	86.8	2.8
67.	43	0.3	0.8	90.0	90.4	85.1	4.9

* i.e. the escaped fluid regarded as water to be added to the water contents of the cooled side.

Of course, we took only those experiments into account in which all the parameters but the temperature gradient were identical. If the amount of escaped water is not neglected (a fully justified procedure), the coefficient of correlation amounts to $+0.58 \pm 0.14$.

Table II

No.	Duration of experiment hrs.	Temperature gradients C°/mm	Amount of fluid escaped ml	Percentage of water content on cooled side with escaped fluid*	Water content on heated side	Percentage difference in water contents
1.	16	3.0	0	71.5	70.4	+1.1
2.	9	1.0	0	70.8	70.9	−0.1
3.	0.5	1.0	0	74.2	74.2	0
4.	1	1.0	0	74.2	74.5	−0.3
5.	21	3.0	2.5	69.0	65.0	+4.0
6.	2	3.0	0	75.0	74.1	+0.9
7.	2	3.0	0	68.5	70.2	−1.7
8.	0.25	3.0	0	71.0	71.4	−0.4
9.	3.5	3.0	1 drop	73.0	71.4	+1.6
10.	18	1.5	0	71.6	69.8	+1.8
11.	26	1.0	0	77.4	77.2	+0.2
12.	23	1.0	0	75.2	71.0	+4.2
13.	7.5	2.0	0	75.4	74.4	+1.0
14.	17	2.0	0	73.3	68.5	+4.8
15.	22	3.0	0	67.5	67.0	+0.5
16.	22	1.0	0	70.7	69.0	+1.7
17.	22	1.0	0	73.5	71.8	+1.7
18.	23	1.5	0	73.8	70.6	+3.2
19.	23	1.0	2.2	71.2	70.5	+0.7
20.	22	2.0	0	73.3	69.5	+3.8
21.	44	1.0	0	77.0	75.7	+1.3
22.	44	0.8	0.3	78.9	77.5	+1.4
23.	64	0.8	0	75.5	75.7	−0.2
24.	39	1.0	0	79.4	79.1	+0.3
25.	39	0.9	0	79.4	78.5	+0.9
26.	47	1.0	0	77.7	78.9	−1.2
27.	47	1.0	0.2	78.8	78.3	+0.5
28.	47	1.0	0.2	76.6	76.5	+0.1
29.	42	0.8	0	78.9	77.7	+1.2
30.	42	1.0	0.6	79.0	79.2	−0.2
31.	42	0.6	1.5	80.8	80.5	+0.3
32.	42	0.6	1.0	77.8	78.5	−0.7
33.	91	0.6	0	74.7	72.6	+2.1
34.	91	0.6	0	74.6	74.6	0
35.	91	0.8	0	76.1	72.5	+3.6

* i.e. the escaped fluid regarded as water to be added to the water contents of the cooled side.

The effect varied also according to variety. The difference between the cool and warm side amounted to 5.4 per cent in one (Jonathan), to 3.1 per cent in another (Rozmaring) variety of apple.

1b) A total of 35 experiments was made with slices of potato (Table II). The cooler side contained more water (including water that had trickled out) in 25 cases, there was no change in 2 cases, and the warmer side contained more

Table III

No.	Duration of experiment hrs.	Temperature gradients C°/mm	Amount of fluid escaped ml	Percentage of water content on cooled side with escaped fluid*	Water content on heated side	Percentage difference in water contents
1.	71	0.5	0.6	90.2	89.8	+0.4
2.	71	0.5	2.6	90.9	89.8	+1.1
3.	71	0.5	0.4	90.5	90.4	+0.1
4.	23	0.8	0.0	89.5	87.6	+1.9
5.	23	1.0	1.0	90.0	90.2	−0.2
6.	23	0.8	0.6	88.9	88.1	+0.8
7.	45	0.9	2.0	88.8	88.4	+0.4
8.	45	0.9	0.8	89.4	84.7	+4.7
9.	45	1.2	0.9	89.6	87.8	+1.8
10.	72	0.7	1.0	87.2	86.8	+0.4
11.	72	0.9	0.2	89.8	83.7	+6.1
12.	72	0.8	0.2	85.4	80.9	+4.5
13.	43	0.8	1.8	91.7	90.4	+1.3
14.	43	1.0	2.2	91.7	91.3	+0.4
15.	43	0.9	3.6	91.6	90.8	+0.8
16.	44	0.7	0.1	90.9	91.5	−0.6
17.	44	0.9	0.2	91.7	88.7	+3.0
18.	44	0.9	0.1	92.0	91.4	+0.6
19.	28	0.8	0.0	89.5	90.2	−0.7
20.	28	1.0	0.0	90.0	89.9	+0.1
21.	28	1.0	0.0	89.9	89.5	+0.4
22.	94	0.6	2.0	90.0	92.9	−2.9
23.	94	0.8	0.2	90.5	90.7	−0.2
24.	94	0.8	1.0	89.2	91.4	−2.2
25.	56	0.7	0.5	85.5	83.8	+1.7
26.	56	0.6	0.3	85.0	83.7	+1.3
27.	56	0.8	0.5	87.2	87.2	0.0

* i.e. the escaped fluid regarded as water to be added to the water contents of the cooled side.

water in 8 cases. On the average, the cool side contained 1.1 per cent more water than the warm side. Water escaped only in 9 cases, with a maximum of 2.5 ml.

1*c)* Twenty-seven experiments were made with slices of carrot (Table III). The cooler side contained more water (including water that had trickled out) in 20 cases, there was no change in one case, and the warmer side contained more water in 6 cases. On the average, the cool side contained 0.9 per cent more water than the warm side. Fluid escaped in all but 4 cases. The maximum was 3.6 ml, with a mean value of 0.8 ml. The freezing-point depression amounted to 0.20° C.

1*d)* A total of 9 experiments was made with slices of pumpkin. There was more water in the cooled parts in all cases; minimum, 0.4 per cent; maximum, 2.6 per cent; mean 1.7 per cent. Fluid (0.1 to 4.2 ml) escaped in all cases.

1*e)* Six experiments, each, were made with kohlrabi, cucumber and onion. It was practically always the cool side which contained more water. The number of experiments was too low to admit of statistical evaluation.

2. Altogether 14 experiments were made with agar-agar gel. We observed no difference in the water contents of the two sides, nor did water escape in any case. Synthetic sponge was used in 7 experiments. According to the refractometer, the sugar solution was significantly more diluted on the cool side.

3. Altogether 32 experiments were made with intact potato tubers (Table IV). The cooled part contained more water in 22 cases, there was no change in 3 cases, and the heated part contained more water in 7 cases. The mean value of surplus water on the cool side amounted to 0.8 per cent.

Discussion

Our experiments with sections of plant tissue and intact potato tubers have clearly demonstrated that the temperature gradient changes the water concentration in the tissues by driving the fluid from the warmer to the cooler parts. The fact that differences in temperature mobilize the water of living plant tissues means that heat is capable of performing work in biological structures. The effect is presumably proportional to the gradient. However, apart from the temperature difference, the very structure of the tissue, too, influences the result, as proved by variations that depended on the direction of the section and the variety of fruit employed. Ignorance of all factors involved prevents us from establishing quantitative relationships in this respect.

As regards the mechanism of the phenomenon, we ascribe the observed effect to thermo-osmosis due to differences *i.e.* vapour pressure. "Thermic humidity conductance" (LIKOV [30]) and distillation through vapour space, as indi-

Table IV

No.	Heating °C	Cooling °C	Time of experiment hrs.	Water contents		Percentage difference in water contents
				warm	cold	
1.	40	18	4	79.0	79.4	+0.4
2.	40	17	6	79.4	78.7	−0.7
3.	40	16	6	77.7	78.3	+0.6
4.	40	16	12	78.3	77.3	−1.0
5.	40	20	45	73.5	73.5	0
6.	38	14	26	75.2	73.0	−2.2
7.	41	16	64	73.8	74.6	+0.8
8.	40	17	69	74.7	75.0	+0.3
9.	38	16	89	73.4	74.3	+0.9
10.	41	18	66	76.2	75.9	−0.3
11.	39	17	65	79.8	81.7	+1.9
12.	38	18	89	80.3	80.0	−0.3
13.	39	16	71	75.5	77.6	+2.1
14.	40	18	66	78.4	79.5	+1.1
15.	35	17	66	74.5	79.2	+4.7
16.	40	17	64	79.2	80.8	+1.6
17.	43	17	72	74.8	76.3	+1.5
18.	38	16	72	76.0	77.0	+1.0
19.	37	15	71	74.7	74.2	−0.5
20.	39	15	66	76.9	78.3	+1.4
21.	37	16	45	77.8	78.3	+0.5
22.	38	17	92	75.0	75.5	+0.5
23.	39	16	69	72.7	75.5	+2.8
24.	39	16	70	75.0	75.6	+0.6
25.	40	16	75	77.2	79.3	+2.1
26.	40	17	90	78.5	80.0	+1.5
27.	40	18	66	79.4	79.8	+0.4
28.	39	17	74	78.7	81.9	+3.2
29.	40	19	67	78.8	77.6	−1.2
30.	40	20	72	79.6	79.6	0
31.	40	19	95	76.6	76.6	0
32.	43	19	71	74.5	74.7	+0.2

cated by the behaviour of the synthetic sponges and the intercellular presence of air in the plants, may play a certain role in the mechanism in question.

The problems under review might be easier if we could measure the amount and concentration of intra- and extracellular fluids, further the tempera-

ture of the cells or of their components by means of micro-thermoelements or by means of their radiated (infrared) heat. Experiments in this direction and their extension to animal tissues are planned.

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F. VETŐ,
Orvostudományi Egyetem, Biofizikai Intézet, Pécs, Hungary

THE EFFECT OF 15 MeV ELECTRON RAYS ON THE ACTIVITY AND EXCITABILITY OF THE ISOLATED FROG HEART

By

J. TIGYI

INSTITUTE OF BIOPHYSICS, MEDICAL UNIVERSITY, PÉCS

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The effect of 15 MeV betatron β-rays on the isolated frog heart has been investigated. Regular contraction was observed during irradiation in 12 per cent of the experiments. However, both amplitude and frequency were considerably lower than the original ones.

During irradiation a decrease of the threshold stimulus of the ventricles isolated by Stannius' ligature was demonstrable in the majority of cases.

The experiments provided data to the relation between radiation effect and excitation, and render the assumptions of the semiconductor theory more plausible.

The effect of the radiation of radioactive isotopes on the isolated frog heart was investigated in our institute many years ago [1, 2, 3]. We do not wish to mention in this connection the debate about the early experiments of ZWAARDEMAKER [4] because in those the amount of radioactivity was at least three magnitudes smaller than in our experiments. Experiments in which the magnitude of radioactivity used was approximately equal to ours have been made also in other institutes in the last ten years [5, 6].

All these experiments contain many uncertainties. Nobody has succeeded in determining the necessary cofactors, the presence of which, together with the sufficient intensity of radiation, cause the positive effect. In our opinion — as explained in our paper — the experiments made with the aid of an external source of radiation greatly help to clarify the problem, since

1. the possibility of bringing any kind of trace element or compound as impurities into the heart is certainly excluded;

2. as to the action mechanism of irradiation, these experiments could give an answer to the question whether it is the corpuscles or quants themselves or the recoil ionisation of the nucleus, causing a very great specific ionisation, which is effective;

3. if the external source is a particle accelerator in which the energy of particles is variable within a wide range, there is a possibility of studying the energy dependence of the effect.

In the present study we wished to clarify the question whether external β-radiation had an effect on the isolated frog heart.

The support of the IAEA offering us facilities for using in our experiments the betatron of the Radiological Institute of the University of Rome, gave us an opportunity to carry out this plan.

Methods

Straub's heart preparation of the frog Rana esculenta was used in these experiments; irradiation was performed after stopping the hearts with K-rich solution. The stopping solution contained 0.17 g NaCl, 0.30 g KCl, 0.30 g $CaCl_2$, 0.02 g $NHCO_3$ in 100 g water, as described in a previous paper [1].

In the second part of the experiments cardiac arrest was achieved by ligature of the atrioventricular border (Stannius' 2nd ligature) and after this the threshold stimulus voltage of the arrested heart ventricles was examined with single condenser discharges. The capacity of the condenser was 0.1 μF and the charging voltage could be varied with an accuracy of 0.05 V.

The radiation source was a Siemens betatron. The apparatus was used with a radiation-conducting tube of 3 cm diameter. The effective dose was checked with a Baldwin dosimeter. The hearts were placed in the centre of the distal end of the radiation-conducting tube. After switching on the apparatus, the dose rate was indicated by a dosimeter built into the apparatus. The maximal dose rate was 500 rad/min. The energy of β-rays was variable between 3—16 MeV.

Results

Our first question was, what reactions the arrested heart preparations show under circumstances exactly identical with those applied in our previous study, with the difference that in the present experiments they were exposed

Table I

	positive	negative	summarized
Number of preparations	8	58	66
Number of experiments	14	234	248

to the β-radiation of the betatron whereas formerly to that of radioisotopes. After preparation, the hearts were active with normal Ringer solution for 30 to 60 minutes; after this the normal Ringer solution in the Straub-cannula was replaced by a K-rich solution, the action of the heart being registered continuously. Full arrest ensued after 3 to 5 systoles; subsequently after a pause of 60 ± 5 sec. the β-radiation was switched on, then again a pause of 60 ± 5 sec. followed, and after that the exchange of the K-rich solution for normal Ringer's solution. After 3—5 similar exchanges the experiments were repeated 4 to 5 times. 250 irradiation experiments were made with 66 preparations in the same way. The results are shown in Table I.

As an example, Fig. 1 shows (a) one of the best and (b) one of the worst positive experiments.

1. Total restitution of heart action was not observable in any of the experiments. The amplitude was, at the most, half, the frequency about $^1/_5$, of the original values.

2. After the beginning of irradiation the arrested heart reacts with a latency period of a few seconds, moreover after the cessation of irradiation 1—2 systoles could be observed in a few cases.

The majority (88 per cent) of the frog hearts arrested with this method did not visibly react to external β-irradiation of such energy and intensity. The doubtlessly positive experiments do not allow the statement that external β-radiation does not affect the action of the heart.

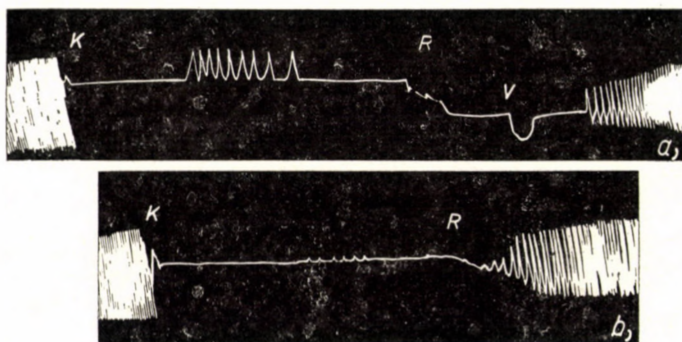

Fig. 1. The effect of 15 MeV electron-rays on the arrested frog heart.
a) One of the best experiments. *b)* One of the worst positive experiments

The positive experiments were invariably observed with 16 MeV irradiation but — in knowledge of the apparatus — this does not mean that irradiation with higher energy is more effective than with smaller energy, because the possible maximal dose rate at 15 MeV is 500 rad/min, and at 3 MeV about 1/10 of that. Thus, it is not possible to draw conclusions as to energy dependence.

In the second group of experiments the question was raised, whether some other change in the properties of the isolated frog heart could be observed in response to irradiation. In other experiments made in our institute we found [7] that the excitability of skeletal muscles irradiated by β-radiation of ^{80}Sr—^{90}Y changes significantly after relatively little doses, therefore we investigated how betatron irradiation influenced the excitability of the heart ventricle. The electrical excitability of the hearts arrested with K-rich solution is very low, they are not excitable with an electric impulse under 100 V, therefore cardiac arrest was produced with Stannius' 2nd ligature. It is known that the automacy of the ventricles stopped in such a way results in spontaneous contractions. In our experiments which lasted, at the most, 40 minutes after

the ligature, we could not observe spontaneous contractions. The threshold stimulus voltage was determined in the following way. Starting with a voltage undoubtedly above the threshold, single condenser discharge impulses were given every 5 seconds. After every 3rd stimulus the voltage was decreased by 0.1 V as in the following voltage series: 1.2—1.2—1.2, 1.1—1.1—1.1, 1.0—1.0—1.0, 0.9—0.9—0.9, *etc.*

The experiment was performed in the following manner. First the constancy of the threshold stimulus voltage was checked in a series of three 2-minute periods, and when it had proved constant (the same number of contraction was shown in all three periods; see Fig. 2) we began to investigate the

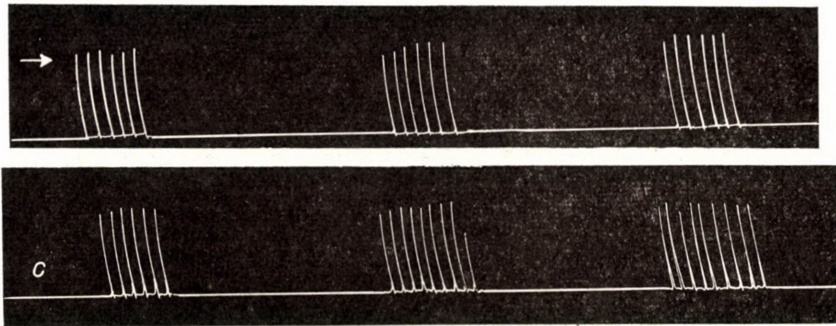

Fig. 2. The effect of 15 MeV electron-rays on the excitability of the isolated frog heart ventricle. Above: three stimulation periods without irradiation. Below: three stimulation periods, during the second one irradiation with an intensity of 500 rad/min

effect of irradiation. Similar stimulation series of 3 times 2 minutes were made as before, but during the second minute period the heart was irradiated with 15 MeV 500 rad/min of β-radiation.

Fig. 2 shows a typical experiment as an example of the 20 experiments made with such a method. An equal number of contractions can be seen in all three 2-minute periods in the upper part of Fig. 2, thus the threshold stimulus is constant. In the lower part, the first series shows a threshold stimulus equal to the above. The second series was recorded during irradiation, and the decrease of threshold stimulus is clearly visible (+ 3 contractions). In the third series — also without irradiation — this decrease in excitability continued.

A considerable decrease of threshold stimulus was observed in 14 of the 20 experiments, either immediately during or within 2 minutes after irradiation. Set out in detail, of 14 positive experiments a decrease of the threshold stimulus could be demonstrated in 8 cases during irradiation and in 6 cases during the next 2-minute period of stimulation. No effect of irradiation was noted in the other 6 experiments.

Discussion

Irradiation with 15 MeV β-rays of hearts inhibited with a K-rich solution showed that no activating effect could be observed in the majority of the experiments, but in a small percentage (12 per cent of the preparations, and 6 per cent of the experiments) an activating effect was doubtlessly present. The question is, what accounted for this small percentage of positive experiments. As an explanation, in the first place, the following two hypotheses are advanced.

1. The maximum intensity of radiation was just at the lowest limit of the minimal effective value. On the other hand, the threshold stimulus voltages of the hearts are known to show a great individual dispersion. Thus it is possible that in the case of the positive experiments (12 per cent) the threshold of the radiation stimulus was equal to, or a little lower than, the maximum intensity which could be delivered by the apparatus. With the other hearts (negative experiments) the threshold was higher than that intensity. This is also indicated by the observation that in 4 of the 8 positive cases the effects were observed twice, and in one case even 3 times.

Though in the study of the action-mechanism of radiation taking into account the energy absorbed by a whole organ can often be misleading, it may still offer some information. In the present experiments the absorbed dose was determined according to the following consideration. The mass absorption coefficient of the 15 MeV-electron rays is 0.7 g/cm^2 [8]. A frog heart, which weighs about 100 mg, may be regarded as an absorption-layer with a surface of 1 cm^2 and with a surface-density of 0.1 g/cm^2. Thus the absorbed dose:

$$D = I_0 - I = I_0 - I_0^{e-0.7 \cdot 0.1} = I_0 \left(1 - \frac{1}{1.07}\right) = 0.07 \ I_0,$$

which is about 7 per cent of the total dose. Since in the first part of our experiments the hearts were irradiated with a dose-rate of 500 rad/min for one minute, we can estimate the absorbed dose to be about 35 rad.

If we compare this energy absorption with an effective electrical stimulus, *e.g.* when a 0.1 μF condenser charged to 1 Volt is discharged, it will cause a contraction. The change of the electrical energy in this case is

$E = {}^1/_2 Q \cdot U = {}^1/_2 10^{-7}$ Coul. 1 Volt $= 0.5 \cdot 10^{-7}$ V Coul $= 0.5$ erg,which is 7000 times less than the change in radiation energy (see Ernst, [9]).

In this energetistical conception we must take into consideration that the radiation gained from the betatron is not constant, but represents radiation impulses of a duration shorter than 10^{-5} sec, with a frequency of 50 cycles. It is known that in the case of the electrical impulses of such extremely short duration the threshold-stimulus voltage is much higher than with longer im-

pulses (low frequency). So the above detailed energetistical views can supply only a rough information about the comparison of the radiation energy with the electrical energy necessary for stimulation. In any case, it would be interesting to perform these experiments with a constant β-radiation source, so that a real comparison could be made concerning the stimulatory effect of irradiation.

2. Radiation itself is not able to cause an activating effect on the heart, only together with the sensitizing factor which is contained in the heart. As it has been shown, this sensitizing factor can also be a trace element [10]. If such a sensitizing factor is assumed the present experiments show that it must be searched for among the components existing originally in the heart (it might be taken up in the alimentary way), since in the present experiments invariably the same chemicals were used. At this moment we have no concrete data on the exact properties of this sensitizing factor.

The positive experiments gave an answer to our former problem, establishing that in the experiments made with radioactive isotopes it was not the recoil ionisation that caused the effect, but the emitted corpuscles and quants themselves. The radiation of the artificial radioactivity generated by the 15 MeV betatron β-radiation is — according to our calculations and measurements — many magnitudes below the effective intensity, therefore it is negligible.

The second part of the experiments shows that the change of the excitability of the ventricles separated by Stannius' 2nd ligature is really more sensitive, although less marked test than the activation of potassium-inhibited hearts. If it is considered that the threshold stimulus voltage of the hearts arrested with a K-rich solution is very high, this fact will be more convincing. In any case the fact that irradiation for two minutes (cca. 70 rad; see LEBEDINSKY [11]), causes a well-defined diminution of the threshold stimulus in two-thirds of the experiments, lends support to the view that there are such ionic or electronic processes in the radiation effect as are in connection with the processes of excitation (see ERNST [12]).

The decrease of excitability caused by the radiation effect can give certain information about the reactivation mechanism of the heart. Restarting of the arrested heart action will take place when, in response to irradiation, the threshold stimulus has decreased so much that the impulse generated in the sinus or in the atrium is able to elicit a systole.

In the future, first of all, the following experiments seem to be necessary:

1. Performance of these same experiments with a β-radiation source of higher intensity (dose rate).

2. Determination of the energy dependence of the effect.

3. The application of a constant, not impulse-like, source of radiation possibly with low frequency (10—50) modulation.

4. Search for further more sensitive parameters of heart-action, which would make it possible to study similar physiological effects of radiation.

Acknowledgements

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József TIGYI,
Orvostudományi Egyetem Biofizikai Intézete, Pécs

CORRELATION BETWEEN THE ACTIVITIES OF COLLAGENMUCOPROTEINASE AND TRYPSIN

By

ILONA BANGA

FIRST DEPARTMENT OF PATHOLOGY AND EXPERIMENTAL CANCER RESEARCH, MEDICAL UNIVERSITY, BUDAPEST

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Tissues containing collagen (Achilles tendon, tendon of rat tail), if existing in the form of fibres, cannot be denaturated by dehydration with alcohol, acetone and ether or by treatment with dry heat at 110° C, as to render them digestible by proteolytic enzymes. Collagenmucoproteinase (CMPase) is able to dissolve the proteins of the collagen only if the tissues are ground and pulverized. Grinding and pulverization are, however, not equivalent to denaturation. A theory is given for the explanation of this phenomenon.

Tests made with 10 different trypsin preparations have proved that the activity of trypsin on one of its specific substrates, N-benzoyl-DL-arginine-β-naphthylamide (BANA), is not the same as its activity on the pulverized Achilles-tendon. It follows that the activity of trypsin is different from that of CMPase, while most trypsin preparations contain a certain amount of this enzyme.

CMPase acts differently on native collagen and metacollagen which latter may be regarded as a partially denatured collagen. The examined trypsin preparations displayed, apart from CMPase activity, different degrees of metacollagenase activity.

The existence and the mechanism of CMPase are studied and analyzed on the evidence of experimental results and literary data.

Introduction

It was reported in earlier publications [1, 2, 3, 4, 5] that an enzyme capable of digesting native collagen can be isolated from human and animal pancreas. Exposed to the action of this enzyme, namely the collagenmucoproteinase (CMPase), isolated rat tail tendon fibres disintegrate into filaments and are torn up in the course of chemical contraction-relaxation [2]. Another effect of the enzyme is that enhancing the swelling of the fibres in acetic acid, and that it is impossible to recover fibres from the acetic-acid gel by the addition of NaCl or alcohol. While only 5 to 10 per cent of the protein of native collagen fibres are lyzed by the enzyme, 30 to 35 per cent of the protein is dissolved out of the pulverized Achilles tendon. Certain authors [22, 23] attribute this phenomenon to the fact that dehydration (alcohol, acetone) or pulverization and grinding may cause a denaturation of the collagen protein. Since ground and pulverized Achilles tendon is digested by various trypsin preparations as well, the said authors are not convinced of the existence of CMPase as a separate, specific enzyme but attribute the observed process to the action of trypsin.

— The present study was, therefore, undertaken with a view to solving two problems. 1. Does the heat engendered by grinding and pulverization, and does the preceding process of drying by acetone or alcohol-ether so denature the fibres as to make them digestible by enzymes? 2. Does CMPase really exist, in other words is there an enzyme specific for collagen, or, else, is trypsin able to split off a polypeptide from pulverized collagenous tissues that has to be considered collagen on account of its high hydroxyproline contents?

HODGE *et al.* [6], in their electron microscopic studies have found that, after treatment of tropocollagen solutions with trypsin, the action of ATP failed to produce the typical segment-long-spacing (SLS) fibrils, and that, instead, the monomers of the individual segments appeared in the solution. They explained the phenomenon by assuming that the trypsin dissolves out a tyrosine-containing polypeptide which keeps the collagen molecules together in a longitudinal direction, and splitting off of this polypeptide inhibits the normal development of fibrils. The question is whether this phenomenon is due to trypsin or to CMPase, *i.e.* a specific enzyme.

Methods and results

Conditions under which CMPase digests collagenous tissues

We studied digestion by collagen on two kinds of substrates: bovine Achilles tendon and collagenous fibres isolated from rat tail tendon. On the basis of their hypro contents, some 92 to 96 per cent of both kinds of tissue consist of collagen protein. Both substrates were used in the native state, *i.e* the tissues were minced and then homogenized with water or a buffer in a waring blendor. This procedure failed to yield the desired result in respect of the Achilles tendon: the fibres did not separate, so that incubation with the enzyme did not give a uniformly fine suspension, the fibres remained clustered. It was, on the other hand, possible to obtain from the rat tail tendon fibres measuring between 0.06 and 0.12 mm in diameter. These were accepted as native collagen fibres.

The homogenized bundles of fibres were dehydrated with alcohol, acetone and ether, and dried at 100° to 110° C for 16 to 24 hours. Then both the native and the denatured dried fibres were ground to fine powder in a hammer mill. All preparations were incubated with 0.02 mg/ml of CMPase isolated by us; buffer was prepared of a mixture of 25 ml of 0.2 *M* Tris (hydroxymethyl-aminomethane) and 40 ml of 0.1 *N* HCl, of pH 7.4, at 25° C and 37° C. Dissolution of the collagen was determined as described earlier [3].

Table I shows the results for the Achilles tendon. The dissolution of differently pretreated specimens was studied. Results obtained in buffer without CMPase served as controls, those obtained after the addition of the enzyme

Table I

Digestion of differently pretreated Achilles tendon by collagenmucoproteinase (CMPase)

Pretreatment	Percentage dissolution of Achilles tendon after 30 min. incubation	
	N/10 pH 7.4 Tris buffer	0.02 mg/ml CMPase in pH 7.4 buffer
1. Disintegrated in blendor, weighed in the humid state	5	5
2. Disintegrated in blendor, acetone-dried	10	10
3. Disintegrated in blendor, alcohol-dried	10	10
4. Disintegrated in blendor, treated with acetone and dried at 110° C	10	10
5. As under (2), ground in hammer mill	10	30—35
6. As under (3), ground in hammer mill	10	30—35
7. As under (4), ground in hammer mill	10	30—35

Table II

Digestion of rat tail tendon by collagenmucoproteinase (CMPase)

Pretreatment	Percentage dissolution at 25° C after incubation of 30 minutes	
	In veronal-acetate buffer; pH 7.4	In 0.02 mg/ml CMPase solution
1. Isolated bundles of native collagen fibres	5	6
2. As under (1), ground in hammer mill	15	50—60

indicated the activity of the latter. Neither disintegration in the blendor, nor dehydration with acetone or alcohol, nor heat treatment at 110° C sufficed to make the fibres digestible by CMPase, whereas a grinding of the dehydrated fibres in a hammer mill (with or without previous heating) made them digestible by the enzyme at the rate observed earlier [3].

The results for rat tail tendon fibres are listed in Table II. They made it even more obvious that the susceptibility of the fibres to enzymatic action is not due to denaturation by acetone, alcohol or heat, but to their pulverized condition. We tested the collagen fibres as they were removed from the rat tail tendon without any pretreatment, and found that they were resistant to enzymatic action both in a fresh and in a dried condition. Their dissolution began only after their having been pulverized. A protein (for distribution see Table II) was demonstrable in the supernatant; it contained 14 to 16 per cent of hypro.

Specific activity of various trypsin preparations on N-benzoyl-DL-arginine-β-naphthylamide (BANA), and their CMPase activity on Achilles-tendon powder

We acquired in the world market 10 different highly purified trypsin preparations: (1) *Worthington* trypsin, twice crystallized, salt-free; (2) *Worthington*, twice crystallized + 50% $MgSO_4$; (3) *Armour Tryptar*; (4) *Trypure Novo* (3×28); (5) *Choay* trypsin dialyzed and lyophilized; (6) *Merck* trypsin; (7) *Light* trypsin; (8) *Richter* trypsin; (9) trypsin received from Dr. J. A. NORTHROP;

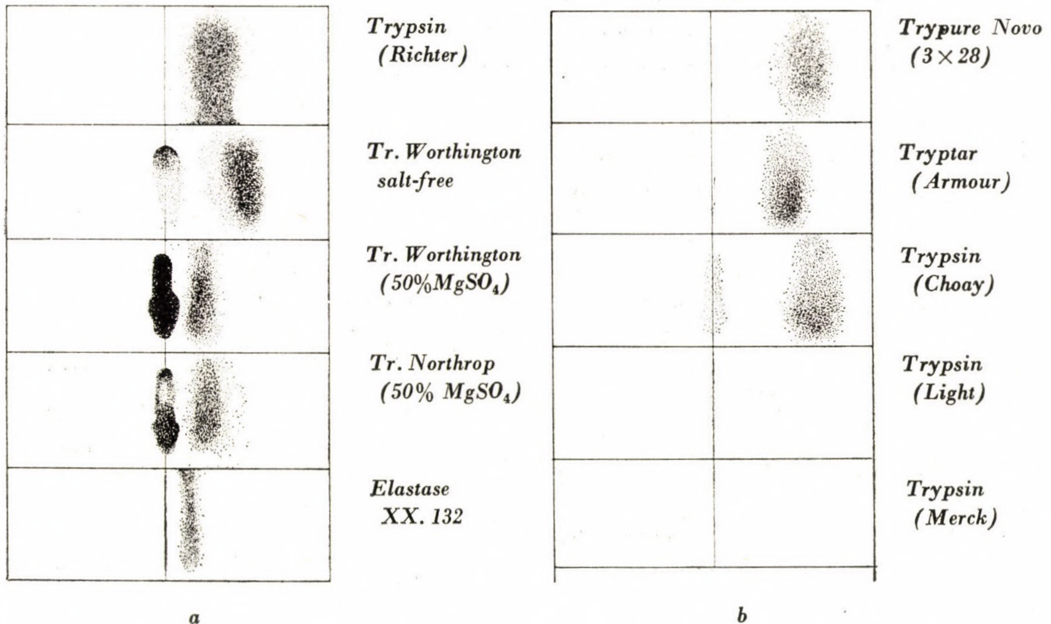

Fig. 1a, 1b. Paper electrophoretic patterns of various trypsin preparations. Dose: 2 mg. Veronal buffer; pH 8.6; $\mu = 0.1$; 110 V; 20^h. Acid fuchsin stain. All preparations migrated towards the negative pole

(10) *Boehringer* trypsin. We determined their homogeneity by means of paper electrophoresis at both pH 6.5 and pH 8.6. We obtained clearly distinct separate protein spots at both values, and failed to observe any change in the enzyme at the alkaline pH. This seems to be in contradiction to the finding of LEWIS *et al.* [7] who did not apply an alkaline pH at the electrophoretic separation of elastase, trypsin and chymotrypsin, because trypsin, according to them, is labile at pH-values over 7.0. Figs 1/a and 1/b present the electrophoretic patterns of the examined preparations, all of which had been recrystallized twice. Homogeneous elastase served the purpose of comparison. Run under identical experimental conditions, the major part of the different pre-

parations migrated toward the cathode; their mobility was, however, not identical and some of the preparations were, moreover, not homogeneous, those in particular which contained salt ($MgSO_4$). The salt-free *Worthington* trypsin, as also the *Choay* trypsin contained — as did the salt-containing preparations — a non-moving component. The crystalline trypsin (containing 50 per cent of $MgSO_4$) we had received from Dr. NORTHROP had likewise a non-moving protein

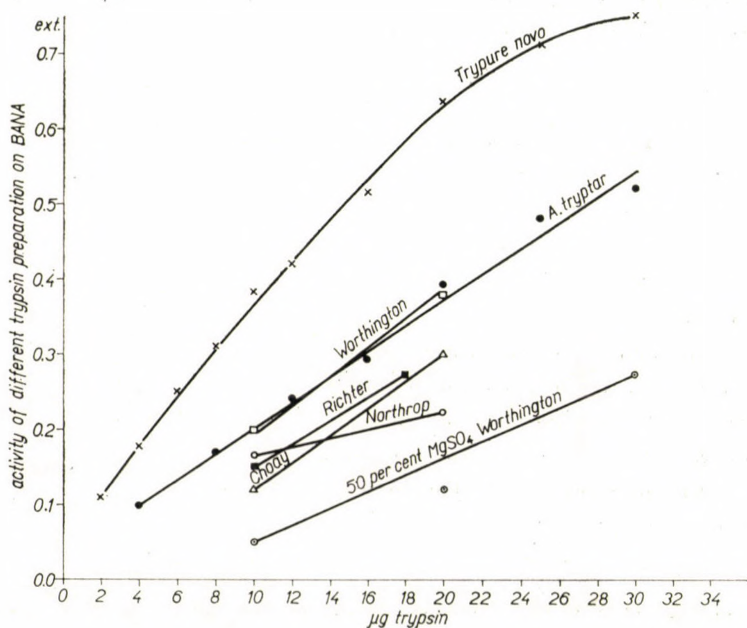

Fig. 2. Activity of various trypsin preparations on BANA as substrate. Ordinate: extinction measured with the method of BLACKWOOD *et al.* [9] Abscissa: trypsin protein in μg

component. An amount of 2 mg of the *Light* and the *Merck* trypsins yielded no stainable protein component and chemical analysis revealed in them 90 per cent of inorganic impurities and only 10 per cent of protein, a fact that explains our failure to demonstrate their protein electrophoretically. The mobility and the protein spot of homogeneous elastase proved to be different from those of the trypsin preparations, although — according to literature — its isoelectric point is about 9.5 [7,8]. It follows that crystalline trypsin preparations consist, in the main, of a protein whose isoelectric point is higher than that of elastase. Our previous electrophoretic studies [2, 3] showed the mobility of CMPase to be greater than that of elastase, a property which made it possible to separate it from elastase. It would seem that most trypsin preparations contain a protein fraction possessing CMPase activity.

Our next group of experiments was designed to find out whether there exists a correlation between the activity of trypsin as observed on its specific substrate, *i.e.* BANA, and its activity as observed on pulverized Achilles tendon. The first step was to plot the curve of enzymatic activity for each trypsin preparation at identical BANA-concentration and under equal experimental conditions. The method of BLACKWOOD and MANDL [9] was used for the determination of tryptic activity. Fig. 2 presents the concentration curve of 7 trypsin preparations. The *Light* and *Merck* trypsins showed no measurable activity in the concentrations applied; the preparation of *Boehringer* had the

Table III

Activity of elastase preparations on BANA as substrate

Preparations	Examined quantity μg	Ext. 550 μm
590530 non-crystalline	20	0.34
$213/F_3$ crystalline	20	0.25
XX/132, electrophoretically homogeneous elastase	20	0.01
XX/132, electrophoretically homogeneous elastase	50	0.10
XX/132, electrophoretically homogeneous elastase	100	0.25
Electrophoretically homogeneous elastomucoproteinase	50	0.08
Electrophoretically homogeneous elastomucoproteinase	100	0.22
Trypure Novo 3×28 (control trypsin)	20	0.61

same activity as that of the *Choay* trypsin, so that these preparations are not represented in the curve. The employed 20 μg amount of elastase, irrespective of whether it had been obtained with the method of HALL and CZERKAWSKI [10], by means of starch gel electrophoresis [11], or by means of Sephadex chromatography as applied by LOEVEN, failed to display activity against BANA. Table III shows the activity on BANA of various elastase preparations as compared with that of the most active trypsin preparation (*Trypure Novo* 3×28). Pure elastase and elastomucoproteinase preparations revealed a minimum activity even at a concentration of 100 μg. This method is eminently suitable for the differentiation of elastase and trypsin activity.

Fig. 3 and Table IV show that, tested on pulverized Achilles tendon, nearly all trypsin preparations possessed different degrees of collagen-digesting capacity. Quantities of 10, 20, 40 and 100 μg were compared; gravimetric measurements were made whenever dissolution reached a high degree within 30 minutes. The degree of dissolution was determined with our own method [3] except in the case of low concentrations (10, 20 μg) where the amount of dissolved protein in the supernatant was estimated with Folin's reagent by

the method of LOWRY *et al.* [12], against a standard curve for which the measured protein contents were checked with micro-Kjeldahl. It is only within a comparatively narrow range of concentrations that trypsin preparations are in a linear relation to the degree of collagen digestion (see curve No. 1 in Fig. 3), and so activities had to be computed from the values within the said range. We regarded as zero value the point at which the continuation of the straight

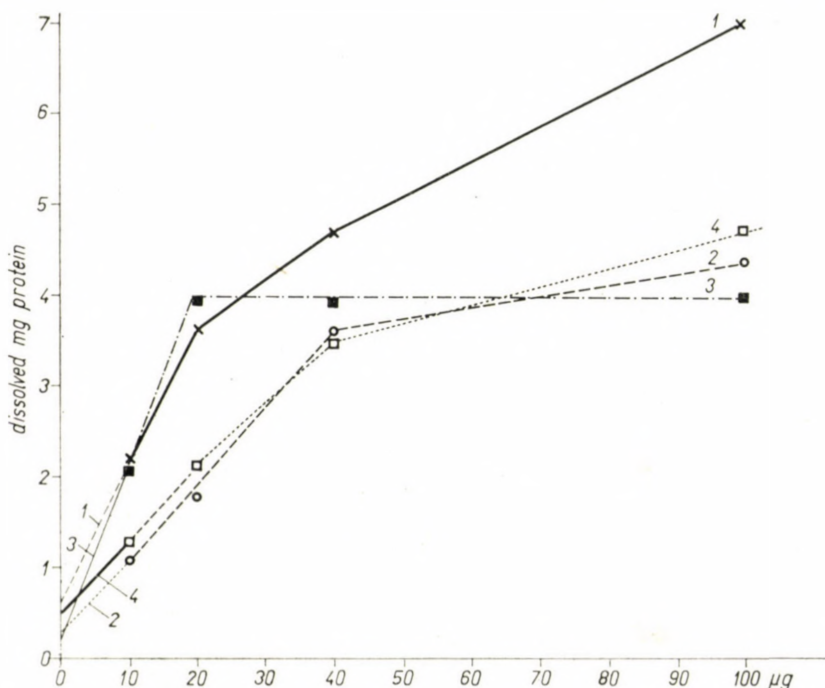

Fig. 3. Collagenmucoproteinase activity of different trypsin preparations on pulverized Achilles tendon. *1* = *Trypure Novo* 3×28; *2* = *Armour Tryptar*; *3* = *Worthington*-saltless; *4* = *Choay*

part of the curve intersected the ordinate, and deducted the value corresponding to the distance between the point of intersection and the true 0-point of the coordinates. Fig. 3 presents the activity of four trypsin preparations at 25° C on Achilles-tendon powder as substrate. Table IV illustrates the activity of 9 trypsin preparations in CMPase units, at 37° C and 25° C. Some preparations *(Trypure Novo, Armour Tryptar* and *Worthington)* displayed different activities at the different temperatures, but no differences were observed in 6 preparations. These results are, thus, in contradiction to the general notion that collagen protein is denatured at 37° C and, therefore, highly susceptible to proteolytic enzymes.

Table IV

Collagenmucoproteinase (CMPase) activity of different trypsin preparations at 37° C and 25° C

Gravimetric method: a concentration curve was plotted for each preparation; the points which lay in the same straight line, were extrapolated to zero enzyme concentration as shown in Fig. 3; the unit of CMPase per 1 mg of enzyme protein was then computed after deduction of the zero value

Preparations	CMPase unit/mg	
	at 37° C	at 25° C
Trypure Novo (3×28)	108	155
Armour Tryptar	220	75
Worthington (saltless)	265	185
Northrop	110	105
Choay	65	77
Richter	120	110
Merck	16	15
Light	10	10
Boehringer	75	78

Table V

Relative activity of different trypsin preparations on BANA and Achilles-tendon powder as substrates

(For method and computation see text)

Preparations	Relative activity (25° C)	
	BANA	Achilles tendon
Trypure Novo (3×28)	100	88
Armour Tryptar	63	40
Worthington (saltless)	58	100
Northrop	46	40
Choay	34	42
Richter	41	54
Merck	0	13
Light	0	5
CMPase	30	100

Table V shows the correlation between the relative BANA activity of the trypsin preparations and their relative CMPase activity as determined on pulverized Achilles tendon. The observed highest activity was accepted as having a value of 100 in respect of both substrates, and all other activities

were referred to this standard. If the relative activities coincide, it means in our opinion that the collagen-digesting capacity of the trypsin preparations is identical with the proteolytic activity of the specific enzyme (on BANA), and that, consequently, both must operate through the same mechanism. It is, however, clear from Table V that the relative activities were different on the two substrates. *Trypure Novo* (3×28), showed the highest activity (100) on BANA as substrate; *Worthington* (saltless) trypsin showed the highest activity on Achilles tendon, while its activity on BANA was 58 only. CMPase, isolated by us, exhibited an activity of 30 on BANA, and one of 100 on Achilles tendon. It would follow from these figures that the activity of trypsin, as observed on BANA, and the CMPase activity of the examined preparations are different enzymatic manifestations, regulated by different mechanisms.

Comparisons between the collagenmucoproteinase and metacollagenase activity of different trypsin preparations

Metacollagen means a kind of collagen we obtained from collagenous tissues either by 2 to 10 min. treatment at 67 to 70° C or by means of the contraction-relaxation phenomenon produced in a 40 per cent solution of potassium iodide at 20° C [13, 14, 15]. Metacollagen, derived from the fibres of rat tail tendon by way of chemical relaxation, does not lose its fibrillar structure, whereas gelatin has no structure and forms a gel in water. We regard metacollagen as partially denatured collagen whose physico-chemical properties differ from those of the native product. We compared pulverized metacollagen, derived from Achilles tendon, with powder obtained by grinding

Table VI

Collagenmucoproteinase(CMPase) and metacollagenase activity of different trypsin preparations

(For method and computation see text)

Preparations	Specific enzymatic activity	
	CMPase	Meta-collagenase
Trypure Novo (3×28)	110	1150
Armour Tryptar	220	600
Worthington (saltless)	270	750
Northrop	110	460
Choay	70	750
Richter	170	1100
Merck	10	200
Light	5	50
Collagenmucoproteinase	270	1400

untreated Achilles tendon, on the basis that, if a pulverization of the Achilles tendon is equivalent to its denaturation, trypsin preparations must digest Achilles tendon powder and pulverized metacollagen in the same manner. Table VI makes it clear that this was not the case. The same trypsin preparation displayed a metacollagenase activity 3 to 10 times higher than its CMPase activity as measured on pulverized collagen. Specific enzymatic activity is expressed as the amount (g) of collagen digested with our earlier described method [3] by 1 mg of enzyme protein within 30 minutes. CMPase activity, as registered in Table VI, is referred to the pulverized Achilles tendon of young cattle as substrate, while metacollagen obtained from the same powder served as substrate for the measurement of metacollagenase activity. The metacollagen in question was produced as follows. Ten g of Achilles-tendon powder was suspended in 150 ml of distilled water at 75° C, and then kept at a temperature of 70° C for 10 minutes. It was then rapidly cooled, filtered, washed with 100 ml of cold distilled water, dehydrated with acetone and dryed at 110° C. We observed the highest metacollagenase activity with the CMPase preparation we had isolated by means of starch electrophoresis. Its value was 1400 as against an activity of 270 determined on Achilles-tendon powder. This would mean that the proteolytic activity of CMPase is more than 4 times as high on metacollagen as on collagen. The various trypsin preparations exhibited different activities against both Achilles tendon powder and metacollagen. If the relative activities of the individual trypsin preparations are compared (Table VI), they show no agreement; the activity of the preparations differed according to whether collagen or metacollagen was the substrate. CMPase alone displayed maximum activity on both substrates. The CMPase activity of *Worthington* saltless trypsin was equal to the activity of the CMPase isolated by us, while its activity on metacollagen amounted to 54 per cent only, as against the 100 per cent activity of our CMPase. Of the examined trypsin preparations, it was *Trypure Novo* which most approached the metacollagenase activity of CMPase; its activity was considerable on Achilles tendon and BANA substrate alike. The results recorded in Table VII allow the conclusion that CMPase activity and metacollagenase activity are not identical. It follows that pulverized Achilles tendon and metacollagen derived from it are not just denatured collagen proteins but in certain respects different substances.

Discussion

The first question was whether the human and animal pancreas synthetized an enzyme capable of digesting, splitting or attacking native collagen. The answer to this question has in fact been supplied in an earlier paper [2] in which we demonstrated that native, *i.e.* freshly isolated and untreated,

collagen fibres, if exposed to the action of CMPase obtained from the pancreas, undergo changes that can be determined by the method of chemical contraction-relaxation. The enzymatic activity manifested itself furthermore with a change in the fibres' capacity of swelling. Why is it then that the enzyme lyses only a minimum amount of collagen protein if the fibres are in the native state, whereas as much as 30 to 35 per cent of it within half an hour if the fibres are

Table VII

Comparative data regarding the relative collagenmucoproteinase(CMPase) and metacollagenase activities of different trypsin preparations

Preparations	Relative activity	
	CMPase	Meta-collagenase
Trypure Novo (3×28)	88	82
Armour Tryptar	40	43
Worthington (saltless)	100	54
Northrop	40	33
Choay	42	54
Richter	54	79
Merck	13	20
Light	5	5
CMPase	100	100

pulverized (ground) before being treated with the enzyme? (Kühn *et al.* [16], using acid-insoluble collagen and a highly concentrated trypsin, demonstrated 79 per cent digestion in 24 hrs.) We suggest that CMPase produces the same effect on fibres and on pulverized collagen. There exists a barrier which prevents the split-off element — termed by us [17] mucoid$_2$ — to escape from the interior of the fibres as long as the latter are intact. The barrier will be broken down in the process of grinding and pulverization, so that mucoid$_2$ gains free access to the solution. The appearance of dissolved collagen in the supernatant does not, therefore, depend on the fibres being denatured; it is just a matter of diffusion. The barrier in question may consist of oriented interfibrillar mucopolysaccharides [18], but may likewise consist of certain perifibrillar membranes.

The second problem was whether the pancreas synthesized a separate enzyme, namely collagenmucoproteinase, or whether trypsin itself acted on collagen in the observed manner. The experiments have made it clear that the action of the trypsin preparations on the BANA, *i.e.* a specific synthetic peptide, is not identical with their action on collagen. The different trypsin preparations either contain different amounts of CMPase by way of impurity or, else,

the molecules of trypsin have two active groups; one that is responsible for the splitting of BANA, and another that splits off a peptide molecule from the collagen. RAMACHANDRAN and KARTHA [19] further RICH and CRICK [20] visualize the structure of the tropocollagen molecule as a helical helix composed of three polypeptide chains. HODGE *et al.* [6] suggest that peptide chain appendages with high tyrosine contents extend from these molecules which ensure a longitudinal connection of the molecules in the course of fibrillogenesis. The action of trypsin would, according to this theory, consist in splitting off these appendages, so that no structure like the native fibrils can be formed. Although KÜHN *et al.* [16] refuse this explanation of tryptic action, they admit nevertheless that the collagen remaining after a treatment with trypsin contains considerably less hexose and hexosamine than untreated collagen. We think that enzymatic activity, as observed in the experiments of HODGE *et al.* [6] and KÜHN *et al.* [16], was really the activity of CMPase, an enzyme contained in all trypsin preparations. $Mucoid_2$, split off the pulverized Achilles tendon by CMPase, may be regarded as a polypeptide with high hypro-contents, characteristic of collagen. It contains moreover a neutral heteropolysaccharide component which is in covalence with the polypeptide fraction. The peptide chain appendages may be identical with $mucoid_2$, and this the more so as fibrils failed to develop in our experiments if they had been exposed to the action of CMPase [1, 21]. Relying on the various literary data we feel, therefore, justified in suggesting that an enzyme displaying collagenase activity is synthesized by the pancreas, while the observed mechanism of its action admits of the conclusion that the properties of this enzyme are the same as those of CMPase as described in several earlier papers [1, 2, 3, 4, 17, 21].

Acknowledgement

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Ilona BANGA,
Budapesti Orvostudományi Egyetem I. sz. Kórbonctani és Kísérleti Rákkutató Intézete, Budapest.

EFFECT OF INHIBITORS ON THE ACTIVITY OF TRYPSIN AND COLLAGENMUCOPROTEINASE

By

ILONA BANGA and JOLANDA MAYLÁTH-PALÁGYI

FIRST DEPARTMENT OF PATHOLOGY AND EXPERIMENTAL CANCER RESEARCH, MEDICAL UNIVERSITY, BUDAPEST

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The effect of three trypsin inhibitors on the enzymatic activity of two trypsin preparations has been studied. The specific activity of trypsin was determined on N-benzoyl-DL-arginine-β-naphthylamide (BANA), that of collagenmucoproteinase (CMPase) on Achilles tendon powder.

The BANA-activity and the CMPase activity of the same trypsin preparation were differently affected by the inhibitors, which justifies the assumption that the two activities are different.

It is evident from the results that pure, twice crystallized trypsin preparations are not homogeneous.

Introduction

Most of the current trypsin preparations display also collagenmucoproteinase (CMPase) or so-called collagenase activity [1]. The earlier observation that the activity of these preparations on BANA (N-benzoyl-DL-arginine-β-naphthylamide), the specific substrate of trypsin is different from their CMPase activity as determined on pulverized Achilles tendon (collagen), justifies the conclusion [1] that trypsin preparations are contaminated with CMPase or, else, that trypsin contains two active groups, one of them responsible for BANA activity, the other for collagenase activity.

The problem whether activity on BANA is identical with or different from that on Achilles tendon can, among others, be determined by observing the effect of trypsin inhibitors. If, under identical conditions, inhibitors produce the same inhibitory effect on both activities, the active principle and the mechanism must be the same in both cases, whereas, if the same inhibitor produces different effects on the two activities, they must be due to different active groups and have different mechanisms.

Material

Three trypsin inhibitors,* made by the *Worthington* Co. were used in the present experiments: 1. highly purified ovomucoid; 2. crystallized soybean inhibitor; 3. non-crystallized soybean inhibitor. The trypsin preparations were 1. *Worthington*'s trypsin (twice crystallized, saltless), a preparation which showed a 100 per cent CMPase activity in earlier experiments; 2. *Trypure Novo* (3×28) which proved to have the highest BANA activity.

* We are indebted for the inhibitors and the trypsin preparations to Dr. MANDL, Columbia University, New York.

Method and results

The BANA activity of both trypsin preparations could be well defined with the method of BLACKWOOD and MANDL [2]. Of the ten different trypsin preparations tested in a previous experiment [1] it was *Trypure Novo* (3 × 28) which displayed the highest activity on BANA as substrate. The dose of the

Table I

Inhibition of activity of Worthington's trypsin on BANA in McIlvaine's buffer

Enzyme (20 μg)	Ratio of enzyme and inhibitor	Inhibitor and percentage inhibition		
		Ovomucoid	Soybean cryst.	Soybean non-cryst.
Worthington's	1 : 0.2	28	35	27
trypsin	1 : 0.4	56	47	33
	1 : 0.6	70	59	52
	1 : 0.8	78	67	75
	1 : 1	84	75	79

Table II

Inhibition of activity of Trypure Novo on BANA, in McIlvaine's buffer

Enzyme (20 μg)	Ratio of enzyme and inhibitor	Inhibitor and percentage inhibition		
		Ovomucoid	Soybean cryst.	Soybean non-cr.
Trypure Novo	1 : 0.2	28	21	19
	1 : 0.4	40	52	36
	1 : 0.6	67	72	48
	1 : 0.8	77	80	65
	1 : 1	80	90	78

trypsin preparations was constant (20 μg) throughout, while the amounts of the inhibitors were so chosen as to give different concentrations, the highest being a trypsin:inhibitor ratio of 1 : 1. The effect of different concentrations of the three inhibitors on the BANA activity of the two trypsin preparations is listed in Tables I and II. It can be seen that it was only the crystallized soybean inhibitor which produced different effects on the two preparations, while there was no essential difference between the inhibition values in the other two cases. Figs. 1, 2 and 3 present in a diagrammatic form the inhibition values for both trypsin preparations.

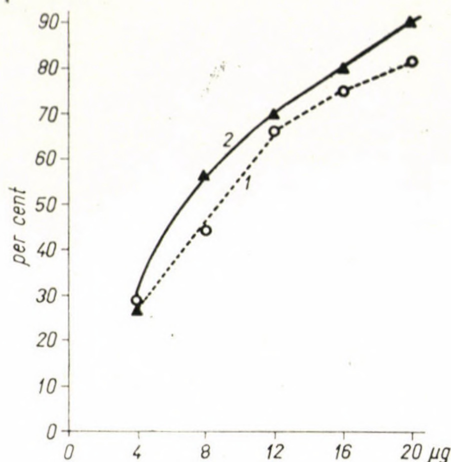

Fig. 1. Effect of ovomucoid on the activity on BANA of *Trypure Novo* and *Worthington*'s trypsin. *1* = 20 μg of *Trypure Novo* ; *2* = 20 μg of *Worthington*'s trypsin. Abscissa: inhibitor (μg); ordinate: inhibition (%)

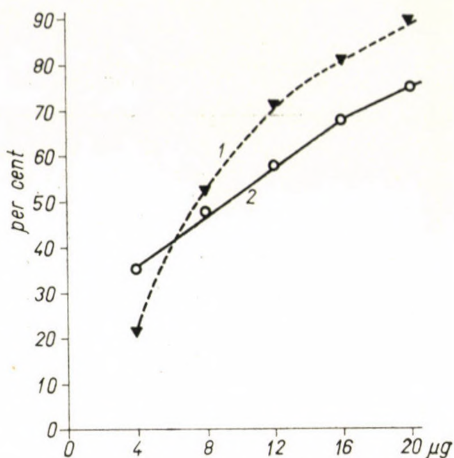

Fig. 2. Effect of crystallized soybean inhibitor on the activity on BANA of *Trypure Novo* and *Worthington*'s trypsin. *1* = 20 μg of *Trypure Novo* ; *2* = 20 μg of *Worthington*'s trypsin. Abscissa: inhibitor (μg); ordinate: inhibition (%)

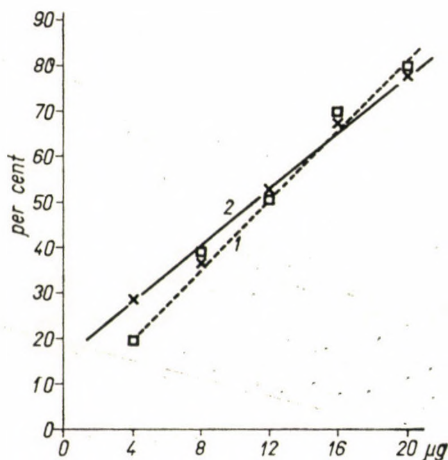

Fig. 3. Effect of non-crystallized soybean inhibitor on the activity on BANA of *Trypure Novo* and *Worthington*'s trypsin. *1* = 20 μg of *Trypure Novo* ; *2* = 20 μg of *Worthington*'s trypsin. Abscissa: inhibitor (μg); ordinate: inhibition (%)

The value of CMPase activity were determined on pulverized Achilles tendon with the technique of BANGA *et al.* [3] under the experimental conditions described previously [1]. The maximum inhibitor ratio was 1 : 1. In view of the gravimetric method used, the amount of enzyme was 100 μg, in a total volume of 2.5 ml. The results are listed in Table III.

Table III

Inhibition of CMPase activity of Worthington's trypsin on pulverized Achilles-tendon in McIlvaine's buffer

Enzyme	Ratio of enzyme and inhibitor	Inhibitor and percentage inhibition	
		Ovomucoid	Soybean
Worthington's trypsin	1 : 0.1	0	0
	1 : 0.2	0	0
	1 : 0.4	0	0
	1 : 0.8	10	10
	1 : 1	15	15

As regards ovomucoid, there was a considerable difference between its inhibitory effect on BANA activity and on CMPase activity. While the values for BANA activity were between 28 and 56 per cent with enzyme-inhibitor ratios of 1 : 0.2 and 1 : 0.4 (Tables I, II), such concentrations had no effect

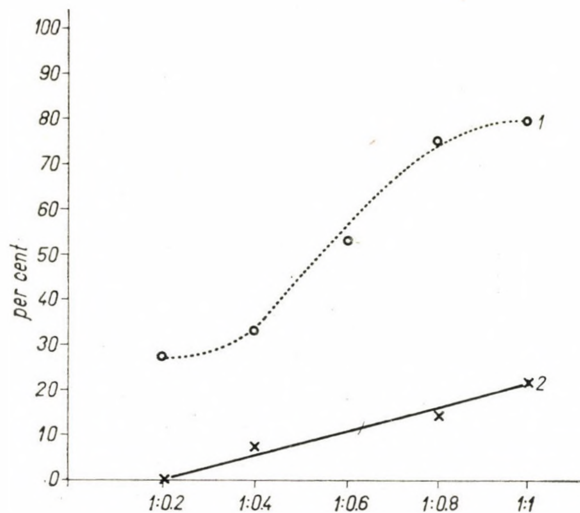

Fig. 4. Effect of soybean inhibitor on the specific activity of *Worthington*'s trypsin on BANA respectively on pulverized Achilles tendon. *1* = BANA, *2* = Achilles tendon. Abscissa: ratio of enzyme and inhibitor; ordinate: inhibition (%)

on CMP-ase activity (Table III). At concentrations of 1 : 0.8 and 1 : 1, inhibition of BANA activity was 77 and 84 per cent, respectively (Tables I, II), while that of CMPase activity was 10 to 15 per cent, respectively (Table III).

As to the soybean inhibitor, at concentrations of 1 : 0.2 and 1 : 0.4, inhibition of BANA activity was 19 and 52 per cent, respectively (Tables I, II),

whereas inhibition of CMPase activity was nil (Table III). With an enzyme: inhibitor ratio of 1 : 1, inhibition of BANA activity was between 75 and 90 per cent (Tables I, II), whereas that of CMPase activity, 15 per cent (Table III).

It is, thus, obvious that trypsin activity on BANA was considerably counteracted at comparatively low concentrations of the inhibitors, while they produced no effect on CMPase activity. Fig. IV presents an illustration of this difference. With an enzyme/inhibitor ratio of 1 : 1, inhibition of activity

Table IV

Inhibitory effect of ovomucoid and soybean on CMPase activity in different buffers

Enzyme	Ratio of enzyme and inhibitor	Inhibitor and percentage inhibition					
		Tris buffer pH 7.4		Na_2CO_3-HCl buffer pH 7.4		McIlvaine's buffer pH 7.2	
		O	S	O	S	O	S
Worthington's	1 : 0.1	0	0	0	0	0	0
trypsin	1 : 0 2	11	0	0	0	0	0
	1 : 0.4	15	7	12	0	0	0
	1 : 0.8	43	14	50	46	10	10
	1 : 1	58	22	60	69	15	15

on BANA reached a value of 80 per cent against only 22 per cent in respect of CMPase activity.

Since, according to the method of Blackwood and Mandl [2] trypsin activity on BANA has to be estimated in McIlvaine's phosphate buffer, we employed the same buffer for the determination of CMPase activity so as to facilitate a comparison of the results.

In another group of experiments we examined in two other buffers, Tris and Na_2CO_3—HCl, the effect of two inhibitors on the CMPase activity of *Worthington*'s trypsin, measured on pulverized Achilles tendon as substrate. Results are assembled in Table IV. It can be seen that ovomucoid had approximately the same inhibitory effect in the two buffers, and that it had practically no effect in McIlvaine's buffer. Soybean inhibitor produced no marked inhibition either in Tris or in McIlvaine's buffer, not even at higher concentrations. It was only in the buffer Na_2CO_3—HCl that, at concentrations of 1 : 0.8 and 1 : 1, that inhibition reached 46 and 69 per cent, respectively.

Discussion

Of three trypsin inhibitors used, it was the crystallized soybean inhibitor the effect of which on the activity of trypsin on BANA was different accordingly to whether *Trypure Novo* (3 × 28) or *Worthington*'s twice crystallized saltless trypsin was employed. This indicates a quantitative difference between the active groups of the two preparations, and constitutes additional proof of the fact that the different trypsin preparations are not homogeneous.

The fact that the activity of trypsin on BANA was differently inhibited than the CMPase activity has confirmed our earlier experiments which showed that the two activities were due to different active principles and were governed by different mechanisms. Ovomucoid and soybean inhibitor affected CMPase activity very slightly, but considerably inhibited the effect on BANA of the same trypsin preparation.

It follows that, as has already been suggested [1], the specific activity of trypsin preparations is not identical with their CMPase activity; each of the two activities is bound to a different protein component of the enzyme, or there are two different active groups in trypsin. Had there been no difference between the two, both would have been equally inhibited at identical concentrations of the same inhibitor. We feel justified in suggesting that the pancreas of humans and animals synthetizes an enzyme which is capable of digesting native collagen, an enzyme which is in close affinity with, but not the same as, trypsin.

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Ilona Banga, Jolanda Mayláth-Palágyi,
Budapesti Orvostudományi Egyetem I. sz. Kórbonctani és Kísérleti Rákkutató Intézete, Budapest.

STUDIES OF THE SPECIFICITY OF MUSCLE CHOLINESTERASES

I. THE ROLE OF ACTIVE ANIONIC SITES

By

Á. Szöőr, A. Kövér and Ö. Pohánka

INSTITUTE OF PHYSIOLOGY, MEDICAL UNIVERSITY, DEBRECEN

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Comparative studies have shown that muscle cholinesterases differ in their hydrolytic activities. On decreasing the pH of the incubation mixture from 8.0 to 5.5 the hydrolysis of dimethylaminoethyl acetate, expressed in percentage of acetylcholine breakdown, increased about sevenfold by using of true cholinesterase, and doubled by applying of myosincholinesterase. At identical conditions the hydrolysis of dimethylaminopropyl acetate expressed in percentage of acetylcholine breakdown tended to decrease slightly in the case of true cholinesterase, whereas it increased eight- to ninefold in that of myosincholinesterase.

Decamethonium proved to be a more potent inhibitor of both cholinesterases than pentamethonium, but no significant differences could be demonstrated between the two enzymes as regards the inhibition.

On the basis of the original terminology put forward by Mendel and Rudney [1] the cholinesterases isolated from different tissues may be divided into two large groups, true cholinesterases and pseudo cholinesterases. Other terms have also been suggested to facilitate differentiation [2, 3, 4, 5, 6]. The cholinesterases belonging to both groups catalyze essentially the same reaction [7]. In the hydrolytic activity active anionic and esteratic sites play an important role [8, 9]. The differences between the true and the pseudo cholinesterases, that manifest themselves apart from substrate specificity in substrate inhibition and affinity to specific inhibitors, may probably be attributed to differences in the structure of active centres, or in their intramolecular arrangement. On this basis they may be surmised to play different physiological roles.

In the striated muscle of rabbit the difference in the physiological role played by myosin possessing cholinesterase activity, and by the true cholinesterase that can be isolated and purified from the sarcoplasmic protein fraction, is suggested also by the difference observed in their localization [10]. To obtain some data useful in experiments *in vivo* it seemed necessary to study the specific properties of the two enzymes *in vitro*, *i.e.* the properties of the active centres involved in the hydrolytic activity and their intramolecular distribution.

The present paper reports on studies concerning the properties of the active anionic sites involved in enzymatic activity.

Materials and methods

Assay of enzyme activity. As myosin had to be used at high concentrations, in the case of a manometric method the error due to adsorption had to be taken into account [11]. In the case of the electrometric method [12, 13] the buffer capacity of the high protein concentration makes unreliable the results based on shifts in pH. For this reason the photometric method seemed to be the most suitable for assaying enzyme activity. In our investigations the principle of enzyme assay has been based on the determination of the substrate concentration before and after incubation. For this, HESTRIN's method [14] was used determining the ester linkages. This procedure was more advantageous than the other photometric procedures described in the literature, because it can be used extensively. In calculating the results we took into account the substrate breakdown, autohydrolysis measured without enzyme, under identical conditions. It was minimal under pH 7.4 and increased over pH 7.4. In the incubation mixtures the substrate concentration was $1.10 \cdot 10^{-3}$ *M*. The colour intensities corresponding to the concentration of the substrates were estimated in a Havemann photometer, at 530 mμ. In the range of pH 5.5 to 7.0 a 0.01 *M* phosphate buffer, in the pH 7.4 to 8.0 range a 0.01 *M* veronal-Na—HCl buffer (final concentrations) served to ensure the proper reaction. The experiments were carried out at 37° C, the duration of incubation was 1 hour. The specific activity was expressed as μg Ach/mg protein N/hour. Nitrogen was determined by micro-Kjeldahl method. The pH of the incubation mixture was controlled before and after incubation by means of glass electrodes. No shift in pH was observed.

Substrates, inhibitors. The substrates and inhibitors used in the experiments were dissolved in distilled water immediately prior to use. Acetylcholine chloride was obtained from *Hoffmann—La Roche* Ltd. Basel. Pentamethonium and decamethonium preparations made by *Light* Co. were used. Dimethylaminoethyl acetate hydrochloride and dimethylaminopropyl acetate hydrochloride were prepared in our laboratory [15].

Enzymes. The myosin preparations were obtained from striated muscles of rabbit according to GUBA—STRAUB method [17], as modified by WEBER [16]. Prior to the extraction of myosin the muscle homogenate was washed with chilled distilled water then with 0.05 *M* KCl, according to SZENT-GYÖRGYI [18]. Before estimating enzyme activity, the purified myosin preparation was diluted to 120 to 140 mg N per 100 ml. True cholinesterase was purified from the sarcoplasmatic extract obtained at pH 7.3 with 0.05 *M* KCl solution. True cholinesterase Fr. II was used in a solution containing 25 to 30 mg N per 100 ml [19].

Results

Dimethylaminoethyl acetate at the optimal pH of cholinesterases is a neutral molecule, which gains a positive charge on decreasing the pH [20]. Thus, the Coulomb force arising between the dimethylaminoethyl acetate and the active anion groups of the enzyme may be increased by lowering the pH. The measure of the increase depends also on the number and distribution of the active anion groups of the enzyme. As a result of this phenomenon the cholinesterases hydrolyse less dimethylaminoethyl acetate (as expressed in per cent of acetylcholine breakdown) near the optimal pH value than at a lower pH. In such cases the electrostatic pull developing between the substrate (which turns positive in character) and the active anion groups promotes the formation of the enzyme-substrate complex and the orientation of the substrate molecule on the surface of the enzyme. Since the positive charge of the quaternary amine radical is independent of the pH, the pH-dependent hydrolysis of the tertiary amine ester has been expressed as related to the acetylcholine breakdown measured under the same conditions. As a result, the hydrolysis of the tertiary amine ester depends only on the change in the positive character of the substrate

and on the number of the active anion groups of the enzyme, or on their relative positions, as well as eventually on the relative spatial arrangement of the anion groups and the ester-splitting ones functioning at the given pH.

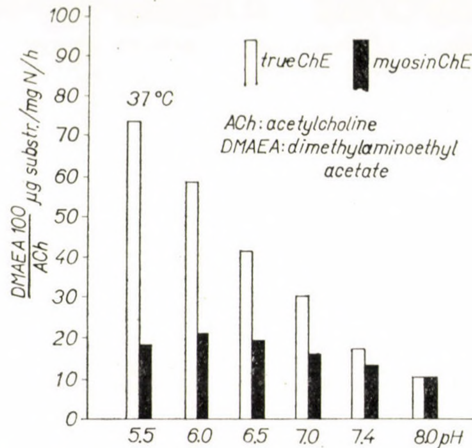

Fig. 1. Comparative enzymatic hydrolysis of dimethylaminoethyl acetate at different pH's. The percentage values shown on the ordinate have been computed on the basis of the hydrolysis of the two substrates, acetylcholine and dimethylaminoethyl acetate

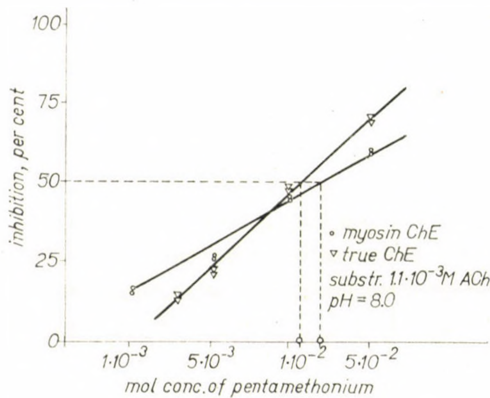

Fig. 2. Inhibition of myosincholinesterase and true cholinesterase activity by pentamethonium, at pH 8.0. Duration of preincubation with inhibitor, 30 minutes

Fig. 1 shows the hydrolysis of dimethylaminoethyl acetate, expressed in percentage of acetylcholine breakdown by true and myosincholinesterase, at different pH values.

When the pH was decreased from 8.0 to 5.5, the breakdown of dimethylaminoethyl acetate by true cholinesterase increased from 10 per cent to 75 per cent, while in the case of myosin the increase of activity was hardly twofold.

The data obtained in this experiment suggest that the difference in the increase of hydrolytic activity between the two enzymes may be attributed to a difference in their active anionic structure.

On grounds of the evidence presented by BERGMANN and SEGAL [21] we studied the role played by the active anion groups. We preincubated the enzymes with positively charged terminal bisquaternary amine inhibitors of different chain lengths and examined their effects on the hydrolytic activity.

Fig. 2 illustrates one of our experiments carried out with pentamethonium at inhibitor concentrations on the abscissa. The percentage inhibition is pre-

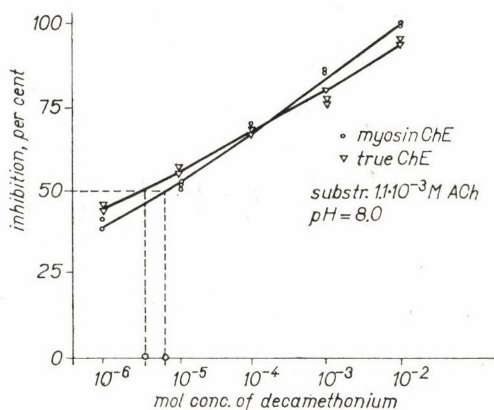

Fig. 3. Inhibition of myosincholinesterase and true cholinesterase by decamethonium, at pH 8.0. Preincubation with inhibitor, 30 minutes

sented on the ordinate. Acetylcholine served as substrate. In the case of both enzymes the inhibitor concentrations causing 50 per cent inhibition are also shown. According to the results the lower pentamethonium concentration inhibited in a lesser measure, and the higher one in a more marked measure the activity of true cholinesterase than that of myosincholinesterase. The I_{50} value of true cholinesterase was at $1.66 \cdot 10^{-2} M$ pentamethonium concentration, while that of myosincholinesterase at $1.15 \cdot 10^{-2} M$. The difference was not considered significant.

The decrease of the pH from 8.0 to 6.0 of the incubation mixture had no substantial influence on the inhibitory action of pentamethonium.

Decamethonium inhibited the activity of both enzymes at much lower concentrations (Fig. 3). $5.0 \cdot 10^{-6} M$ decamethonium produced a 50 per cent inhibition of true cholinesterase activity, while the I_{50} value for myosincholinesterase was at $7.5 \cdot 10^{-6} M$.

According to these experiments, inhibition increases with lengthening the carbon chain. Decamethonium inhibited both enzymes at much lower concentrations than pentamethonium, but no significant difference in the measure

of inhibition between the two enzymes could be observed with either of the inhibitors.

To study the arrangement of the active anion and ester-splitting groups, the experiments with dimethylaminoethyl acetate were repeated at pH 8.0, and 5.5 with dimethylaminopropyl acetate too.

Fig. 4 shows the hydrolysis of dimethylaminopropyl acetate, expressed in percentage acetylcholine breakdown, by true and myosincholinesterase.

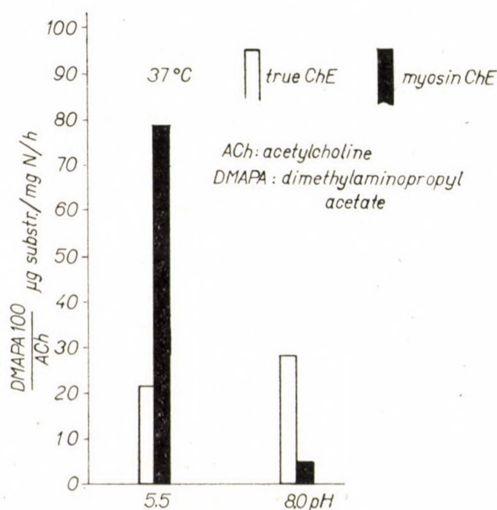

Fig. 4. Enzymatic hydrolysis of dimethylaminopropyl acetate at pH 5.5 and pH 8.0. The percentage values shown on the ordinate have been computed on the basis of the hydrolysis of the two substrates, acetylcholine and dimethylaminopropyl acetate

The pH-dependence of the enzymatic hydrolysis of the tertiary amine ester was significantly different from that of dimethylaminoethyl acetate. On decreasing the pH from 8.0 to 5.5 myosincholinesterase split 8 to 9 times the original amount of dimethylaminopropyl acetate, at identical conditions the activity of the true cholinesterase tended slightly to decrease.

Discussion

As to the substrate specificity of myosincholinesterase, KÖVÉR and KOVÁCS [22] showed that myosincholinesterase prepared from striated rabbit muscle broke down acetyl-beta-methylcholine like acetyl-choline, the hydrolysis of butyrilcholine increased with the increase of substrate concentration, and at the highest concentration employed ($10^{-2}M$) the measure of hydrolysis was approximately the same as at the optimal acetylcholine concentration and

otherwise under the similar conditions. On the basis of these properties, too, myosincholinesterase is different from the purified, true cholinesterase.

These earlier experiments, too, suggested the important role of the protein structure of cholinesterase, as far as typing is concerned. This was indicated also by the observation that the myosincholinesterase prepared from heart muscle was different in substrate-specificity from the myosincholinesterase of striated muscle.

The present findings agreed well with those just discussed, although in another relation. On the basis of the results now obtained we think that one of the significant differences between the two kinds of muscle cholinesterases is to be sought in the spatial arrangement of the negative charges adjacent to the ester-splitting groups. As to the relation between the active anion and ester-splitting groups we accept the view [20, 23] according to which the Coulomb force arising between the active anion group of the enzyme and the cationic head of the substrate promotes the activity of the ester-splitting group. In the case of the two enzymes used, the different hydrolysis of dimethylaminoethyl acetate and dimethylaminopropyl acetate shown at different pH's suggests that the distance between the cationic head and the esteratic bond creates optimal conditions for the active centres of true cholinesterase using dimethylaminoethyl acetate, and for those of myosincholinesterase applying dimethylaminopropyl acetate.

In our experiments the distance between the active anion groups proved to be the same in the case of both enzymes. According to BERGMANN *et al.* [21, 24], the bisquaternary amine inhibitors can adapt themselves to the anion groups on the surface of the cholinesterase molecule. Owing to the close spatial proximity of the anion groups, however, the inhibitor molecule must bend for being adapted. Bending becomes much easier as the length of the chain increases, because in the case of a short chain the deviation from the angle of normal combination is necessarily greater. This may explain why decamethonium is a more potent inhibitor than pentamethonium.

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Árpád SZÖŐR, András KÖVÉR, Ödön POHÁNKA,
Orvostudományi Egyetem Élettani Intézete, Debrecen.

STUDIES OF THE SPECIFICITY OF MUSCLE CHOLINESTERASES

II. THE ROLE OF THE ESTERATIC SITE

By

Á. Szöőr, A. Kövér and T. Kovács

INSTITUTE OF PHYSIOLOGY, MEDICAL UNIVERSITY, DEBRECEN

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In the course of comparative studies of muscle cholinesterases, the hydrolysis of alcohol or acyl homologue aliphatic esters has been subjected to investigation. The hydrolysis of these esters by true- and myosincholinesterase increased in proportion with the size of the substrate molecules.

True cholinesterase hydrolyzed the ethyl chloroacetate about fifty times more rapidly than ethyl acetate, while myosincholinesterase hydrolyzed both substrates at the same activity.

The interaction between cholinesterases and the simple aliphatic- or the cholinester substrates has been studied by Wilson [1, 2, 3], who could show that the most important factor in the interaction is the Coulomb force active between the negatively charged groups of the enzyme and the positively charged groups of the substrate [1]. The combination of cholinesterases with uncharged, *e.g.* simple aliphatic ester molecules can be ascribed to the much weaker dispersive, van der Waals force [2]. In the formation of the enzyme-substrate complex, a minor role is played by the esteratic site of enzyme, which catalyses first of all the splitting of the ester linkage [3].

In the present experiments we have studied the effects on the enzymatic hydrolysis of simple, aliphatic esters with different length of the carbon chain in the ester alcohol or acyl components. Further we have investigated the change of the enzymatic hydrolysis increasing the electrophilic character of carbonyl group in the simple, aliphatic ester, by building-in a chlor atom into the methyl group of the acetate.

Materials and methods

Enzyme assay. In accordance with earlier experience [5] and in view of the high concentration of myosin necessarily present, we used a photometric method for assaying enzyme activity. Substrate concentration was determined before and after incubation according to Hestrin [6]. In the incubation mixtures the concentration of the substrates was 1.1×10^{-3} *M*. The colour intensity corresponding to the substrate concentration was measured in a Havemann photometer, at 530 mμ. The experiments were carried out at 37.8° C, incubation lasted 1 hour. At around the optimal pH of the cholinesterases, the non-enzymatic hydrolysis of the simple aliphatic esters is relatively high. This makes it rather difficult, even if suitable control tests are made, to evaluate the relatively slight enzymatic hydrolysis. Autohydrolysis could diminished by assaying activity at pH 7.4, at a lower than the optimal one. This way we

obtained reproducible results, although the measure of enzymatic hydrolysis decreased. The pH was ensured by veronal Na—HCl buffer of 0.01 *M* at a final concentration. The measure of hydrolysis was expressed as the quantity of substrate hydrolyzed in 1 hour by 1 mg of enzyme protein N. The pH of the incubation mixture was controlled with glass electrode before and after incubation. No shift in pH was noted.

Substrates. The simple aliphatic esters have been prepared from the suitable alcohol and acid, or acid-chloride components, at our laboratory. Purity was controlled on the basis of the boiling point. Ethyl chloroacetate was prepared from monochloracetic acid and ethanol, by boiling in the presence of concentrated sulphuric acid. The pure preparation was obtained by distillation at 142° C. The simple aliphatic esters dissolve poorly in water and therefore we applied a small quantity of sodium dodecyl sulphate, to increase the solubility.

Enzymes. Myosin was prepared from striated muscles of rabbit according to GUBA—STRAUB method [8], as modified by WEBER [7]. Prior to the extraction of myosin the muscle homogenate had been washed with chilled distilled water, then with 0.05 *M* KCl solution. Before assaying enzyme activity, the purified myosin preparation had been diluted to 120 to 140 mg N per 100 ml. True cholinesterase was prepared from sarcoplasmatic extract obtained with 0.05 *M* KCl solution at pH 7.3 [4]. The purified true cholinesterase, Fr. II, was used in solution containing 25 to 30 mg N per 100 ml.

Experimental

With the two types of muscle cholinesterase, we studied the enzymatic hydrolysis of simple aliphatic esters first by using esters containing the same acid component and alcoholic components of different chain lengths. The results are presented in Fig. 1.

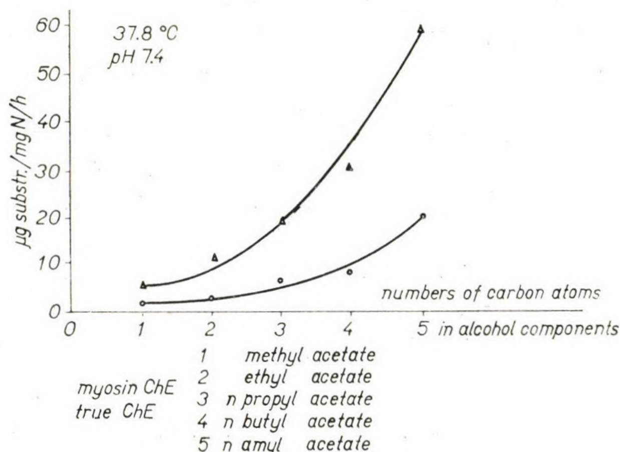

Fig. 1. Enzymatic hydrolysis of alcohol homologue esters. The ordinate shows the rate of breakdown, in substrate/mg protein N/hour units. The abscissa shows the number of $-CH_2$-groups of the alcohol components of the substrate

The data in Fig. 1 indicate that the alcohol homologue esters with a long carbon chain, high molecular weight and large size were hydrolyzed several times better than those with a short carbon chain and small size. There was,

however, no difference between the two muscle cholinesterases as regards the increase in hydrolysis depending on molecular size.

On the basis of literary data it may be supposed that the changes in the size of the alcohol components of the simple aliphatic esters were causing the above mentioned phenomenon not through the activity of esteratic site, but through the difference in the dispersion forces active between substrate and enzyme.

However, the changes induced in the size and properties of the acyl groups of the simple aliphatic esters may directly influence the hydrolytic

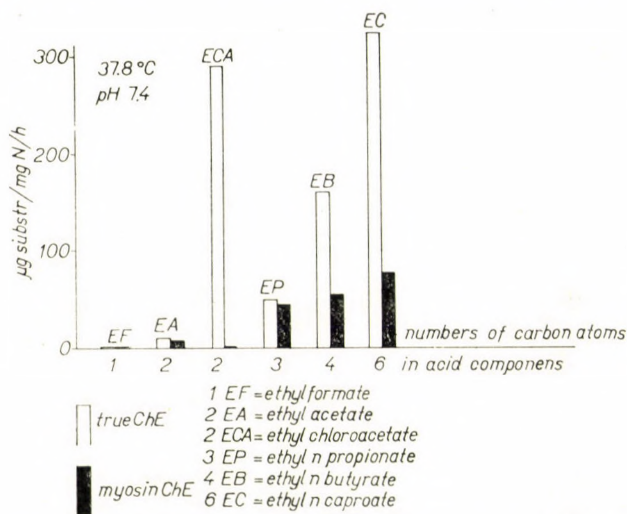

Fig. 2. Enzymatic hydrolysis of acyl homologue esters and ethylchloroacetate. Ordinate: hydrolysis in substrate/mg protein N/hour units. Abscissa: number of carbon atoms in the acyl components of the substrates

activity of the esteratic groups. Fig. 2 shows the results of the experiments with acyl homologue esters, as well as with ethyl chloroacetate.

The results in Fig. 2 indicate that the hydrolysis of the acyl homologues increased proportionately to the increase in their carbon atom number. This was somewhat more marked in the case of true cholinesterase than in that of myosincholinesterase. The difference was not significant statistically. However, the substitution of the methyl radical of the acyl group of ethylacetate with chlorine resulted in a highly significant increase of true cholinesterase activity, while under such conditions there was a decrease in the activity of myosincholinesterase. The phenomenon observed cannot be explained by the difference in molecular weight between ethylacetate and ethylchloroacetate; it may be probably due to the structural differences in the esteratic groups of the two enzymes.

Discussion

The hydrolysis of simple aliphatic esters is promoted by the van der Waals force, arising between the enzyme and substrate molecules close-set to each other [2]. The force exerts its action so that a shift is resulted in the electronic structure of the substrates near the enzyme, which leads to the development of a temporary dipole. This "momentary electrical field" suffices to evoke an inductive effect in the closely lying molecules. Thereby the simple aliphatic esters behaving electroneutrally, and cholinesterases become capable

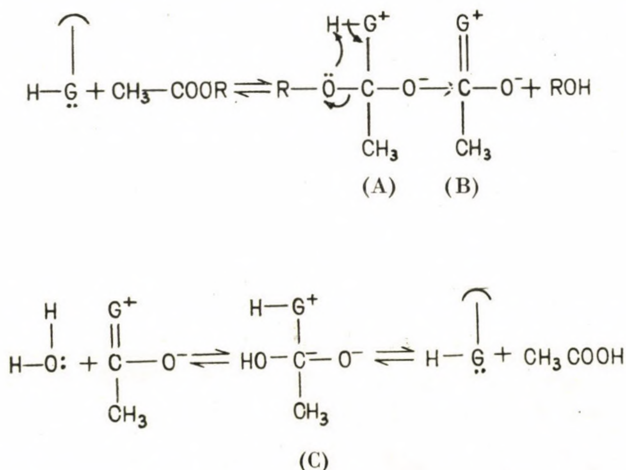

Fig. 3. Mechanism of the hydrolytic reaction catalyzed by the cholinesterases

of uniting to form enzyme-substrate complexes. As a result, the cholinesterases can hydrolyze to a small extent, also the simple aliphatic esters. The van der Waals force is several times weaker in order of magnitude than the Coulomb force. This explains why the electroneutral molecules are hydrolyzed at a much slower rate than those of cationic character. The size of the molecules is not indifferent, either. The large substrate molecules are linked firmly to the surface of the enzyme and, as a result of this, they are hydrolyzed in an increased rate. The changes in the dispersive forces acting between the simple ester molecules of different size and the cholinesterases produce no significant differences in the specific, hydrolytic activity of the muscle cholinesterases.

The carbonyl groups of the ester molecules that may be used as substrate are definitely polar in character. It has been shown that a covalent bond may be formed between the carbon atom of the electrophilic carbonyl group in the substrate and the basic radical of the esteratic site of the enzyme [9, 10]. The carbon-bound groups are influencing the electrophilic character of the substrate molecule, which is reflected also in the charge of the carbonyl oxygen. If the

electrophilic character of the carbon is increased by a group, the electrons of the oxygen are attracted to an increased extent, as a result of which the charge of the oxygen decreases. This change weakens the negativity of the oxygen atom, reducing the strength of the connexion between the carbonyl group of the substrate and the positive radical of the esteratic group of the enzyme.

The mechanism of enzymatic hydrolysis is shown in Fig. 3 [12].

The esteratic site is symbolized by the H—$\underset{\cdot\cdot}{G}$ sign. The dissociable H designates the acid, $\underset{\cdot\cdot}{G}$ the basic group. The structure designated $\underset{\cdot\cdot}{G}$ is supposed to be capable of electron-transport [10, 11], which is proved also by the fact that if the covalent bond between this group and the substrate is broken up, the electrons become rearranged. In the first step of the hydrolytic reaction a connexion is formed between the carbonyl group of the substrate and the esteratic group of the enzyme (A), then the ester molecule becomes so re-arranged that with the formation of acyl-enzyme (B) the alcohol component is split off. The reaction ends with water uptake, when the enzyme gets back its hydrogen and the acyl group is split off from the complex in the form of acid (C).

On the basis of our experiments with ethyl chloroacetate we assume that the substitution with a chlorine atom can enhance the hydrolytic activity of the enzymes acting as electron donors. In the case of true cholinesterase (among the muscle cholinesterases) this requirement is provided for. This may be the explanation of the fact that ethylchloroacetate is hydrolyzed in a different measure by the two muscle cholinesterases.

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Árpád Szöőr, András Kövér, Tibor Kovács,
Orvostudományi Egyetem Élettani Intézete, Debrecen.

COMPARISON OF THE ANTIGENIC PROPERTIES OF CHROMEOVALBUMIN AND NATIVE OVALBUMIN ON THE BASIS OF QUANTITATIVE PRECIPITATION

By

MARY KÁVAI and L. KESZTYÜS

INSTITUTE OF PATHOPHYSIOLOGY, MEDICAL UNIVERSITY, DEBRECEN

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The results of the quantitative precipitation analysis of chrome-labelled ovalbumin [11] are presented. It is pointed out that labelling with chrome in 15 and 25 mol proportions has no substantial influence on the antigenicity and reactivity of the protein molecule.

The chemical labelling of some protein is usually accompanied by changes, qualitative or quantitative, in its immunological properties. FRANCIS *et al.* [1] iodinated antiovalbumin antibodies and found that 4 atoms I/mol antibody did not change the reacting antibody content, whereas more intense iodination gradually diminished the amount of antibodies precipitated by the native antigen. PRESSMAN *et al.* [2] have shown in connexion with the iodination of antiserum and certain gamma globulin fractions that over 9 atoms I/mol antibody the antibodies were seriously damaged, due partly to the incorporation of iodine and partly to its oxidative action.

Iodination of the antigen over the value of 5 to 10 atoms I/mol antigen likewise diminishes the formation of linkages between the original antibody and the iodinated antigen [3]. The diiodotyrosine formed acts as a new chemospecific determinant group and possesses specific antibody-binding power [4].

It has been known for long from the investigations of LANDSTEINER [5, 6] and PAULING [7] that chemical compounds linked to native proteins, by forming special and extremely specific determinant groups, alter the original antigenicity of the protein.

On the basis of all these the question arose whether chromatization of ovalbumin will change the combining power of the product, will there be new specific determinant groups, or some other changes in antigenicity. To find the answers, we have made quantitative precipitation tests with native and chrome-labelled ovalbumin, as well as with antiovalbumin and antichromeovalbumin produced in response to the parenteral administration of the former material.

Materials and methods

Antigen. This was prepared by COLE's method [8], recrystallized 4 times (referred to in the following as N-OA). It consisted of one single fraction electrophoretically. As determined by OUDIN's [9] linear, simple, tube gel diffusion antigen analysis, the main component of oval-

bumin mobility contained 1 to 2 per cent of two contaminating components, showing conalbumin and ovomucoid mobility at immune-electrophoresis [10].

Ovalbumin, labelled with chrome in various ways, in alkaline media, prepared according to KÁVAI and KESZTYÜS [11]. Labelling was carried out with 15, 25, 50 and 75 atom Cr^{3+} per ovalbumin molecule. Correspondingly, in the following these will be referred to as 15 Cr-OA, 25 Cr-OA, 50 Cr-OA and 75 Cr-OA.

Antiserum. Sera from rabbits immunized with N-OA and 15 Cr-OA (in the following, N-OA-IS, 15 Cr-OA-IS).

Quantitative precipitation was carried out by the technique of KABAT and MAYER [12]. 0.5 ml of antiserum was used, the protein content of the precipitate formed was determined at 280 and 210 mμ [13] in an *Unicam* spectrophotometer. The method is suitable for measuring mixed proteins, since it gives the protein content not on the basis of absorption by cyclic amino acids, which occur in different proportions in albumin and globulin, but on grounds of absorption by peptide linkages. The disadvantage is that the method is highly sensitive, so that dilute systems have to be used to obtain a measurable extinction. The supernatants were subjected to the usual precipitation tests and were analyzed also by OUDIN's [9] gel diffusion method. In the latter case the procedure was as follows. The supernatants were divided into two parts, one part was mixed with agar, analyzed for antibody content, and antigen sufficient to form a minimal precipitate was layered over it, while the other part of the supernatants was analyzed for antigen content, and therefore it was layered over antibody mixed with agar.

Isotope analysis of quantitative precipitation. In the case of antigen labelled with Cr^{51}, the precipitate formed was tested for activity. The precipitate was washed by the usual method. The washing fluids were pooled, and the precipitate was dissolved in normal sodium hydroxide. By a modification of the method of MAURER and TALMAGE [14] the activities of the washings, supernatant and precipitate were determined in tubes similar in size and containing the same volume of NaI crystals, by means of a scintillation detector. After assaying its activity, the precipitate was tested for protein content.

Results

Fig. 1 shows the quantitative precipitation of one of the native antiovalbumin rabbit sera with antigens N-OA, 15 Cr-OA, 25 Cr-OA and 15 ^{51}Cr-OA.

The three curves are similar in shape in the zone of excess antibody. Curves 2 and 3 run under curve 1. Curves 2 and 3 reach the peak on adding higher antigen-N and show more of antibody. In 8 N-OA-IS we demonstrated 1 to 3 per cent more antibody with 25 Cr-OA than with N-OA. In the excess antigen zone the curves obtained with Cr-OA deviate more markedly from curve 1, with these antigens more precipitate was formed and it was not completely dissolved.

Isotope analysis of the changes in the amount of antigen contained in the precipitate shows (broken curve No. 2) that until the maximum has been reached the total ^{51}Cr-OA is demonstrable in the precipitate. After the maximum has been reached, more and more of it passes over into the supernatant, but the antigen content of the precipitate continues to increase and decreases only in the case of extreme antigen excess.

Fig. 2 shows the dependence of the antibody-antigen ratio (mol ratio) on the amount of antigen.

The data in Fig. 2 indicate that with the increase in the amount of antigen the antibody-N-antigen-N content of the precipitate decreases. If the corresponding points are connected, a straight line results. According to the

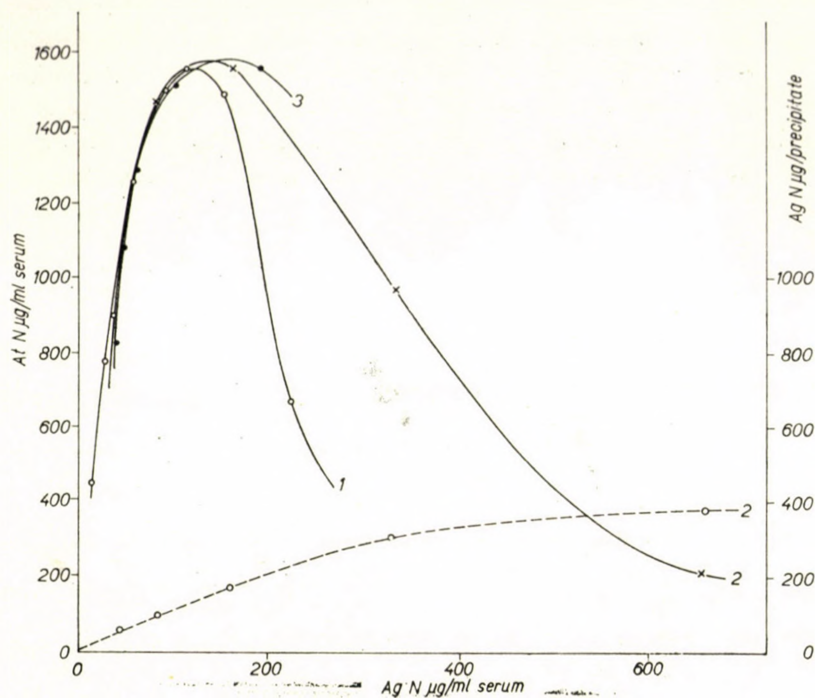

Fig. 1. Quantitative precipitations of N-OA-IS + N-OA and different Cr-OA preparations. Curve *1:* precipitation with N-OA. Curve *2:* precipitation with 15 Cr-OA. Curve *3:* precipitation with 25 Cr-OA. Broken-line curve No. *2:* antigen-N in precipitate obtained with 15 ^{51}Cr-OA, as determined by isotope analysis

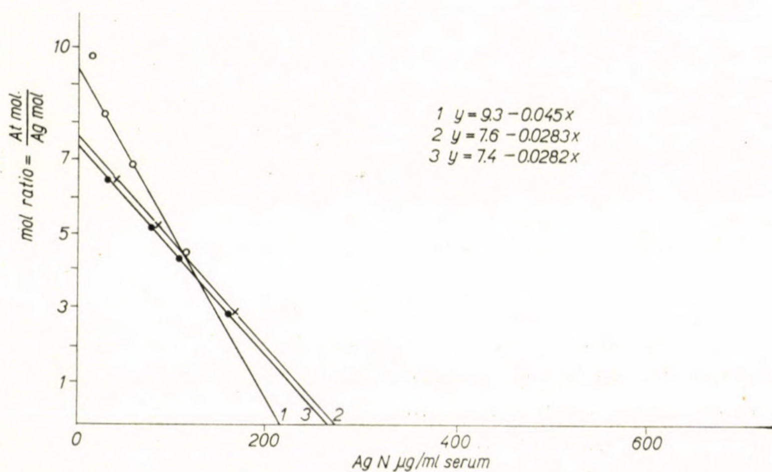

Fig. 2. Antibody-antigen ratios of quantitative precipitations of N-OA-IS + N-OA and different Cr-OA preparations, plotted against antigen concentration. Straight lines *1*, *2* and *3* show the mol ratios vs. antigen concentration in the case of precipitation obtained with ovalbumin, and chrome-ovalbumin preparations, respectively. The graphically obtained equations of each curve are also shown

general equation for the straight line, y = a-bx. In our case y = antibody-N: antigen-N ratio in the precipitate; x = antigen-N content in the precipitate; "a" and "b" are constants, of which "a" means the point at which the straight line meets the "y" axis and "b" means the slope of the straight line. Constants "a" and "b" are the characteristics of the antigen-antibody system. The value

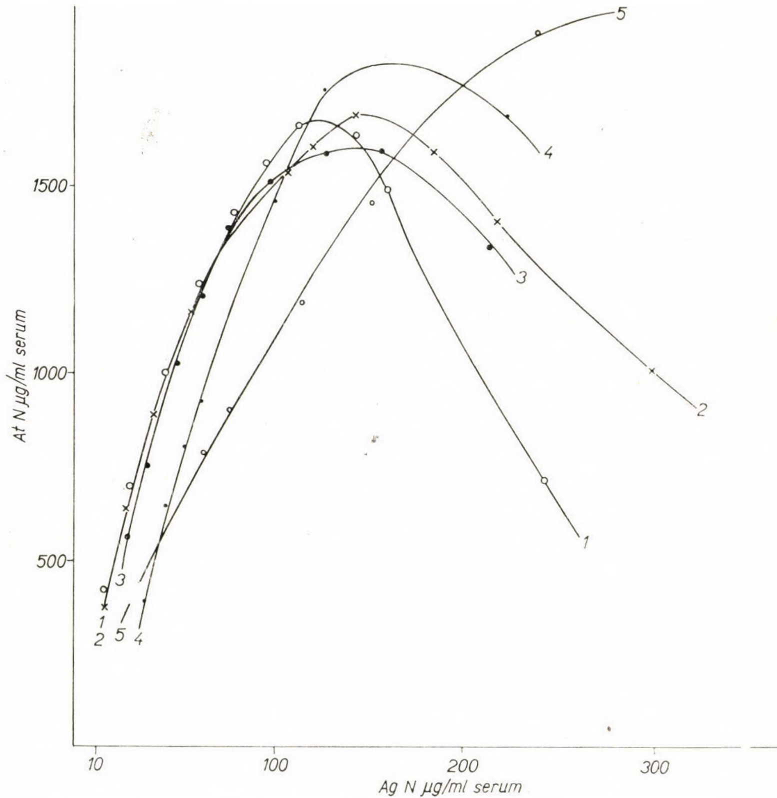

Fig. 3. Quantitative precipitation of 15 Cr-OA-IS with N-OA and with different Cr-OA antigens

of "a" shows in what proportions the antigen and antibody tested can combine with each other. The higher the combining power, the higher the value of constant "a" in the corresponding equation. The value of "b" is influenced by the antibody content of serum. The relationship between them is reversed; the higher the antibody content of serum, the lower the "b" value. On the basis of these constants two different antigens and sera, respectively, can be compared.

The equation of the straight line can be obtained from the data at our disposal by computing the regression coefficient mathematically [15] or gra-

Table I

Tubes No.	1	2	3	4	5	6	7	8	9	10	11	12
15 ^{51}Cr-OA μg N	20.5	41	82	164	328	656	20.5	41	82	164	328	656
15 ^{51}Cr-OA ml	0.1	0.2	0.4	0.8	0.16	0.32	0.1	0.2	0.4	0.8	0.16	0.32
15 ^{51}Cr-OA Imp/min	610	1,220	2,440	4,880	9,760	19,520	610	1,220	2,440	4,880	9,760	19,520
0.9% NaCl ml	1.4	1.3	1.1	0.7	1.34	1.18	1.4	1.3	1.1	0.7	1.34	1.18
Serum ml	0.5 ml antiovalbumin rabbit serum						0.5 ml normal rabbit serum					
Activity of precipitate, c. p. m.	650	1,260	2,415	4,541	5,869	3,686	—	—	—	—	—	—
Activity of supernatant, c. p. m.	—	—	—	310	3,935	15,663	661	1,114	2,495	4,920	9,630	19,121
Activity of washing fluid, c. p. m.	—	—	—	—	27	229	82	75	87	119	275	750
Ag μgN in precipitate	21.7	42.4	81	153	192	121	—	—	—	—	—	—
Total μgN in precipitate	470	767	865	635	295	190	—	—	—	—	—	—
At μg N	450	726	783	482	103	66	—	—	—	—	—	—
Mol ratio	6.6	5.3	2.8	—	—	—	—	—	—	—	—	—
Sup. + Ag	+	+	—	—	—	—	—	—	—	—	—	—
Sup. + Serum	—	—	—	+	+	+	—	—	—	—	—	—

phically. In the case of a smaller number of experimental data the latter method is the one more suitable.

Comparison of the constants of equations 1, 2 and 3 reveals that in 2 and 3 the value of "a" is lower. This means that chrome treatment reduces the combining power of the protein. On the other hand, the decrease in the "b" value means that with the chrome-labelled protein more antibody can be recovered from the same serum.

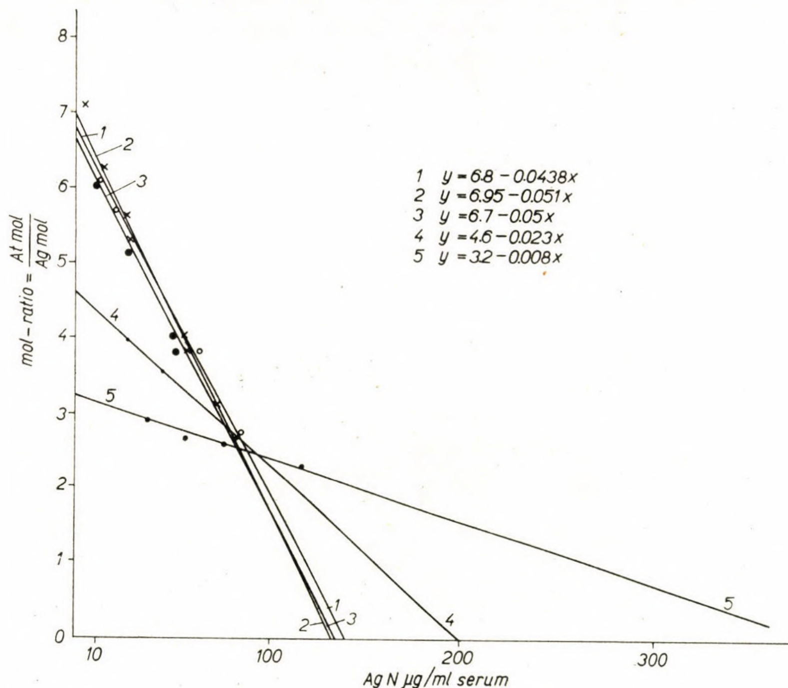

Fig. 4. Antigen-antibody ratios of precipitations with 15 Cr-OA-IS + N-OA, as well as different Cr-OA antigens, plotted against antigen concentration

In Table I are shown the numerical data for the quantitative precipitation reaction involving the use of antigen ^{51}Cr-OA and serum N-OA-IS.

The data in Table I indicate that 15 ^{51}Cr-OA does not precipitate with normal rabbit serum, the activity observed in the supernatant + washing fluid is equivalent with the activity of the antigen added to the serum. The activity of the precipitate in the 15 ^{51}Cr-OA + N-OA-IS system indicates that in the presence of excess antibody the total amount of antigen is contained in the precipitate, and no activity is demonstrable in the supernatant. However, in the presence of excess antigen also the activity of the supernatant suggests that antigen is present in it. In the supernatant the two contaminating components of the antigen, demonstrated by OUDIN's method, do not interfere

with the analysis made by the usual technique, owing to their low concentrations; precipitation occurs only in the positive tubes. With OUDIN's method similar results are obtained concerning the OA fractions of the native and labelled OA-preparations in the supernatant and added to it. While the presence of contaminants could not be established in any of the supernatants, the antibodies produced against them were demonstrable in every one of them. This indicates that the antigenic contaminations, if representing 1 to 2 per cent of the employed amount of antigen, do not separate the antibody produced against them.

We have made similar studies with antichromeovalbumin immune serum. The results are presented in Fig. 3 and Fig. 4.

The data in Fig. 3 show the precipitation reaction between 15 Cr-OA-IS and the native, as well as differently chrome-labelled ovalbumin preparations. The precipitations obtained with native ovalbumin, 15 Cr-OA and 25 Cr-OA are quantitatively comparable. In the case of more intense chrome treatment (see curves 4 and 5) the precipitation is different in character. In Fig. 4 the mol ratio values are plotted against the antigen concentrations. It can be seen that the straight lines of the mol ratios for the precipitations obtained with native ovalbumin and with the two, less intensely chrome-treated, preparations Cr-OA [1, 2, 3], are nearly identical. The value of constant "a" is highest with the homologous — 15 Cr-OA — antigen. When precipitation is carried out with N-OA, the value of constant "a" is lower, and in the case of 25 Cr-OA still lower.

Discussion

A comparison of the antigenic activities of native and chrome-labelled ovalbumin revealed that chrome treatment at alkaline reaction at 15 and 25 mol ratios had but a slight influence on the original antigenicity of ovalbumin. The difference observed may manifest itself in the following way. In the case of *native antiovalbumin* the results of the quantitative precipitation tests indicated a reduction of the combining power of the chrome-treated albumin. The same quantity of antigen reacted with less antibody. This phenomenon may be explained by assumming that some determinant group was partially damaged, or that chrome treatment altered the distribution of charges on the surface of the protein molecule. It cannot be claimed that a determinant group has fallen out, because the same amount of antibody can be demonstrated with chrome-ovalbumin, as in the homologous system, although only after large amounts of antigen have been added. 15 Cr-OA and 25 Cr-OA yielded 0 to 3 per cent more antibody; this may have been due to aspecific globulin linkages, inasmuch as the phenomenon exceeds the limits of error of the method.

The results obtained with the *antichrome-ovalbumin immune serum* suggest that in the course of chrome treatment no new specific determinant group

has developed, because the same amounts of antigen could be demonstrated with the native and the chrome-treated antigens. More intense treatment with chrome caused a marked decrease in reactivity and an increase of the aspecific reaction. According to PRESSMAN [16] in the case of chemically modified proteins the change in the antigen-antibody reactions should be sought in a change of the protein-protein interaction. PRESSMAN was the first to describe that in homohapten heteroprotein precipitation, iodination of the antibody increased the demonstrable antibody content.

Finally, by measuring the activity of ^{51}Cr-ovalbumin we could follow up the fate of the precipitating antigen. Our results and those published by WORMALL [4] and FARR [17], obtained with ^{131}I-labelled antigen, showed a measurable activity in the supernatant in the antibody excess zone, too. With antigen labelled with ^{51}Cr, no activity indicative of the presence of antigen was demonstrable in the supernatant, either in the zone of antibody excess or in the zone of equivalence.

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Mária KÁVAI, Lóránd KESZTYÜS,
Orvostudományi Egyetem Kórélettani Intézete, Debrecen.

TRANSBRONCHIAL DETERMINATION OF LEFT INTRAATRIAL PRESSURE IN DOGS

By

A. Naszlady

DEPARTMENT OF EXPERIMENTAL MEDICINE, NATIONAL INSTITUTE OF CARDIOLOGY; EXPERIMENTAL RESEARCH LABORATORY, MEDICAL UNIVERSITY BUDAPEST

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A method has been described for reaching the left atrium from the trachea with a cervical opening. A cannula with a mandrin is introduced into the trachea and guided through the left ventral end of the carina in slightly ventro-caudal direction. By employing a suitable instrument it is thus possible to determine the pressures in the left heart.

Shortly after the catheterization of the right half of the heart had gained ground in the wake of Cournand's [1] pioneer work, investigators began to search for means of gaining access to the chambers of the left heart. Various methods were tried. Zimmermann *et al.* [2] attempted to reach them retrogradely from the aorta; Ponsdomenech and Nunez [3] experimented with the puncture of the left ventricle; Björk *et al.* [4] punctured the left atrium percutaneously from behind; Radner [5] reached the left heart from suprasternal approach; Ross [6] and many workers after him reached the left heart from the right atrium through the septum.

The very multitude of these procedures reveals the truth that none of them is fully satisfactory; under given experimental conditions, the one is impracticable, the other counterindicated, the third inconvenient, etc.

It occurred in the course of studying the pathogenesis of pulmonary oedema that we were faced with the necessity of ascertaining pressure in the left atrium. The retrograde procedure and right-side transseptal catheterization seemed to be inexpedient because of the risk of changing the haemodynamic conditions, while all percutaneous methods involved the risk of injuring the pulmonary tissues. Besides, all these procedures require radiological control. The bronchoscopic transbronchial method, as applied by Facquet *et al.* [7], presupposes long experience and cannot be used in the case of crossed circulation. It was after such antecedents that the idea of a direct puncture through the tracheal stump had emerged.

Experimental

Mediastinal topography is favourable for transbronchial direct puncture. The major arterial trunks are contiguous to the left atrium anteriorly, the diaphragm and the left ventricle from below, the left primary bronchus under the aortic arch and partly the ramifying pulmonary artery from above. That portion of the left primary bronchus which lies next to the carina tracheae abuts on the left atrium from above and behind. It follows that if a slightly ventro-caudally directed puncture is made through the carina, immediately

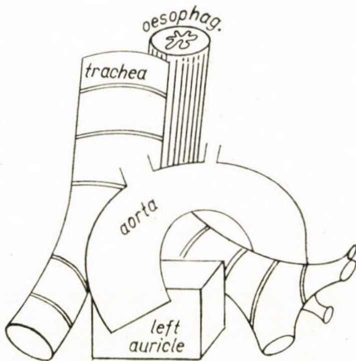

Fig. 1

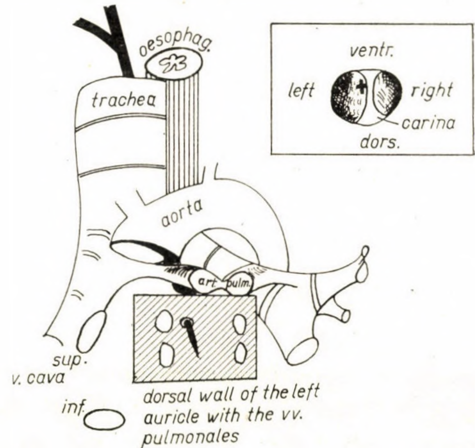

Fig. 2

to the left of its ventral end, the cannula will penetrate that wall of the left atrium which is between the pulmonary veins. (The needle may pierce one of these veins.) (Figs 1, 2).

A Y-shaped trocar, provided with a mandrin, was used in the present study, but large-diameter needles for lumbar puncture can likewise be employed. After the trocar has reached the left atrium (the tool can be pushed forward easily and without resistance in this case), the mandrin has to be withdrawn. Blood of vivid red colour at its tip shows that the end of the trocar is in a cavity filled with saturated blood. This cavity must be the left atrium or one of the pulmonary veins. Should the trocar have advanced as far as the left ventricle, the pressure will tell it, and a careful withdrawal of the instrument will suffice to correct its position. After removing the mandrin, the trocar has to be filled with heparinized physiological saline and, this done, closed by a rubber cork. The left branch is connected to a manometer which indicates changes in intra-atrial pressure. An L-shaped cannula, inserted in the stump of the trachea, ensures respiration and, by pressing it against the wall, keeps the trocar fixed.

We used 16 mongrel dogs of 8 to 12 kg body weight for these experiments. The position of the trocar was verified post mortem in every case.

The tip of the trocar was situated in the left atrium in all cases but one when it was in the left upper pulmonary vein. Neither haemopericardium nor haemothorax was observed in any case. A slight haematoma (about 6 to 8 ml) was encountered in two dogs between the lamellae of the bronchopericardial membrane. Pressures were easy to register in every case (H_2O-manometer + Kymograph).

Transbronchial puncture of the atrium in itself did not give rise to pulmonary oedema in the control experiments. We instituted also experiments in which right-side catheterization was combined with the above-described procedure; p.c. (wedge) pressure yielded the same values as those obtained by the direct puncture of the left atrium.

It seems, in consideration of these results, justified to conclude that the method under review is reliable and can be employed whenever the cervical part of the trachea has to be laid open. It is in these cases much simpler than other current methods since radiology, catheterizaton and bronchoscopy can be dispensed with.

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Attila Naszlady,
Országos Kardiológiai Intézet Kórélettani Osztálya, Budapest.

THE EFFECT OF LARGE DOSES OF HISTAMINE ON THE PULMONARY CIRCULATION IN THE DOG

By

J. Litwin

DEPARTMENT OF HUMAN PHYSIOLOGY, SCHOOL OF MEDICINE, WARSAW, POLAND

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The circulatory effects of histamine, administered intravenously in doses of 15 and 50 μg/kg, were studied in anaesthetised and artificially ventilated dogs. Histamine was shown to elicit a biphasic response of pulmonary blood vessels. The first phase appeared shortly after the injection of the amine and consisted of a rise in pulmonary arterial pressure and an increase of pulmonary venous blood flow. Calculated pulmonary vascular resistance remained either unchanged or decreased slightly, in the majority of experiments. These changes coincided with the rapid fall of systemic arterial pressure and were usually accompanied by a slight acceleration of the heart rate and a transient increase of ventricular contractile force. A distinct rise of systemic venous pressure as well as of femoral venous blood flow appeared at the same time. After 30 sec. to 2 min. a second phase followed, consisting of a slight decrease of pulmonary arterial pressure, strong reduction of pulmonary venous flow and a considerable and consistent increase of calculated pulmonary vascular resistance. The above changes were accompanied by a marked reduction of the ventricular contractile force, secondary lowering of systemic venous pressure and a considerable reduction of femoral venous flow. In contrast to the immediate effects of histamine, the secondary phase is protracted and the return of pulmonary pressure, flow and resistance towards the control levels parallels the recovery of systemic arterial pressure.

Although extensive experimental evidence has been accumulated concerning the action of histamine on the pulmonary vessels, the nature of this action is not fully elucidated.

There can be little doubt that histamine produces a rise in pulmonary arterial pressure, followed frequently by a secondary fall. This was repeatedly demonstrated in dogs [6, 7, 11, 14, 18], rabbits [6], cats [3, 6, 8] and guinea pigs [9]. These findings seemed to indicate a vasoconstrictor action of histamine on the pulmonary vessels. The assumption was further supported by the results of perfusion experiments which offered direct evidence of a local vasoconstrictor effect of histamine in the lungs of dogs [1, 5, 12], cats [12, 15], rats [10] and monkeys [4].

On the other hand, Stornstein, Cudkowicz and Attwood [16] demonstrated that the calculated pulmonary arterial resistance followed the changes in cardiac output evoked by histamine, in other words that no active vasoconstriction ensued from the administration of histamine.

In view of these conflicting results the need for further investigations arose. The experiments to be reported in this paper had the purpose of establishing the vasomotor effects of large doses of histamine on the pulmonary vessels

of artificially ventilated dogs. A multichannel recording system was employed providing for simultaneous and continuous measurement of the systemic and pulmonary circulatory parameters.

Methods

The experiments were performed on mongrel dogs weighing 14 to 17 kg. Morphine sulphate (2 mg/kg, subcutaneously) and chloralose (70 mg/kg, intravenously) were used as anaesthetics. The chest was opened in the left fifth intercostal space and the lungs were ventilated by means of a Starling Ideal Pump. In the basic series of experiments the following measurements were recorded on a four-channel Sanborn Polyviso apparatus: *a)* aortic blood pressure by a Statham transducer from a catheter inserted through one carotid artery; *b)* pulmonary arterial blood pressure by a second transducer from a catheter, inserted through the left upper lobar artery; *c)* ventricular contractile force by means of a Walton strain gauge arch sutured to the surface of the right ventricle; and *d)* pulmonary venous blood flow by the method described by AVIADO and SCHMIDT [2]. The outflowing blood from the vein of the left lower lobe was collected in a collapsible rubber reservoir and returned instantaneously to the femoral vein by means of a Dale—Schuster pump. The action of the pump was adjusted in such a way as to secure continuous emptying of the reservoir. A Shipley—Wilson rotameter was inserted into the tubing connecting the outflow side of the pump to the femoral vein. Vascular resistance of the left lower lobe was calculated by dividing mean pulmonary arterial pressure by blood flow. Pulmonary venous pressure of the left lower lobe was ignored in these calculations since this pressure was unchanged under the influence of histamine.

In other experiments the following measurements were recorded together with the measurements of systemic arterial blood pressure and ventricular contractile force: *a)* left atrial pressure by a Statham transducer from a catheter inserted through the left upper lobar vein; *b)* inferior vena cava pressure by another Statham transducer from a catheter inserted through the femoral vein; and *c)* femoral venous blood flow by means of a method similar to that applied for measuring pulmonary blood flow.

In all experiments manuronate 10 mg/kg or heparin 400 I. U./kg intravenously was used as an anticoagulant. Histamine was administered intravenously in doses of 15 and 50 μg/kg. These doses regularly produced a profound and prolonged systemic hypotension.

Results

Systemic arterial pressure. The changes in systemic arterial pressure were uniform. Following the injection of histamine an abrupt fall in pressure appeared, amounting to a mean decrease of more than 30 per cent after 15 sec. With histamine doses of 15 μg/kg, maximal lowering of pressure occurred after approximately 1 min, the pressure being decreased by 46 per cent on the average. With histamine doses of 50 μg/kg, maximal lowering of pressure was observed after 1 to 3 min, and it amounted to a mean decrease of 50 per cent of the preinjection level. Later the pressure was observed to return gradually to the initial level.

The responses of systemic arterial pressure to histamine are summarized in Table I.

Pulmonary arterial pressure. Histamine produced usually a biphasic response of pulmonary arterial blood pressure. Immediately after the injection of histamine, a marked rise of pressure appeared attaining the maximum after approximately 15 sec. At this time it amounted to a mean increase of 10 per

Table I

Summary of responses to histamine

Dose of histamine μg/kg	No of experiments	Control. Mean ± S. E. (Range)			% Δ 15–30 sec. after histamine. Mean ± S. E. (Range)				% Δ 1–3 min. after histamine. Mean ± S. E. (Range)			
		SABP mm Hg	PABP mm Hg	PBF ml/min.	SABP	PABP	PBF	PVR	SABP	PABP	PBP	PVR
15	5	102±11 (60 to 120)	18±3.8 (12 to 30)	121±5.6 (108 to 136)	−36±4.4	+10±2.5	+13±4.1	0±4.5	−46±5	−9±5	−48±7.9	+64±13
50	5	108±12 (80 to 150)	20±2.4 (14 to 25)	110±4.2 (96 to 120)	−34±2.6	+10±4.5	+15±2.5	−6±1.9	−50±4.4	−3±4.1	−65±8.2	+258±50

SABP — Systemic Arterial Blood Pressure
PABP — Pulmonary Arterial Blood Pressure
PBF — Pulmonary Venous Blood Flow (One Lobe)
PVR — Pulmonary Vascular Resistance

cent of the initial level. The rise in pressure lasted about 1 min. and was followed, in most experiments, by a slight but prolonged secondary fall. One to 3 min. after the injection of histamine, pulmonary arterial pressure was reduced by 9 or 3 per cent, on the average, depending upon the dose of histamine administered. The return of pulmonary arterial pressure to the initial level paralleled the recovery of systemic arterial pressure.

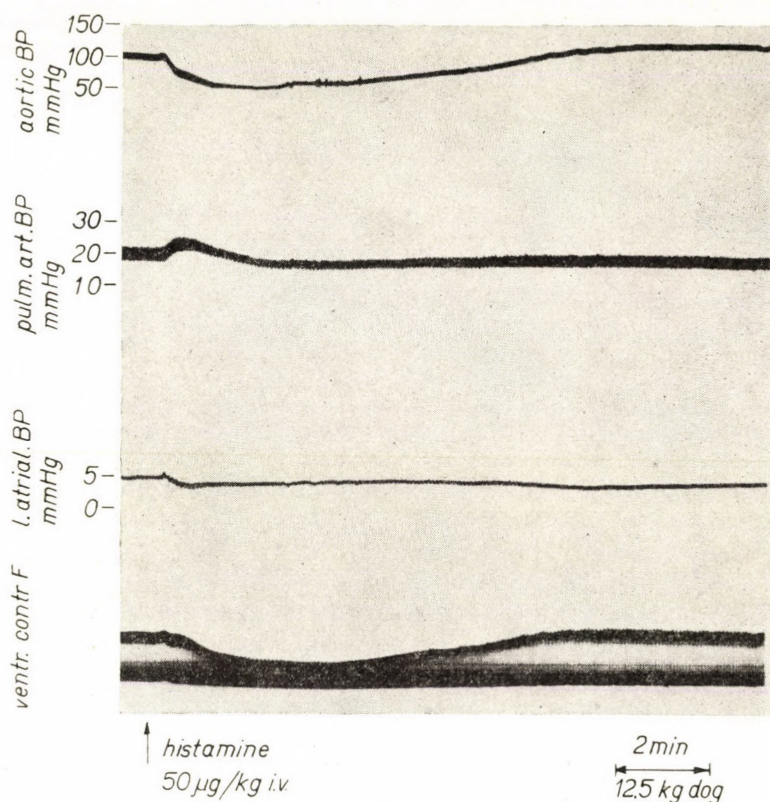

Fig. 1. Effect of a large dose of histamine on pulmonary arterial blood pressure and ventricular contractile force

The responses of pulmonary arterial pressure to histamine are summarized in Table I.

Figs 1 and 2 present recordings in which the biphasic response of pulmonary arterial pressure to histamine is clearly demonstrated.

Left atrial pressure. A slight lowering of this pressure was observed concurrent with the fall of systemic arterial pressure. It usually remained reduced during the entire period of secondary pulmonary hypotension elicited by histamine. The return of left atrial pressure to the initial level paralleled the recovery of pulmonary arterial pressure.

A typical response of left atrial pressure to histamine is shown in Fig. 1.

Pulmonary venous flow. A consistent biphasic response of pulmonary venous flow was observed after the injection of histamine.

The immediate effect of histamine consisted of a marked increase of blood flow lasting approximately 1 min. This increase of flow concurred with the rapid fall of systemic arterial pressure and the primary rise of pulmonary

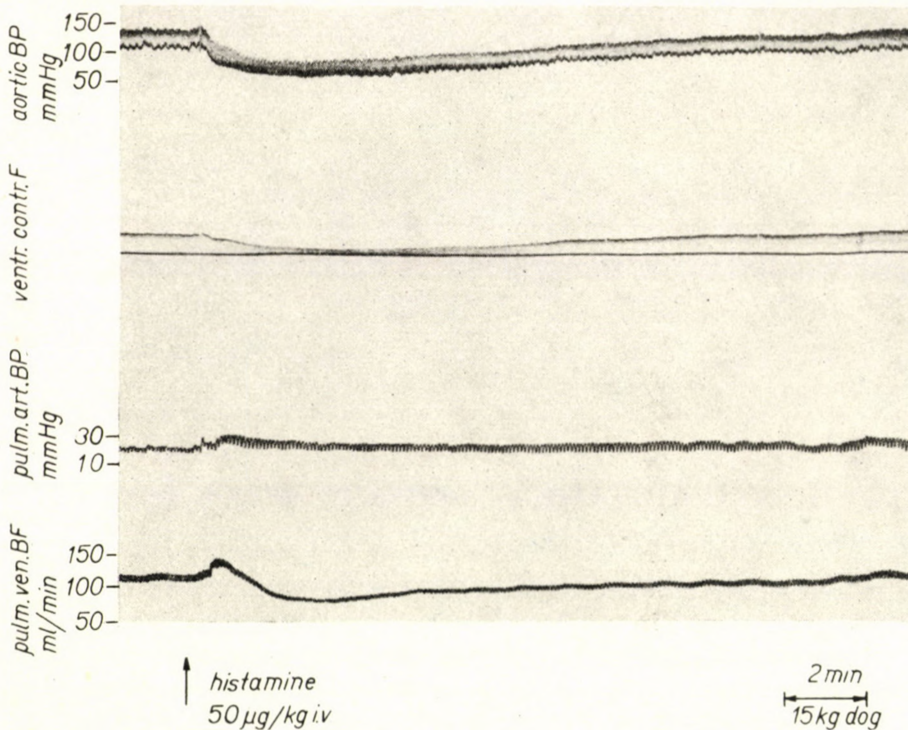

Fig. 2. Effect of a large dose of histamine on pulmonary venous blood flow

arterial pressure. Fifteen sec. after the injection of histamine, pulmonary blood flow increased by 13 or 15 per cent of the initial level, on the average, depending upon the dose of histamine administered. At the same time, systemic arterial pressure was on the average reduced by 36 and 34 per cent, respectively, whereas pulmonary arterial pressure was increased by 10 per cent of the pre-injection level.

The primary increase of pulmonary blood flow was followed by an intense and prolonged reduction of flow. The minimum appeared 1 to 3 min. after the injection of histamine. At this time, pulmonary venous flow was on the average reduced by 48 and 65 per cent, respectively, depending on the dose of

histamine. The secondary fall of pulmonary venous flow coincided with the maximum of systemic hypotension and the secondary lowering of pulmonary arterial pressure. The return of pulmonary flow to the preinjection level ran parallel to the recovery of both pulmonary and systemic arterial pressure.

The effects of histamine on pulmonary venous flow are summarized in Table I.

Fig. 2 shows a typical record demonstrating the biphasic response of pulmonary venous flow to the injection of histamine.

Calculated pulmonary vascular resistance. Inconsistent changes of pulmonary vascular resistance were observed in the period of enhanced pulmonary flow. Smaller doses of histamine produced only insignificant changes of resistance. In some instances, a moderate increase of resistance was noted, and it was slightly lowered in others. Larger doses of histamine caused more uniform changes of resistance. It was moderately decreased in all experiments immediately following the injection of histamine, the mean decrease being 6 per cent of the preinjection level.

On the other hand, in the period of secondary reduction of pulmonary flow, pulmonary vascular resistance increased considerably and consistently, after both dose levels of histamine. The maximum increase in resistance was noted 1 to 3 min. after the administration of histamine. At this time pulmonary resistance was increased by 64 per cent on the average and even as much as 258 per cent, depending upon the dose of histamine applied. Afterwards, pulmonary resistance was observed to return gradually to the initial level, this coinciding with gradual recovery of pulmonary venous flow as well as pulmonary and systemic arterial pressure. The changes in resistance were determined mainly by the changes in pulmonary blood flow.

Experimental data concerning the effects of histamine on pulmonary vascular resistance are summarized in Table I.

Ventricular contractile force. Histamine usually elicited a biphasic change of ventricular contractile force. Immediately after the injection, a transient increase appeared in the majority of experiments, associated with some acceleration of cardiac rhythm. The positive inotropic effect of histamine concurred with a rapid fall of systemic arterial pressure, primary augmentation of pulmonary flow and the rise of pulmonary arterial pressure. In the experiments in which no positive inotropic action was observed, ventricular contractile force was practically unaltered or decreased slightly.

After 30 sec. to 1 min., a secondary decrease of ventricular contractile force followed. The decrease was usually most marked 3 min. after the injection of histamine. At this time, the amplitude of ventricular contractions was reduced by 30 to 60 per cent of the control. The recovery of ventricular contractile force was gradual and paralleled the recovery of both systemic arterial pressure and pulmonary blood flow.

The effect of histamine on ventricular force is shown in Figs. 1, 2 and 3.

Systemic venous pressure and flow. The effects of histamine on systemic venous pressure were always biphasic. A slight rise of pressure, lasting about 1 min., appeared in the inferior vena cava immediately after the injection of

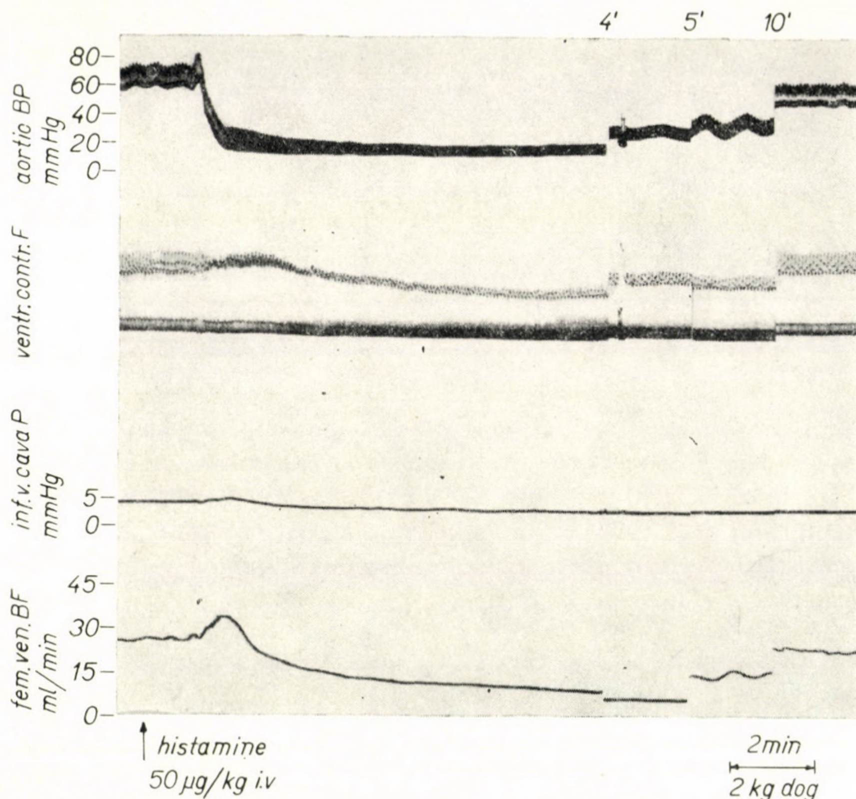

Fig. 3. Changes in systemic venous pressure and femoral venous blood flow elicited by a large dose of histamine

histamine. This was followed by a prolonged, though moderate, secondary decrease, which paralleled systemic hypotension.

The response to histamine of femoral venous blood flow was also consistently biphasic. A marked increase of flow occurred shortly after the injection of histamine, concurring with a rapid fall of systemic arterial pressure. After 1 to 2 min., a pronounced reduction of blood flow appeared, to persist during the entire period of systemic hypotension. The return of flow to the preinjection level followed the recovery of systemic arterial pressure.

Fig. 3 presents a typical response to histamine of systemic venous pressure and blood flow.

Discussion

The experimental evidence presented proves that two phases may be distinguished in the response of pulmonary circulation to large doses of histamine.

The first phase appears immediately after the injection of histamine and coincides with a rapid fall of systemic arterial pressure. It consists of a rise in pulmonary arterial pressure, lowering of left atrial pressure and an increase of pulmonary venous blood flow. Calculated pulmonary vascular resistance is either unaltered or decreases slightly during this phase. These changes are mostly accompanied by a moderate increase of ventricular contractile force and acceleration of cardiac rhythm. Systemic venous pressure and femoral venous blood flow are both elevated. The duration of the primary phase lasts usually for from 30 sec. to 2 min.

The second phase consists of a slight secondary lowering of pulmonary arterial pressure, a pronounced reduction of pulmonary venous blood flow and a considerable increase of calculated pulmonary vascular resistance. Left atrial pressure remains slightly lowered throughout this phase. Ventricular contractile force as well as systemic venous pressure and femoral venous blood flow decrease markedly. It should be added that the maximum of the above effects coincides with the maximum of systemic hypotension. In contrast to the immediate effects of histamine, the secondary phase is protracted and the return of all parameters to the initial levels parallels evidently the recovery of systemic arterial pressure.

The above findings support in principle the results of earlier investigations in which the biphasic effect of histamine on pulmonary blood vessels was originally demonstrated [6].

However, the main advantage of the experimental technique applied in the present study lies in that it allows an adequate evaluation of the relative importance of the various haemodynamic factors involved in the action of histamine on pulmonary circulation.

On the basis of our findings the immediate effect of histamine on pulmonary blood flow and vascular resistance may be interpreted as resulting from an increase of cardiac output, indicated by the augmentation of pulmonary venous flow. The increase of cardiac output is most probably due to the enhanced venous return as well as the positive inotropic effect of histamine. The increase of venous return may be concluded to from the distinct rise of systemic venous pressure and the marked augmentation of femoral venous flow. The increase of cardiac output and pulmonary blood flow is most likely the cause of the rise in pulmonary arterial pressure. There is no consistent evidence of pulmonary vasoconstriction in this period since pulmonary vascular resistance was mostly unchanged or even slightly lowered.

The mechanism of the secondary effect of histamine on the pulmonary vessels is a complex one. Secondary lowering of systemic venous pressure appears, paralleled by a marked reduction of femoral venous flow. This indicates pooling of blood in the periphery and a diminished ventricular filling resulting in decreased cardiac output. At the same time, a considerable decrease of ventricular contractile force occurs. This may be due to: 1. the diminished ventricular filling, and/or 2. a secondary impairment of cardiac function consequent upon the reduced systemic arterial pressure and diminished coronary blood flow. The decrease of ventricular contractile force may contribute to the reduction of cardiac output. It should be added that such a reduction is proved by the dramatic fall in pulmonary blood flow.

However, pulmonary arterial pressure is maintained at a level only slightly below normal throughout this phase. This leads to a tremendous rise in pulmonary vascular resistance. As a matter of fact, it can be doubled or even tripled in the period of minimum systemic pressure.

The rise in resistance seems to justify the assumption that large doses of histamine are causing pulmonary vasoconstriction. Such an assumption would be in agreement with the results of perfusion experiments in which histamine was demonstrated to exert a local vasoconstrictor effect on pulmonary vessels.

On the other hand, WILLIAMS [17] and GALLETTI, SALISBURY and RIEBEN [13], have shown that pulmonary arterial pressure and resistance depend upon the inflow into the pulmonary artery, the resistance decreasing with the augmentation of inflow and vice versa. In view of these findings, the results of the present experiments may be explained without postulating an active vasoconstriction in the pulmonary area. In fact, the considerable rise in pulmonary vascular resistance induced by histamine was shown to be determined mainly by the decrease in pulmonary blood flow, the changes in pulmonary arterial pressure being of minor importance. Therefore, the assumption seems to be justified that the increase of pulmonary resistance is mainly secondary to the systemic circulatory events resulting in a decrease of blood inflow into the pulmonary artery. Such an interpretation, however, does not rule out the possibility that the eventual vasoconstrictor action of histamine may play a contributory role in eliciting the rise of pulmonary vascular resistance.

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Jerzy Litwin,
Department of Human Physiology, School of Medicine, Warsaw, Krakowskie Przedmieście 26/28, Poland.

AVOIDING CONDITIONED REFLEX IN BLIND RATS AND RATS DEPRIVED OF VIBRISSAE

By

L. Korányi, E. Endrőczi and K. Lissák

INSTITUTE OF PHYSIOLOGY, MEDICAL UNIVERSITY, PÉCS

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Avoiding conditioned reflex has been established and extinguished in white rats. Motor processes were promptly coordinated in the course of learning.

Enucleation of the eyes and removal of the vibrissae provoked a temporary disturbance of movements, followed by a gradual improvement of spatial orientation. Removal of the said analyzers transitorily inhibited the acquired motor process without destroying it.

Extinction of the conditioned reflex showed that internal inhibition was most vigorous in blind animals deprived of vibrissae; also blind animals with intact vibrissae displayed stronger internal inhibition than the controls.

No essential change in the somatomotor process was observed after the avoiding conditioned reflex had been restored.

The sensory analyzers through which temporary connections are established vary according to the phylogenetic grade of the animal species. The establishment of temporary connections on which the mechanism of learning relies is closely correlated with spatial and temporal orientation. The various analyzers of the organism play different roles in these processes, but are capable of replacing one another. Small [1], relying on the evidence of maze experiments with white rats, was of the opinion that the mechanism of spatial orientation depended on tactile stimuli. This theory was based on the observation that blind rats, deprived of their olfactory area, do not lose the acquired spatial orientation [2, 3, 4]. The vibrissae of rats may play a certain role in orientation, although — according to the evidence of Vincent's experiments [5] — this role is insignificant. Bogardus and Henke [6] suggest that the movements of the head and the resulting tactile stimuli are of importance in the incipient phase of learning and are no longer required once the acquired movements have become automatic.

The present experiments were designed to study the effect of the elimination of the visual analyzer and certain tactile receptors in connection with the avoiding conditioned reflex.

Material and method

Thirty white male rats from our own strain, ranging in weight from 120 to 150 g, were used. Conditioned reflex was established in the following manner. The animals were placed in a box with a floor surface of 27 × 35 cm and a height of 50 cm. One side of the box was

made of plexiglass. The floor consisted of unisolated, hardware rods 3 mm in diameter, placed at 0.5 cm intervals. Receiving an electric shock on their paw, conducted by the grid floor, the animals took refuge on a shelf which, with a surface of 7 × 8 cm, was attached to the centre of the 27 cm long inner side of the box. The height of the shelf was 15 cm in the present experiments.

As an unconditioned stimulus, direct-current electric shock (80 V) paired with a bell signal was applied. First, the animals received on a shock every second until they found the means of escape by jumping upon the shelf. This happened, as a rule, within 20 to 30 sec. Association of the electric shock with the bell signal was then begun. A bell was presented for 10 sec. at one-minute intervals, and an additional electric shock was administered during each of the last 2 seconds. The animals were allowed to remain on the shelf 20 seconds after the termination of the last bell signal and were then replaced to the floor of the box. It took 5 to 10 trials for the reflex to develop, while the period of latency diminished to between 1 and 3 seconds. As soon as this had been achieved, the time of the electric shock was brought forward, with the bell signal still lasting 10 seconds. We were thus able to perform 20 trials per day per animal, *i.e.* a total of 100, before the beginning of the essential part of the experiment. On the average, every third association was reinforced. Then the rats were divided at random into three equally populated groups (*A*, *B*, *C*). The eyes of the members of groups *A* and *B* were enucleated, the animals of group *B* were moreover deprived of vibrissae, while the rats of group *C* served as controls.

Results

Since one edge of the shelf was attached to the inner side of the box, the animals had three alternatives of how to jump on the shelf. During the first associations, *i.e.* when the animals still leaped on the shelf under the effect of the electric shock, none of the three available sides of the shelf was preferred. After about 10 to 15 trials the rats always chose the same side for the jump, usually that on which the first conditioned jumps had been made or, else, that which enabled them to reach the shelf in the shortest time. It should be noted that both the box and the shelf were perfectly regular geometrical patterns, so that the animals could jump upon the shelf at any of its sides with equal ease. We lifted the animals from the shelf and placed them at random anywhere on the floor on the second and third experimental day. Then diagrammatic records were drawn about the escaping routes taken by the animals from their place on the floor to the point from which they jumped onto the shelf. It was observed that, from wherever they started, they always chose the same side of the shelf and the shortest path leading to it.

The vibrissae of a half of the members of group B were removed in the intertrial interval on the day preceding the operation (group Bl). It elicited a short orienting and searching reaction which lasted 1 to 2 minutes. The movements of these animals became extremely rapid, but the time required for the performance of the conditioned avoiding reflex became considerably longer in all animals but one, and they were rather uncertain when making the first jumps (for instance, some fell back from the shelf, others missed it, *etc.*). However, all such initial disturbances ceased after 8 to 10 associations.

This was followed by the enucleation of both eyes of the members in groups A, B1 and B2, and it was at this time that the vibrissae in group B2 were cut off.

Members of group A remained practically unchanged in respect of both spatial orientation and conditioned reflex. Animals of group B2, on the other hand, displayed diminished orienting reaction when placed on the box on the day after operation; and did not show conditioned reflex reaction. However, the difference between members of group A and those of group B gradually disappeared. The difference between the two groups was that the stereotype of group B had to be changed during subsequent days of the experiment, and the first and second conditioned stimuli had always to be paired with electric shock for the animals to respond. But, beyond this, neither a change in the

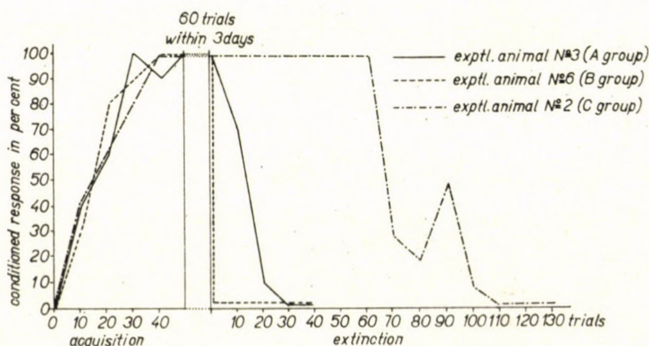

Fig. 1. Establishment and extinction of conditioned reflex in a rat, each, of experimental groups *A*, *B* and *C* (For details see text)

situation of the bell nor our having turned the animals by their tail and let them drop to the floor of the box immediately before the sound of the bell was presented, occasioned any essential disturbance in respect of spatial orientation. The animals performed the reflex as usual. We recorded also in this case the route from the random starting point to the point of jump; the diagrams so obtained were similar to those made prior to operation. Only two animals, one of group B1 and one of group B2, were exceptions; these had become completely disoriented.

In attempt to study the tactile discriminative capacity of enucleated animals during the intertrial period the grid floor of the box was covered with a pasteboard for the duration of 8 associations. While animals of the control group continued to perform the conditioned reflex as before, members of group A and B failed to show any conditioned reflex performance, but they performed the reflex again as soon as we removed the pasteboard.

As regards extinction, it took in the control group some 60 to 80 non-reinforced trials for the inhibition of the conditioned reflex to begin, and complete extinction was achieved after 100 to 140 trials, while internal inhibition developed after 5 to 13 non-reinforced trials in group A. It has been noted

in the foregoing that the stereotype of group B was changed in the postoperative period inasmuch as the first and second acoustic stimuli had to be reinforced by electric shock in order to make the animals perform the avoiding

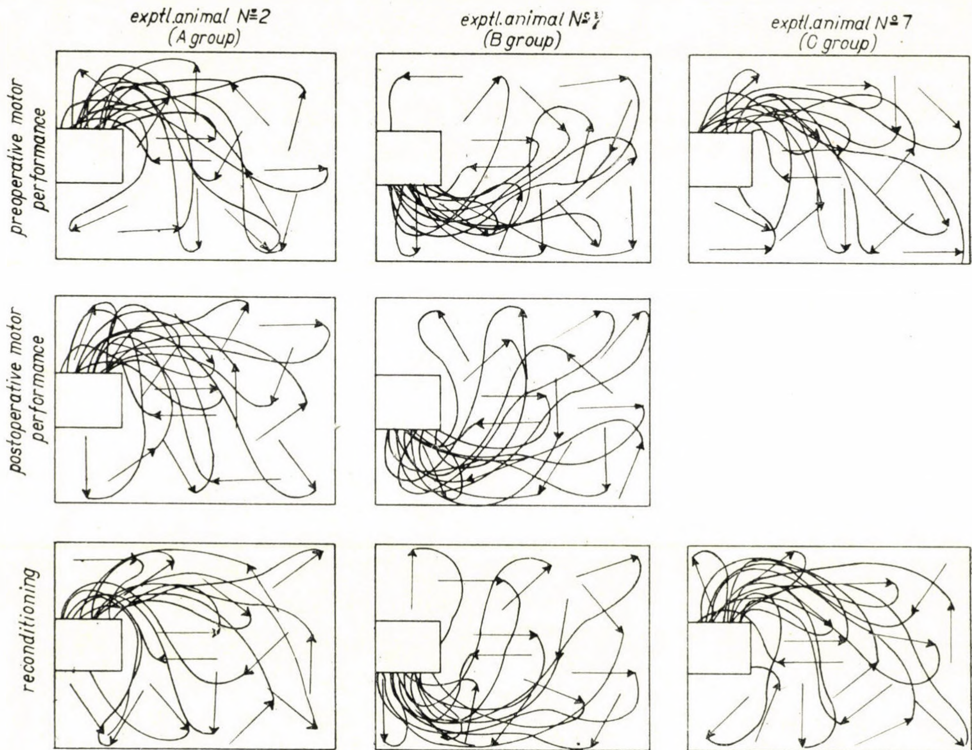

Fig. 2. Spatial orientation of and routes taken by a rat, each, of the three experimental groups in a situation governed by the avoiding conditioned reflex. Arrows point to three random spots from which the animals started in performing the conditioned reflex action

conditioned reflex. It is, thus, understandable that members of group B failed to leap in the extinction experiments (Fig. 1).

As the next step, the members of groups A and B were habituated without the administration of conditioned stimulus during 20 minutes on each of 4 or 5 consecutive days. The time of the orienting, searching activity and washing automatisms became longer, and the behaviour of the animals, which contained comparatively few motor elements, gradually changed into the normal behaviour of caged rats.

Extinction was followed by the restoration of the conditioned reflex. It took 4 to 14 associations for the members of groups A and B_1, and 1 to 3 for the controls. Diagrams, prepared after the re-establishment of the conditioned reflex and showing the escaping routes performed by the animals from their

random place on the floor to the point of jump, did in no way differ from the two previous diagrams. Only the afore-mentioned two rats in group B were exceptions in this respect, they continued to be completely disoriented (Fig. 2).

Discussion

The spatial orientation and the motor pattern accompanying with the avoiding conditioned reflex activity in rats has a rule in its development which is not only characteristic to the conditioned reflex performance developed actually but in the reconditioned situation, too. The escaping routes and the places of jumping on to the shelf resemble well in the first, as well as, in the second conditioned reflex activity. It would follow that not the somatomotor pattern itself but the temporary connection between it and the conditioned auditory stimulus was inhibited, so that the somatomotor pattern remained intact during the period of extinction. The fact that the conditioned reaction subsided more promptly in groups A and B than in the controls points to a process of more intensive inhibition.

Our results in respect of groups A and B are in harmony with those of BERITOFF [7] and AYRAPETYANS [8], and justify the conclusion that the elimination of the visual and certain tactile analyzers disturbs spatial orientation for a short time only and does not essentially affect the mechanism of learning based on spatial orientation. On the other hand, LASHLEY and BALL [9] demonstrated that a removal of the visual and olfactory cortex led to grave disorders in this respect. There occurred, however, no disorder after the ascending spinal tracts had been divided [10]. All these findings favoured the conclusion that the mechanism of acquiring spatial orientation relied on cerebral processes. HUNTER [11] claimed that sensory analyzers constituted equipotential factors in the mechanism of spatial orientation, and that, after the removal of a peripheral afferent channel, the remaining channels were capable of completely replacing it, a process of compensation which takes place in the cerebral cortex. The experiments of BERITOFF *et al.* [12, 13, 14] have shown that in the dog the labyrinthine receptors are involved in spatial orientation, and have further proved that — by innumerable steps of chainlike conditioned reflex reactions — the different sensory analyzers capable of substituting one another are governing the mechanism of spatial orientation.

As regards now the question of the factors responsible for the intensified internal inhibition in animals deprived of sensory analyzers, WATSON [2] nearly 60 years ago suggested that a decrease in motivation played a significant role in such cases. It follows that the intensification of internal inhibition regarding conditioned reflexes is not due to the lack of sensory analyzers in these animals but to a decrease in motivation, correlated with their emotional behaviour.

Present knowledge is still deficient in this field, and it is difficult to tell how the elimination of sensory analyzers affects the processes of motivation and, through the latter, the trend of facilitatory and inhibitory processes involved in the establishment of temporary connections.

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Lajos Korányi, Elemér Endrőczi, Kálmán Lissák,
Orvostudományi Egyetem Élettani Intézete, Pécs.

EFFECT OF INTRACEREBRALLY ADMINISTERED CHOLINERGIC AND ADRENERGIC DRUGS ON NEOCORTICAL AND ARCHICORTICAL ELECTRICAL ACTIVITY

By

E. ENDRŐCZI, G. HARTMANN and K. LISSÁK

INSTITUTE OF PHYSIOLOGY, MEDICAL UNIVERSITY, PÉCS

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In cats under urethane anaesthesia it has been observed that cholinergic agents (eserine and carbaminoylcholine) injected into the area of the rostral thalamus increase the synchronizing activity of the neocortex and facilitate the formation of barbiturate spindles. Cholinergic substances injected into the reticular formation of the rostral mesencephalon caused neocortical desynchronization and inhibited the formation of barbiturate spindles. Carbaminoylcholine and eserine injected into the ventrolateral reticular formation gave rise to temporary theta activity in the dorsal hippocampus. If injected into the brain stem and posterior hypothalamus, or administered intravenously, adrenaline and nor-adrenaline enhanced both the synchronization and the desynchronization caused by cholinergic drugs, though not consequently so in every case. Carbaminoylcholine and eserine injected unilaterally into the septum evoked hippocampal theta activity on the homolateral side. The intrahippocampal injection of acetylcholine leads to desynchronization, but its bilateral injection results in a temporary generalized seizure activity.

Tubocurarine injected into the hippocampus evoked spike activity. In response to the unilateral injection of the drug, spike activity appeared sooner in the neocortex than on the contralateral side in the dorsal hippocampus. Spike activity was inhibited by the intrahippocampal or intraseptal injection of acetylcholine. A similar effect has been observed following the injection of a 2 per cent KCl solution.

During the past decade numerous investigations have shown that the intravenous injection of cholinergic drugs resulted in EEG desynchronization, while the administration of atropine gave rise to high-amplitude slow activity. It was also observed that anticholinergic drugs caused a dissociation of the behavioural state and the electrophysiological pattern [1, 2, 3, 4]. The administration of adrenergic drugs evokes an EEG arousal reaction through the reticular formation system of the brain stem. This does not occur after the reticular formation of the rostral mesencephalon has been severed [5, 6, 7]. Opinions diverge as to the mechanism by which cholinergic and adrenergic drugs influence the function of the brain stem and as to whether a chemical mediation would play a role in the transmission of impulses in that structure [1, 2, 5, 6].

In the present experiments in cats anaesthesized with urethane we have studied the effect on the electrical activity of cholinergic and adrenergic drugs injected through a cannula into the archicortex.

Materials and methods

Cats of either sex, weighing 2.0 kg on the average, anaesthesized with 1.0 g/kg urethan intraperitoneally, were used. The electrical activity of the neocortex was recorded by means of silver-ball electrodes, while subcortical electrical activity by means of stainless-steel enamel insulated bipolar electrodes having a distance between the tips 1 mm; the free electrode tip was measured about 0.5 mm. In some cases combined glass cannula and electrode units were used. For this purpose a glass capillary, about 0.1 to 0.15 mm thick was inserted into a stainless-steel tube of 0.2 mm lumen, coated with enamel for insulation, except for the last 0.5 mm on the tip. Chemical stimulation was performed through the glass cannula, while the steel electrode served for recordings of electrical activity. In other experiments a glass capillary placed between bipolar metal electrodes was used for the purpose of infusion. The glass capillary was attached to a polyethylene tube filled beforehand with the adequate fluid. The doses were administered by means of a microinjector and varied usually from 5 to 10 μl in volume. To avoid overpressure, the upper end of the polyethylene tube was left patent through which any fluid in excess could flow back. The chemical stimulants were acetylcholine, carbaminoylcholine, eserine sulphate, d-tubocurarine, adrenaline—HCl, nor-adrenaline—HCl, 2 per cent KCl, injected into the corresponding area at neutral pH.

The electrodes and cannulas were placed subcortically under stereotactic control. After having completed the experiment, the brain of the animal was perfused with 40 per cent formalin and the location of the electrodes or the cannula was determined in histological sections [8].

Results

Effects of acetylcholine, carbaminoylcholine and eserine sulphate

In cats anaesthesized with urethane the electrical activity of the neocortex is desynchronized and neither high-amplitude slow activity nor spindle formation is observable [9]. Carbaminoylcholine or acetylcholine (2 to 5 μg, in 5 to 10 μl volume) injected into the anterior group of thalamic nuclei gave rise to 8 to 12 c/sec. spindle activity of an average duration of 5 to 15 minutes. Injection of 10 μg eserine sulphate produced a similar effect. Barbiturate doses not giving rise to marked spindle formation produced long-lasting hypersynchronization when injected intravenously after eserine or carbaminoylcholine had been injected into the anterior thalamus. In these experiments, 0.5 to 1.0 mg/kg body weight sodium hexobarbital was injected intravenously; this dose did not give rise to spindle formation in the cats under urethane anaesthesia. The intrathalamic injection of cholinergic drugs preceded the intravenous administration of the barbiturate by from 0.5 to 1.0 minute. The control experiments proved that there was no summation of the effects if the interval between the two intravenous injections of threshold barbiturate doses exceeded 15 minutes. If injected into the intralaminar group of thalamic nuclei, eserine or carbaminoylcholine alone in several cases caused desynchronization, but significantly enhanced at the same time the spindle-forming activity of hexobarbital.

Injection of cholinergic drugs into the reticular formation of the brain stem resulted in neocortical desynchronization. The effect of the cholinergic substances injected into different mesencephalic areas and into the area of the dorsal or ventral tegmentum developed within a matter of seconds and lasted

in the case of carbaminoylcholine 25 to 30 minutes, and in that of eserine 15 to 20 minutes. During that period sodium hexobarbital caused no spindle-formation, in spite of its having been injected intravenously in increasing doses of 20 μg to 5 mg/kg. The carbaminoyl-choline injected into lower brain stem areas

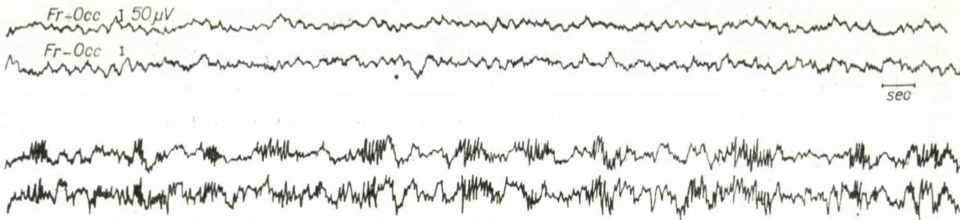

Fig. 1. Appearance of spindles in cortical records following injection of 5 μg carbaminoyl-choline into the rostral thalamic nuclei

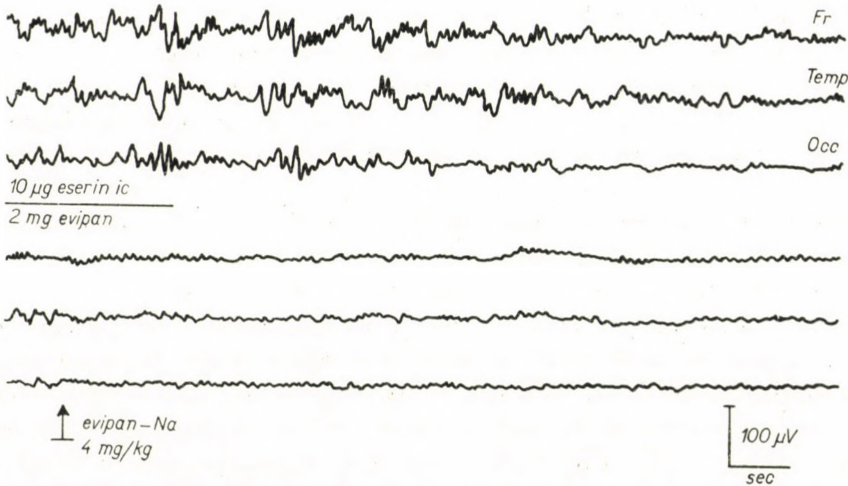

Fig. 2. Inhibition of barbiturate spindle formation by 10 μg of eserine injected into the rostral mesencephalic reticular formation bilaterally

resulted in a cortical hypersynchronization followed within 30 seconds by desynchronization. In these cases chemical stimulation was applied to the lateral part of the reticular formation at the level of the ponto-mesencephalic junction (Figs. 1 and 2).

Effects of adrenaline and nor-adrenaline

No change in the EEG activity of the cats under urethane anaesthesia resulted from adrenergic drugs injected into the reticular formation of the brain stem in doses of 2 to 10 μg. In contrast to this, the intravenous adminis-

tration of 10 μg of adrenaline definitely increased the spindle formation evoked by the cholinergic chemical stimulation of the rostral thalamus, as regards both amplitude and duration. On the other hand, the intravenous administration of such an adrenaline dose significantly increased the duration of the desynchronization evoked by the cholinergic stimulation of the mesencephalic reticular formation. In some cases 2 μg of adrenaline injected directly into the posterior hypothalamic area increased the spindle formation evoked from the rostral thalamus, but this was demonstrable only in 2 cases out of 7. No significant difference could be noted between the adrenaline and nor-adrenaline effects as regards intravenous administration (Fig. 3).

Effect of cholinergic drugs on archicortical electrical activity

The electrical activity of the dorsal hippocampus in the cat under urethane anaesthesia is characterized by slow, 1 to 2 sec waves, with low amplitude and fast activity [10]. Following the injection of 5 to 10 μg of eserine or carbaminoylcholine into the ventrolateral reticular formation of the mesencephalon, marked theta activity, 5 to 10 minutes in duration and 5 c/sec in average frequency, could be observed. Injection of these drugs into other brain stem structures produced no significant changes in dorsal hippocampal activity. In contrast to this, marked theta activity was evoked by the drugs if they were injected into the medial septum. The injection of 5 μg of carbaminoylcholine into the unilateral septum caused homolateral theta activity, whereas the contralateral side showed desynchronized electrical activity.

If injected directly into the dorsal hippocampus (areas CA_1 and CA_2), 2 to 5 μg doses of acetylcholine caused a transient desynchronized activity, 3 to 5 minutes in duration. No spike discharges or slow activity could be noted. The electrical activity of the contralateral side was similarly desynchronized. Neocortical electrical activity remained unchanged, or showed a transient desynchronization.

Generalized spike activity developed if the two consecutive injections of acetylcholine had been given bilaterally, at a 1-minute interval. Twenty to 30 seconds following the injection into the contralateral side exclusively that side showed marked seizure activity, lasting for about 1 to 1 ½ minutes.

Effect of tubocurarine on archicortical electrical activity

As injected in 10 to 20 μg doses into the dorsal hippocampal areas CA_1 and CA_2, tubocurarine gave rise to rhythmic spike activity after a latency period of 2 to 3 minutes. The discharges occurred with a delay of a few seconds both in the neocortex and reticular formation, but the delay in the contralateral hippocampus was significant, being several minutes in duration in most

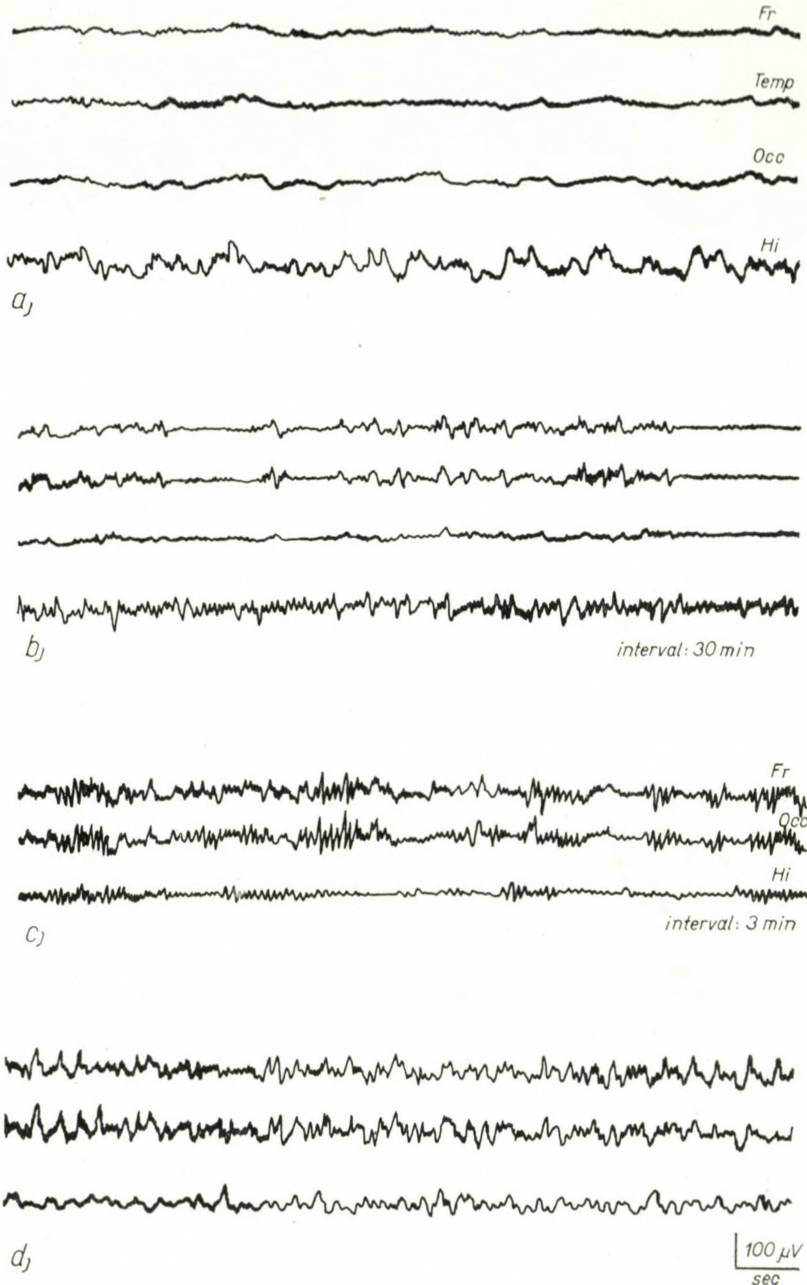

Fig. 3. Showing combined effects of eserine (10 μg) and nor-adrenaline (5 μg) on barbiturate spindles

a : control. *b* : 3 minutes after the intravenous administration of 1.5 mg/kg sodiumhexobarbital. *c* : same as *b*, but 30 min. later, following injection of 10 μg of eserine into the rostral thalamus. *d* : prolongation of slow waves by nor-adrenaline injected into the posterior hypothalamus

cases. The contralateral hippocampal spikes appeared later than the neocortical ones, and were also smaller in amplitude than those on the injected side.

As injected into the unilateral dorsal hippocampus, 10 μg of tubocurarine caused locally desynchronization, followed by the appearance of rhythmic spike activity. In this early phase the tubocurarine injected into the contralateral side produced no effect, although by then the spike activity could also be observed in the neocortex. The inhibition of the convulsive tendency of the

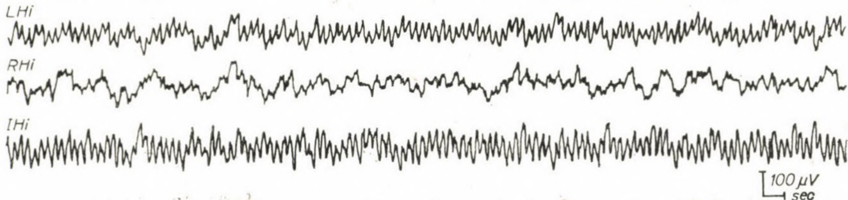

Fig. 4. Hippocampal theta activity 2 minutes after the injection of 5 μg of carbaminoylcholine into the right septum. The third record corresponds to the interhemispherical hippocampal lead

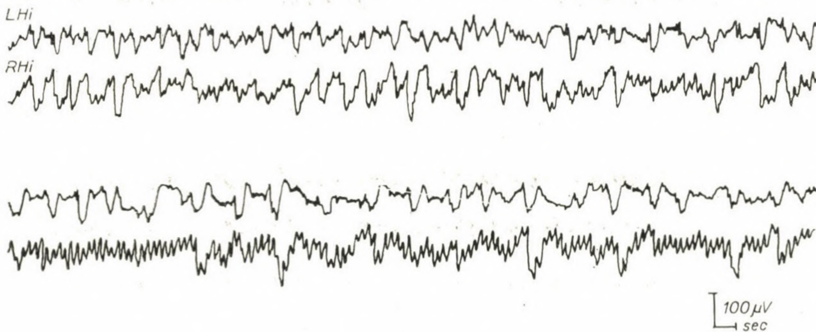

Fig. 5. Right hippocampal theta activity following injection of 10 μg of eserine into the right septum

contralateral hippocampus was transient lasting 5 to 10 minutes in most cases. Subsequently the rhythmic spike activity appeared also on that side, but its appearance and intensity depended on the degree of anaesthesia.

Thus, the tubocurarine-induced seizure activity was inhibited by 5 μg of acetylcholine injected into the same side, but even more so by that injected into the contralateral side. This phenomenon manifested itself partly with a decreased amplitude of spikes or with a complete disappearance of spike activity, as well as by desynchronization. Similar results were obtained by the use of 10 μl of a 2 per cent KCl solution. Inhibition of the seizure activity was characterized by that it lasted half to one minute and could best be observed in the initial phase of the tubocurarine-induced seizure activity. A similar in-

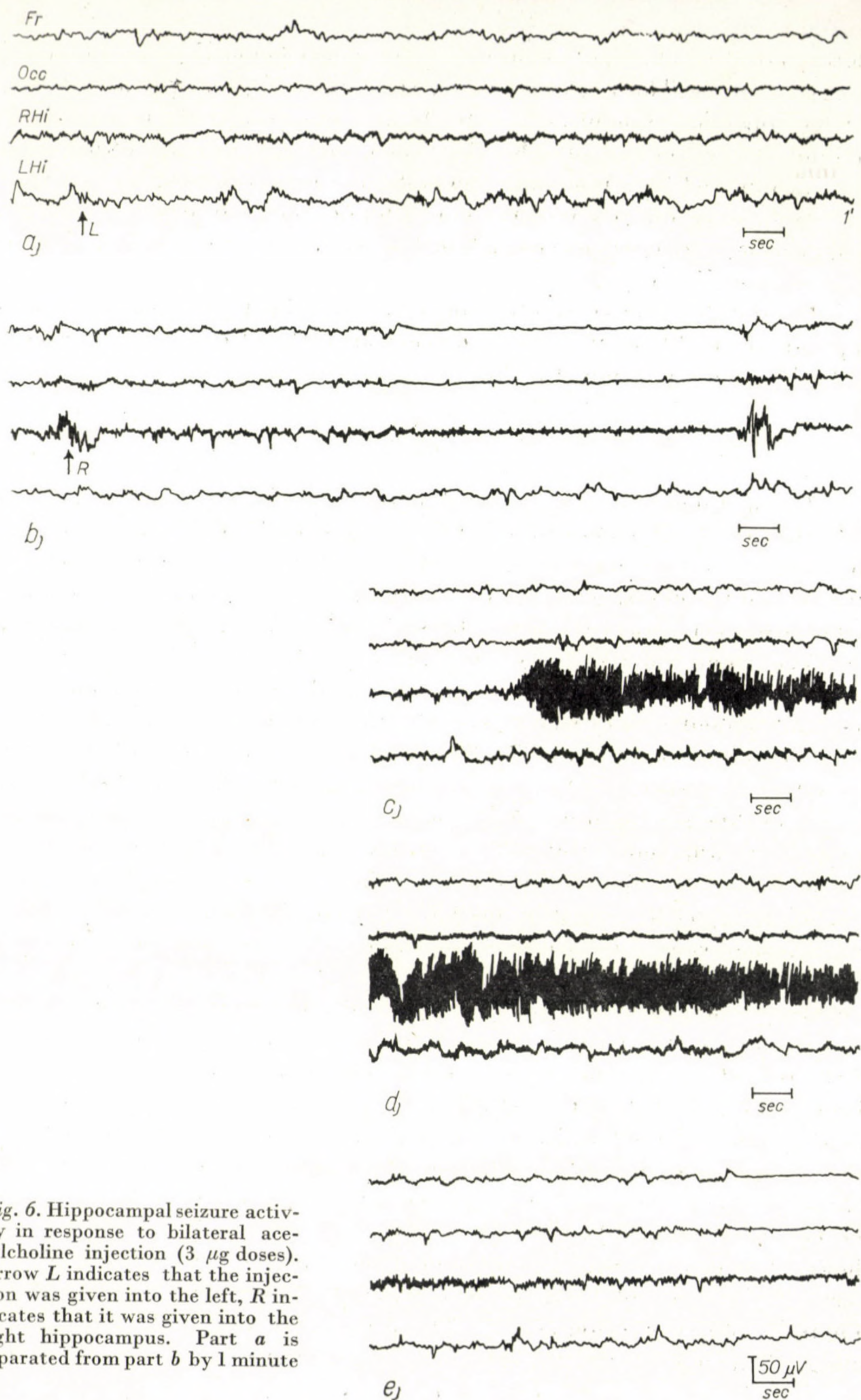

Fig. 6. Hippocampal seizure activity in response to bilateral acetylcholine injection (3 µg doses). Arrow *L* indicates that the injection was given into the left, *R* indicates that it was given into the right hippocampus. Part *a* is separated from part *b* by 1 minute

hibitory effect was brought about by 5 μg of acetylcholine injected unilaterally into the septum. The responses to repeated intrahippocampal acetylcholine and KCl injections diminished gradually and seizure activity increased after the inhibitory effect had subsided. This sometimes was so marked that inhibition was followed by a temporary generalized seizure activity.

As injected directly into the dorsal hippocampus in doses of from 2 to 10 μg, neither adrenaline nor nor-adrenaline had an effect on the tubocurarine-induced seizure activity.

Physiological saline solution injected into the brain stem or into the hippocampus in 10 to 20 μl doses, did not alter the electrical activity of the cerebral areas in question (Fig. 7).

Discussion

The neurophysiological evidence obtained during the past decade indicates that the brain stem contains two systems, different from each other in function, but deeply interwoven structurally. The action of one is identical with that of the non-specific diffuse activator system, and causes EEG desynchronization [11], while the other, becoming organized at a lower brain stem level, synchronizes neocortical electrical activity [12, 13, 14]. The latter system apparently has connexions with the anterior and midline thalamic nuclei, low frequency stimulation of which gives rise to recruiting responses through the thalamo-cortical connexions [15]. The responses to cholinergic drugs injected into different areas of the brain stem and thalamus indicate that both systems possess cholinergic receptors. While cholinergic drugs injected into the area of the rostral thalamus resulted in a synchronization and increased the formation of barbiturate spindles, carbaminoylcholine or eserine injected into the area of the reticular formation inhibited the spindle-forming activity of barbiturates and caused EEG desynchronization. At lower brain stem levels the cholinergic drugs evoked desynchronization after having caused an initial synchronization; this suggested stimulation of a functionally mixed structure. This assumption is in harmony with those data, according to which stimulation of the reticular formation at the pontomesencephalic junction results in synchronization [12, 13]. It is likely that the rostral and intralaminar "nuclei", the thalamus and the low brain stem reticular formation constitute a cholinergic synchronizing system overlapped topographically by the non-specific diffuse activator system, so that it would be extremely difficult to separate the two functionally. As injected into the ventral tegmentum, carbaminoylcholine and eserine evoked hippocampal theta activity, which, according to electrophysiological observations, is facilitated through the afferent pathways running across the septum [16, 17]. This is indicated also by the fact that cholinergic drugs injected into the septum result a similar effect, confirming thereby

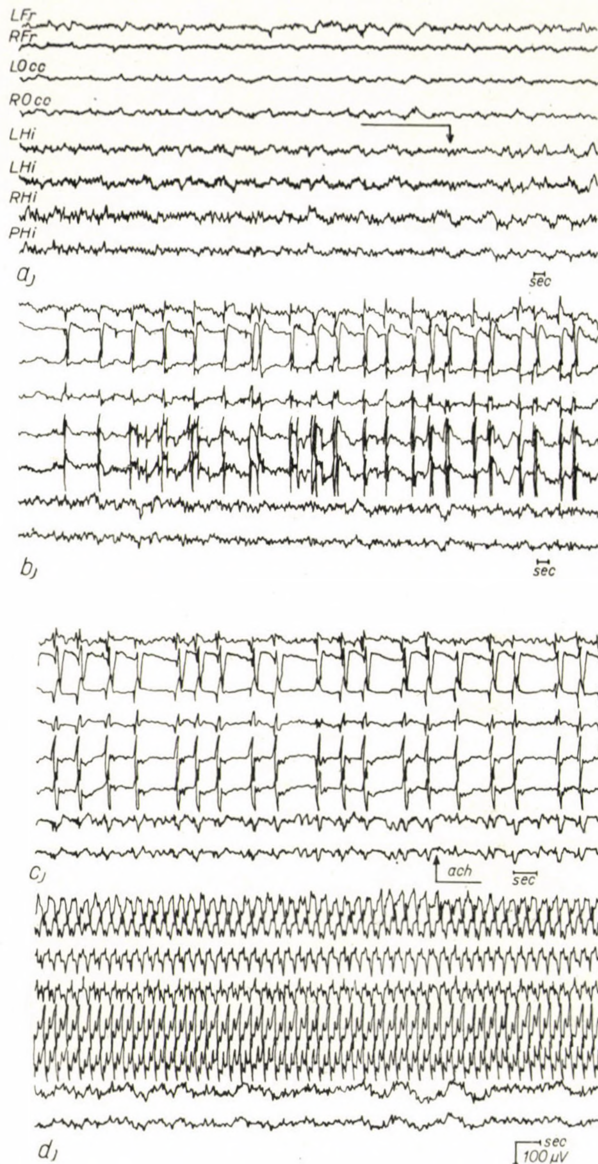

Fig. 7. Seizure activity induced by intrahippocampal injection of tubocurarine (left side). Note early appearance of spikes in homolateral hippocampal records and neocortical leads. Spike activity appears after a delay in the contralateral hippocampal records, and the injection of acetylcholine (5 μg) into the contralateral hippocampus suppressed the coming spike activity. Parts *a*, *b*, *c* and *d* are separated by intervals of 2 minutes

also our earlier pertinent observations [18, 19]. The fact that acetylcholine injected into the dorsal hippocampus evokes neither theta, nor spike activity, calls attention to two considerations: 1. The organisation of theta activity

depends on the septal afferent system, as it has been indicated recently by microelectrode and toposcopic experiments [20, 21]. 2. Spike activity cannot be considered to be based exclusively on cholinergic receptors.

Studies of the neuronal connexions of the archicortex by means of intra- and extracellular microelectrode leads indicate that at least two different mechanisms must be assumed to exist in the integration of hippocampal inhibition. One mechanism may be due to a long-lasting depolarization developing on the basal dendrite network of pyramidal cells, that is essentially a catelectrotonic inhibition [23]. Other authors ascribe inactivation to an interneuronal inhibitory mechanism [24, 25, 26]. The afferentation of the latter is facilitated through the system of the fornix and is correlated with theta activity. In our present experiments acetylcholine temporarily inhibited the tubocurarine-released seizures accompanied by desynchronization. Considering that the theta waves are a summation in time and space of post-synaptic potentials (wavelets), the desynchronization brought about by cholinergic drugs might be a desintegrating function of such an organization. There is no definite answer yet to the question as to whether the inhibition is based on the mechanism of prolonged desynchronization, or is effected through the collaterals. The latter possibility seems to be corroborated by the fact that the response to a cholinergic stimulation of the septum is similar to the response to acetylcholine injected into the hippocampus.

The prolonged desynchronization taking effect in the pyramidal cells plays presumably a substantial role in the rhythmic spike discharges, which appear with great regularity at 3 to 8 sec. intervals, depending on the quality of the experimental preparation. In extracellular microelectrode leads this auto-regulative spike activity regulation develops with latencies of 100 to 300 msec; here a lasting depolarization on the body of the pyramidal cells is held responsible for the long refractory period [23]. Under our experimental conditions this rhythmic spike activity could be observed also during urethane anaesthesia, and in an urethane anaesthesia it never turned into generalized seizure activity.

The inhibitory nature of the interhemispheral hippocampal connexions is indicated by our observation, that it was only after a long latency, after the unilateral injection of tubocurarine the spike activity appeared on the contralateral side, although it could be observed at the neocortical and reticular formation levels. At the same time, contralaterally administered acetylcholine during the phase of desynchronization evoked locally a generalized seizure activity. This seems to indicate that the simultaneous appearance of the depolarization induced locally by acetylcholine and that released reflectorily from the contralateral side, facilitates spike formation.

In view of the fact that the afferents between the bilateral hippocampi are connected in the first place with the pyramidal and granular cells of the

CA_4 area, which have on the one hand the lowest tendency to seizure activity, and, on the other, which are the sites from where the presumably inhibitory interneurones (mossy fibres, collaterals of Schaffer) run to the pyramidal cells of the areas CA_1, CA_2 and CA_3, then we might claim that the phenomenon of interhippocampal inhibition observed by us has, to some extent, a structural substrate [25, 26, 27]. The correlation between cholinergic inhibition and theta activity is remarkable insofar as the same afferents bringing about theta activity produce at the same time an inhibition of the tubocurarine spikes.

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Elemér Endrőczi, Géza Hartmann, Kálmán Lissák,
Orvostudományi Egyetem Élettani Intézete, Pécs

THE ROLE OF CENTRAL NERVOUS ACTIVATING AND INHIBITORY STRUCTURES IN THE CONTROL OF PITUITARY-ADRENOCORTICAL FUNCTION. EFFECTS OF INTRACEREBRAL CHOLINERGIC AND ADRENERGIC STIMULATION

By

E. Endrőczi, G. Schreiberg* and K. Lissák

INSTITUTE OF PHYSIOLOGY, MEDICAL UNIVERSITY, PÉCS

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The changes in pituitary-adrenocortical activity evoked by cholinergic and adrenergic drugs injected into various areas of the diencephalon and the brain stem have been investigated under chronic experimental conditions in cats. Carbaminoylcholine and eserine injected into the septum, preoptic region, anterolateral hypothalamus or into the dorsal tegmental area, were found to inhibit pituitary-adrenal activity. Cholinergic chemical stimulation of the medial and caudal hypothalamus, as well as of the posterior hypothalamus and ventral tegmentum resulted in an increase of ACTH secretion. Adrenaline, nor-adrenaline and ephedrine increased ACTH secretion only when injected into the area of the posterior hypothalamus and ventral tegmentum.

The suppression of adrenocortical activity in response to the cholinergic stimulation of the inhibitory structures of the forebrain could be blocked by the adrenergic stimulation of the posterior hypothalamus.

No close parallelism could be demonstrated between the behavioural reactions evoked by chemical stimulation and pituitary-adrenal function. This would indicate that chemical stimulation of the diencephalon and the brain stem area makes it possible to separate endocrine regulatory processes from the complex behavioural and emotional reactions.

Many observations made in recent years have indicated that the subcortical structures can both facilitate and inhibit pituitary-adrenocortical activity [1, 2, 3, 4]. While the electrical stimulation of the forebrain rhinencephalic structures, *e.g.* hippocampus, septum, anterior and lateral hypothalamus, inhibits adrenocortical hormone secretion, that of the basal hypothalamus, posterior subthalamus and rostral mesencephalon increases ACTH secretion. The mode of action and the internal structural correlations of the activator and inhibitory effects of the brain stem, diencephalon and rhinencephalon are largely unknown. In the present paper we shall report on some experimental results concerning pituitary-adrenal activity in the course of stimulations with intracerebrally injected cholinergic and adrenergic agents.

Materials and methods

Cats of either sex, 65 in number and weighing 1.6 to 3.0 kg, were used. To apply chemical stimulation, glass cannulas attached to a polyvinyl tube were implanted into different parts of the brain, by means of a stereotactic apparatus. The outer diameter of the capillary

* Present address: Department of Neurology, Medical University No. 1, Moscow, USSR

was 0.4 mm, the internal one varied from 0.1 to 0.12 mm. The diameters of the polyvinyl tube attached to it were similar and the tip of the tube was sealed with rapidly hardening wax at the end of the experiment. The cannula was held in place by means of acrylate, the 1 cm long end of the polyvinyl tube was pulled through the closed wound surface and fixed to the suture. In most cases one cannula, in others four cannulas were implanted in one animal. The internal capacity of the cannula, controlled prior to implantation, varied from 5 to 8 μl.

The chemical stimulants used were eserine sulphate, acetylcholine and carbaminoylcholine hydrochloride, adrenaline, nor-adrenaline hydrochloride and ephedrine. Physiological saline, adjusted to pH 7.3—7.4 with a bicarbonate solution, was used as the solvent. The active agents were administered in 10 μl volumes, by means of a microsyringe. According to the control examinations with dye or China ink, the volume diffusion of 10 μl of fluid corresponds to an area about 3 mm in diameter [5].

Chemical stimulation was applied 5 to 7 days after operation. The cholinergic agents were injected at 15-minute, the adrenergic agents at 3 consecutive 15-minute intervals. The changes in behaviour were recorded, then 45 to 60 minutes after the last injection the cats were anaesthesized with intraperitoneally injected pentobarbital. This was followed by the examination of pituitary-adrenal function. As described in detail earlier, corticosteroids were determined by a physico-chemical method after having collected adrenal venous blood for 60 minutes [6, 7]. Cannulas were implanted in the brain of the control animals, too, but they received 20 μl of a physiological saline solution prior to study. In these animals the cannulas were located in the same way as in the experimental animals.

At the end of the experiment the brain was perfused with 40 per cent formalin, then the location of the cannulas was determined in frozen sections [8].

Results

Considering that the volume and diffusion of the fluid employed with chemical stimulation make no point-like structural localization possible, the results are discussed according to regional anatomical units. Evaluation was made by comparing the data with the adrenocortical secretion values for the control cats treated with physiologic saline solution. In view of the fact that the corticosteroid content of adrenal venous blood ranged from 43 to 54 μg/kg body weight/hour in the 11 control cats, values under 40 μg or over 60 μg were considered to represent significant changes. We had to adopt this method of evaluation, because under the experimental conditions described no group statistical evaluation was possible.

Changes in pituitary-adrenocortical activity and in behaviour in response to the injection of cholinergic drugs

As injected into the septum and in the area of the antero-lateral hypothalamus, 5 μg of carbaminoylcholine and 10 μg of eserine sulphate, respectively, evoked in 4 to 5 minutes a marked behavioural reaction, characterized by menacing snarling, spontaneous rage reaction and attacks against animals and man. The motor reaction manifested itself first of all in movements in the contralateral direction. While the controls showed no behavioural reaction or any deviation from cats treated in other brain areas as regards adrenocortical hormone secretion, the injection of cholinergic drugs caused a significant decrease of corticosteroid secretion (Table II).

Table I

The effect of physiological saline injection into different mesencephalic and diencephalic areas

No.	Total corticosteroids μg/kg b.w./hour	Location*
1	48.0	BS
2	54.0	POR
3	52.0	SO
4	52.0	PA
5	47.0	TC
6	53.0	MB
7	43.0	MB
8	52.0	PH
9	44.0	RF
10	50.0	RF
11	54.0	VT

* BS = basal septal area
POR = preoptic region
SO = supraoptic nucleus
PA = paraventricular area
TC = tuber cinereum
MB = mammillary bodies
PH = posterior hypothalamus
RF = reticular formation
VT = ventral tegmental area

Table II

The effect of carbaminoylcholine and eserine injected into the septal area, preoptic region and anterolateral hypothalamus

No.	Total corticosteroids μg/kg b.w./hour	Drugs*	Location**
1	8.4	CCh (5 μg)	BDB
2	15.2	CCh (5 μg)	BDB
3	18.8	CCh (5 μg)	BS
4	22.0	CCh (5 μg)	BS
5	22.0	CCh (5 μg)	MS
6	20.0	Es (10 μg)	MS
7	28.0	Es (10 μg)	POR
8	32.0	Es (10 μg)	POR
9	27.0	CCh (5 μg)	MFB
10	37.0	CCh (5 μg)	MFB
11	35.0	CCh (5 μg)	MFB

*CCh = carbaminoylcholine
Es = eserine sulphate
**BDB = Broca's diagonal band
BS = basal septal area
MS = medial septal area at the level of anterior commissure
POR = preoptic region
MFB = medial forebrain bundle

The most intense and diffuse behavioural reactions were evoked by the injections of carbaminoylcholine and eserine into the medial and caudal group of hypothalamic nuclei. After an initial phase of restlessness these cats were running around in circles, obviously agitated, wildely mewing and trying to run away when touched. As to the vegetative signs, dilatation of the pupils,

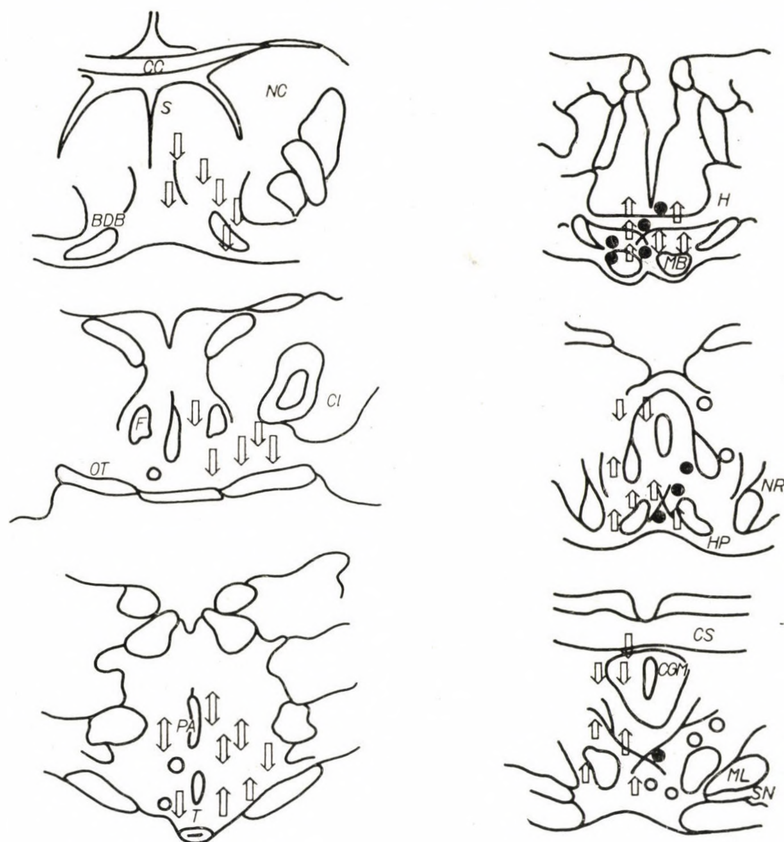

Fig. 1. Location of injections in different areas of the brain. White arrows indicate suppression or increase of pituitary-adrenocortical activity in response to cholinergic drugs. Bidirectional arrows indicate that the intracerebral injections of cholinergic drugs produced insignificant effects. Empty circles: negative results from injections of adrenergic drugs. Black circles: enhanced pituitary-adrenocortical activity following adrenergic stimulation

urination and increased respiration were observed. About 15 to 20 minutes after injection in most of the cats behaviour was characterized by an escape reaction, and during that period numerous reactions of "hallucinative" character were also observable. The animal stared motionless at some point in space, then jumping suddenly aside, escaped from that area. In some cases the escape reaction turned into marked catatony, with unusually intense respiratory activity. In spite of these intense changes of behaviour, in some cats no

characteristic alterations in pituitary-adrenocortical activity could be demonstrated. Thus, no increase of corticosteroid secretion resulted from injecting the cholinergic drugs into the area of the supraoptic or paraventricular nuclei, although the behavioural reaction of these animals was marked. A moderate or definite increase was observed when the drugs were injected into

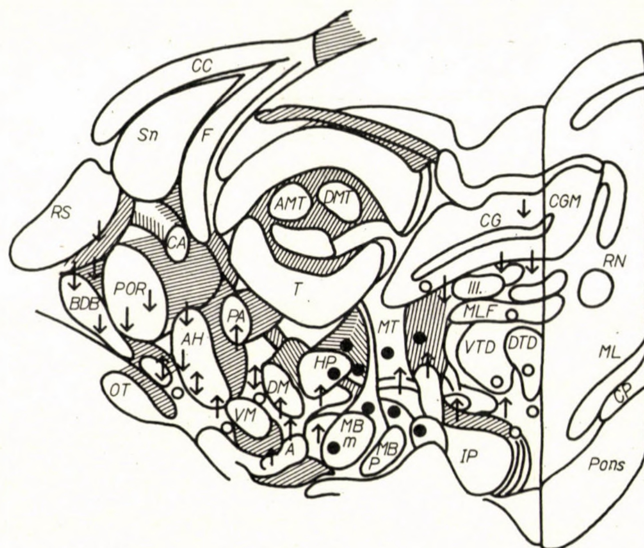

Fig. 2. Reconstruction of hypothalamic and midbrain structures, showing the points and effects of cholinergic and adrenergic chemical stimulations. The abbreviations used are as follows:
A = arcuate nucleus; AH = anterior hypothalamus; AMT = anterior medial thalamic nuclei; BDB = Broca's diagonal band; CA = anterior commissure; CC = corpus callosum; CGM = central gray matter; CP = cerebral peduncle; DM = dorsomedial hypothalamic nucleus; DTD = dorsal tegmental decussation; DMT = dorsal medial thalamic nuclei; F = fornix; HP = posterior hypothalamic area; IP = interpeduncular area; MBm = medial group of mammillary body; MBp = posterior group of mammillary body; MT = mammillo-thalamic tract; MLF = medial longitudinal fascicle; ML = media lemniscus; PA = paraventricular nucleus; OT = optic tract; POR = preoptic region; RN = nucleus ruber; Sn = splenium; VM = ventromedial hypothalamic nuclei; VTD = ventral tegmental decussation. The arrows indicate the response to cholinergic, the circles that to adrenergic] stimulation

the area of the tuber cinereum, premammillary area or posterior hypothalamic area. The behavioural reaction to the chemical stimulation of the posterior hypothalamus was characterized by coordinated fear and escape reactions, while in response to the stimulation of the more rostral and the anterior groups of nuclei this reaction was accompanied by many non-coordinated motor reactions and vegetative signs (salivation, urination) (Table III).

The cholinergic drugs injected into the mesencephalic area caused opposite changes in emotional behaviour and in pituitary-adrenocortical activity, if we analyze the above phenomena according to dorsal and ventral areas. While

injection into the area of the ventrolateral reticular formation and ventral tegmentum markedly increased adrenocortical secretion and caused coordinated fear or escape reactions, stimulation of the central gray matter and dorsal tegmentum resulted in coordinated rage reaction and purposeful aggressivity.

Table III

The effect of carbaminoylcholine and eserine injected into the medial and posterior hypothalamic areas

No.	Total corticosteroids µg/kg b.w./hour	Drugs	Location
1	47.0	CCh (5 µg)	PA
2	54.0	CCh (5 µg)	PA
3	56.0	CCh (5 µg)	SO
4	55.0	Es (10 µg)	SO
5	67.0	CCh (5 µg)	TC
6	65.0	Es (5 µg)	TC
7	75.0	Es (10 µg)	PM
8	82.0	CCh (5 µg)	PM
9	76.0	CCh (5 µg)	MB
10	84.0	CCh (5 µg)	MB
11	67.0	CCh (5 µg)	PH
12	72.0	CCh (5 µg)	PH
13	77.0	CCh (5 µg)	PH

CCh = carbaminoylcholine
Es = eserine sulphate
PA = paraventricular area
SO = supraoptic nucleus
TC = tuberal region including ventromedial nucleus
PM = premammillary region including arcuate nucleus
MB = mammillary bodies
PH = posterior hypothalamus
n = no significant difference from control values

Stimulation of the latter area led to a decrease of adrenocortical hormone secretion, stimulation of the area of the ventral tegmentum evoked a marked startle reaction, often followed by escaping associated with fear reaction. In such a period the animal was running to and fro in the observation room and tried to hide under some furniture. When stimulation was applied near the dorsal tegmental area, the animal sat quietly in its place, but attacked immediately any animal or man going near to it. Behaviour was characterized by continuous angry snarling.

Table IV

The effect of carbaminoylcholine and eserine injected into the mesencephalon

No.	Total corticosteroids μg/kg b.w./hour	Drugs	Location
1	64.0	CCh (5 μg)	RF
2	68.0	CCh (5 μg)	RF
3	85.0	CCh (5 μg)	RF
4	73.0	Es (10 μg)	RF
5	59.0	Es (10 μg)	RF
6	78.0	CCh (5 μg)	VT
7	85.0	CCh (5 μg)	VT
8	76.0	CCh (5 μg)	VT
9	79.0	CCh (5 μg)	VT
10	34.0	CCh (5 μg)	DT
11	32.0	CCh (5 μg)	CGM
12	29.0	CCh (5 μg)	CGM
13	36.0	Es (10 μg)	DT
14	30.5	Es (10 μg)	DT

CCh = carbaminoylcholine
Es = eserine sulphate
RF = reticular formation
VT = ventral tegmental area
DT = dorsal tegmental area
CGM = central gray matter

Changes in pituitary-adrenocortical activity and in behaviour in response to stimulation with adrenergic drugs

The changes in pituitary-adrenocortical activity observed after the injection of adrenaline, nor-adrenaline and ephedrine are summed up in Table V. The first 9 animals showed no significant change in response to adrenergic drugs injected into the anterior and medial hypothalamic areas. The second group of 13 animals showed excessively high secretory values when the posterior hypothalamic and ventral tegmental areas had been stimulated, but no substantial change resulted when the drugs had been injected into the central gray matter of the mesencephalon or into the reticular formation at the level of nucleus ruber.

For adrenergic chemical stimulation 3 times 2 μg doses had been injected, but in the case of nor-adrenaline even 0.5 μg proved to be effective. On the other hand, 0.5 μg of adrenaline or ephedrine, injected into the posterior hypothalamic area, produced no changes.

Table V

The effect of adrenaline, nor-adrenaline and ephedrine injected into different hypothalamic and mesencephalic areas

No.	Total corticosteroids μg/kg b.w./hour	Drugs	Location
1	48.0*	Adr. (2 μg)	SO
2	51.5*	Adr. (2 μg)	PA
3	49.5*	Noradr. (2 μg)	PA
4	52.0	Noradr. (2 μg)	DT
5	54.5*	Adr. (2 μg)	RF
6	50.0*	Noradr. (2 μg)	RF
7	53.0*	Noradr. (2 μg)	VT
8	54.0*	Adr. (2 μg)	VT
9	49.0*	Eph.	VT
10	98.0	Adr. (2 μg)	PH
11	105.0	Noradr. (2 μg	PH
12	91.0	Eph.	PH
13	116.0	Noradr. (2 μg)	MB
14	89.0	Noradr. (0.5 μg)	PH
15	132.0	Noradr. (2 μg)	VT
16	96.5	Eph.	VT
17	82.0	Adr. (2 μg)	VT
18	89.5	Noradr. (2 μg)	VT
19	51.0*	Adr. (0.5 μg)	PH
20	54.0*	Adr. (0.5 μg)	PH
21	48.0*	Eph. (0.5 μg)	PH
22	76.0	Noradr. (0.5 μg)	PH

Adr. = adrenaline
Noradr. = nor-adrenaline
Eph. = ephedrine
SO = supraoptic nucleus
PA = paraventricular area
DT = dorsal tegmental area
RF = reticular formation
VT = ventral tegmental area
PH = posterior hypothalamus
MB = mammillary bodies
* = no significant difference from control values

As little as 2 μg of nor-adrenaline sufficed to evoke a behavioural reaction characterized by coordinated, purposeful (target-motivated) aggressive reaction, on injection into the posterior hypothalamic area. Similar doses of adrenaline or ephedrine produced no such response. Unlike cholinergic stimu-

lation, nor-adrenaline evoked no vegetative reaction; when left alone, the animals were sitting quietly, but immediately attacked, snarling angrily, any animal or man trying to go near them.

Suspension of the effect of forebrain inhibitory structures by adrenergic stimulation of the brain stem

In these experiments two cannulas each had been implanted bilaterally into the basal septal, posterior hypothalamic and ventral tegmental areas. Through the forebrain cannulas 5 μg of carbaminoylcholine, through the brain stem cannulas 10 to 15 minutes later 2 μg of nor-adrenaline were injected. Administration of the latter drug was repeated twice at 15-minute intervals. The behavioural reactions evoked by carbaminoylcholine were alleviated by the nor-adrenaline markedly in some animals, and slightly in others. This applied first of all to the restlessness and spontaneous rage reactions, while the target-motivated (purposeful) aggressive reaction persisted in these animals. In response to the combined treatment, the corticosteroid content of adrenal venous blood increased in a variable measure, but never decreased, as it was the case when cholinergic drugs alone had been applied to the basal septal area.

Table VI

The blocking action of posterior hypothalamic injections of adrenergic drugs upon the inhibitory effect of carbaminoylcholine injected into the septal area and antero-lateral hypothalamus

No.	Total corticosteroids μg/kg b.w./hour	Drugs	Location
1	87.0	CCh (5 μg)	BS
		Adr (2 μg)	PH
2	79.0	CCh (5 μg)	BS
		Noradr (2 μg)	PH
3	96.0	CCh. (5 μg)	BS
		Noradr. (2 μg)	PH
4	112.0	CCh. (5 μg)	BS
		Noradr. (2 μg)	PH
5	68.5	CCh. (5 μg)	BS
		Noradr. (2 μg)	PH

CCh = carbaminoylcholine
Adr = adrenaline
Noradr. = nor-adrenaline
BS = basal septal area
PH = posterior hypothalamus

Discussion

The changes in pituitary-adrenocortical activity observed following cholinergic stimulation agreed well with our earlier data achieved with electrical stimulation [1, 4]. While the chemical and electrical stimulation of the septum, Broca's diagonal band, preoptic region, anterolateral hypothalamus [1] suppressed pituitary-adrenocortical activity, the chemical or electrical stimulation of the posterior hypothalamus, the basal and caudal hypothalamic nuclei and the ventral areas of the rostral mesencephalon brought about a marked increase in ACTH secretion. In our conditioned reflex experiments [1] we have already pointed to the significant role played by the forebrain inhibitory structures in the organization of discriminatory inhibition, and our recent investigations have indicated that stimulation of these structures enhances habituation, while after their destruction the defensive conditioned reflex as a whole is damaged [10]. All these phenomena have been illuminated by the present experiments from another angle and it seems that forebrain inhibition extends also to pituitary-adrenocortical activity, the function having the leading role in the organization of general systemic adaptation.

The data for cholinergic stimulation suggested the presence of cholinergic receptors in the inhibitory and activating mechanisms of the diencephalon and brain stem, though they supplied no final answer to the nature of impulse transmission. While cholinergic stimulation of the posterior hypothalamus and ventral tegmentum caused a definite increase in adrenocortical hormone secretion, it was from these same areas that adrenergic stimulation evoked the greatest increase of ACTH production. Thus this nervous structure might contain in a complex fashion the cholinergic and adrenergic receptors, and stimulation of either of them results in responses of a similar nature.

As pointed out in our previous studies concerning neuroanatomical correlations [1], the inhibitory system of the forebrain blocks through the medial forebrain bundle the activating influence of the brain stem; this has been observed in both electrophysiological [11, 9] and behavioural studies. In the afferentation of the forebrain inhibition, a role of fundamental importance is played by the orbito-frontal cortex, the rhinencephalon and, through its reciprocal connexions, by the reticular formation of the brain stem. Electrophysiological observations indicate that the afferents coming from the archicortex and amygdaloid nucleus play a less significant role than the reciprocal brain stem connexions of the basal forebrain structures, though the problem requires further investigation [11].

Adrenergic stimulation of the posterior hypothalamus and ventral tegmentum resulted in excessive ACTH secretion, but such stimulation of the mesencephalic reticular formation induced no such secretory change. This confirms the earlier observation that the subthalamic and posterior hypothala-

mic areas contain adrenergic receptors [12, 13], and that tritium-labelled noradrenaline is accumulated exclusively in these neurones. No answer can be given to the question concerning the role played by the diencephalic adrenergic mechanism in the regulation of ACTH secretion by the anterior pituitary. Nevertheless, it seems unlikely that a chemical mediator of such nature would be the final link in the hypothalamo-pituitary neurohumoral connexion [14, 15, 18]. It may be assumed that adrenergic chemical stimulation participates, *via* interneuronal connexions or eventually by direct stimulation, in the release of the corticotropin releasing factor, whose existence seems to be confirmed by recent evidence. It was remarkable that, like electrical stimulation chemical stimulation of the hypothalamo-pituitary neurosecretory system produced no significant change in ACTH secretion [1]. This is another observation at variance with the hypothesis that vasopressin or oxytocin would play a significant role in the control of pituitary-adrenocortical activity [4, 15].

The changes in behaviour and the emotional changes evoked by chemical stimulation did not uniformly produce an increase of ACTH secretion. On the contrary, in some cases just the opposite occurred. This suggests that by means of cholinergic and adrenergic stimulation it may become possible to study the endocrine organization in itself within the many-sided integrative hypothalamic activity. At the same time, these observations also indicate that the emotional reaction developing under natural conditions and together with it the stress reaction require the organized, uniform function not only of the diencephalic, but also of the extrahypothalamic structures.

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Elemér Endrőczi, Grigorij Schreiberg, Kálmán Lissák,
Orvostudományi Egyetem Élettani Intézete, Pécs

ELECTROPHYSIOLOGICAL AND PHARMACOLOGICAL ANALYSIS OF THE EFFECT OF γ-AMINOBUTYRIC ACID AND PICROTOXIN ON THE INHIBITORY MECHANISM OF THE POSTERIOR ADDUCTOR IN LAMELLIBRANCHIATA

By

A. Puppi

INSTITUTE OF PHYSIOLOGY, UNIVERSITY MEDICAL SCHOOL, PÉCS

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Gamma aminobutyric acid (GABA) produces the same effect in *Lamellibranchiata* as in other animals. Low concentrations (10^{-6} weight per cent), applied to the visceral ganglion, inhibit the tone of the posterior adductor. Applied to the cerebral ganglion, GABA inhibits its activity and so increases the tone of the posterior adductor.

Low concentrations of GABA (10^{-6} weight per cent) have a diphasic effect on the ganglia, bioelectric activity is first stimulated and then inhibited. High concentrations (10^{-3} weight per cent) promptly inhibit the bioelectric potentials.

Picrotoxin is an antagonist of GABA.

In earlier reports [9] we have dealt with the cholinergic, catecholaminergic and 5-hydroxytryptaminergic mechanism of the tone of the posterior adductor in *Lamellibranchiata*, and the effects of curarization and nicotinization of the muscular and neural apparatus as also their treatment with monoiodoacetic acid and 2,4 dinitrophenol. The present investigations were designed to observe the effect of γ-aminobutyric acid (GABA).

The presence and properties of "Factor I" and GABA in animal organisms have scarcely been studied. Koshtoyants [7] claims that GABA produces, *via* the cerebral ganglion, a marked effect on the periodic motor activity of mussels. Its presence must be assumed since its specific action can be traced through the whole animal kingdom,from the protozoans to the mammals. This assumption has been borne out by our observations.

Koshtoyants and Kokina [6] demonstrated that GABA and β-alanine inhibited the rhythmic activity of *Paramecium caudatum*, an animal without nervous system. Vereshtchagin and Sutinsky [12] showed that GABA and β-alanine inhibited the motor activity of insects, and suppressed the bioelectric activity in the nervous system of these animals. Florey [3] suggests that, in crustaceans, "Factor I" inhibits the claw-opening reflex at the level of neuromuscular mediation, and also inhibits cardiac and intestinal contraction. Brockmann *et al.* [2] demonstrated that extracts of mammalian brain decreased the tonicity of the claw muscles in the crab. Vereshtchagin *et al.* [13] found that GABA inhibited bioelectric activity in the nervous system of caterpillars.

Methods

The tone of the posterior adductor of *Lamellibranchiata* as also the mechanism of its inhibition are regulated by the cerebral and the visceral ganglia. Myography has been applied in the present study of the role played by the individual elements, while a simultaneous registration of the bioelectric activities of the cerebral and visceral ganglia served to analyze the activity of neural elements.

Myograms were made by attaching the posterior valve (separated from the anterior adductor) to the pen arm of a kymograph which recorded the processes occurring in the posterior adductor. For electrophysiological analysis, the action potentials of the cerebral and visceral ganglia were recorded synchronously by means of bipolar silver electrodes and an ink-writing oscillograph. The action potentials of the cerebral ganglion were led off from the cerebrovisceral connective, those of the visceral ganglion from the motor fibres running from the visceral ganglion to the muscle.

It may be argued that the action of agents applied to the cerebral ganglion is disturbed if impulses are passing through the connective from the visceral to the cerebral ganglion, and that, similarly, the action of agents applied to the visceral ganglion is impaired by the fact that the nerves running to the muscle contain also fibres which pass to the posterior adductor without interruption. We have to remember that we are here concerned with deviations from normal electrograms, further, that one has to reckon with the said disturbing factors also in the case of a ganglionic lead.

The amplifying frequency varied from 0.2 to 75 Hz in our experiments. The active chemical agents were applied in doses of 0.1 ml 10^{-6} or 10^{-3} weight per cent concentrations per g of body weight, by the aid of soaked minute cotton swabs placed on the visceral or the cerebral ganglion.

The circulation of the animals was normal throughout. The visceral ganglion and the posterior adductor being next to each other, the swab placed on the former produced an effect on the latter too. For reflex stimulation of the muscle a slide inductor (4 V) was used. The stimulating electrodes were applied to the posterior margin of the mantle which is rich in nerve endings.

The myographic results were evaluated by comparisons with control curves as also on the basis of the time characteristics of muscular changes.

Results

Myographic observations in connection with GABA. GABA was used at concentrations of 10^{-3} and 10^{-6} w/w. Applied to the visceral ganglion, high and low concentrations produced opposite effects.

As expected, low concentrations decreased the tonicity of the posterior adductor. After 20 sec. stimulation, relaxation began 152 per cent, and terminated 76 per cent, sooner. The decrease in tonicity amounted to 121 per cent after the division of the connective, *i.e.* the ratio, time required for the development of tone and tone intensity was 21 per cent more in the test animals than the corresponding value in the controls. The contractions consisted almost exclusively of tetanic components, tonic components were negligible, the pessimum phenomenon, *i.e.* the gradual change of the contraction-enhancing effect of the current into inhibition causing relaxation was observable. The tetanic components were, thus, markedly phasic. Relaxations were rapid and of the phasic type.

High concentrations of GABA (10^{-3} w/w) produced the opposite effect. The tone persisting usually for an average of 14 minutes remained unchanged for several hours after the application of GABA at a concentration of 10^{-3}.

This phenomenon was supposed to be due to that concentrated GABA acts as a metabolic poison.

GABA applied to the cerebral ganglion seemed to inhibit its activity as far as could be judged from the elevated tone of the posterior adductor. This effect manifested itself with a decreased tetanicity of the muscle, always supposing that decreased activity of the cerebral ganglion leads to increased

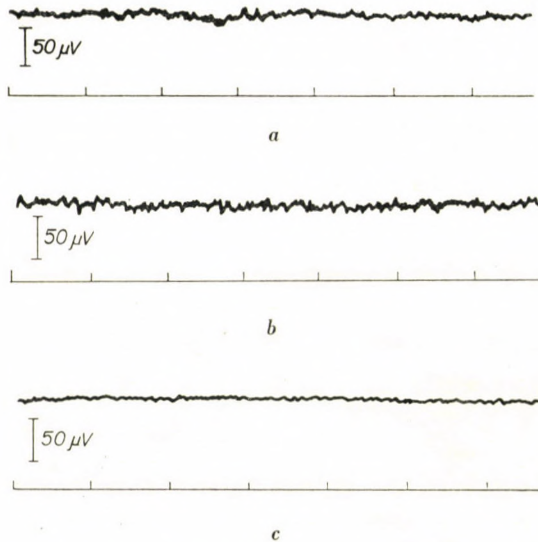

Fig. 1. Biopotentials of the cerebrovisceral connective following application to the cerebral ganglion of GABA at a concentration of 10^{-6}
a = control; b = at 1 min.; c = at 3 min.

tonicity in the posterior adductor. The intensity of this effect seemed to be in direct relation to the concentration of GABA.

Increase in tonicity after stimulation for 20 sec. amounted to 98 per cent at high, and to 56 per cent at low, concentrations (10^{-5} and 10^{-8} w/w, respectively). The effect of a preceding elimination of the cerebral ganglion was still felt after a transection of the connective. In this case the tone was 82 per cent more intense than under physiological conditions.

Electrophysiological observations in connection with GABA and picrotoxin. GABA is known [4, 8, 10] to inhibit rhythmic bioelectric activity not only in vertebrates and invertebrates but also in organisms devoid of nervous apparatus. Data in this respect are lacking in regard to molluscs.

We applied GABA to the cerebral ganglion at low and high concentrations (10^{-6} and 10^{-3} w/w). Low concentrations strengthened the bioelectric activity of the nerve in the first phase (Fig. 1/b), but activity began to decrease in the second minute and soon disappeared altogether. Fig. 1/c shows connec-

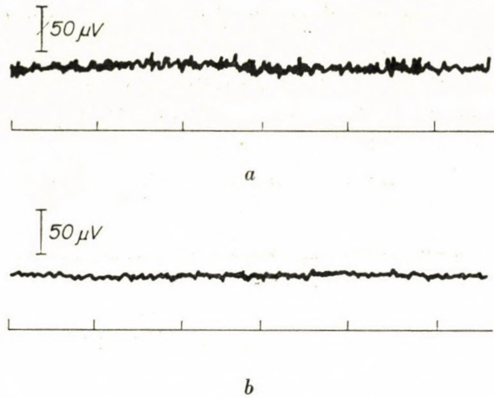

Fig. 2. Biopotentials of the cerebrovisceral connective following application to the cerebral ganglion of GABA at a concentration of 10^{-3}
a = control; b = after treatment

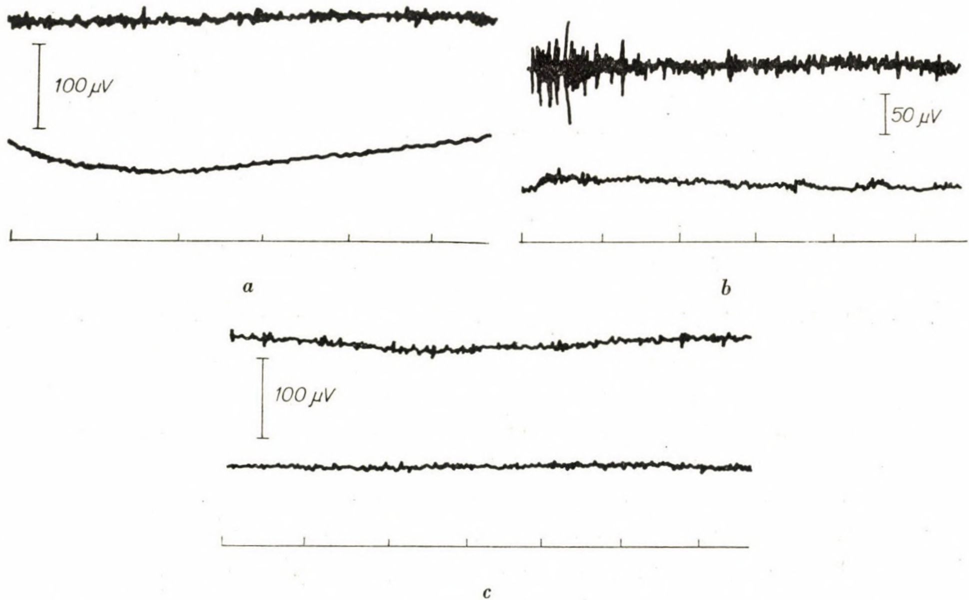

Fig. 3. Cerebral (above) and visceral (below) biopotentials following application of picrotoxin and GABA to the cerebral ganglion
a = control; b = picrotoxin (concentration, 0.05 *M*); c = GABA (concentration 10^{-3})

tival activity at 3 minutes. Low concentrations of the substance have, thus, a diphasic effect. No such effect was observed after the application of high concentrations (Fig. 2/b). Connectival activity began to diminish immediately after treatment with GABA, a sign of its prompt inhibitory action. Application to the visceral ganglion gave the same results, with the difference that the first (stimulative) phase lasted somewhat longer.

The existence of an antagonism between GABA and picrotoxin has been pointed out by several authors [1, 3, 5]. The effect of GABA has hardly been studied in connection with molluscs, and the same applies to picrotoxin. RICHARDS [11] claims that it does not affect the pedal ganglion.

Picrotoxin applied to the cerebral ganglion at a concentration of 0.05 *M* was found to strengthened connectival biopotentials (Fig. 3/b). Spindle-shaped bundles of impulses, indicative of increased activity, were often ob-

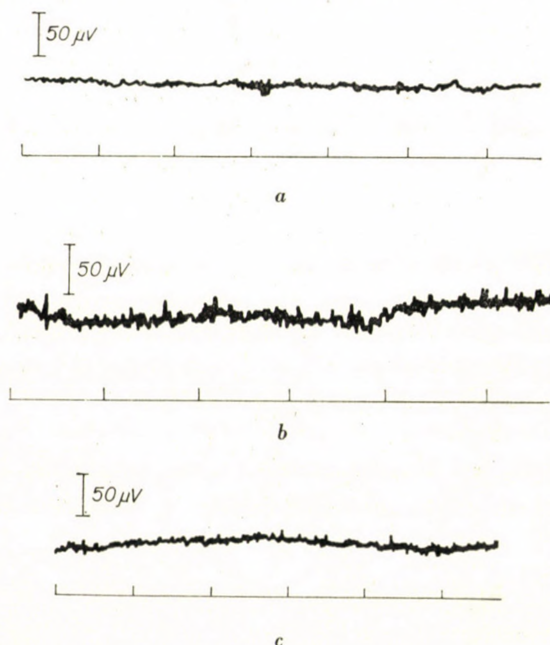

Fig. 4. Biopotentials of the visceroefferent nerve following application of picrotoxin and GABA to the visceral ganglion
a = control; *b* = after treatment with picrotoxin (0.05 *M*); *c* = after treatment with GABA (10^{-3})

served. This shows that picrotoxin is a powerful stimulant in molluscs as well. Records of the impulses conducted by the nerve running from the visceral ganglion to the posterior adductor (visceroefferent nerve) showed a simultaneous strengthening of the biopotentials and a synchronous appearance of the impulse bundles of the connective. GABA, applied to the ganglion after the impulses had been intensified by picrotoxin, caused a considerable decrease of activity, thus showing that the antagonism between the two substances is quite as marked in *Lamellibranchiata* as in other animals (Fig. 3/c).

Certain differences were observable nevertheless. The effect of GABA, as described in the foregoing paragraph, lasted in our experiments not longer than 60 to 90 seconds after which bioelectric activity once more attained

the high level reached under the effect of picrotoxin. Application of picrotoxin to the visceral ganglion gave similar results (Figs 4/a, 4/b, 4/c) with the difference that the counteraction of GABA was weaker in this case.

Discussion

It is clear from the foregoing that GABA causes inhibition from both the bioelectric and the myographic angle. Af low concentrations, its inhibitory effect manifested itself in the second phase. This myographically observed effect is presumably bilateral: GABA inhibits the neuromuscular synapses (or perhaps the very muscle fibres), and, by inhibiting the activity of the visceral ganglion, extinguishes the impulses which keep up tonicity.

Although high concentrations of GABA suppress the activity of the visceral ganglion promptly and almost completely, the tone still remains stronger than normal. We explain this phenomenon by assuming a non-specific toxic effect of GABA on the muscle elements, resulting in muscular parabiosis. It follows that GABA has the same effect on molluscs as on other animals. Low concentrations, applied to the visceral ganglion, inhibit the tone of the posterior adductor. The inhibitory effect of GABA on the central ganglia has been confirmed by the electrophysiological findings.

An analysis of the effects exerted at different concentrations of GABA shows that the stronger the inhibition of the cerebral ganglion, the more striking the tonic properties of the posterior adductor will become.

*

The experiments described above were conducted at the Physiological Institute of the Zhdanov University, Leningrad.

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András Puppi
Orvostudományi Egyetem Élettani Intézete, Pécs.

THE EFFECT OF COMBINED HYDROCORTISONE AND REPEATED FORMALIN STRESS ON ADRENAL CORTICOSTERONE AND ALDOSTERONE PRODUCTION

By

P. Vecsei-Weisz, K. Farkas, Veronika Kemény and D. Tanka

STATE INSTITUTE OF RHEUMATOLOGY AND BALNEOLOGY BUDAPEST

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Adrenocortical hormone production has been estimated by determining the hormone content from the incubation fluid of surviving adrenal slices. In rat experiments it has been found that, following treatment with large doses of hydrocortisone, beside the decrease of corticosterone production the production of aldosterone was also markedly decreased. When hydrocortisone treatment was combined with repeated formalin stress, corticosteroid production was mostly the same as following formalin injections alone; aldosterone and in a smaller measure also corticosterone, production were higher than in the animals treated with hydrocortisone alone. Atrophy of the adrenals was not significantly influenced by simultaneous formalin and hydrocortisone treatment. In the rats treated with hydrocortisone and formalin the glomerular zone was broader and the sudanophobic zone narrower than in the rats treated with hydrocortisone alone.

A fearsome, though fortunately rare complication of chronic corticosteroid therapy is the decrease of endogenous steroid production, adrenal insufficiency. This becomes manifest mostly after the withdrawal of exogenous steroids and in severe cases may kill the patient. Although this adrenal insufficiency following steroid withdrawal was described shortly after steroid therapy had gained wide-spread acceptance [1, 2, 3, 4], the mechanism of its development is still unclear. For example, it is unclear what changes take place in the production of aldosterone beside the usually emphasized and investigated glycocorticoid insufficiency. The pertaining evidence published thus far is rather contradictory. While Farrel [5] (1956) found no decrease in the aldosterone level of adrenal venous blood in dogs following chronic cortisone administration, some clinical observers [6, 7] noted a marked decrease of aldosterone excretion in the course of steroid treatment. One of us (Farkas [8]) found at autopsy of patients died after steroid withdrawal a mostly atrophied fascicular zone beside a normal glomerular zone; in one case the latter zone was also atrophied. The contradictory data induced us to subject the question to an experimental study. We wished to investigate in addition, in the search for therapeutic methods, the problem as to how an eventual hypoproduction of aldosterone would be influenced by simultaneous stress released by injections of formalin. In earlier experiments [9, 10, 11] we showed namely that following the repeated administration of formalin adrenal aldosterone production increased. Recently, this observation has been corroborated by Fachet *et al.* [12].

Methods

Male albino rats from the same stock, weighing 80 to 120 g, were used. One group was treated for 5 or 8 days with hydrocortisone acetate *(Richter*, Budapest) subcutaneously, a second group received in addition but at different points of time 0.5 ml of a 2 per cent formalin solution. Formalin treatment lasted 5 days in every case; *i.e.* the animals treated with hydrocortisone for 5 days received formalin from the beginning, those treated with hydrocortisone for eight days were given formalin after 3 days of hydrocortisone pretreatment. Twenty-four hours after the completion of treatment the animals were killed by decapitation, the adrenals were removed and cleansed without delay, cut into four parts and tested for corticosteroid, first of all aldosterone and corticosterone production by GIROUD's method [13]. For that purpose the specimens of adrenal tissue were incubated at 38° C in Krebs—Ringer bicarbonate solution containing 200 mg glucose per 100 ml. After the first 30 minutes the incubation fluid was decanted and the steroid production by the surviving adrenals was characterized by assaying the steroid content of the next, 2-hour, incubation fluid. Except for a few cases, at least six adrenals from 3 animals were incubated together and each column in Fig. 1 and Fig. 2 represents the steroid production by the adrenals of several animals.

After incubation the incubation fluid was tested for corticosteroids by chromatography. The incubation fluid (mostly 10 ml of Krebs—Ringer bicarbonate solution through which 95 per cent O_2 and 5 per cent CO_2 had been flown) was alkalinized with 0.5 ml *n* NaOH solution and shaken out immediately with about 20 ml of chloroform. Extraction with chloroform was repeated twice and the pooled chloroformic extracts were vacuum-distilled under 50° C, at indifferent gas tension. The extracts were applied to Whatman No. 1 filter paper strips. At first a "purifying" chromatography was done in the Bush—A [14] (ligroin: methanol : water 100 : 85 : 15) system, at room temperature, for 12 to 24 hours. As a result of this the impurities of the extract (first of all lipids) together with the mobile phase left the about 20 cm long strip used by us. Then chromatography was carried out in the Bush B 5 [14] (benzene : methanol water 100 : 55 : 45) system, at 38° C for 1 hour, after vapour saturation lasting for ½ to 1 hour. Subsequently the strips were put back into the Bush A system for "intensification". This, as we have shown [15, 16] leaves the position of the corticosteroids unchanged, but increases significantly the tetrazolium-reducing power of the steroids and thus enhances the sensitivity of corticosteroid assays. This "intensifying reaction" was described first by BUSH [17], who traced it back, erroneously, only to contaminations in the tetrazolium salts. The "intensifying" chromatography was carried out at room temperature for 4 to 12 hours. Then the chromatograms were developed with alkaline tetrazolium blue, the coloured formazan spots eluted and the steroid concentration was determined spectrophotometrically. Further details are to be found in our previous paper [18].

For histological study the specimens of adrenal tissue were fixed in 4 per cent formalin, frozen, and the sections cut were stained with Sudan IV for lipids.

Results

a) Functional results. The results for corticosterone are shown in Fig. 1, those for aldosterone production in Fig. 2. First of all, aldosterone production changed markedly; it decreased significantly following hydrocortisone treatment, to traces in some cases. On the other hand, the rats treated with hydrocortisone and formalin showed marked aldosterone production in most cases. Some groups produced more aldosterone physiologically, they behaved like the rats treated with formalin alone [9, 10, 11].

The changes in corticosterone production were similar to those in aldosterone production, but the differences were less significant. In the rats subjected to hydrocortisone treatment for 5 days, corticosterone production decreased to lesser extent than aldosterone production did.

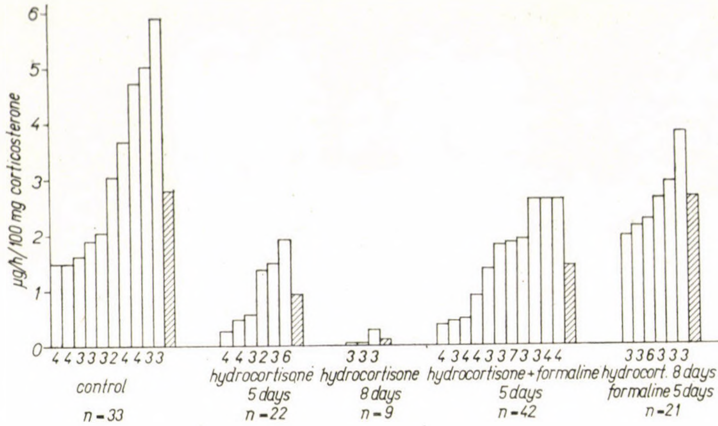


Fig. 1. Adrenal corticosterone production. Each empty column represents the results for groups with usually three rats. The number of the rats is shown under the column. Except for the six-rat groups, both adrenals were incubated. The shaded columns indicate the mean values. *n*: number of rats in each group. The values for both hydrocortisone-treated groups significantly differ from the controls ($p < 0.01$). Similarly significant differences can be found between the groups subjected to 8-day hydrocortisone, and 8-day hydrocortisone + formalin treatment. In the case of 5 day treatment, the difference is on the border of significance

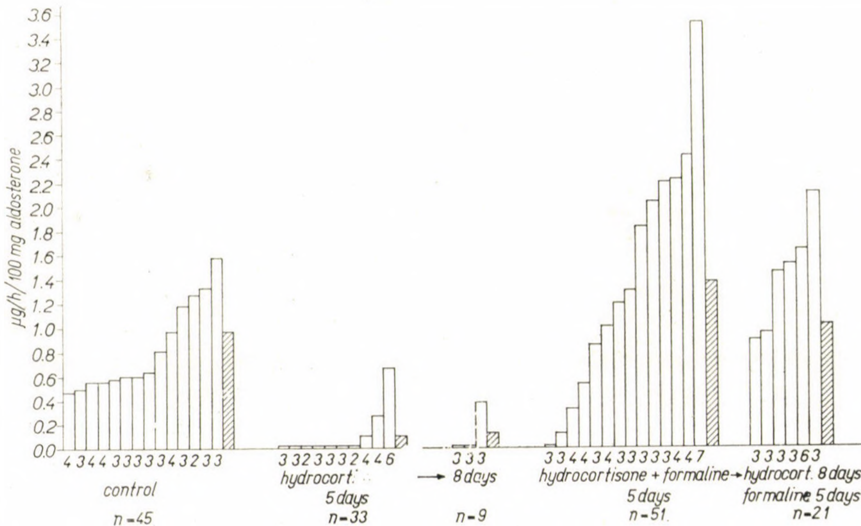


Fig. 2. Adrenal aldosterone production. Symbols and signs as in *Fig. 1.* The values for hydrocortisone treated rats are significantly different from the control values and from the results for the rats treated with hydrocortisone and formalin ($p < 0.01$)

After extracting and running the incubation media of the surviving adrenals from the animals treated with hydrocortisone and formalin, a spot reacting with tetrazolium appeared regularly. This spot, which on running in the Bush

B 5 system took place between cortisone and corticosterone, near the cortisone spot. The intensity of this spot has not been estimated quantitatively.

b) Morphological results. Fig. 3 illustrates the values for adrenal weight. Adrenal atrophy was similar to, or even more marked in the animals treated with both hydrocortisone and formalin than in those treated with hydrocortisone alone.

The adrenals from 12 hydrocortisone-treated and from 11 hydrocortisone and formalin-treated rats (5-day experiments) have been studied histologically. The results are shown by microphotographs No. 1 and No. 2. As com-

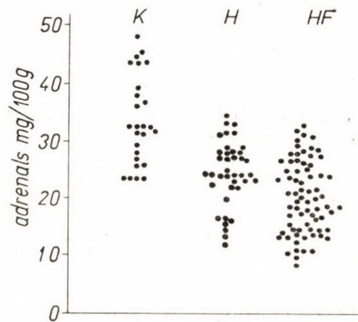


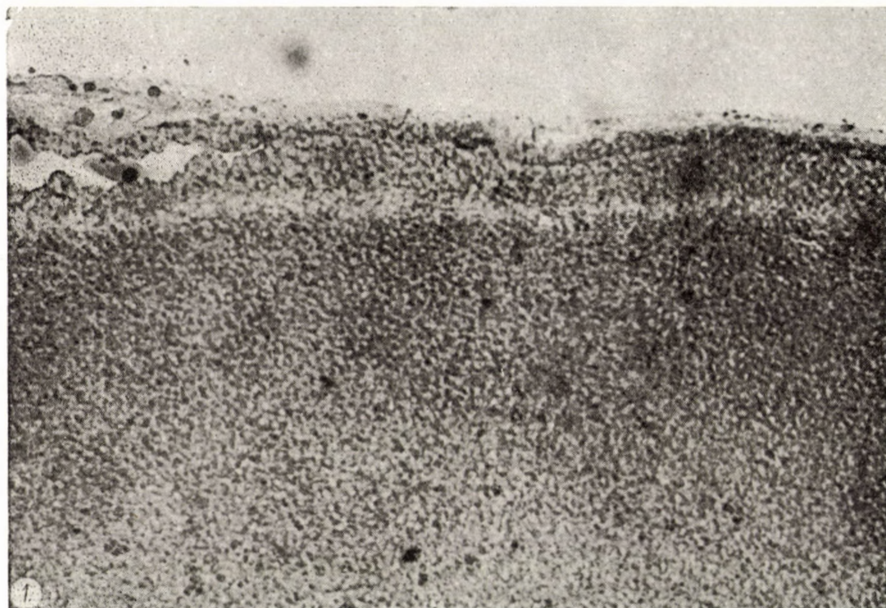
Fig. 3. Adrenal weights (left + right adrenal). K: control animals. H: hydrocortisone-treated rats. HF: hydrocortisone + formalin-treated rats. The results for the latter two groups differ significantly from the control values. Adrenal weights in the groups treated for 5 days and 8 days behaved in the same way, and are therefore shown together

pared with the controls, no definite differences were found in the lipid contents of the different strata. However, whereas in the rats treated with hydrocortisone alone the sudanophobic zone between the glomerular and fascicular zones was marked, or eventually widened, in the rats subjected to combined hydrocortisone and formalin treatment that zone was excessively narrowed as a result of a widening of the glomerular zone.

Discussion

It was remarkable that following hydrocortisone treatment the decrease of aldosterone production exceeded the decrease of corticosterone production, well-known from the literature. This result is at variance with that found by Das Gupta [19], who reported that aldosterone production did not decrease in corticoid-treated rats. This may be attributed to the facts that Das Gupta used smaller steroid doses than we did and determined by biological titration the aldosterone-like activity of adrenal venous blood and not the steroid content of the incubation fluid of surviving adrenal tissue. From our results we

draw the conclusion that, at least in some of the hormone-withdrawal syndromes, first of all following steroid treatment with large doses the adrenal insufficiency involves an equal diminution of glycocorticoid and aldosterone production, like in Addison's disease. Although some clinical reports [2] have mentioned disturbances of electrolyte metabolism similar to that found in



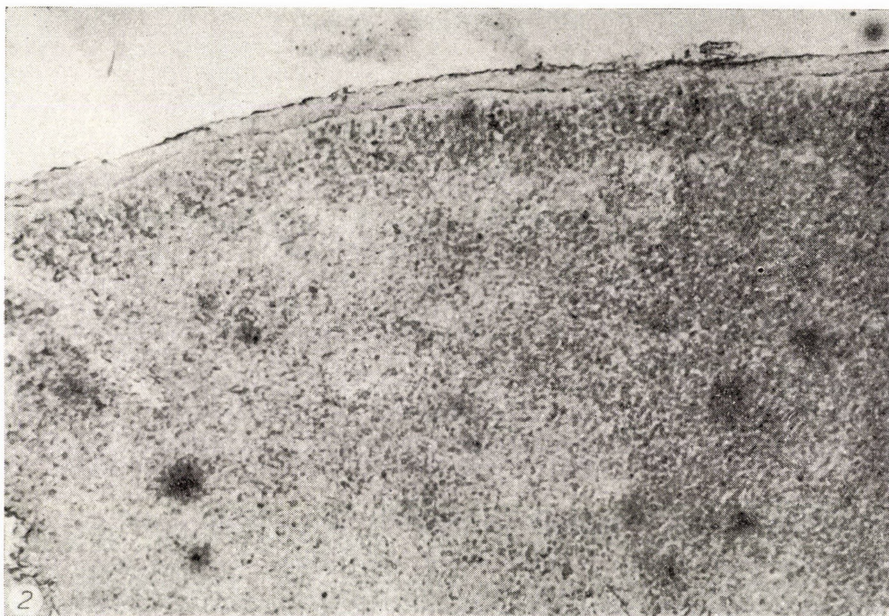
Microphotogram No. 1. Adrenal from hydrocortisone-treated rat

Addison's disease, our experimental results call for further and extensive clinical investigations.

It is to be emphasized that in the case of the combined hydrocortisone and formalin treatment it was not the aldosterone production decreasing effect of hydrocortisone, but the opposite effect of formalin that prevailed. Formalin treatment acts in the direction of normalization also in the case of corticosterone production. (We could not even expect to get so high corticosterone values as those for aldosterone production; corticosterone production does not increase even when formalin alone is administered [9, 11].) This result is believed to be important because it may show the means of prevention of adrenal insufficiency due to hormone withdrawal. Such experiments are in progress.

As to the morphological results, the changes in adrenal weight indicated once again [20, 21] that no close relationship can be found between the morphological and the functional tests. Similar conclusions may be drawn from

some other histological results, too thus, in the rats treated with hydrocortisone alone the lipid contents of the glomerular and fascicular zones did not decrease in a measure corresponding to the functional changes. Our results for the fascicular zone are at variance with certain data in the literature [22]. This may be due to the fact that in our experiments adrenalectomy took place 24 hours



Microphotogram No. 2. Adrenal from rat subjected to combined hydrocortisone and formalin treatment

after the last injection of hydrocortisone. In harmony with the functional results is our observation (microphotos 1 and 2) that in the rats given combined hydrocortisone and formalin treatment the glomerular zone was widened at the expense of the sudanophobic zone.

To galley-proof. However, it has been published recently, that after treatment with hydrocortisone in dog aldosterone secretion decreased [Farrell G.: Rec. Progr. Horm. Res. **19**, 367, (1963) in discussion]. This date agrees with our experiences on rats.

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Pál Vecsei (Weisz), Károly Farkas, Veronika Kemény, Dezső Tanka, Országos Rheuma- és Fürdőügyi Intézet, Budapest.

INVESTIGATIONS CONCERNING THE ALDOSTERONOTROPIC EFFECT OF ACTH

By

P. VECSEI-WEISZ and VERONIKA KEMÉNY

RESEARCH LABORATORY, INSTITUTE OF RHEUMATOLOGY AND BALNEOLOGY, BUDAPEST

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The effect of endogenous ACTH hyperactivity on the production of aldosterone and corticosterone in surviving adrenal slices of white rats has been examined. The following results were obtained.

Corticosterone production was considerably increased by a single injection of formalin as also by asphyxia, while the level of aldosterone showed no significant change.

Corticosterone production considerably increased and that of aldosterone was unchanged in compensatory hypertrophy, 5 days after the removal of the other adrenal.

It is suggested that ACTH secretion has no regulatory effect on aldosterone synthesis. The question as to whether results concerning the production of aldosterone in surviving sections of the adrenal gland can be accepted as valid for conditions *in vivo* is answered in the affirmative on the evidence of literary data and the results of the present experiments.

The mechanism which regulates the secretion of aldosterone is still unelucidated. The kidney [1, 2, 3], the pineal body and the subcommissural organ [4, 5, 6], some undefined neurosecretions accumulating in the posterior lobe of the pituitary [7, 8], as also the humoral influence of the anterior pituitary have, according to the present state of our knowledge, to be taken into consideration as regulatory factors. As regards the hormones produced by the anterior pituitary, it is — apart from the rather controversial role of the growth hormone [9, 10] — the role of ACTH which has to be taken into account in the first place. The involvement of ACTH in the regulation of aldosterone secretion seems to be supported by the considerations: *a)* some of the ACTH-preparations promote the production of aldosterone *in vivo* and *in vitro* [11, 12, 13, 14]; *b)* the secretion of aldosterone diminishes after hypophysectomy [15, 16]; *c)* acute stressor effects (such as surgical interventions, psychic influences, *etc.)*, by enhancing the production of ACTH, increase the urinary output of aldosterone in human subjects [17, 18, 19, 20]. There are, on the other hand, literary data contradicting this role of ACTH, such as *a)* hypophysectomy does not cause atrophy of the zona glomerulosa in which aldosterone is produced [21]; *b)* stimuli known to promote the secretion of aldosterone produce this effect in hypophysectomized animals as well [16, 22]; *c)* the aldosterone secretion-promoting humoral principle which can be transferred by means of crossed circulation has no significant ACTH-like effect, *i.e.* it does not perceptibly increase the secretion of glycocorticoids [23].

In view of these contradictory reports, we have attempted to shed fresh light on the problem under new experimental conditions in the rat.

Since according to data in the literature and our earlier experiments the different ACTH preparations differ widely in their aldosteronotropic effect [11, 12], and having no synthetic material [24] at our disposal, it was not by means of administering ACTH that we studied the action of this agent, but by creating experimental conditions under which an increased production of endogenous ACTH could be expected.

1. We observed the effect of various acute stresses. Although it was clear that acute stresses increased the excretion of aldosterone [18, 19, 20], we wanted to ascertain whether increased urinary output was due to an increase in aldosterone secretion or a decrease of its metabolism.

2. We examined the production of aldosterone under conditions of compensatory adrenal hypertrophy.

Material and method

White male rats of the same stock, weighing between 80 and 120 g, were used.

1. Acute stress

a) Stress was induced by a single intramuscular injection of 0.5 ml of 2 per cent formalin. Adrenocortical function was tested at 10, 30 and 90 minutes, 5 and 24 hours.

b) In order to bring about asphyxia, another kind of stress, we placed 3 rats, each, in closed glass vessels of about 1200 ml capacity. The hypercapnic hypoxia developed in the closed space induced pronounced dyspnoea and excitement in the animals. They lay suddenly down at the bottom of the vessel after the period of excitement was over and would have promptly died if the vessels had not been opened. We transferred the animals, therefore, to open air immediately after the termination of the phase of excitement (which usually occurred 20 minutes after the beginning of the experiment).

Adrenocortical function tests were carried out 10, 30, 60 and 120 minutes after the termination of the experiment.

c) Bleeding as stress was likewise applied. Some of the rats were anaesthesized with intraperitoneal pentobarbital, 5 mg per 100 g body weight. Five minutes later the saphenous vein was prepared, 1000 I. U. of heparin injected and drawing of blood through a cannula introduced into the femoral vein was begun immediately and continued until 30 minutes after the beginning of anaesthesia, when adrenocortical function was tested. The total amount of withdrawn blood was 3 to 4 ml.

Another group of animals was anaesthesized with ether. All other experimental conditions were the same as those in the preceding group. It should be noted that neither the conditions of anaesthesia nor the quantity of withrawn blood could be controlled as satisfactorily as in the pentobarbital group.

Having previously observed that in the formalin and asphyxia groups the effect of acute stress reaches the peak in 30 minutes, it was at this time only that function tests were performed in the exsanguinated animals.

2. Compensatory hypertrophy

The left adrenal was removed in a group of animals under ether anaesthesia, and function tests were carried out on the contralateral, hypertrophic gland 5 days after the adrenalectomy.

Function tests

The animals were decapitated and their blood was collected in heparinized centrifuge tubes. Each tube contained the pooled blood of three animals, so that the figures for corticosterone levels express the mean value of the pooled plasmas.

Immediately after decapitation, the adrenal glands were removed, carefully cleaned, weighed, sliced and — in accordance with GIROUD's technique [25] — incubated at 38° C in Krebs—Ringer's bicarbonate solution which contained 200 mg per 100 ml of glucose and had been perfused with a mixture of 95 per cent O_2 and 5 per cent CO_2. The first phase of incubation lasted 30 minutes. The incubating medium was then changed, and the second phase lasted 2 hrs. No ACTH was added to the medium. Values for corticoid were expressed in μg per hour per 100 mg of adrenal gland. Since at least 6 glands of 3 animals were incubated together, the figures express mean values.

The determination of the aldosterone and corticosterone contents of the incubating solution and the pooled blood plasma was made with the method described in earlier publications [26, 27]. STUDENT's "t"-test was used for the statistical evaluation of results. Differences with $p < 0.05$ or less were accepted as significant.

Results

I *a)* Values obtained at different times after a single injection of formalin are illustrated in Figs. 1/a, 1/b and 3. The level of corticosterone in peripheral blood as also the production of corticosterone in the adrenal slices were

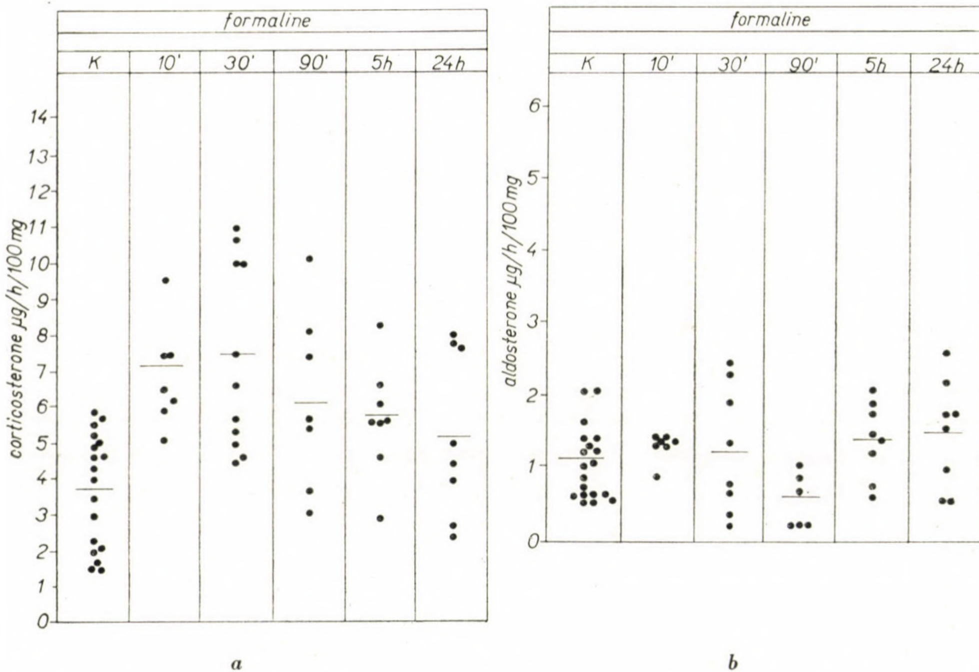

Fig. 1a, 1b. Effect of a single dose of formalin on the production of corticosterone and aldosterone in surviving adrenal slices. There was no significant increase in the production of aldosterone. K = control animals

found to have increased [28]. Although the values showed great scattering, the increase was significant statistically. Aldosterone production remained practically unchanged.

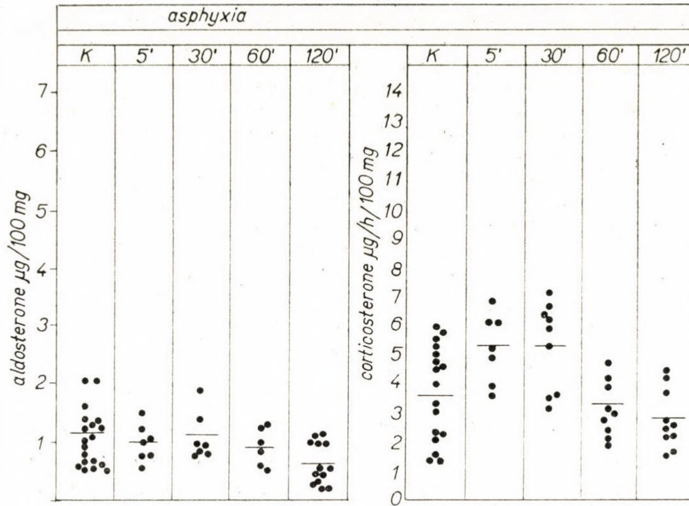

Fig. 2. Effect of asphyxia on aldosterone and corticosterone production in surviving adrenals. There was no significant increase in the production of aldosterone. K = control animals

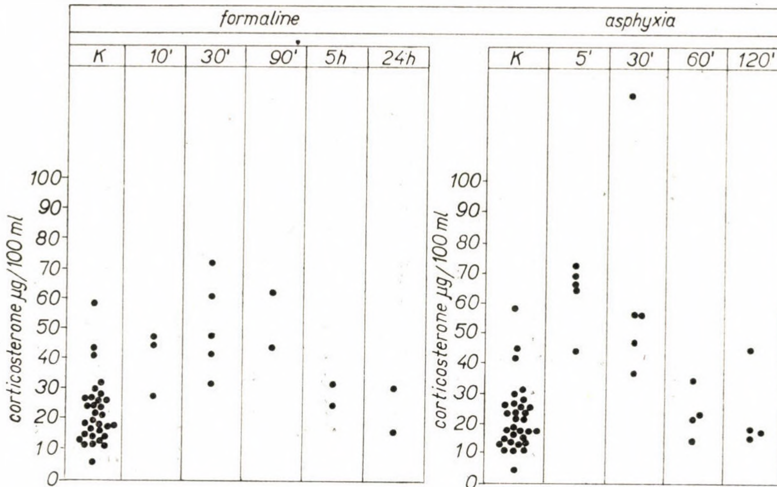

Fig. 3. Corticosterone in peripheral blood after the injection of formalin and after asphyxia. K = control animals

I *b)* Figs. 2 and 3 present the effects of asphyxia. Results in this respect were similar to those observed in formalin stress, with the difference that the values for corticosterone returned to the physiological level more rapidly. A marked decrease in aldosterone production occurred in 120 minutes, as shown in Fig. 2.

I *c)* Fig. 4. illustrates results obtained after blood loss under pentobarbital anaesthesia. There was a considerable difference between the production

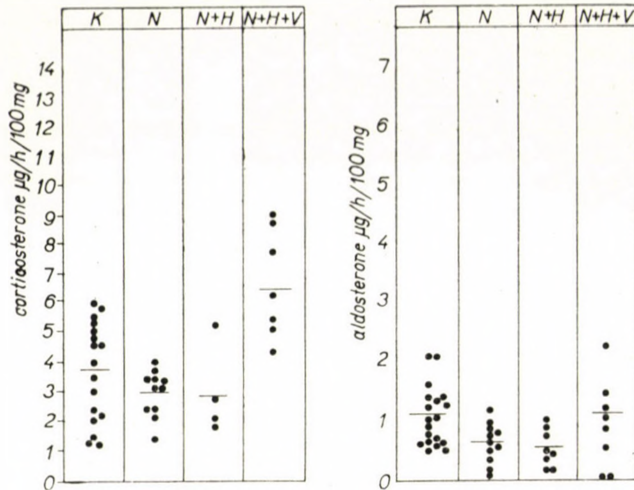

Fig. 4. Effect of pentobarbital anaesthesia and bleeding on the corticosterone and aldosterone production in surviving adrenal slices. K = control animals; N = pentobarbital anaesthesia, N + H = pentobarbital anaesthesia combined with administration of heparin; N + H + V = pentobarbital + heparin + bleeding. For doses and time factors see text

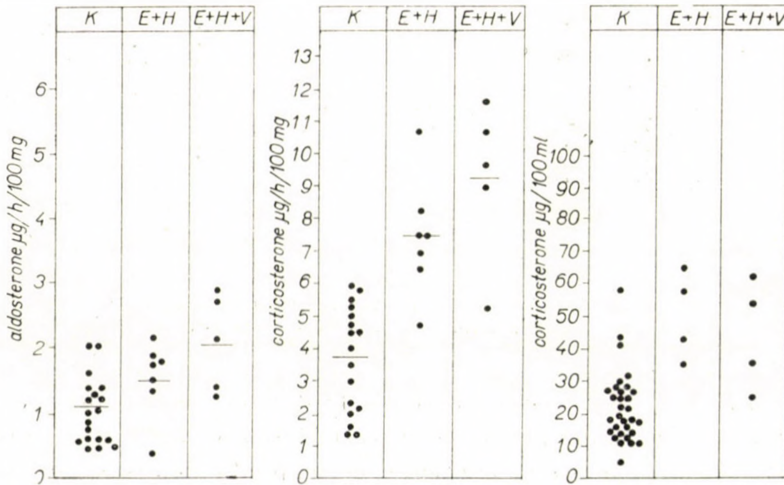

Fig. 5. Effect of ether anaesthesia and bleeding on aldosterone and corticosterone production in adrenal slices and on the corticosterone level in peripheral blood. K = control animals; E + H = ether anaesthesia and heparin administration; E + H + V = ether anaesthesia + heparin + bleeding. (The statistically insignificant rise of aldosterone production in group E + H was due presumably to that some anaesthesized animals may have lost blood after the administration of heparin.)

of corticosterone and that of aldosterone. The number of determinations concerning the level of corticosterone in peripheral blood was not sufficient to warrant conclusions. While the production of corticosterone seemed to have

considerably increased, no comparable increase in the production of aldosterone could be registered. The reliability of these results is, however, challenged by the observation that pentobarbital alone reduces the production of aldosterone under the given experimental conditions. This is why we felt induced to study the effect of bleeding under ether anaesthesia.

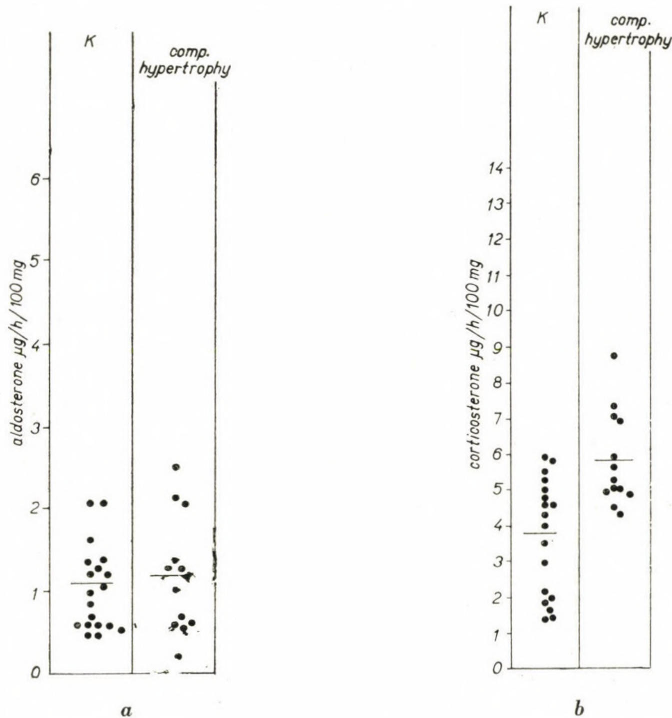

Fig. 6a, 6b. Aldosterone and corticosterone production in the remaining hypertrophic gland after adrenalectomy. The difference in respect of corticosterone is significant

I *d)* Results obtained in animals bled under ether anaesthesia are shown in Fig. 5. Bleeding promotes the production of aldosterone under the given experimental conditions and aldosterone production was not substantially affected by the stressor effect of ether alone, as it had not been by formalin or asphyxia, either.

In view of the data in the pentobarbital group, and in consideration of literary data [29] according to which no change in the production of aldosterone manifests itself within half-an-hour after the injection of heparin, we refrained from making experiments in which ether anaesthesia would have been applied without the use of heparin.

II. Fig. 6 presents the results obtained in animals with compensatory adrenal hypertrophy. In agreement with our earlier observations and some

literary data [30, 31] the amount of corticosterone produced by the remaining gland showed a considerable increase 5 days after the removal of the other adrenal gland, a phenomenon due to ACTH hyperactivity. No analogous increase was observed in respect of aldosterone production.

Discussion

Let us first discuss the question as to whether results concerning the production of aldosterone, observed in surviving sections of the adrenal gland, can be accepted as valid for conditions *in vivo*. That such results *in vitro* in respect of corticosterone can be applied to conditions *in vivo* has been made clear by VAN DER VIES in 1960 [32]. With a view to promoting a solution of the problem, we present in Table I literary data together with the results of our

Table I

Literary data concerning changes in aldosterone secretion

	Nature of treatment, stress, disease	Examined *in vivo*	Adrenal slices, *in vitro*
1	Reduced uptake of sodium	Increased level in peripheral blood. Secretion increase, as determined in humans with the isotope dilution method (42). Aldosterone level in the adrenal venous blood of rats shows likewise increase [16]	Increase [43]
2	Increased uptake of potassium	Increase [51]	Increase [44]
3	Renal hypertension	Increase (in dogs) [45]	Increase [44]
4	Administration of angiotensin	Increase (in dogs) [45]	Increase [46]
5	Administration of heparin	Decrease (in humans) [47]	Decrease [48]
6	Conn's syndrome	Increase [42]	Increase (surgical material) [49]
7	Chronic treatment with formalin	Increase of aldosterone level in adrenal venous blood [50]	Increase [40, 41]
8	Barbiturate anaesthesia	Decrease (in dogs) [38]	Decrease (according to data in the present paper)
9	Bleeding	Increase [25, 36]	Increase (see present paper)

Table II

Summary of results obtained in the present experiments

Mobilization of ACTH by means of	Corticosterone production	Aldosterone production
acute formalin stress	increased	No significant increase
asphyxia	increased	
ether anaesthesia	increased	
compensatory hypertrophy	increased	
bleeding	increased	increased*

* Further investigations are necessary for definite conclusions concerning aldosterone production (see Fig. 5).

present and earlier investigations. It will be seen from the Table that the procedure under review is quite as justified in respect of aldosterone as it is in that of corticosterone. Examination of surviving sections of the adrenal gland has various advantages. First, prompt decapitation shortens the time of manipulating the animals; second, it is possible to study the aldosterone production of a greater number of glands simultaneously, a facility in the determination of steroids.

Although the hyperproduction of glycocorticoid (corticosterone) invariably pointed to the presence of ACTH after acute stress, there was in general no hyperproduction of aldosterone observable in our experiments, which appears to contradict the observations of authors according to whom the urinary output of aldosterone rises after acute stress [17, 18, 19, 20]. It is obvious that those authors were not dealing with increased secretion but a diminished metabolism of aldosterone. This assumption is supported by literary data [33, 34].

In contrast to the other forms of acute stress, bleeding promoted the secretion of aldosterone in our experiments. That haemorrhage has an effect of this nature has repeatedly been described [35, 36]. Our observations are in agreement with the theory [37] that it is not *via* ACTH that exsanguination gives rise to an increased secretion of aldosterone. Bleeding produced no such effect in the animals anaesthesized with pentobarbital, a phenomenon due to the fact that pentobarbital, in itself, inhibits the production of aldosterone and, thus, presumably affects the regulatory mechanism.

Our observations in connection with compensatory hypertrophy were surprising insofar as neither ACTH (the increased secretion of which was clear from the enhanced production of corticosterone) nor other aldosteronotropic

factors gave rise to a compensatory increase in the production of aldosterone. It would follow that the mechanisms which govern the secretion of aldosterone are less sensitive than those responsible for the production of corticosterone. The phenomenon, as observed by us, *viz.* that a circumscribed destruction of the adrenal induced hypoaldosteronism in lack of compensatory mechanism, is noteworthy from a clinical point of view. (Of course, additional clinical investigations will have to be made in order to arrive at definite conclusions regarding this question.)

The reduced aldosterone secretion during pentobarbital anaesthesia, as observed by us, is in harmony with the report of Davis [38]. On the other hand, Gláz [39] failed to observe any change in the production of aldosterone in rat experiments made with a technique similar to ours, with the exception that she administered 4 mg per 100 g of pentobarbital as against our dose of 5 mg, and performed the tests 15 (and not 30) minutes after the beginning of the anaesthesia.

We want it to be clearly understood that our above findings in connection with formalin injections refer to acute treatment only. We demonstrated in 1959 [40] that chronic formalin treatment leads to increased aldosterone secretion. It is, however, clear that — as proved by the failure to achieve increased production of corticosterone — there is in such cases no prolonged hyperproduction of ACTH. Our results concerning chronic formalin treatment have recently been confirmed by Fachet *et al.* [41].

It seems to be obvious from both our present and earlier experiments that, following acute and chronic treatments, there is frequently a discrepancy between the production of corticosterone and that of aldosterone. It happens that no hyperproduction of aldosterone can be observed when increased secretion of corticosterone points to ACTH hyperactivity. The opposite may occur in cases of chronic treatment with formalin, *i.e.* hyperproduction of aldosterone without an increase in corticosterone secretion. It would follow that the regulation of aldosterone secretion is not connected with the secretion of ACTH. We have found that the administration to rats of large doses of hydrocortisone decreases the production of aldosterone in the surviving adrenal [27]. This need not be interpreted as a proof in favour of the role played by endogenous ACTH. There are, apart from the well-known blockage of ACTH secretion, two other interpretations which are likewise in harmony with our findings, *viz.* (1) exogenous hydrocortisone may have a feedback effect also on the mechanisms which govern the secretion of aldosterone; (2) the mineralocorticoid effect of hydrocortisone might become operative if large doses are administered, and it is known that mineralocorticoids (*e.g.* DOCA) tend to reduce the production of aldosterone.

All we can infer from our results is that ACTH has no aldosteronotropic effect. Our experiments have failed to elucidate the problem of aldosterone

regulation, and we are still unable to offer a clear interpretation of certain results, for instance, why pentobarbital decreases the secretion of aldosterone, or why its production diminishes 120 minutes after asphyxia.

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Pál Vecsei (Weisz), Veronika Kemény;
Országos Rheuma és Fürdőügyi Intézet,Budapest

Pathophysiologia

EFFECT OF CARDIOPATHOGENIC DIET ON THE SERUM LIPOPROTEIN LEVEL IN THE COCK

By

T. Tóth and J. Sós

INSTITUTE OF PATHOPHYSIOLOGY, MEDICAL UNIVERSITY, BUDAPEST

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The value of lipoalbumin + lipoprotein $\alpha-1$ in the serum of normal cocks of domestic fowl is 64.2 per cent, that of the β-fraction 26.5 per cent; the quotient of the two values, 2.42. The corresponding values in the serum of birds kept on a cardiopathogenic diet are 31.1 per cent and 55.4 per cent, with a quotient of 0.55. Such a diet raises the level of the chylomicron fraction considerably, while that of lipoprotein α 2 remains unchanged.

Anitchkow [1] was the first to show that excessive doses of cholesterol induce atherosclerosis. Gofman *et al.* [2] pointed to a change in serum lipoproteins as a characteristic feature of experimental and most cases of human atherosclerosis. The proportion of the alpha fraction (consisting of finely dispersed giant lipoprotein molecules) and the beta fraction (consisting of giant lipoprotein molecules of coarser dispersity) undergoes a change in favour of the latter. D_2 hypervitaminosis and sodium are known to promote vascular lesions elicited by a diet rich in cholesterol [3, 4]. Neutral fats produce a similar effect, while — according to Nishida *et al.* [5] — administration of excessive doses of protein inhibits the development of vascular changes.

The effect of a cardiopathogenic diet containing cholesterol, fat, vitamin D_2 and salt as atherogenic components, on the cardiac and vascular apparatus of various domestic animals was investigated and found to induce serious cardiac and vascular damage in rats [7, 8], dogs [9] and cocks [6]. In order to throw light on the mechanism of these phenomena, changes, as produced by the said diet in the serum lipoprotein level of cocks of Leghorn fowl, have been investigated in the present study.

Material and methods

The lipoprotein contents of the serum of 15 test and 11 control cocks sacrified after 4 months of cardiopathogenic diet were estimated electrophoretically: 0.05 ml and 0.03 ml of serum from each animal were dropped on paper (*Whatman* 1), and the strips were simultaneously run in a veronal buffer of pH 8.6, at 110 V and 12 to 18 mA during 20 hours. The papers were then dried at 110° C for 10 minutes after which the strips with 0.05 ml of serum were stained with Sudan black—B, and those with 0.03 ml with fuchsin. The strips dyed with Sudan were cut into sections of 5 mm, the dye was extracted with methanol containing 20 per cent acetic acid and estimated photometrically at a wavelength of 575 mμ in a "*Uvifot*" apparatus. The proportions of the individual fractions are given in per cent.

Results

In the fuchsin-stained strips: albumin, alpha-1, alpha-2, beta and gamma globulin could be differentiated. In the Sudan-stained strips, it was possible to distinguish a lipoprotein fraction before the albumin zone, and furthermore a lipoprotein fraction which ran parallel to the albumin and alpha-1 globulin, the latter was the principal component of lipoproteins in the control animals. It was not always possible to separate the albumin and alpha-1 fractions

Table I

Distribution of lipoprotein fractions in control cocks

No.	Prealbumin	Albumin + alpha-1	beta globulin
1	3.2	61.2	35.6
2	8.1	77.3	14.6
3	13.2	67.9	18.9
4	15.5	61.5	23.0
5	16.7	58.3	25.0
6	8.0	60.0	32.0
7	7.0	82.9	10.1
8	4.7	67.4	27.9
9	11.2	54.6	34.2
10	1.0	60.1	30.9
11	4.8	55.4	39.8
Mean value:	9.3±4.4	64.2±9	26.5±4.2

within it, and they are, therefore, evaluated together. Measurable amounts of lipoprotein corresponding to alpha-2 globulin were not observed. The fraction of beta lipoprotein, running parallel to beta globulins, as also that of chylomicrons, wich remained practically at the starting line, were clearly distinguishable.

The amount of chylomicrons was nearly as large as that of the fraction albumin + alpha-1 globulin in the serum of normal animals; it showed great variations not only from animal to animal but even within one and the same cock, for the momentary size of this fraction depends on absorption and mobilization. As a general rule, it can nevertheless be claimed that the mean chylomicron level in the serum of birds kept on a cardiopathogenic diet was 3.1 times that of the normal controls. The percentual interproportion of lipoproteins will be presented in the following without including chylomicrons.

The average distribution of other lipoproteins in the control animals was, 9.3 per cent in the prealbumin fraction (minimum 3.2 maximum 16.7),

64.2 per cent (minimum 54.6, maximum 82.9 per cent) running with albumin and alpha-1 globulin; 26.5 per cent (minimum 10.1, maximum 39.8 per cent) beta lipoproteins migrating with beta globulins. The distribution of lipoproteins in the serum of normal animals is shown in Table I.

The serum of test animals revealed hyperlipaemia. The distribution of the lipoprotein fractions was, before the albumin 12.4 per cent (maximum 19 per cent, no measurable value in one instance); in the fraction albumin + alpha-1 globulin 31.1 per cent (maximum 41.7, minimum 10.6 per cent). Beta lipoproteins 55.4 per cent (maximum 73.5, minimum 43.3 per cent). Table II shows the distribution of lipoproteins in the serum of animals kept on the cardiopathogenic diet.

Table II

Distribution of lipoprotein fractions in cocks kept on a cardiopathogenic diet

No.	Prealbumin	Albumin + alpha-1	beta globulin
1	12.3	41.7	46
2	6.3	40.3	43.4
3	14.3	36.4	49.3
4	11.3	27.5	61.2
5	13.8	39.5	46.7
6	13.4	34.1	52.5
7	19	37	44
8	—	26.5	73.5
9	18.2	10.6	71.2
10	11.4	40.2	48.4
11	13.6	24.2	62.2
12	14.2	34.7	51.1
13	10.8	28.8	60.4
14	11.7	16.2	72.1
15	16.2	29.3	54.5
Mean value	12.4 ± 4.5	31.1 ± 8.6	55.4 ± 10.4

Discussion

Fractions containing giant molecules of lipoproteins became predominant in the serum of animals kept on a cardiopathogenic diet, a phenomenon in agreement with data concerning atherosclerotic processes. The ratio $\frac{\text{albumin} + \text{alpha 1}}{\text{beta}}$ was 2.42 for the controls, and 0.55 beta for the test cocks.

Beside the increase of the beta fraction, it is mainly the increase in the amount of alpha-2 lipoproteins that is considered characteristic of the development of myocardial infarction [10, 11, 12]. Recently significance has been ascribed to the joint increase of the chylomicrons and the fraction alpha-2. ALBRINK [13, 14] attaches greater importance to the increase of these triglyceride-carrying fractions than to that of the cholesterol-binding beta fraction, and now the triglyceride content of the serum is regarded as of an importance at least equal to the level of blood cholesterol. MAGRASSI and RAIMONDI [15] observed the simultaneous increase of the fractions alpha-2 and beta in cases of acute experimental myocardial infarction.

As regards the increase in the chylomicrons and the beta fraction, the results of our animal experiments are in agreement with the human observations. While we, too, found the triglyceride contents to have increased, no such tendency was observable in respect of the fraction alpha-2. This may, of course, be a specific feature of the cock, or may be due to the composition of the diet, a problem that requires further investigation.

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Tamás TÓTH, József SÓS,
Budapesti Orvostudományi Egyetem, Kórélettani Intézet, Budapest.

DIE WIRKUNG VON MAGNESIUM AUF DIE NEUROGENE UND ALIMENTÄRE HYPERTONIE BEI RATTEN

Von

J. RIGÓ und I. SZELÉNYI

Technische Assistenten: M. JÓNA und B. RÓZSAHEGYI

PATHOPHYSIOLOGISCHES INSTITUT DER MEDIZINISCHEN UNIVERSITÄT BUDAPEST

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Neurale Belastung hat zur Progression der auf alimentären Wege ausgelösten Hypertonie geführt.

Mit magnesiumreicher Diät konnte sowohl die alimentäre als auch die neurogene Hypertonie abgewehrt werden. Bei den mit kardiopathogener Diät gefütterten und neuraler Belastung ausgesetzten Tieren bleibt die Blutdruckerhöhung aus, wenn die kardiopathogene Diät mit dem Fünffachen der normalen Magnesiummenge angereichert wird.

Die mit kombinierter alimentärer und neurogener Belastung herbeigeführte Blutdruckerhöhung läßt sich durch Darreichung magnesiumreicher Diät normalisieren.

Auf die Prozesse des Organismus wirkt sich die Umwelt teils durch Reize über das Nervensystem, teils durch die im Verdauungstrakt zur Resorption kommenden Stoffe aus. Bei der Entwicklung der Hypertonie fällt beiden Faktoren eine wesentliche Rolle zu. Die Wichtigkeit der Umweltreize ist allgemein bekannt. Nach LANG [16] sind die sich häufig wiederholenden oder permanent vorhandenen Reize in hohem Maße an der Entwicklung der Hypertoniekrankheit beteiligt. Aus den Arbeiten von MILLS [20] wissen wir, daß die Hypertonie durch die Stressoren ausgelöst oder verschlimmert werden kann. SÓS und Mitarbeiter [11, 28, 29, 34] haben Hypertonie bei Ratten auf neurogenem und alimentärem Wege zustande gebracht. Dieselben Autoren beobachteten bei Ratten Hypertonie nach kardiopathogenen Diät [32, 33]. Ein Hauptfaktor der kardiopathogenen Diät ist die Armut an Magnesium [29, 25]. In der Literatur finden wir Angaben über die blutdrucksenkende Magnesiumwirkung. Wie HADDY [11] feststellte, sinkt der Blutdruck nach intravenöser Einspritzung von 10%igem Magnesiumsulfat. KELLY, CROSS und TURTON [15] beobachteten gleichfalls Blutdrucksenkung bei Hypertonikern nach Verabreichung von Magnesiumsulfat. Dieselbe Erscheinung teilte AFANASSEWA [1] mit.

Von diesen Beobachtungen ausgehend, untersuchten wir an Ratten die Wirkung der magnesiumreichen Diät bei den mit neurogener und kardiopathogener Diät (S. 65) zustande gebrachten Hypertonien. Wir suchten die Frage zu beantworten, welchen Schutz die magnesiumreiche Diät bei den mit neurogenen und alimentären Wirkungen herbeigeführten Hypertonien gewährt. Weiterhin wünschten wir festzustellen, wie sich die mit Magnesium angereicherte Diät auf die bereits entstandene Hypertonie auswirkt.

Versuche

Die Versuche wurden an 102 Albinorattenmännchen vorgenommen, die wir in 9 Gruppen einteilten. Die Tiere der ersten vier Gruppen bekamen die übliche semisynthetische Normalnahrung [31]. Die Tiere der folgenden fünf Gruppen erhielten die mit S. 65 bezeichnete kardiopathogene Diät. Die Diät der Tiere der II., IV., VI. und VIII. Gruppe sowie nach Entwicklung der Hypertonie auch die der IX. Gruppe wurde mit dem Fünffachen der normalen Magnesiummenge angereichert, so daß jedes kg der Nahrung 28 g wasserfreies $MgCl_2$ enthielt. Die Tiere der entsprechenden Gruppen (der III., IV., VII. und VIII.) wurden täglich zweimal jeweils 15min

Tabelle I.

Die Wirkung von Magnesium auf die alimentäre und neurogene Hypertonie von Ratten

Gruppe	Zahl der Tiere	Diät + Behandlung	Blutdruck mmHg
I	10	Semisynthetische Normaldiät	110±5
II	10	Semisynthetische Normaldiät + Mg	110± 5
III	12	Semisynthetische Normaldiät + Reizung	160±10
IV	10	Semisynthetische Normaldiät + Reizung + Mg	110± 5
V	10	Kardiopathogene Diät	165± 5
VI	10	Kardiopathogene Diät + Mg	110± 5
VII	14	Kardiopathogene Diät + Reizung	185±10
VIII	10	Kardiopathogene Diät + Reizung + Mg	110± 5
IX	16	Kardiopathogene Diät + Reizung, nach 4 Wochen + + Mg	150± 6 110± 5

einem Licht- und Schallreiz ausgesetzt, den wir in Intervallen von 5 min mit einem Stromstoß assoziierten. Die Tiere der IX. Gruppe bekamen 28 Tage lang kardiopathogene S. 65-Diät und wurden gleichzeitig neuraler Belastung ausgesetzt. Vom 28. Versuchstage an erhielten die Tiere die mit dem Fünffachen der normalen Magnesiummenge angereicherte kardiopathogene S. 65-Diät.

In Tabelle I sind die Angaben über die Anzahl und Behandlung der einzelnen Tiergruppen zusammengefaßt. Wasser konnten die Tiere ad libitum trinken. Den Blutdruck der Ratten kontrollierten wir wöchentlich mit der in unserem Institut gebräuchlichen Blutdruckmeßapparatur [8].

Ergebnisse

Bei Versuchsbeginn betrug der Blutdruck der Tiere einheitlich 110 ± 5 mm Hg.

Unter Wirkung der neuralen Belastung ist der Blutdruck bei den Ratten gegen Ende der 7. Woche auf 160 ± 5 mm Hg gestiegen. Wurde die Diät der einer Reizung ausgesetzten Tiere mit dem Fünffachen der normalen Magnesiummenge angereichert, so schwankte der Blutdruck ungeachtet der Reizung bis zuletzt innerhalb der Grenzen der normalen Blutdruckwerte. Bei keinem einzigen Tier ist der Blutdruck über den Wert von 115 mm Hg hinausgegangen.

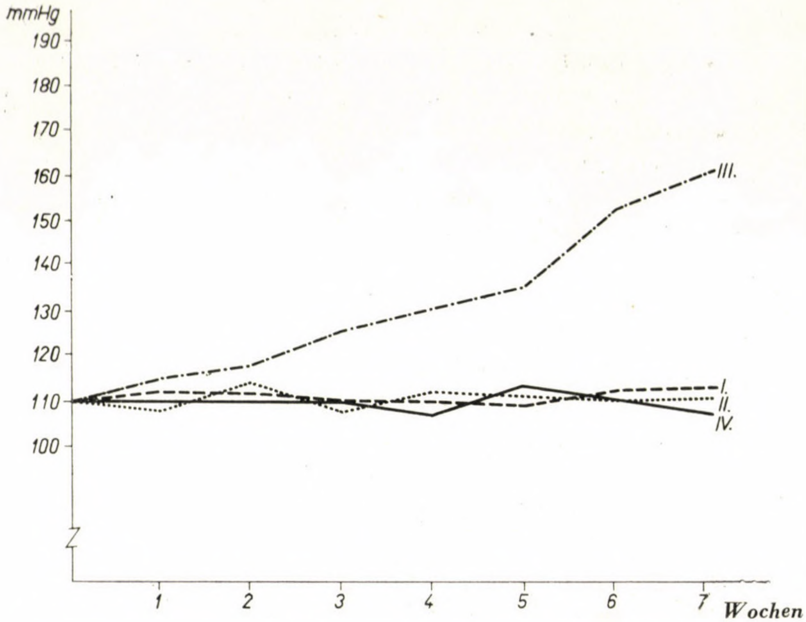

Abb. 1. Die Wirkung von neurogener Belastung $+MgCl_2$ auf den Rattenblutdruck. Die römische Zahlen bezeichnen die Gruppeneinteilung der Tabelle

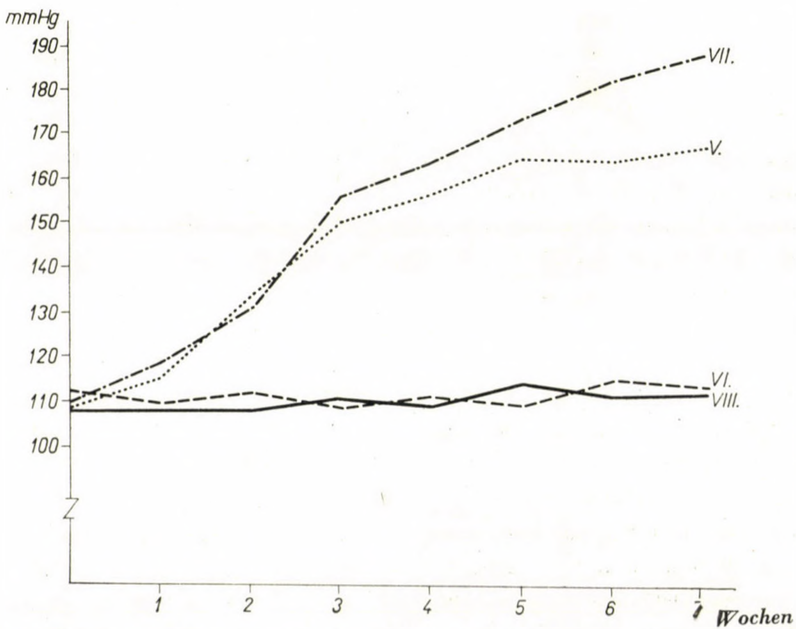

Abb. 2. Die Wirkung von $MgCl_2$ auf die neurogene und alimentäre Hypertonie der Ratten. Die römischen Zahlen bezeichnen die Gruppeneinteilung der Tabelle

Aus diesen Tatsachen darf geschlossen werden, daß das Magnesium der unter Wirkung der neurogenen Belastung zur Entwicklung kommenden Blutdruckerhöhung gegenüber eine Schutzwirkung ausübt. Die Ergebnisse veranschaulicht Abb. 1. Bei den mit normaler, semisynthetischer Diät gefütterten Ratten hat die magnesiumreiche Diät keine Blutdrucksenkung herbeigeführt. Die Blutdruckwerte der Tiere lagen zwischen 105 und 110 mm Hg und entsprachen damit denen der Kontrolltiere. Abb. 1 zeigt auch die diesbezüglichen Resultate.

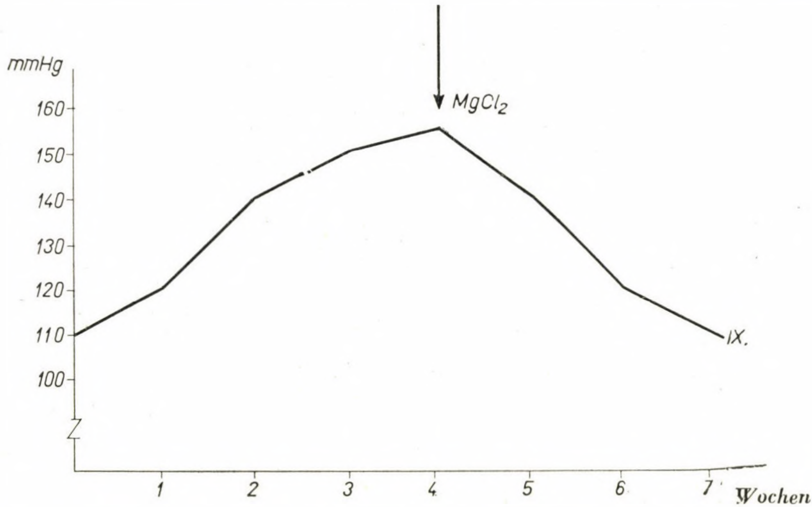

Abb. 3. Die Wirkung von $MgCl_2$ auf die alimentär und neurogen hervorgerufene Blutdruckerhöhung

Im weiteren untersuchten wir die Wirkung der kardiopathogenen S. 65-Diät, die zur Hypertonie führte; der Blutdruck schwankte zwischen 160 und 170 mm Hg. Wurde die kardiopathogene Diät mit Magnesium angereichert, so blieb der Blutdruck der Tiere auf dem Normalwert (durchschnittlicher Blutdruckwert 111 $\pm$ 4 mm Hg).

Wendeten wir neben der kardiopathogenen S. 65-Diät auch eine neurale Belastung an, so stieg der Blutdruck der Tiere beträchtlich: der durchschnittliche Blutdruckwert betrug 186 $\pm$ 7 mm Hg. Dieser Wert übersteigt wesentlich die Werte der nur mit kardiopathogener Diät gefütterten oder der nur einer neuralen Belastung ausgesetzten Tiere. Kam neben der kardiopathogenen Diätbehandlung neurale Belastung zur Anwendung und wurde außerdem die Diät der Tiere mit Magnesium angereichert, so trat keine Veränderung im Blutdruck der Ratten ein. Im Verlauf des Versuches lag der Blutdruck der Tiere stets zwischen 105 und 115 mm Hg.

Wie aus den experimentellen Ergebnissen hervorgeht, wird die blutdruckerhöhende Wirkung der mit kardiopathogener Diät hervorgerufenen alimen-

tären Faktoren von Magnesium abgewehrt, das gegen die neben der Diät angewendete neurale Belastung Schutz bietet (Abb. 2).

Die Tiere der IX. Gruppe wurden bis zum 28. Tage mit kardiopathogener Diät gefüttert und auch neural belastet. Der Blutdruck der Tiere betrug im Durchschnitt 155 mm Hg. Hiernach wurde die kardiopathogene Diät mit Magnesium angereichert. Zwei Wochen später hatte sich der Blutdruck dieser Tiere vollständig normalisiert: ihr Blutdruckwert war 110 $\pm$ 5 mm Hg. Diese Ergebnisse zeigen, daß die magnesiumreiche Diät imstande ist, auch die bereits entwickelte Hypertonie zu bessern (Abb. 3).

Besprechung

Diese Resultate unterstützen die in unserem Institut früher erzielten Ergebnisse, da es gelungen ist, bei den Versuchstieren Hypertonie sowohl auf neurogenem wie auf alimentärem Wege zustande zu bringen. Wurden die mit kardiopathogener Diät gefütterten Tiere einer neuralen Belastung ausgesetzt, so war der Blutdruck der Tiere wesentlich höher als bei den nur einer neurogenen oder nur der alimentären Wirkung ausgesetzten Tieren. Die Ergebnisse bestätigen die früheren Beobachtungen von GÁTI *und Mitarbeitern* [9]. Wahrscheinlich handelt es sich bei der Pathogenese der Hypertonie um die Kombination dieser beiden Faktoren. Laut HALL [12] vermögen die Stressoren allein nur mittelmäßige Hypertonie auszulösen. Nach MILLS [20] kann die Hypertonie durch Stressoren ausgelöst oder verschlimmert werden. Unsere vorangegangenen Untersuchungen haben gezeigt, daß unter Wirkung der neuralen Belastung neben der Hypertonie auch die Herzmuskelveränderungen progrediieren [27].

Nach den Beobachtungen von ALBERT *und Mitarbeitern* [2] ist der Magnesiumspiegel im Blutserum der Hypertoniker signifikant niedriger als im Blutserum normaler Individuen. MADER und ISERI [19] stellten Hypertonie bei jugendlichen Personen fest, bei denen der Magnesiumspiegel unter dem Normalwert lag. BECKER und HAMANN [4] fanden bei essentieller Hypertonie gleichfalls einen niedrigeren Serum-Magnesiumspiegel. Laut HAURY und CANTAROW [13] sinkt der Magnesiumspiegel im Serum bei den mit Vasospasmen einhergehenden Erkrankungen. Nach der Beobachtung von WALKER [36] ist der Serummagnesiumspiegel bei Hypertonie normal oder eher unter dem Normalwert, während dieser Wert in der renalen Phase ansteigt.

Laut HADDY [11] sinkt der Blutdruck im Verlauf einer Infusion von 10%iger Magnesiumsulfatlösung; den Effekt führt er auf die vasodilatatorische Magnesiumwirkung zurück. Nach FRIEDMANN [5] sinkt bei einem 6 mg%igen Serummagnesiumspiegel der Blutdruck normaler Tiere. Im Rahmen unserer Versuche ist der Magnesiumspiegel unter Wirkung der Diät nur mäßig

gestiegen [24], was das Ausbleiben der bei normalen Tieren beobachteten Blutdrucksenkung zu bestätigen scheint.

Nach KELLY, CROSS und TURTON [15] nimmt die renale Blutströmung nach Verabreichung von Magnesium zu. Laut GILLENWATER [10] dilatieren unter Wirkung von lokal angewendetem Magnesium die Nierengefäße, und die Nierendurchblutung steigert sich. Eine Schutzwirkung des Magnesiums stellten auch wir bei den unter Wirkung der kardiopathogenen Diät zur Entwicklung kommenden Nierenschädigungen fest [26]. Für die Wirkung von Magnesium auf den Elektrolythaushalt spricht fernerhin die Feststellung von LUDÁNY [17], wonach der Serum-K-Gehalt nach iv. Einspritzung von Mg zunimmt. Nach eigenen früheren Versuchsergebnissen sank der Kaliumgehalt im Herzen der mit kardiopathogener Diät gefütterten Tiere nach Darreichung magnesiumreicher Diät nicht, obwohl die Diät kaliumarm war [24]. Kalium wirkt auch nach SÓS [31] antihypertensiv. BACH und SÓS [3] haben nachgewiesen, daß durch die Verschiebung des Na : K-Verhältnisses zugunsten von Kalium die neurogene und renale Hypertonie vermindert werden kann.

Auf Zusammenhänge zwischen dem Magnesium- und Kaliumstoffwechsel deuten die Untersuchungen von MACINTYRE und DAVIDSON [18], die annehmen, daß der Kaliummangel eine Folge der durch den Magnesiummangel beeinträchtigten Mitochondrienfunktion sei.

Unsere Versuchsergebnisse gestatten die Schlußfolgerung, daß durch die Verabreichung einer entsprechenden Magnesiummenge die Entwicklung der Hypertonie verhindert bzw. die bereits entwickelte Hypertonie zurückentwickelt werden kann. Außerhalb der bisher besprochenen Wirkungsmechanismen dürfte dem Magnesium auch als vegetativem Ganglienblocker [4] eine Rolle zufallen. Sein cholesterin- [23] und β-lipoproteidsenkender [21] Effekt kann sich gleichfalls günstig bei der Abwehr von Hypertonien auswirken.

Unsere Ergebnisse scheinen zu bestätigen, daß die von PEVSNER [22] und von SOKOLOWSKIJ [35] empfohlene magnesiumreiche Nahrung bei der Behandlung der Hypertonie vorteilhaft sei.

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János RIGÓ, István SZELÉNYI,
Orvostudományi Egyetem Kórélettani Intézete, Budapest.

EFFECT OF p-N-OXYPHENYLGLYCINE ON THE INULIN CLEARANCE OF RATS

By

Bok Nam Li

INSTITUTE OF PATHOPHYSIOLOGY, MEDICAL UNIVERSITY, BUDAPEST

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Two intraperitoneal injections of p-N-oxyphenylglycine (POG), in doses of 20 mg per 100 g, have been administered to white male rats of 100 to 140 g body weight in order to study the effect of the compound on glomerular activity. Values determined before the injections were used as the control. The clearance of inulin (0.48 ml/minute/100 cm^2) decreased considerably (to 0.3 ml/minute/100 cm^2) under the influence of POG, thus proving that the agent in question is noxious to the glomeruli. POG is an antagonist of tyrosine, and its antimetabolic properties play a decisive role in producing the observed effect.

Introduction

Earlier investigations [1] have shown that p-N-oxyphenylglycine (POG) causes glomerular and tubular lesions presumably by antagonizing tyrosine. The concentrating capacity of the kidney was found to have diminished from the 8th day on, while dilution remained essentially unchanged. Blood pressure of the rats rose in proportion to the daily dose of POG; as a rule, chronic hypertension set in as from the 10th day of treatment.

In consideration of the histologically demonstrated glomerular lesions caused by POG, the question arose as to how the administration of the drug would affect the rate of glomerular filtration.

Material and method

Seventeen white male rats of a body weight between 100 and 140 g, from the Institute's own stock, were used. The test animals served as self-controls inasmuch as their normal inulin clearance values were established before the experiments.

The animals were given an intraperitoneal injection of 20 mg/100 g of POG on each of two consecutive days. The dose was administered in 0.5 ml of physiological saline. Since the suspension was strongly acid, the pH was adjusted to 7.0 by means of 0.1 N sodium hydroxyde.

On the day following the second injection of POG, the rats were transferred to a tube of wire-netting which admitted of slight movements without enabling the animals to change their position. The tube was provided with three apertures which fixed the hind legs and the tail, and ensured the collection of urine.

An apparatus, by means of which 1.5 ml of fluid per hour could be administered by the venous route, was employed for the infusion of inulin. First, a massive dose — 0.3 ml of 3 per cent inulin dissolved in 0.45 per cent NaCl — was administered in a tail vein, in order to ensure a stable plasma level. Subsequently, infusion was started with the same solution.

The urinary bladder was drained an hour after the beginning of the infusion. The urine was collected in calibrated tubes and 0.3 ml samples of blood were taken once or several times from a vein of the hind leg. Coagulation was inhibited by means of heparin.

We added 1.4 ml of physiological saline to each blood sample of 0.3 ml, and obtained the necessary amount of serum by centrifugation. The inulin contents of the serum and the urine were determined according to SCHREINER [2].

Results

Results are listed in Table I.

The value for normal clearance, *i.e.* before treatment with POG, was found to be 0.48 ± 0.15 ml/min./100 cm^2, in agreement with the data of COTLOVE [3], BERGLUND [4], and GRIEBETZ *et al.* [5]. It should be noted that differences in the amount and rate of infusion may change the clearance value, if to a slight extent only.

Inulin clearance showed a considerable decrease after the injections of POG. Except in animals Nos 3 and 8, the difference was significant in every

Table I

No.	Body weight of animal (g)	Body surface (cm^2)	Inulin clearance ml/min/100 cm^2 Before the administration of POG	Inulin clearance ml/min/100 cm^2 After the administration of POG
1	105	228	0.46	0.38
2	105	228	0.32	0.18
3	120	245	0.37	0.36
4	120	245	0.47	0.31
5	130	258	0.59	0.31
6	130	258	0.41	0.19
7	120	245	0.60	0.25
8	130	258	0.34	0.39
9	130	258	0.32	0.21
10	115	240	0.66	0.37
11	130	258	0.62	0.37
12	140	271	0.32	0.24
13	130	258	0.35	0.24
14	120	245	0.85	0.38
15	120	245	0.57	—
16	110	234	0.40	0.32
17	130	258	0.65	0.38

Mean value 0.48 ± 0.15 0.30 ± 0.07

$t = 3.566$

$p > 0.01$

rat. The results for animal No. 15 had to be disregarded because there occurred a serious haemorrhage in this rat after the administration of POG. The difference between clearance values before and after treatment was significant statistically ($p > 0.01$).

Discussion

Histological examinations revealed solely glomerular damage during the first days following treatment with POG, and it was only after the 10th day that tubules began to degenerate. It has been shown by us [1] that the characteristic feature of that glomerular damage consisted in extracapillary exudates, frequently leading to a compression and even destruction of the glomeruli. Since it is chiefly in respect of tubular activity that concentration and dilution tests supply information, the present experiments were made to clarify whether it was possible to recognize early glomerular lesions by means of functional tests. The changes in inulin clearance have proved that two doses of 20 mg/100 g of POG diminish the rate of glomerular filtration very considerably.

POG owes its glomerular effect to its antimetabolic properties. The thyroid gland needs much tyrosine, and it was demonstrated by Sós *et al.* [6] that POG inhibits thyroid activity by antagonizing tyrosine. The kidney is similar to the thyroid gland inasmuch as it needs much tyrosine for its normal functioning [7]. It was therefore justified to expect that treatment with POG would, apart from damaging the thyroid, lead to renal injury. That POG acts as an antimetabolite is further supported by experiments now in progress in which we have succeeded in inhibiting the hypertension brought about by POG by administering a three- to five-fold dose of tyrosine.

There exist additional antimetabolites which have been found to damage the kidney, *e.g.* methyl-tryptophan, a tryptophan antagonist [8], and the aminonucleoside 6-dimethylaminopurine, 3-amino-D-ribose [9]. All this shows that inhibition of renal amino acid or purine metabolism gives rise to grave renal damage.

Let us finally remember that POG is widely used in photography. It is evident from the foregoing that direct contact with POG, its absorption through the skin, may become dangerous, a consideration that might justify the introduction of suitable safety measures.

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РЕЗЮМЕ

ВЫЗВАННАЯ ТЕМПЕРАТУРНЫМ ГРАДИЕНТОМ МОБИЛИЗАЦИЯ ЖИДКОСТИ НА БИОЛОГИЧЕСКИХ ОБЪЕКТАХ

Ф. ВЕТЁ

В опытах на различных растительных тканях (яблоко, картофель и т. д.) при действии температурного градиента в 0,2—3,0° С, в течение $^1/_4$—95 часов отмечался значительный сдвиг содержания воды, а именно, более холодная часть ткани содержала на несколько процентов больше жидкости, чем теплая часть. Итак, более высокая температура может также в биологических системах вызывать перемещение жидкости на места с более низкой температурой, подобно тому, как это наблюдалось в опытах по термоосмозу на модели. Полученные данные показывают, что температура предположительно играет роль в мобилизации жидкости в клетках и тканях. Этот вопрос имеет большое биологическое значение в научном объяснении «активной» отдачи воды клетками.

ДЕЙСТВИЕ ЭЛЕКТРОННОГО ИЗЛУЧЕНИЯ В 15 МЭВ НА ФУНКЦИЮ И ВОЗБУДИМОСТЬ ИЗОЛИРОВАННОГО СЕРДЦА ЛЯГУШКИ

Й. ТИДИ

Исследовалось действие β-излучения в 15 Мэв на изолированное сердце лягушки и было установлено, что в 12% случаев, когда приостановка сердца вызывалась раствором с большим содержанием калия, в течение облучения наблюдались регулярные сокращения. Амплитуда и частота контракций, однако, значительно меньше естественных сокращений.

В течение облучения у большинства сердец можно было выявить снижение порога возбудимости желудочка, изолированного наложением лигатуры.

Эксперименты предоставили новые данные о взаимосвязи между действием излучения и возбуждением. Они подкрепляют положения теории о полупроводимости возбуждения.

ИССЛЕДОВАНИЕ ВЗАИМОСВЯЗИ МЕЖДУ АКТИВНОСТЬЮ КОЛЛАГЕНМУКОПРОТЕИНАЗЫ И ТРИПСИНА

И. БАНГА

1. Имеющие вид волокон ткани, содержащие коллаген (Ахиллесово сухожилие, сухожилие крысиного хвоста), после обезвоживания спиртом, ацетоном или эфиром, или в сухом состоянии, термообработкой при температуре 110° С не удалось денатурировать так, чтобы они смогли подвергнуться перевариванию с помощью протеолитических ферментов. Только после размола и пульверизации удалось выявить, что при воздействии коллагенмукопротеиназы коллагеновый белок растворяется. Это действие, однако, не представляет собой денатурацию.

2. Исследование 10 различных трипсиновых препаратов показало, что активность трипсина, измеренная на специфическом субстрате BANA (У-бензоил-DL-аргинин-β-нафтиламид) не тожественна с активностью трипсина, определенной на порошке Ахиллесова сухожилия. Значит, активность трипсина и коллагенмукопротеиназы не идентичны,

но с другой стороны, большинство трипсиновых препаратов содержит большее или меньшее количество коллагенмукопротеиназы.

3. Активность коллагенмукопротеиназы, проявленная по отношению к нативному коллагену, не тожественна с активностью метаколлагеназы. Метаколлаген можно рассматривать как частично денатурированный коллаген. Исследованные трипсиновые препараты показали наряду с коллагенмукопротеиназной активностью также метаколлагеназную активность различной степени.

4. На основании полученных результатов и литературных данных обсуждаются существование и механизм действия коллагенмукопротеиназы.

ДЕЙСТВИЕ ИНГИБИТОРОВ НА АКТИВНОСТЬ ТРИПСИНА И КОЛЛАГЕН-МУКОПРОТЕИНАЗЫ

И. БАНГА и Й. МАЙЛАТ-ПАЛАДИ

1. Исследовалось тормозящее действие трех различных ингибиторов трипсина на ферментативную активность двух трипсиновых препаратов. Специфическая ферментативная активность трипсина измерялась на синтетическом субстрате BANA N-бензоил-DL-аргинин-ß нафтиламид), а активность коллагенмукопротеиназы на Ахиллесовом сухожилии.

2. Результаты показали, что исследованные ингибиторы в различной степени тормозят активность BANA или коллагенмукопротеиназную активность одного и того же трипсинового препарата. Из этого авторы делают вывод, что активность двух энзимов различна, и действующие группы не тожественны.

3. Полученные результаты доказывают, что чистые, двукратно кристаллизованные трипсиновые препараты не гомогенны.

ИССЛЕДОВАНИЕ СПЕЦИФИЧНОСТИ МЫШЕЧНЫХ ХОЛИНЭСТЕРАЗ

I. Роль активных анионных групп

А. СЁР, А. КЁВЕР и Э. ПОХАНКА

1. При сравнительном исследовании мышечных холинэстераз авторы обнаружили разницу в их гидролитической активности. Гидролиз ацетата диметиламиноэтила, выраженный в процентах разложения ацетилхолина, при снижении pH инкубационной смеси с 8,0 до 5,5, в случае исследования истинной холинэстеразы ускорился приблизительно в семь раз исходной величины, а в случае миозинхолинэстеразы — до двукратной величины. При использовании в качестве субстрата ацетата диметиламинопропила гидролиз выраженный в процентах разложения ацетилхолина, в случае истинной холинэстеразы в небольшой степени снизился, а в случае миозинхолинэстеразы повысился до восьми-девятикратной исходной величины.

2. Декаметоний оказался в случае исследования обоих мышечных холинэстераз гораздо более эффективным ингибитором, чем пентаметоний, но в отношении торможения между двумя энзимами значительного различия не наблюдалось.

ИССЛЕДОВАНИЕ СПЕЦИФИЧНОСТИ МЫШЕЧНЫХ ХОЛИНЭСТЕРАЗ

II. Роль группы энзимов, расщепляющих сложные эфиры

А. СЁР, А. КЁВЕР и Т. КОВАЧ

При сравнительном исследовании мышечных холинэстераз авторы изучали гидролиз спиртовых или ациловых гомологичных простых эфиров. Расщепление гомологичных простых эфиров ускорялось при применении обоих исследуемых энзимов, соответственно величине молекул субстратов.

Галогенизированный, замещенный простой эфир, хлорацетат этила, расщеплялся истинной холинэстеразой прибл. в 50-раз быстрей, а миозиновой холинэстеразой прибл. с такой же скоростью, как и соответствующий простой эфир — этилацетат.

СРАВНЕНИЕ АНТИГЕННЫХ СВОЙСТВ ХРОМОВАЛЬБУМИНА И НАТИВНОГО ОВАЛЬБУМИНА НА ОСНОВАНИИ КОЛИЧЕСТВЕННОГО ОСАЖДЕНИЯ

М. КАВАИ и Л. КЕСТЮШ

Сообщается результат количественного анализа, методом осаждения, меченного хромом овальбумина (11). Установлено, что хромирование в соотношении 15 и 25 молей не оказало существенного влияния на антигенное свойство и реактивную способность белковой молекулы.

ИЗМЕРЕНИЕ ДАВЛЕНИЯ В ЛЕВОМ ПРЕДСЕРДИИ СОБАК ТРАНСБРОНХИАЛЬНЫМ ПУТЕМ

А. НАСЛАДИ

Через трахею, вскрытую на шее около конца **Carina ventralis** на левой стороне, немного в вентрально-каудальном направлении, уколом можно попасть в полость левого предсердия, и при помощи соответствующего пункционного прибора можно измерить давление в полостях левой половины сердца.

ДЕЙСТВИЕ БОЛЬШИХ ДОЗ ГИСТАМИНА НА ЛЕГОЧНОЕ КРОВООБРАЩЕНИЕ У СОБАК

Й. ЛИТВИН

На анестезированных собаках с искусственным дыханием изучалось действие гистамина на кровообращение, гистамин вводился внутривенно, в дозах 15 и 50 микрограмм на кг веса тела. Гистамин вызывал двухфазный ответ со стороны легочных сосудов. Первая фаза наступила вскоре после введения гистамина и выражалась в повышении давления в легочных артериях и увеличении кровотока в легочных венах. В большинстве опытов вычисленное сопротивление легочных сосудов оказалось неизмененным, или оно слабо снизилось. Эти изменения совпадали с быстрым снижением системного артериального давления и обычно сопровождались слабым ускорением сердечного ритма и временным усилением силы сокращения желудочков. В это же время наблюдается выраженное увеличение системного венозного давления а также и ускорение кровотока в бедренных венах. Вторая фаза последовала через 30 сек. — 2 мин., она выражалась в следующем: слабое снижение давления в легочных артериях, сильное уменьшение кровотока в легочных венах и выраженное и продолжительное увеличение вычисленного легочного сосудистого сопротивления. Эти изменения сопровождались значительным снижением силы сокращения желудочков, вторичным снижением системного венозного давления и выраженным уменьшением кровотока в бедренной вене. В противоположность быстронаступившему действию гистамина, вторая фаза является продолжительной, величины давления, кровотока и сопротивления в легочных сосудах возвращаются к исходному (контрольному) уровню параллельно с восстановлением системного артериального давления.

УСЛОВНЫЙ ОБОРОНИТЕЛЬНЫЙ РЕФЛЕКС У СЛЕПЫХ И БЕЗУСЫХ (VIBRASSAELESS) КРЫС

Л. КОРАНИ, Э. ЭНДРЁЦИ и К. ЛИШШАК

У крыс альбиносов авторы вырабатывали и угашали условные оборонительные рефлексы. В ходе исследования наблюдалось быстрое координирование двигательных процессов.

После вылущения глазного яблока и удаления волос над губами появились только временные расстройства и наблюдалась постепенно улучшающаяся реакция ориентации

в пространстве. Удаление указанных анализаторов не влияло на уже усвоенный двигательный процесс, а обусловливало только временное торможение его.

В ходе угасания выработанного условного рефлекса было отмечено, что процесс внутреннего торможения интенсивнее всего проявляется в той подопытной группе, в которой кроме удаления зрительного анализатора отрезали также волосы усов. По сравнению с контрольными группами повышенное внутреннее торможение наблюдается также у животных после вылущения глазного яблока.

При восстановлении подвергнутого торможению оборонительного условного рефлекса было отмечено, что в отношении соматомоторного процесса не наступает существенного изменения.

ДЕЙСТВИЕ, ПРИМЕНЯЕМЫХ ВНУТРИЦЕРЕБРАЛЬНО, ХОЛИНЕРГИЧЕСКИ И АДРЕНЕРГИЧЕСКИ ДЕЙСТВУЮЩИХ СРЕДСТВ, НА ЭЛЕКТРИЧЕСКУЮ АКТИВНОСТЬ НОВОЙ КОРЫ И АРХИКОРТЕКСА

Э. ЭНДРЁЦИ, Г. ХАРТМАНН и К. ЛИШШАК

В экспериментах на кошках в уретановом наркозе было установлено, что введенные в область рострального таламуса холинергически действующие вещества (эзерин и карбамилхолин) повышают синхронизационную деятельность неокортекса и облегчают образование барбитуратового «spindle». Введенные в ретикулярную формацию рострального среднего мозга холинергические вещества обусловливали десинхронизацию неокортекса и торможение образования барбитуратового «spindle». После впрыскивания карбамилхолина и эзерина в вентролатеральное ретикулярное образование наблюдалась в дорсальной части аммонова рога тета активность. Впрыскивание адреналина и норадреналина в ствол мозга и в задний гипоталамус или их внутривенное введение повышало как десинхронизационное, так и синхронизационное действие холинергически действующих средств, хотя их эффект не наблюдается во всех случаях.

Впрыскивание карбамилхолина и эзерина в перегородку одной стороны вызывает на гомолатеральной стороне тета-активность гиппокампа. Внутригиппокампальное введение ацетилхолина обуславливает десинхронизацию, но в случае билатерального введения, можно было вызвать временно генерализованную спазматическую активность.

Введение тубокурарина в аммонов рог вызывает «spike»-активность. После введения тубокурарина в одну сторону гиппокампа «spike»-активность появляется в неокортексе раньше, чем на контралатеральной стороне. Впрыскивание ацетилхолина в гиппокамп или в перегородку обуславливает торможение «spike»-активности. Подобное действие наблюдается также после введения 2%-ного раствора KCl.

РОЛЬ АКТИВИРУЮЩИХ И ЗАДЕРЖИВАЮЩИХ СТРУКТУР ЦЕНТРАЛЬНОЙ НЕРВНОЙ СИСТЕМЫ В РЕГУЛИРОВАНИИ ФУНКЦИИ СИСТЕМЫ ГИПОФИЗКОРА НАДПОЧЕЧНИКОВ

Э. ЭНДРЁЦИ, Г. ШРЕЙБЕРГ и К. ЛИШШАК

Влияние внутримозгового холинергических и адренергических химических раздражений

Авторы исследовали на кошках изменение функции системы гипофиз-кора надпочечников, обусловленное действием холинергических и адренергических веществ, инъицированных в различные области промежуточного мозга и ствола мозга. Установлено, что введение карбамилхолина и эзерина в перегородку, в **regio praeoptica**, в антеролатеральный гипоталамус и в область дорсальной покрышки (**tegmentum**) задерживает функцию системы гипофиз-кора надпочечников. Холинергическое химическое раздражение медиального и нижнего гипоталамуса, а также области заднего гипоталамуса и вентральной покрышки (**tegmentum**) обуславливало повышение секреции АКТГ. Адреналин, норадреналин и эфедрин вызывали повышение секреции АКТГ только в случае введения в область заднего гипоталамуса и вентральной покрышки.

Торможение функции коры надпочечников, вызванное холинергическим раздражением тормозящих структур переднего мозга, предотвращается адренергическим раздражением заднего гипоталамуса.

Между реакциями поведения, вызванными химическими раздражениями, и функцией системы гипофиз-кора надпочечников, не удалось выявить точного соответствия. Это обстоятельство указывает на то, что химическое раздражение областей промежуточного мозга и ствола мозга делает возможным отделение эндокринных регулирующих процессов от комплексных реакций поведения и от эмоциональных реакций.

ЭЛЕКТРОФИЗИОЛОГИЧЕСКИЙ И ФАРМАКОЛОГИЧЕСКИЙ АНАЛИЗ РОЛИ γ-АМИНОМАСЛЯНОЙ КИСЛОТЫ И ПИКРОТОКСИНА В МЕХАНИЗМЕ ТОРМОЖЕНИЯ ТОНУСА ЗАДНЕГО СФИНКТЕРА ПЛАСТИНЧАТОЖАБЕРНЫХ (LAMELLIBRANCHIATAE)

А. ПУППИ

Действие γ-аминомасляной кислоты на пластинчатожаберных (**Lamellibranchiatae**), согласно полученным результатам, одинаково с ее действием, на животных других групп. При умеренной концентрации (10^{-6} вес%), в случае апликации на висцеральный узел, она задерживает тонус заднего сфинктера. Задерживая активность церебрального узла γ-аминомасляная кислота повышает тонус заднего сфинктера.

При низкой концентрации (10^{-6} вес%) в действии γ-аминомасляной кислоты наблюдаются две фазы. В первой фазе она стимулирует, а во второй — тормозит биоэлектрическую активность. В большей концентрации (10^{-3} вес%) она сразу же тормозит биоэлектрические потенциалы.

Пикротоксин является эффективным возбуждающим средством, и следовательно, его можно считать антагонистом γ-аминомасляной кислоты.

ДЕЙСТВИЕ ОДНОВРЕМЕННОГО ПРИМЕНЕНИЯ ГИДРОКОРТИЗОНА И ПОВТОРНОГО «СТРЕССА» (ВОЗДЕЙСТВИЯ ФОРМАЛИНОМ) НА ОБРАЗОВАНИЕ КОРТИКОСТЕРОНА И АЛЬДОСТЕРОНА НАДПОЧЕЧНИКАМИ

П. ВЕЧЕИ (ВЕЙС), К. ФАРКАШ, В. КЕМЕНЬ и Д. ТАНКА

Авторы характеризовали продукцию гормонов надпочечниками путем определения синтеза гормонов инкубированными, переживающими срезами надпочечников, причем, для определения гормонов использовалась инкубационная жидкость. В опытах на крысах авторы нашли, что после дачи больших доз гидрокортизона, наряду со снижением образования кортикостерона, наблюдается также весьма значительное снижение синтеза альдостерона. Если одновременно с дачей гидрокортизона применялся также повторный «стресс» (введение формалина), то в большинстве случаев образование кортикостероидов достигало такие же величины, как после введения исключительно только формалина. Синтез альдостерона, но в меньшей мере также образование кортикостерона были более высокими, чем у животных, получавших только гидрокортизон. На атрофию надпочечников одновременное введение формалина и дача гидрокортизона не оказывали существенного влияния. По данным гистологических исследований у крыс, получавших одновременно как гидрокортизон, так и формалин, — по сравнению с животными, получившими только гидрокортизон — наблюдается расширение клубочковой зоны и сужение суданофобной зоны.

ИССЛЕДОВАНИЯ ВЛИЯНИЯ АКТГ НА СИНТЕЗ АЛЬДОСТЕРОНА

П. ВЕЧЕИ-ВЕЙС и В. КЕМЕНЬ

Авторы исследовали влияние чрезмерной продукции АКТГ на синтез альдостерона надпочечниками. Опыты проводились на белых крысах. При измерении продукции кортикостероидов с помощью срезов надпочечников (альдостерона и кортикостерона) были получены следующие результаты:

1. Острый формалиновый стресс и асфиксия, как предполагалось, в значительной мере повышали секрецию кортикостерона; в противоположность этому продукция альдостерона существенно не повышалось.

2. При компенсационной гипертрофии (спустя 5 дней после удаления одного надпочечника) выделение кортикостерона значительно повышается, в то время как выделение альдостерона остается неизменным.

3. Из полученных результатов авторы делают вывод, что регулирование секреции альдостерона не зависимо от эндогенного синтеза АКТГ. Они исследовали и детально разработали также вопрос о том, является ли исследование выделения альдостерона срезами надпочечников характерным для прижизненных условий. На основании литературных данных и опытов (исследование действия кровопускания) авторы приходят к выводу, что «in vitro» тест применим для исследования условий in vivo.

ДЕЙСТВИЕ КАРДИОПАТОГЕННОЙ ДИЕТЫ НА СОДЕРЖАНИЕ ЛИПОПРОТЕИДОВ В СЫВОРОТКЕ ПЕТУХОВ

Т. ТОТ и Й. ШОШ

В сыворотке здоровых петухов содержание альбумин + альфа-1-липопротеидов составляет 64,2%, величина бета-фракции — 26,5%. Частное обеих величин — 2,42. У петухов, получивших кардиопатогенную диету, величина альбумин + альфа-1 липопротеидов составляет 31,1%, в то время как содержание бета фракции равно 55,4%, а частное обоих значений — 0,55. Под влиянием кардиопатогенного режима питания наступает значительное повышение хиломикронной фракции. Повышения количества альфа-2-липопротеида не отмечалось.

ДЕЙСТВИЕ МАГНИЯ НА АЛИМЕНТАРНУЮ И НЕВРОГЕННУЮ ГИПЕРТОНИЮ У КРЫС

Й. РИГО и И. СЕЛЕНИ

Нагрузка нервной системы обостряла алиментарную гипертонию.

Применением богатой магнием диэты удалось предотвратить как алиментарную, так и неврогенную гипертонию. У животных, содержавшихся на кардиопатогенном режиме питания, комбинированном с невральной нагрузкой, при добавлении пятикратного количества нормальной дозы магния, повышения кровяного давления не наблюдается.

Повышение кровяного давления, вызванное комбинированием алиментарной и нейрогенной нагрузки, можно нормализировать с помощью богатой магнием диэты.

ДЕЙСТВИЕ p-N-ОКСИФЕНИЛГЛИЦИНА НА ИНУЛИНОВЫЙ КЛИРЕНС У КРЫСЫ

БОК НАМ ЛИ

Действие двукратного введения p-N-оксифенилглицина (POG (20 мг/100 г внутрибрюшинно) на функцию клубочков, исследовалось в автоконтрольных опытах на 100—140 граммовых белых крысах-самцах. Применением метода инулинового клиренса было установлено, что нормальные величины клиренса (0,48 мл/мин/100 см²) в значительной мере снизились после введения POG (0,3 мл/мин/100 см²). Этим доказывается разрушающее действие POG на клубочки. В вызванном эффекте играет роль тирозинантиметаболическое действие POG.

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DR. ISTVÁN WENT

1899—1963

The whole Hungarian Medical profession is deeply moved by the death of Professor István Went. The loss is particularly great for his disciples, whose spiritual leader he has been. His exceptional personality has been admired by everybody, but it was certainly the most outstanding for those who as his collaborators belonged to his closest circle.

Professor Went was born in 1899, in Arad. He went to high school in Nagyszeben, Arad and Temesvár, then for one year served at the front in World War I. He was admitted to the medical school of Budapest University in 1918. It was there where he took his doctor's degree in 1923. In the fourth year of his medical studies, in 1921 he joined the staff of the Institute of Bacteriology and General Pathology, University of Budapest, where he worked for 11 years under the guidance of Professor Hugó Preisz. He published his first scientific papers on immunological problems as a medical student. In 1930 he became lecturer at Budapest University, on selected chapters of pathophysiology.

In 1926—27 as holder of a Hungarian State Scholarship, he worked at the Institute of Pathophysiology of the University of Paris, with Professor M. Labbé, investigating problems of serum proteins and lipid metabolism. The next two years, as a scholar of the Rockefeller Foundation, he worked in the United States of America, at the Institute of Physiology, Harvard School of Public Health, under Professor C. K. Drinker, studying problems of circulation and respiration physiology. In 1928 he was elected "Research Fellow in Physiology" at Harvard University. In 1930—31 he visited Northern Europe and Great Britain, as a Rockefeller scholar. For half a year he studied the physico-chemical properties of proteins at the Carlsberg Laboratory of Kobenhaven, under Professor Sörensen.

In 1932 he was appointed professor and director of the Institute of Physiology and General Pathology, University of Sciences, Debrecen. He remained director of this Institute without interruption for 31 years.

The life of István Went was an epos of devotion to duty and love of work. For decades he repeatedly took part in the leadership of the university and in the work of different academic and social committees. In 1942—43 he was dean; in 1956—58 scientific deputy dean, then for three more years scientific vice rector.

In 1952 he was awarded the title Doctor of the Medical Sciences. In 1954 he was elected corresponding member of the Hungarian Academy of Sciences. Four years he took part in the leadership of the Medical Department of the Academy and in the work of the Physiological Committee. He was awarded the title Outstanding Worker of Higher Education in 1953, the Kossuth Prize in 1957 and the Order of Work in 1961.

He published nearly 100 original papers. Outstanding among these are his investigations made in 1922—25, on the basis of which he stated that the immunological phenomena manifesting themselves in different forms *in vitro* were produced by the same kind of plasma protein. The quality of the effect produced by the same "immunoprotein" depends on the conditions prevailing in the reaction mixture. With this work he was among the first authors postulating the unitarian concept of immune substances. His views were fully confirmed by orther investigators in later years.

In the next years he investigated the mechanism of anaphylactic shock and made fundamental observations in this field, too. He was the first to point out that during the anaphylactic reactions of certain viscera not histamine-like, but choline-like substances were released from the tissues. This observation, at variance with the then generally accepted histamine theory of anaphylactic manifestations, has been fully confirmed.

In other studies, in collaboration with L. Kesztyűs, then his assistant, Went succeeded in producing protein compounds of thyroxine, adrenaline and histamine and showing that these complexes acted as chemospecific antigens in immunisation experiments, in the sense that the specificity of the antibodies formed was determined by the radical to the protein. He made the fundamentally important observation, significant also in experimental therapy, that such antibodies reacted with the chemospecific antigens not only serologically, but were capable of combining immunochemically with the factor acting as specific radical and of neutralizing its effect. The neutralisation of histamine by these chemospecific antibodies formed in response to the stimulus of the histamine protein complex, as observed in animal experiments, indicated that this compound might be used as an antihistamine. Clinical experiments on several thousand patients showed that histamine azoprotein can be used with success in the treatment of allergic conditions. The introduction of histamine

azoprotein represented the first exmaple of the use of chemospecific complex antigens as therapeutic agents.

In the past ten years Professor Went, studying the so-called chemical counterregulation, pointed out a new principle of the regulation of vital functions. He proved experimentally that biologically active agents or mediators (histamine, acetylcholine, adrenaline) caused a mobilisation from the tissues of antagonistic, regulatory substances that counteracted the effect of chemical stimulation on the effector cells. He showed that this counterregulatory control serving the regulation of functions was bound to the presence of intact nervous structures. These experiments have placed in a different light the problem of the inverse effect of mediator substances and the hypersensitivity of denervated structures.

Professor Went regularly reported on his new achievements at the Meetings of the Hungarian Physiological Society, of which he was one of the founders, as well as at various international congresses. So for example, he attended in 1954 the Congress of the Polish Physiological Society, in 1956 the 9th International Physiological Congress at Brussels. In 1955 he visited the institutions of the Rumanian Academy of Sciences, and in 1962 he gave a paper at the International Physiological Congress held in Leyden.

Professor Went was an excellent, devoted and beloved teacher. This may be one of the reasons why the text-books written or edited by him are so outstanding among Hungarian medical text-books, from the professional and didactical points of view alike.

István Went was a real scientist. His modest, puritanic personality, his sincere abhorrence from ostentation, the wise understanding of the mistakes and errors of others and, above all, his unbroken love of science and devotion to duty aroused the admiration of everyone knowing him or working with him.

While he had been with us, we had been relying upon him with trust, as on one of the chosen individuals, who had contributed significantly to the advance of physiological research, one of those who had laid the foundations of modern physiological research in Hungary. Now, that he is no longer with us, we are fully aware of the greatness of our loss.

Professor Went has passed away, but his noble personality will not be forgotten and will remain an outstanding example for the present generation and for the generations to come.

HETEROGENEOUS LABELLING OF THE CYTOPLASMIC RIBONUCLEIC ACIDS OF PIGEON PANCREAS

By

MÁRIA SZÉKELY, Ö. GAÁL and B. LOVAS

INSTITUTE OF MEDICAL CHEMISTRY, MEDICAL UNIVERSITY, AND LABORATORY FOR RESEARCH OF CHEMICAL STRUCTURES, HUNGARIAN ACADEMY OF SCIENCES, BUDAPEST

(Received May 30, 1963)

In a cell-free system prepared from pigeon pancreas heterogeneous labelling of the cytoplasmic RNAs was found upon incubation with ^{32}P-orthophosphate.

The presence of the nuclear fraction was not necessary for obtaining incorporation into the cytoplasmic RNA fractions.

The RNA isolated from the large granule fraction showed a much higher radioactivity than did the RNA isolated from the microsome fraction.

Incorporation into RNA of labelled phosphate could also be achieved when the isolated large granule fraction was incubated in the absence of any other cell fraction.

It is assumed that the large granules may play an important role in the RNA metabolism of the pancreatic cell.

Metabolic heterogeneity of RNA has been demonstrated in different animal tissues by studying the incorporation of ^{32}P-orthophosphate or other precursors into different RNA fractions. Heterogeneous labelling was observed in such experiments in RNA fractions separated on the basis of their different extractability [1, 2], solubility [2], or chromatographic properties [3]. On the other hand, differences in the metabolic activities were also found when the incorporation of labelled precursors into the RNAs of different cell fractions were compared. The highest labelling was always observed in the sRNA fraction [2, 4, 5, 6, 7, 8], but the labelling of the RNA of microsomal subfractions [2, 5, 7, 8], attached and free ribosomes [9], or microsomal and postmicrosomal fractions [8, 10], too, proved to be different. Little is known about the metabolism of mitochondrial RNA. In this respect the larger cytoplasmic granules are usually considered as a rather inert fraction of low metabolic activity [10]. However, under appropriate conditions, isolated mitochondria, too, show a high rate of incorporation of radiophosphate into RNA [11, 12].

In the experiments presented here the incorporation of ^{32}P-orthophosphate into the RNAs of different particle fractions of pigeon pancreas was studied in a cell-free system. We were particularly interested in the RNA metabolism of the so called "large granule fraction", an inhomogeneous preparation of larger cytoplasmic particles, which, as was shown in previous

studies [13, 14], displays high RNA content and has an important role in the protein synthesis of pancreatic cells. This particle fraction consists of a mixture of mitochondria and large fragments of the endoplasmic reticulum with attached ribosomes, and can be isolated from a homogenate of pigeon pancreas by differential centrifugation between 3000 and 10,000 *g*. In our experiments incorporation of labelled orthophosphate into RNA occurred to a much higher extent in the large granules than in the microsome fraction.

Methods

Preparation of homogenates. The pigeons were killed by decapitation, the pancreas was removed and used immediately. The tissue was chopped and homogenized in a Potter-type all-glass homogenizer with 2 volumes of 15 per cent sucrose containing 0.03 *M* TRIS buffer of pH 7.4.

Incubation. To the homogenate, KCl and $MgSO_4$ were added in a final concentration of 0.025 *M* and 0.002 *M*, respectively. In several experiments, nuclei and secretory granules were removed from the homogenate before incubation by centrifuging for 10 min. at 1000 *g* or 3000 *g* in an MSE refrigerated centrifuge. When indicated, the incubation mixture contained also 0.005 *M* succinate, 0.007 *M* ribose and 0.003 *M* adenosine-5'-phosphate. The same incubation medium was used in experiments where suspensions of the isolated cell fractions were incubated with labelled phosphate.

^{32}P-orthophosphate was added to the reaction mixture at 0 min., in the amount indicated in the text. The mixture was incubated in air, at 37° C for 30 min., with vigorous shaking.

After incubation the reaction mixture was either diluted with a 15 per cent sucrose solution containing 0.03 *M* unlabelled inorganic phosphate and deproteinized immediately with trichloroacetic acid, or it was fractionated by differential centrifugation and the obtained cell fractions were deproteinized with trichloroacetic acid in the presence of unlabelled inorganic phosphate.

Preparation of the cell fractions. The incubation mixture was diluted twofold with the homogenizing solution. The large granule fraction was obtained by centrifuging the incubated 1000 *g* or 3000 *g* supernatant of the homogenate at 10,000 *g* for 10 min. The sediment was washed twice with the homogenizing solution under the same conditions. The microsome fraction was isolated from the 10,000 *g* supernatant by centrifugation for 60 min. at 105,000 *g* in a *Spinco* Model L ultracentrifuge.

In some experiments isolated particle fractions were used for incubation. In these cases, differential centrifugation was performed essentially in the same way before the experiment. A more dilute homogenate was prepared, the chopped pancreas being homogenized with 4 volumes of the sucrose-TRIS solution, the large granule fraction was sedimented between 3000 and 10,000 *g*, the microsome fraction between 10,000 *g* and 105,000 *g*. The sedimented, washed particles were suspended by gentle homogenization in the homogenizing solution or in the 105,000 *g* supernatant. Incubation was carried out as described above.

Electron microphotographs of the particles were taken with a *Hitachi H* 10 electron microscope. To the suspension of large granules in 15 per cent sucrose, formalin was added in a final concentration of 4 per cent. After shaking thoroughly, the suspension was centrifuged and the sediment fixed with OsO_4 for 2 hours at 4° C at pH 7.2. After fixation the preparation was washed twice and finally suspended in a 2 per cent agar solution [15]. (All the solutions used contained 15 per cent sucrose.) The solidified agar was cut into small cubes, dehydrated with ethanol and embedded into methacrylate. Ultrathin slices were prepared with the aid of a Niklowitz microtome.

Isolation of labelled RNA. Essentially the method of DAVIDSON AND SMELLIE [16] was used. The ethanol precipitate of RNA was washed twice with ethanol and absolute ethanol, dissolved in water and reprecipitated with two volumes of ethanol in the presence of unlabelled inorganic phosphate. After standing for some hours at −12° C, the precipitate was washed as before and dissolved in water. The optical density at 260 mμ was determined and in some experiments an aliquot of this solution was used directly for measurement of radioactivity. This NaCl extraction method yielded about 60 per cent of the total RNA of the particles. No attempts were made for quantitative recovery of RNA. From one experiment to the

other there was a great variation in yield, since during incubation the RNA content of all fractions, but especially that of the supernatant, showed a considerable decrease, caused very probably by the active RNase present in the homogenate.

In several experiments, the RNA precipitate was dissolved in 0.3 *N* KOH and hydrolyzed at 30° C or 37° C for 16 hours. The hydrolyzate was acidified to pH 1 with $HClO_4$, centrifuged, neutralized with KOH and the solution, after removal of the $KClO_4$, was used for the determination of nucleotide content and radioactivity.

In some experiments, fractionation on a Sephadex G 50 column was performed as a final step in the purification of RNA. By this procedure, the purity of the RNA preparations could be tested and, if necessary, the last traces of contaminating small molecular impurities could be removed. 0.4—0.7 ml of the solution, containing approximately 1 mg RNA, was applied

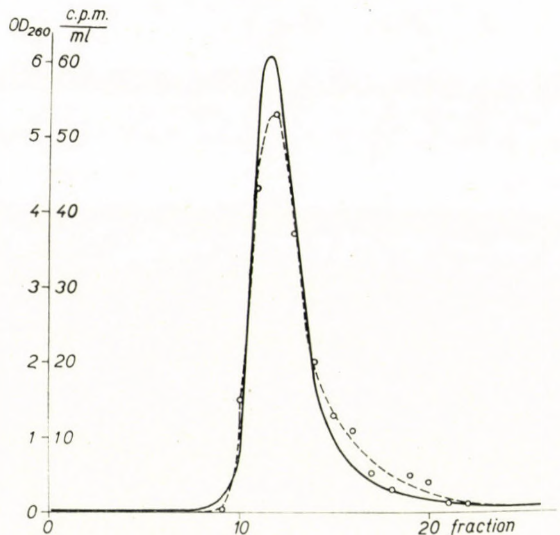

Fig. 1. Sephadex-sieving of isolated labelled RNA. Straight line: optical density at 260 mμ. Dotted line: radioactivity of the fractions, c.p.m./ml

to a 2.2×17 cm column, followed by distilled water. The RNA was recovered between the 20th and 25th ml of the eluate. Radioactivity measurements were made from the RNA-containing and the subsequent fractions; the latter — if present — contained the small molecular compounds. The components of artificial mixtures of unlabelled RNA and labelled inorganic phosphate as well as those of not sufficiently purified RNA preparations could perfectly be separated by this method.

Fig. 1 shows the result of the Sephadex fractionation of a purified labelled RNA preparation. The radioactivity peak agreed well with the OD_{260} curve. In order to prove that the radioactivity measured was indeed due to labelled RNA only, the possibility of the presence of large molecular contaminants had also to be excluded. For this purpose the solution of the purified labelled RNA (1 mg in 0.4 ml) was incubated with 2 μg of crystalline bovine pancreatic RNase at 30° C for 100 min. Before and after RNase treatment the RNA preparation was fractionated on the same Sephadex column. RNase treatment resulted in a broadening of the optical density peak, in a decrease of the maximum value and in a shift of the maximum towards later fractions (Fig. 2A). After prolonged treatment with RNase (16 hours), two flat maxima were obtained, both shifted towards the later fractions, thus indicating the appearance of degradation products of different molecular sizes (Fig. 2B). As could be expected, if only labelled RNA was present, the radioactivity of the Sephadex fractions exactly followed the changes in the optical density curve; the maximum value of radioactivity decreased and the position of the maxima shifted towards the fractions containing the small molecular degradation products.

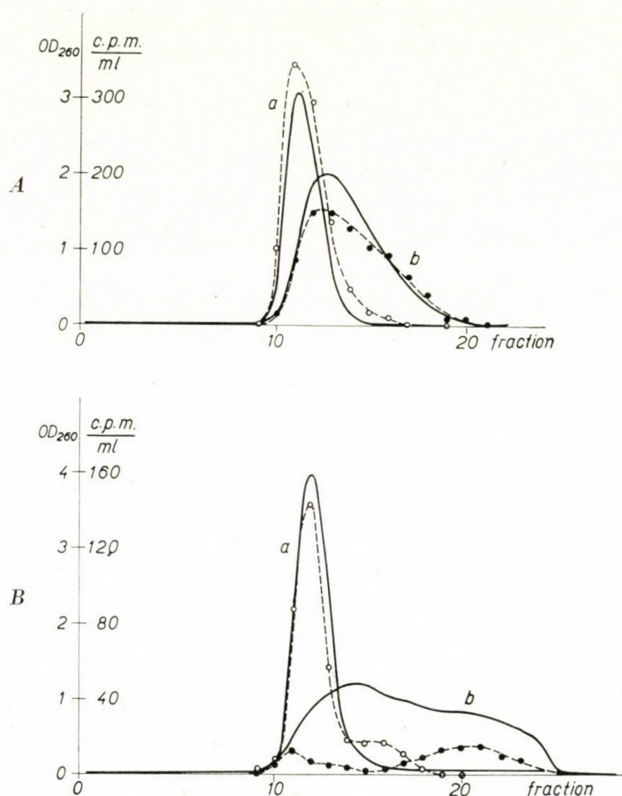

Fig. 2. Effect of RNase treatment on labelled RNA preparation. Curves a: result of Sephadex-sieving before RNase treatment. Curves b: result of Sephadex-sieving after RNase treatment. Straight lines: optical density at 260 mμ. Dotted lines: radioactivity of the fractions, c.p.m./ml. RNase treatment: *A :* incubation for 100 min. with 2 μg crystalline RNase, at 37° C. *B :* incubation for 16 hours with 6 μg RNase at 30° C

Results

Labelling of nucleic acids in a homogenate system. When a whole homogenate of pigeon pancreas was incubated with ^{32}P-orthophosphate for 30 minutes and the total nucleic acid of the homogenate was isolated after incubation, this nucleic acid fraction showed a high specific radioactivity. The labelling of the cytoplasmic RNA was demonstrated by removing the nuclear fraction from the homogenate after incubation, by 10 min. centrifugation at 1000 *g*, and isolating the RNA from the 1000 *g* supernatant. The specific radioactivity of this cytoplasmic RNA fraction was in some experiments practically equal to that of the total nucleic acid fraction, in other cases a somewhat lower labelling was found in the cytoplasmic RNA (Table I). Some variations

in the results may be caused by an incomplete separation of nuclear and cytoplasmic fractions. If a homogenate is centrifuged at 1000 *g* after having been incubated in the presence of K and Mg ions, part of the mitochondria may be lost with the nuclear sediment. Thus, recovery of the cytoplasmic RNA is not always complete.

As can be seen in Table I, the presence of nuclei during incubation was not necessary for obtaining incorporation into cytoplasmic RNA. Although

Table I

Incorporation of radiophosphate into cytoplasmic RNA in the presence and in the absence of nuclei

A whole homogenate, or a 1000 *g* supernatant, as indicated, was incubated at 37° C for 30 min, in the presence of 100 μc ^{32}P-orthophosphate, 10 μmol succinate, in a total volume of 2.5 ml, in experiments 1 and 2. In experiment 3 the radioactivity added was 40 μc, succinate 7.5 μmol, total volume 2.0 ml.

Experiment No.	Incubated system	Nucleic acid fraction isolated	Radioactivity of nucleic acid c. p. m./mg
1.	whole homogenate	total nucleic acid	500
	whole homogenate	cytoplasmic RNA	295
	1000 *g* supernatant	cytoplasmic RNA	215
2.	whole homogenate	total nucleic acid	290
	whole homogenate	cytoplasmic RNA	229
	1000 *g* supernatant	cytoplasmic RNA	238
3.	whole homogenate	total nucleic acid	80
	whole homogenate	cytoplasmic RNA	93
	1000 *g* supernatant	cytoplasmic RNA	42

the cytoplasmic RNA acquired a higher labelling if the whole homogenate was incubated, nevertheless, incorporation of labelled orthophosphate into RNA could also be achieved in a 1000 *g* supernatant, where no nuclei were present. Similar results were obtained with a 3000 *g* supernatant of the pancreas homogenate.

The rate of incorporation of ^{32}P-orthophosphate into RNA was approximately linear for about 30 minutes, later it declined. During incubation, vigorous shaking and good aeration were necessary in order to obtain incorporation, indicating the necessity of respiration for the incorporation process. The addition of an oxidizable substrate, however, was not an absolute requirement, the endogeneous respiration seemed to be sufficient for this process. Inhibition of oxydative phosphorylation by the addition of 10^{-5} *M* 2,4-dinitrophenol decreased the labelling to some extent, but did not abolish it (Table II, exp. 1).

Labelling of the RNA of different cell fractions. If a 3000 *g* supernatant was fractionated by differential centrifugation after incubation with labelled inorganic phosphate, and the RNAs of the different cell fractions were isolated,

Table II

Incorporation of radiophosphate into the RNA of different cell fractions

In experiments 1 and 2 a 3000 *g* supernatant was incubated with 30 μc of ^{32}P-orthophosphate for 30 min. at 37° C, in the presence of 4 μmol adenosine-5'-phosphate, 7.5 μmol D-ribose, 12.5 μmol succinate. In experiment 3 the radioactivity added was 50 μc, the incubation mixture did not contain adenosine-5'-phosphate, ribose or succinate, and the duration of incubation was 45 min. The amount of the 3000 *g* supernatant present in the incubation mixture corresponded to 1.15, 1.3 and 2.25 g pancreas in the experiments 1, 2 and 3, respectively. After incubation, cell fractions were separated by differential centrifugation and the RNA of each fraction was isolated, and the amount of the RNA and its specific radioactivity were measured

Experiment No.	Source of RNA, cell fraction	Total amount of RNA isolated, mg		Specific radio-activity c. p. m./mg RNA
		before incubation	after incubation	
1.	large granules	2.0	1.4	107 (77)
	microsomes	1.5	1.5	81 (53)
	supernatant	0.09	0.05	2540 (250)
2.	large granules	0.70	0.50	309
	microsomes	2.3	2.3	87
	supernatant	0.2	0.09	1180
3.	large granules	4.3	2.0	56
	microsomes	3.2	0.33	22
	supernatant	0.37	0.25	290

In experiment 1, the numbers in parentheses show the radioactivity acquired during incubation in the presence of 10^{-5} *M* 2,4-dinitrophenol.

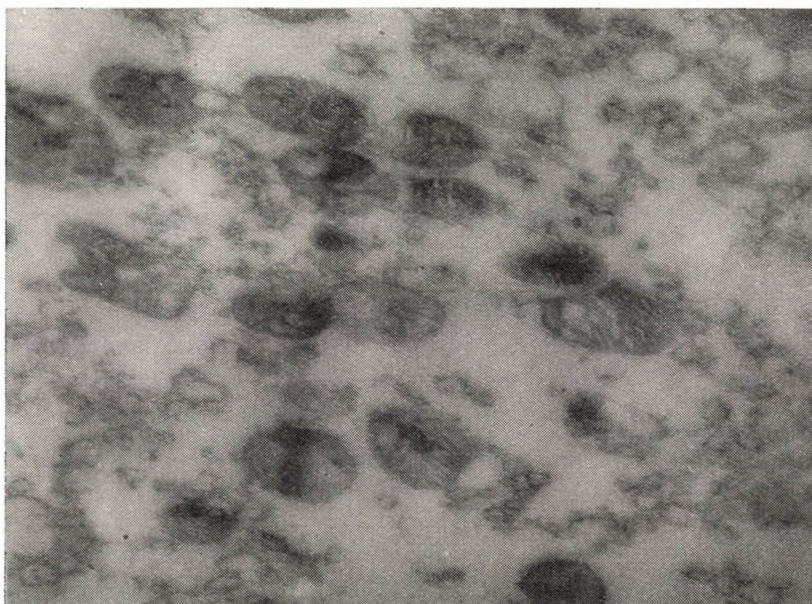

Fig. 3. Electron micrograph of a large granule fraction prepared by differential centrifugation between 1000 *g* and 8000 *g*. Magnification: × 38,800

Table III

Incorporation of radiophosphate into RNA in the large granule fraction

The large granule fraction sedimented between 3000 *g* and 10,000 *g* and washed twice with the homogenizing solution, was suspended in the homogenezing solution. This suspension was incubated with ^{32}P-orthophosphate (30 and 50 μc in experiments 1 and 2, respectively), in the absence and in the presence of cell sap. After incubation the large granule fraction was separated by centrifuging at 10,000 *g* and in experiment 2, the pellet was again washed with the homogenizing solution. The RNA of the granules was isolated andt its specific radioactivity determined

Experiment No.	Incubation	Radioactivity of the RNA of the particles c. p. m./mg
1.	in the absence of cell sap	84
	in the presence of cell sap	145
2.	in the absence of cell sap	84
	in the presence of cell sap	240

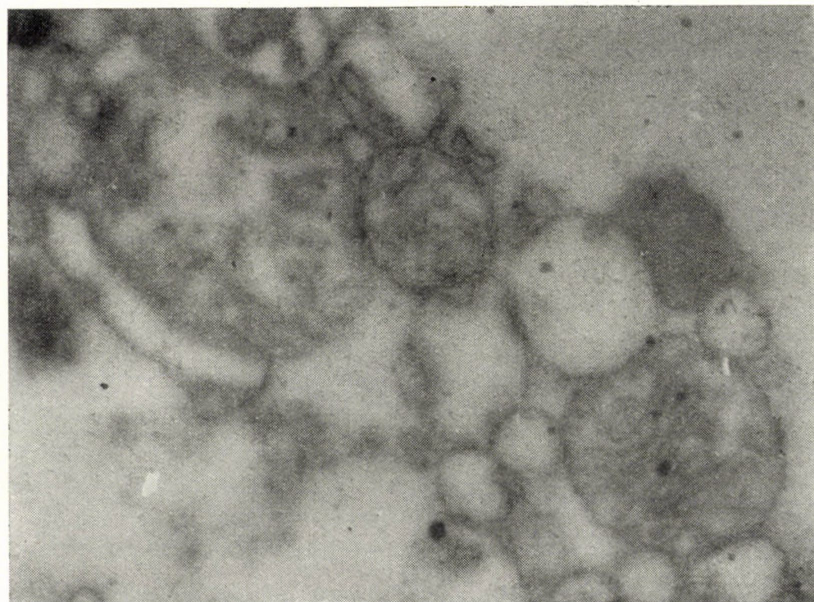

Fig. 4. Electron micrograph of a large granule fraction prepared by differential centrifugation between 3000 *g* and 10,000 *g* Magnification: × 51,000

heterogeneous labelling of the cytoplasmic RNAs could be demonstrated. As expected, the fraction showing the highest specific activity was the RNA prepared from the 105,000 *g* supernatant. There was striking difference in the radioactivity of the RNA of different particle fractions. The labelling of the RNA isolated from the large granule fraction was always much higher than that of the RNA of the microsome fraction (Table II).

Incorporation of labelled orthophosphate into RNA occurred also when the isolated large granule fraction was incubated with ^{32}P-phosphate alone or in the presence of the 105,000 *g* supernatant. The presence of the cell sap increased the incorporation of radiophosphate into the RNA of the particles (Table III). The results were similar when the large granule fraction was prepared by differential centrifugation between 1000 *g* and 8000 *g* or 1000 *g* and 10,000 *g*, instead of between 3000 *g* and 10,000 *g*.

The large granule fractions were studied by electron microscopy. Figs 3 and 4 show electron microphotographs of large granule fractions prepared in the way described above. As can be seen in the pictures, all these fractions consist mainly of a mixture of mitochondrial particles and large reticular fragments with attached ribosomes. Some dense bodies can also be seen in the preparations.

Discussion

The large granule fraction of the pigeon pancreas exhibited in these experiments a very active incorporation of ^{32}P-orthophosphate into RNA. Although no assumptions can be made on the basis of these data concerning the synthesis of different RNA fractions, it can be concluded that the high labelling found in this fraction is the result of a high metabolic activity of the RNA of the large granules. Other causes of the high radioactivity in that fraction could be a contamination with highly labelled sRNA, or a high phosphate turnover caused merely by the structural connection with the oxidative phosphorylating system. These explanations, however, can be excluded with great probability.

The total label introduced by a possible contamination with sRNA may only amount to a small part of the label found in the RNA of the large granules. It can be seen in the experiments shown in Table II that, even in case of a contamination of the large granule fraction by as much as 10 per cent of the supernatant, the amount of the contaminating sRNA would be only about 0.01 mg, and the total radioactivity introduced with this RNA would not exceed the value of 20 c.p.m.

On the other hand, it is very improbable that a high ATP turnover should be the cause of the high labelling of large granular RNA, as in the presence of 2,4-dinitrophenol there was still a difference between the labelling of the RNA of large and small granules. Similar results were obtained in an experiment where ATP labelled with ^{32}P in the two terminal phosphate groups was used instead of ^{32}P-orthophosphate for the labelling of the cytoplasmic RNA.*

* We are indebted to Dr. I. MILE for the preparation of labelled ATP.

Thus, it can be assumed that the large granule fraction contains an RNA which is metabolically highly active. This particle fraction is heterogeneous, but, as was shown earlier [13], its RNA content is too high to be ascribed solely to the presence of ribosomes. It has been suggested that mitochondrial particles, too, may contain a much larger amount of RNA than do the mitochondria of liver or muscle. At present, it cannot be decided whether this metabolically active RNA originates from the mitochondrial particles or from the ribosomes attached to the large reticular fragments present in the large granule fraction. In the latter case, one ought to assume that the ribosomes attached to these fragments are metabolically different from those found in the microsome fraction. Such a metabolic heterogeneity of the ribosomal population may exist, if the lipoprotein membranes exert a strong effect upon the function of the attached ribosomes. (A similar influence of the lipoprotein membranes was suggested by Hendler [17] with respect to the protein synthesizing activity of the ribosomes.) It is also possible that a structural integrity of the membranes is required for active incorporation.

The possibility cannot be excluded, either, that the incorporating activity of the ribosomes attached to different parts of the endoplasmic reticulum is essentially identical. In that case it is only the mitochondrial RNA which could show this high activity in the incorporation process. As the large granular RNA is a mixture of the RNAs of mitochondria and ribosomes, the above assumption would mean that the specific activity values in Table II are only averages and the labelling of the mitochondrial RNA was even higher. In fact, in that case it must be at least 10 times higher than the labelling of the ribosomal RNA.

The larger cytoplasmic granules are usually considered as being practically inert with respect to both protein and RNA metabolism. The present experiments have, however, shown that, at least in the pigeon pancreas, the RNA of the large granule fraction possesses a high metabolic activity and thus, this cell fraction may play an important part in the RNA metabolism of the whole cell.

Acknowledgement

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Mária SZÉKELY, Ödön GAÁL, Béla LOVAS
Budapesti Orvostudományi Egyetem Orvosi Vegytani Intézete és
Magyar Tudományos Akadémia Kémiai Szerkezeti Kutató Intézete, Budapest.

CHANGES OF THE NUCLEIC ACID CONTENT IN THE DENERVATED SUBMAXILLARY GLAND OF THE DOG

By

J. MOLNÁR, A. TIGYI and K. LISSÁK

INSTITUTE OF PHYSIOLOGY, MEDICAL UNIVERSITY, PÉCS

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The changes in the nucleic acid and total N content of the saliva secreted by the denervated and the contralateral submaxillary glands have been studied in the dog. The RNA content showed a marked decrease in both relative and absolute values during the 2nd to 3rd weeks following denervation. The DNA value computed for 100 mg of fresh tissue increased, while the absolute value calculated for the whole gland showed no significant change during the period of observation. No substantial change in nucleic acid content occurred after removal of the superior cervical ganglion. The changes caused by sectioning the chorda tympani were significantly enhanced by superior cervical ganglionectomy. The rate of saliva secretion in response to pilocarpine was reduced after denervation, while the secreted total N/1 g glandular tissue ratio showed a tendency to increase.

Sectioning of the chorda tympani, the principal secretory nerve of the submaxillary gland, results in an atrophy of the gland. The structural changes following denervation are well-known from the light microscopic studies [1, 6]. Similarly, significant advances have been achieved in the interpretation of supersensitivity and the phenomena of "paralytic secretion" [10, 11, 12, 15, 16, 17, 18]. On the other hand, less is known of the chemical changes taking place in denervated glands. Most papers deal with the changes of different enzymes or enzyme systems [25, 26, 28, 29]. BURFORD and HUGGINS [5] have published interesting data concerning the phosphatido-peptide and phospholipid metabolism of the supersensitive submaxillary gland of the cat. The changes in the nucleic acid content have been investigated by RABINOVITCH *et al.* [27] in glands with ligated ducts. Some authors have reported that mucin output increased following denervation [5, 8].

In the first step of the present experiments the effect of the elimination of nervous regulation on the nucleic acid content of the dog's submaxillary gland, as well as on the total nitrogen content of the glandular secretion was studied.

Methods

Adult male dogs were used, divided into three groups. In group 1 the isolable 2 to 3 cm portion of the chorda tympani was excised together with a 2 to 3 cm long portion of the lingual nerve. In group 2 the superior cervical ganglion was removed also. In group 3, only superior cervical ganglionectomy was performed. The operations were carried out under

aseptic conditions, and intravenous hexobarbital anaesthesia. The left gland was used as the control in every case. The animals were subjected to study 14 to 25 days after operation, when the characteristic physiological reactions had already developed [1, 6].

The glands were removed under hexobarbital anaesthesia. Part of the removed gland served for quantitative nucleic acid assay. The acid-soluble fractions and the lipids were removed by the Schmidt—Thannhauser method, as modified in our institute [22]. The dried and powdered gland was incubated with 0.5 *N* KOH at 37° C for 1 hour [1 ml 0.5 *N* KOH/100 mg of wet tissue] [19]. After neutralization with 70 per cent ice cold perchloric acid (PCA) acidification with the same volume of 1 *N* PCA and washing twice with 1 *N* PCA was performed. Deoxyribonucleic acid (DNA) was extracted with 1 *N* PCA, at 80° C for 25 minutes. The quantity of nucleic acids was determined with the aid of U. V. spectrophotometer, at two wave lenghts (*Beckman DU.* Spectrophotometer Model 2400). Ribonucleic acid (RNA) was measured at 260 and 275 mμ [19], DNA at 268 and 284 mμ [30], using RNA (*Fluka* A. G.) and thymic deoxyribonucleate (*L. Light* & Co. Ltd.) as the standards. The results were expressed in μg RNA or DNA/100 mg wet tissue, as well as in mg RNA or DNA/whole gland. In both cases the percentage difference in nucleic acid content between the denervated and contralateral glands was also computed.

Saliva was collected under general anaesthesia. The efferent ducts of the glands were exposed, and the saliva flowing out through an inserted glass cannula from both glands was collected for 1 hour, following the subcutaneous injection of 1 mg/kg body weight of pilocarpine HCl. The samples thus obtained were tested for total N content by the Kjeldahl method.

Results

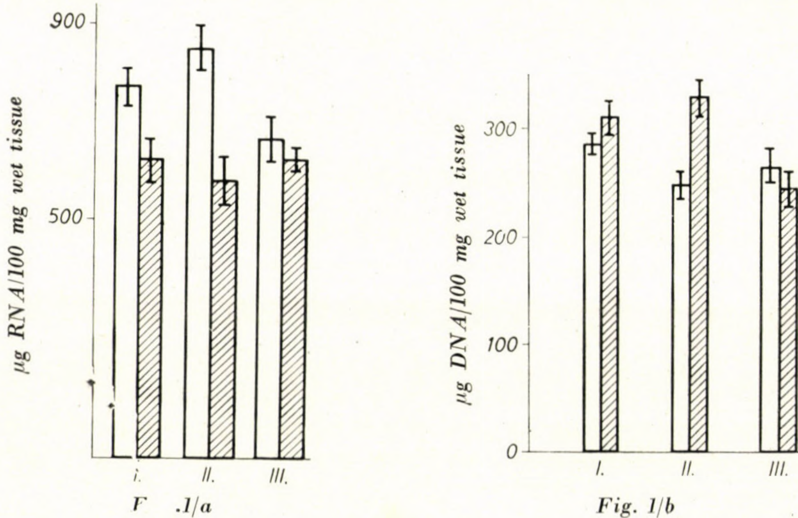
During the 2nd and 3rd weeks after denervation the following changes were noted in the nucleic acid values of the glands computed for 100 mg of wet tissue. In group 1 the denervated glands contained 623 μg RNA/100 mg wet tissue, in comparison with the 774 μg/100 mg value for the contralateral glands ($p < 0.001$). In group 2 the denervated glands showed a value of 581 μg RNA/100 mg wet tissue, in comparison with the contralateral value of 856 μg/100 mg wet tissue ($p < 0.001$). In group 3 there was no significant difference between the two sides: the denervated value was 623 μg/100 mg wet tissue and the contralateral was 666 μg RNA/100 mg wet tissue ($p > 0.2$). Thus, in group 1 the RNA content decreased by 19 per cent, in group 2 by more than 32 per cent in the denervated gland.

In general the DNA content increased in the denervated glands. In group 1 the increase was not significant ($p > 0.05$). In group 2, 318 μg/100 mg wet tissue values were obtained for the denervated gland, in comparison with the 254 μg/100 mg for the contralateral gland ($p < 0.01$), which represents an increase of 24 per cent. In group 3 the difference was not significant statistically.

Fig. 1 shows the RNA and DNA contents of the denervated and the contralateral glands, in terms of μg RNA or DNA/100 mg wet tissue. The columns on the right show the values for the denervated glands.

The changes in the RNA/DNA ratio were characteristic. In group 1 we found a 25 per cent decrease (to 2.06 from 2.73) in comparison with the contralateral glands ($p < 0.01$). In group 2 the decrease was even more marked, exceeding 40 per cent. Beside the decrease in the RNA content of the denervated glands, there was also an increase of RNA in the contralateral glands.

This is obvious if we relate to 100 mg wet tissue the value of 1.85 for the denervated glands and the value of 3.09 obtained for the contralateral gland ($p < 0.001$). Emmelin *et al.* [13] found that the contralateral glands increased in weight. It is remarkable that the increase of RNA content surpassed that of the DNA content. This was indicated also by the fact that in group 3 the



F .1/a *Fig. 1/b*

RNA/DNA ratio was 2.56 on both sides, and this at the same time represented the normal value.

Fig. 2/a shows the numerical values for the RNA/DNA ratio of the contralateral and denervated glands, and Fig. 2/b the percentage difference in the RNA/DNA ratio of the denervated and the contralateral glands.

The mg values for the total nucleic acid content of the glands are also shown. Owing to the heterogeneity of the experimental objects studied we could use only the percentage differences between the two sides. In group 1, the RNA content of the denervated glands was 32 per cent less than that of the contralateral ones. In group 2 the difference amounted to 43 per cent. In group 3, the difference was not unequivocal and was less than 10 per cent. Although showing a slight decreasing tendency in the denervated glands, the decrease of the DNA content never exceeded 10 per cent.

Fig. 3 shows the percentage differences in mg RNA content between the denervated and the contralateral glands.

With the exception of group 3, there occurred a decrease in weight of the denervated glands, reaching 35 per cent in some cases. No decrease in the weight of the gland was found in four animals.

Even if the loss in weight is compared in group 1 with the decrease of RNA content of the denervated glands it exceeded the measure of weight

loss by 14 to 16 per cent, and in group 2, by 22 to 24 per cent. The most uniform picture and the minimal scattering is obtained if the results are evaluated according to those outlined above.

It was the DNA content which was the least affected by the experimental interventions; only with losses exceeding 30 per cent in glandular weight that a decrease about 10 per cent was observed.

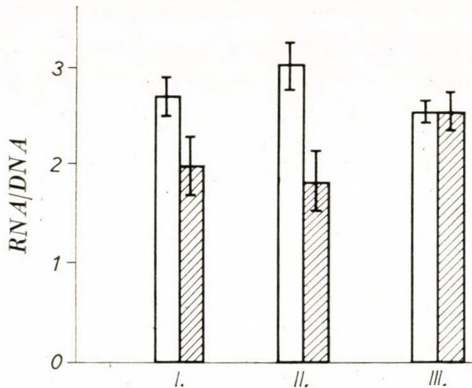


Fig. 2/a

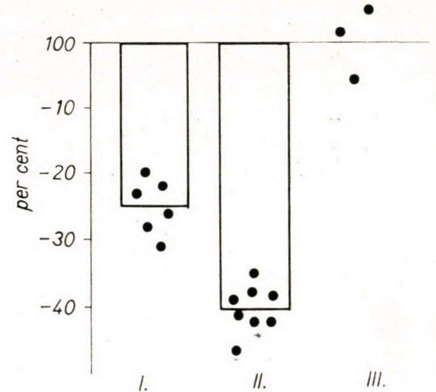


Fig. 2/b

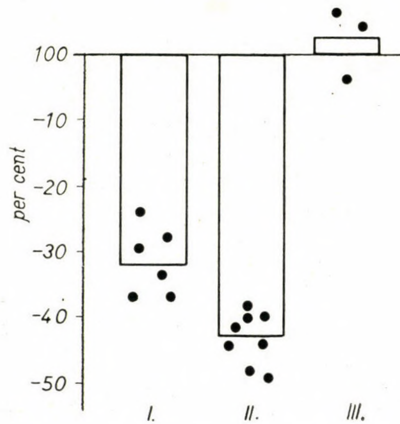


Fig. 3

Except for group 3 the volume of saliva secreted by the denervated glands decreased [14] but most markedly in group 2. The quantitative data concerning the secreted ml-s of the saliva obtained by fractionated collection and their total N values are characteristic in every group.

Figure 4 shows some typical examples. The abscissa indicates time, the left ordinate the volume of saliva output in ml, the right the total N content

of the samples in mg. In the upper part we find the data for the contralateral, in the lower those for the denervated glands. Fig. 4/a contains the data for an animal of group 1, Fig. 4/b those for one of group 2. The solid line represents saliva, the broken one the total N.

In agreement with the data published by other authors [5, 8] it was found that the values for N concentration and total N/1 g gland tissue/1 hour

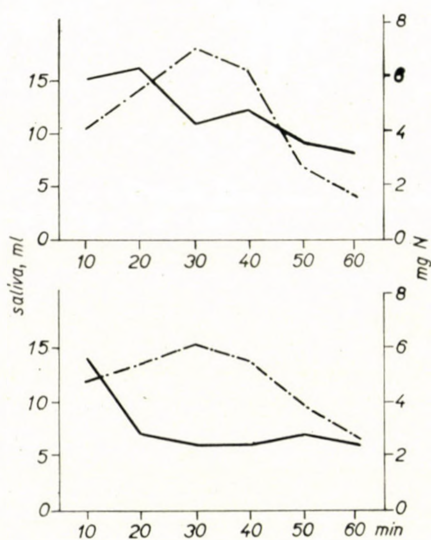


Fig. 4/a

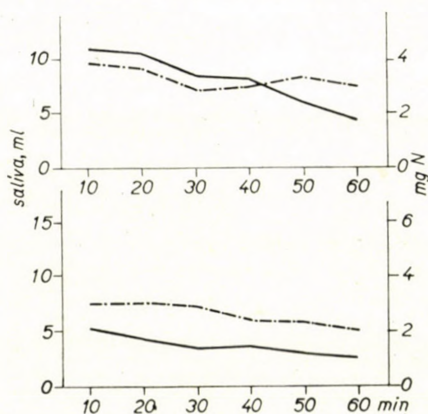


Fig. 4/b

increased in the saliva secreted by every gland denervated at least 3 weeks earlier.

The tables represent the numerical data for glandular weight loss, saliva output and total N content. Table I shows the data of some animals in group 1 and Table II some animals in group 2.

Discussion

The decrease of RNA content following denervation was found to exceed by far the measure of glandular weight loss. On the other hand, total N output showed a tendency to increase. From the point of view of the nucleic acid-protein synthesis relation, it is difficult to interpret this increased protein production associated with an excessive RNA decrease [5, 8].

It has been generally accepted that cells synthetizing protein at a fast rate show a high RNA content. This fact is considered to be one, and at the

Table I/a

Animal	No. of days following denervation	Gland weight, g		Saliva secretion during 1 hour, ml		ml saliva/1 g gland/1 hour	
		C	D	C	D	C	D
a	14	8.88	8.51	64.0	46.5	7.20	5.46
b	21	12.50	8.25	89.0	58.0	7.12	7.03
c	24	6.78	5.93	71.0	46.0	7.75	4.10
d	28	6.23	5.82	54.0	32.0	8.66	5.49

Table I/b

Animal	Total N secretion during 1 hour, mg		Total N, mg per cent		mg total N/1 g gland/1 hour	
	C	D	C	D	C	D
a	24.43	16.50	38.2	35.5	2.75	1.94
b	34.56	33.99	38.8	58.6	2.76	4.11
c	27.84	28.35	39.2	66.6	4.10	4.77
d	15.66	15.04	29.0	47.0	2.51	2.58

Table II/a

Animal	No. of days following denervation	Gland weight, g		Saliva secretion during 1 hour, ml		ml saliva/1 g gland/1 hour	
		C	D	C	D	C	D
e	18	8.19	6.97	69.6	35.3	8.49	5.05
f	21	6.58	4.78	49.1	22.6	7.45	4.77
g	24	5.29	3.63	38.2	17.8	7.21	4.90

Table II/b

Animal	Total N secretion during 1 hour, mg		Total N, mg per cent		mg total N/1 g gland/1 hour	
	C	D	C	D	C	D
e	18.73	14.28	26.9	40.5	2.84	2.05
f	20.14	16.18	41.0	71.6	3.06	3.38
g	7.34	12.12	19.2	68.1	1.38	3.34

D = Denervated submaxillary gland
C = Contralateral submaxillary gland

same time the oldest, of the proofs of the functional relationship between RNA and protein synthesis [3, 4, 7]. The role of RNA in protein synthesis has been subjected to extensive morphological and biochemical studies, but the data concerning the salivary glands are scarce and not unequivocal. Thus, for example GUBERNIEV and ILINA [20] found a 400 per cent increase of ^{32}P incorporation in response to acute pilocarpine treatment in the submaxillary gland of the rat. JUHÁSZ *et al.* [23] observed an increase of the RNA content of the rabbit's lacrimal gland following hormone treatment and the increase went parallel with the lysozyme production of the gland. Such a parallel synthesis of nucleic acids and proteins was not strengthened by the observations of HOKIN and HOKIN [21], whose studies *in vitro* showed no change in RNA content of the rabbit's parotid gland, either in enzyme synthesis, or in the phase of secretion. Similar results have been obtained by DALY and MIRSKY [9] in the mouse pancreas *in vivo.*

Our informative experiments allow no far-reaching conclusions except to justify a finer quantitative and qualitative analysis, of salivary proteins, of the intracellular distribution of the RNA content, and of electron microscopical studies of their ultrastructural changes.

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János MOLNÁR, András TIGYI, Kálmán LISSÁK
Orvostudományi Egyetem, Élettani Intézet, Pécs.

Physiologia

EFFECT OF CHRONIC STRESS ON THE OXYTOCIC AND ANTIDIURETIC ACTIVITY OF THE HYPOPHYSIS IN THE RAT

By

K. FENDLER, GY. TELEGDY and E. ENDRŐCZI

INSTITUTE OF PHYSIOLOGY, MEDICAL UNIVERSITY, PÉCS

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Female albino rats were forced to swim daily until total exhaustion, over a period of 29 days. The animals were divided into groups and were examined at 3 to 4-day intervals for changes in the hypophyseal oxytocic and antidiuretic activities. The oxytocic activity of the rats forced to swim showed a slight decrease on the 6th and 8th days of swimming, followed by a considerable increase on the 12th and 15th days. This 4 to 7-fold increase (as compared with the control values) reached the maximum on the 18th day, then declined gradually on the 22nd and 29th days, but did not return to the initial level during the period of observation. Pituitary antidiuretic activity showed an increase in the animals forced to swim under the above experimental conditions.

Studies of the posterior pituitary and hypothalamic neurosecretion have gained new impetus in recent years, when an increasing body of evidence has been presented indicating the connexion with hypothalamic neurosecretion of certain trophic hormones of the anterior pituitary. It is generally known that acute, non-specific stressor effects evoke a hyperfunction of the hypothalamic-hypophyseal system [23, 25, 28]. It is also known that electrical or chemical stimulation of certain central nervous areas influences the activity of the posterior pituitary [1, 2, 8, 14]. In the light of this evidence one feels inclined to surmise that the change in posterior pituitary activity would appear as part of the organism's general adaptation.

In the present experiments we wished to study the effect of non-specific stress on the hormone content of the posterior pituitary. We employed the method of forcing the animals to swim in cold environment, as a general stress acting upon the hypothalamic-hypophyseal neurosecretory system.

Methods

Seventy adult female albino rats, weighing 130 to 170 g, were used. Thirty-five animals were forced to swim once daily, until totally exhausted. The animals were subjected to examinations in groups of five animals each, on the 6th, 8th, 12th, 15th, 18th, 22nd and 29th days, simultaneously with 3 normal control animals. Eight animals were hydrated once daily and in two groups of four animals each we studied them, together with 3 controls to each group, on the 12th and 18th days of hydration.

Total exhaustion. The animals were forced to swim until totally exhausted once every day, all at the same time, before feeding, in a 50-litre, 45 cm deep container filled with water of 18° C. While the animals were swimming, the temperature of the water did not vary more than $\pm$ 0.5° C.

Hydration. The animals were hydrated daily through gastric tube, administering 7 ml/100 g body weight of tap water. In the controls, the gastric tube was introduced once daily, but no water was given. On the 12th and 18th days four animals, together with 3 controls, were examined for hypophyseal oxytocin content.

Pituitary extraction. The animals were killed by decapitation 2 hours after swimming and the blood was collected in heparinized glass tubes. The hypophysis and endocrine organs were removed and weighed with 0.5 mg precision on torsion scales. The hypophyses from the single groups were pooled and homogenized in 0.25 per cent acetic acid (taking 0.4 ml for each hypophysis) with quartz dust. The materials were stored in a refrigerator at + 4° C overnight, then they were centrifuged. After making the suitable dilutions, the pH was measured, then biological assays were made for oxytocic and antiduiretic activity.

Assay of pituitary oxytocic activity. The extracts were diluted 500-fold and titrated on the surviving rat uterus, as previously described [9, 11], in Sartorius vessels of 5 ml capacity. The largest volume of the extract dilutions of unknown concentration added to the Tyrode's solution was 0.1 ml. As the standard for comparison, synthetic Oxytocin (Richter) was used.

Assay of blood oxytocic activity. The data published in the literature relative to the oxytocic activity of blood vary over a wide range. BISSET and WALKER [3] found 1.7 to 6.8 milliunits/ml, HAWKER *et. al.* [15] 0.06 milliunits/ml, and FITZPATRICK *et al.* [13] 0 to 1.0 milliunit/ml. The divergences in the results are to be ascribed to species and individual differences, as well as to differences in the methods of extraction employed. If we take into account the physiologically active levels described by CSAPÓ [5] and FIELITZ *et al.* [12], the lower values reported by the latter author seem to be acceptable. Taking into consideration the above points of view, the blood samples were tested by two methods. First, we added a maximum volume of 0.3 ml of freshly taken heparinized blood without any extraction to the Tyrode's solution in which the uterus was suspended. The rest of the blood obtained was extracted according to BISSET and WALKER [3] and the extract was subjected to biological assay.

Comparative evaluation of antidiuretic activity. Male dogs, weighing 15 to 20 kg, were anaesthetized with 0.10 g/kg body weight chloralose, the ureters were exposed, and urine was collected through indwelling polyethylene cannulas in a metric flask. Then the saphenous vein was cannulated and 0.85 per cent NaCl solution of 38° C was infused at a constant rate of 100 ml/hour/10 kg body weight. Diuresis was checked by collecting urine every 5 minutes, then, when it was found to be constant in 4 successive five-minute periods, we injected 1 I. U. of posterior pituitary extract (Piton *Organon)* intravenously, to test the animal for ADH sensitivity. After diuresis had normalized, the substances of unknown antidiuretic activity, as well as the dilutions of the extracts, were injected intravenously. The extracts were 50-fold diluted with 0.85 per cent NaCl solution; the single doses varied from 1 to 3 ml. Before injection, the pH of the dilutions was controlled in every case. The changes in diuresis were represented graphically.

Results

Pituitary oxytocic activity

Pituitary oxytocic activity computed for 100 g body weight showed a slight, not significant decrease by the 6th and 8th day, respectively. On the other hand, the animals forced to swim for 12 days showed a definite increase. This increase was even more marked in the animals forced to swim 15 days, and the maximum increment was observed on the 18th day. As compared to that value, those obtained on the 22nd and 29th days were somewhat lower, but still much higher than the initial level. These changes have been summarized in Fig. 1.

Oxytocic activity of blood

The blood samples titrated directly, without extraction, showed activity values ranging from 40 to 250 microunits/ml, the extracted blood values varied from 1 to 2 milliunits/ml. The blood samples from the animals forced to swim and those from the controls equally showed high and low values; no unequivocal changes resulted from the forced swimming.

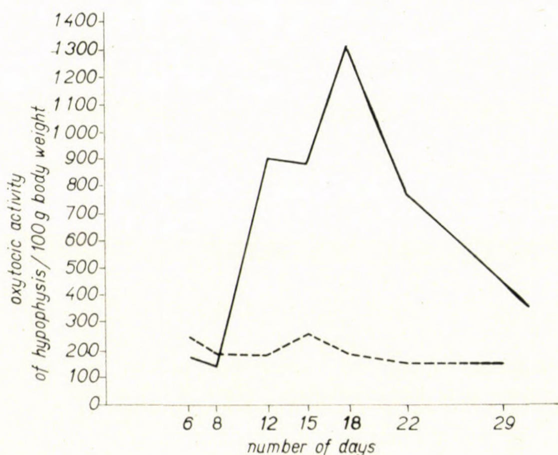

Fig. 1. Broken line: pituitary oxytocic activity of control rats, on the 6th, 8th, 12th, 15th, 18th, 22nd and 29th days. Solid line: pituitary oxytocic activity of rats forced to swim. Oxytocic activity has been computed for 100 g of body weight

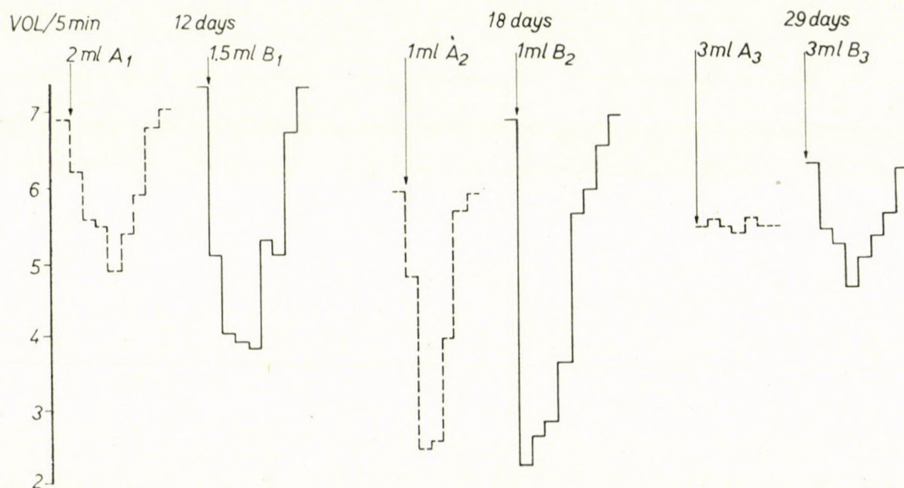

Fig. 2. Antidiuretic activity of pituitary extracts in the dog. A_1, A_2, A_3: extracts of control (not swimming) animals. B_1, B_2, B_3: extracts from animals forced to swim

Effect of hydration

The animals hydrated for 12 and 18 days yielded pituitary oxytocic activity values not different from those shown by the controls.

Antidiuretic activity

Fig. 2 shows the antidiuretic activities of the pituitary extracts obtained on the 15th, 22nd and 29th days. In all three instances the extracts prepared from the animals forced to swim caused a more marked decrease of diuresis than the extracts obtained from the controls. The difference in activity between the control extracts reflects differences in ADH sensitivity of the individual dogs. This has been proved also by the different responses to the 1 I. U. posterior pituitary extract injected with every test.

Discussion

There are no unequivocal data in the literature concerning the changes in the hormone and neurosecretion contents of the neurohypophysis. Eichmer [7], Melandra and Corbetta [19] found the neurosecretion content to decrease after adrenalectomy. On the other hand, Rennels [22] reported that in the rat the same operation caused an increase in the pituitary oxytocin and neurosecretion contents, while dehydration and lactation caused a decrease in the oxytocin, vasopressin and neurosecretory substance contents. Pasqualino and Ragonese [21] observed the release of neurosecretion from the rat's hypophysis in response to hydration. Kovács *et al.* [16], too, described the ADH mobilizing effect of dehydration in the rat. In our earlier experiments [9, 11], castration and oxytocin treatment diminished, oestrogen and progesterone treatment increased the oxytocic activity of the hypophysis in the rat. De Groot and Hartfield [6] observed in the rat during the first 12 hours following bilateral adrenalectomy, splenectomy (as surgical shock) and adrenal demedullation a decrease of neurosecretion, that normalized rapidly, or in the case of the first two operations showed an increase over the initial value on the fourth day. In our experiments the ineffectiveness of hydration has ruled out the possibility that the effects would have been produced by the rats drinking water while swimming. Considering the increasing possibility of separating the posterior pituitary hormones [18, 27], we have to stress that in our experiments the increase of pituitary oxytocic activity was associated with an increase of antidiuretic activity. Both in the experimental animals and the controls the blood oxytocin values varied over wide ranges, indicating that it is unlikely that an increased storage in the neurohypophysis would be accompanied by a simultaneous inhibition of secretion.

Considering that earlier we have found chronic hydrocortisone treatment to increase pituitary oxytocic activity in the rat [10], an alteration of adrenal function might also play a role in the phenomenon investigated. YANG [29] studied the rat's adrenal function under the effect of the same stressors as employed by us, and found no increase of secretion.

According to TELEGDY [26], adrenal secretion decreased during the first 18 days of chronic swimming experiments carried out in 18 °C water. These data make it questionable whether the adrenocortical system would have a role in the phenomenon observed by us.

The experimental results may be interpreted in several ways. The 18° C water, in which the animals were swimming, may undoubtedly act as a cold stressor effect. Changes effected through the thyroid-hypothalamic system may also play a role. There are contradictions in the literature concerning the TSH release caused by oxytocin [4, 17, 20, 24], although this mechanism too, may have its role. At the same time, it cannot be ruled out that the increase in the activity of the hypothalamic-hypophyseal system under the influence of non-specific stressor stimuli [23, 25, 28] would influence the secretion of other anterior pituitary trophic hormones and thus to enhance the organism's general adaptation.

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Kornél FENDLER, Gyula TELEGDY, Elemér ENDRŐCZI
Orvostudományi Egyetem Élettani Intézete, Pécs.

A STUDY ON THE ROLE OF THE RENIN-ANGIOTENSIN SYSTEM IN THE CONTROL OF ALDOSTERONE SECRETION

By

J. SÓLYOM, Susanna KOTRA, Á. SALAMON and J. STURCZ

DEPARTMENT OF PHYSIOLOGY, MEDICAL UIVERSITY, BUDAPEST

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The role of the renin-angiotensin system in the augmentation of aldosterone secretion following acute blood loss has been studied. Aldosterone production of the adrenals was determined *in vivo* and *in vitro*, after bilateral ligation of the renal hili and nephrectomy, respectively. The incerase in aldosterone secretion, following acute haemorrhage, was found to take place also in the absence of the renin-angiotensin system.

From a series of studies of the factors regulating aldosterone secretion, DAVIS *et al.* [10] concluded to the significant role of the renin-angiotensin system in the augmentation of aldosterone secretion. In hypophysectomized dogs the increase in aldosterone production that otherwise follows acute blood loss, failed to occur if the kidneys had been removed. On the other hand, the increase in aldosterone secretion observed following acute haemorrhage [8, 21], during chronic sodium depletion [16, 17, 22], and thoracic caval constriction [7, 10] was found to be accompanied by a rise in renin secretion. Also, the renin fraction of kidney homogenisates, and synthetic angiotensin II increased the rate of aldosterone secretion [1, 2, 6, 15, 18, 19, 20].

The present experiments were designed to test DAVIS' hypothesis. If the renin-angiotensin system plays a significant role in the regulation of aldosterone secretion, bilateral nephrectomy or ligation of the renal hilus might be expected to prevent the elevation of aldosterone secretion subsequent to acute blood loss.

Methods

Experiments were performed on 106 rats of both sexes and of the same breed, weighing from 200 to 250 g. The animals had received a normal diet until 24 hours prior to the experiment, when they were deprived of food. Anaesthesia was induced by urethan, giving 1 g per kg body weight, intravenously.

A) In the first series of experiments the aldosterone level in the venous blood of the adrenals was determined after ligation of the renal hilus.

The kidneys were exposed through an abdominal midline incision, and both renal hili were ligated. An hour later the left adrenolumbar vein was cannulated and collection of blood started. Bleeding was continued during a 60-minute period. The amount of blood removed was not replaced. The same procedure was undertaken in the control animals with the exception that the renal hili were not ligated. About 4 ml of blood was obtained from each animal. The samples from 3 to 4 animals were pooled to obtain sufficient material for aldosterone determination.

B) In a second series of experiments aldosterone production of the adrenals was established *in vitro*. The changes in aldosterone secretion, occurring in response to acute blood loss, were studied in nephrectomized and control animals.

The animals were divided in three groups. *I.* In the first group both adrenal glands were removed and incubated immediately following the abdominal incision. *II.* In the second group, 30 minutes after the incision, blood amounting to 0.5 per cent of the body weight was removed from each animal through the femoral vein. Sixty minutes after the laparotomy the adrenals were removed and incubated. *III.* In the third group of animals the procedures described under II were undertaken and, in addition, bilateral nephrectomy was carried out immediately following laparotomy.

Aldosterone production of the adrenals was examined *in vitro*, by the method of GIROUD et al. [14]. The adrenals were weighed and then quartered. Incubation was undertaken in Krebs—Ringer bicarbonate medium (6.0 ml per 100 mg of adrenal tissue), containing 200 mg per 100 ml of glucose, for two hours at 38° C. A mixture of 95 per cent oxygen and 5 per cent carbon dioxide was bubbled through the solution. Each tube contained about 120 mg of adrenal tissue usually obtained from 3 animals.

Quantitative determination of aldosterone was performed by paper chromatography.

An ethyl acetate extract of the venous blood (*A*), and a chloroform extract of the incubation medium (*B*) were prepared. The extracts were washed with 0.2 *N* sodium carbonate solution and distilled water, then dried over sodium sulphate, and evaporated in vacuum. The dry residue was dissolved in dichloromethane and dropped on Whatman's paper No. 1. The extract was first chromatographed in a benzene-methanol-water system (10 : 8 : 2) for about 16 hours at room temperature [3], then in Bush's B5 system (benzene-methanol-water; 10 : 5 : 5) for about 3 hours at 37° C [4, 5]. Aldosterone was identified and measured by means of aldosterone standards run parallel with the material in question.* On each paper amounts of 1, 2, 4 and 5 μg of aldosterone were dropped and a calibration curve was prepared for the quantitative determination of aldosterone. The chromatograms were developed with alkaline tetrazolium blue (2 mg of tetrazolium blue per 1 ml of distilled water, added to an equal amount of 10 per cent sodium hydroxide). The formazan spots obtained were cut out of the paper, eluted in 1.5 ml of the 7 : 3 mixture of ethyl acetate and methanol, and photometrized in an ELKO III photometer, at a wavelength of 530 mμ.

Results

Series A. — Experiments were carried out in 52 rats arranged in 7 control groups, and 7 experimental groups in which hilar ligation had been undertaken prior to the experiment. Amounts of aldosterone found in the venous blood of the adrenals were converted to 100 g of body weight and one hour.

Mean values for aldosterone secretion were 0.60 ± 0.16 μg/100 g body wt/hour in the control group, and 0.64 ± 0.18 μg/100 g body wt/hour in the experimental group, respectively. The difference between the two values was not significant ($p > 0.5$).

Data obtained in the individual experiments are summarized in Table I, while Fig. 1 represents the mean values and standard deviations.

Series B. — The amounts of aldosterone produced by the adrenals during incubation were converted to 100 mg of adrenal tissue and one hour.

In the first group experiments were performed on 21 animals in 7 series. The average of aldosterone production in this group was 2.31 ± 0.67 μg/100 mg of adrenal tissue/hour.

* The authors are indebted to *Ciba* Ltd., Basel, for the kind supply of aldosterone. cortisone, cortisol, and hydrocortisone.

Table I

Aldosterone levels in the venous blood of the left adrenal in rats with intact kidneys (A), and with the renal hili ligated (B)

The values are expressed in μg and converted to 100 g of body weight and one hour.

A	B
0.33	0.29
0.38	0.35
0.52	0.43
0.59	0.58
0.64	0.68
0.71	1.00
1.04	1.19
Average 0.60 $\pm$ 0.16	Average 0.64 $\pm$ 0.18

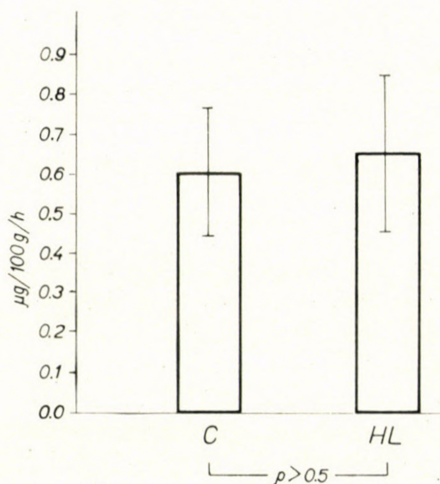

Fig. 1. Mean aldosterone levels and standard deviations in the venous blood of the left adrenal in rats with intact kidneys (*C*), and with the renal hili ligated (*HL*)

The second group consisted of 18 animals divided into 7 series. Mean aldosterone production attained 3.93 $\pm$ 1.42 μg/100 mg of adrenal tissue/hour. The difference between the means obtained in group I and group II was significant ($p < 0.02$).

In the third group, 15 animals in 6 series were studied. The averaged value of aldosterone production was 3.82 $\pm$ 0.81 μg/100 mg of adrenal tissue per hour. The difference between the means in group II and group III was not significant ($p > 0.5$).

Table II

Aldosterone production of the adrenals in vitro, in normal rats (I), in rats subjected to acute blood loss (II), and in nephrectomized rats subjected to acute blood loss (III)

The values are expressed in μg and converted to 100 mg of adrenal tissue and one hour

I.	II.	III.
0.98	2.83	2.27
2.25	2.88	3.69
2.27	3.12	3.97
2.34	3.46	4.14
2.45	3.79	4.27
2.88	4.66	4.57
3.05	6.82	
Average: 2.31 ± 0.67	3.93 ± 1.42	3.82 ± 0.81

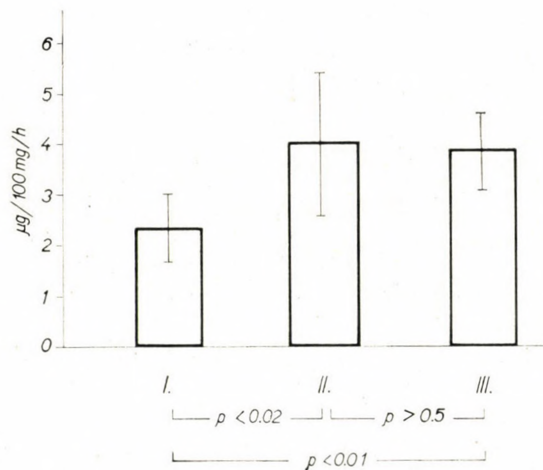

Fig. 2. Mean aldosterone levels and standard deviations in adrenals *in vitro*, of normal rats (I), of rats subjected to acute blood loss (II), and of nephrectomized rats subjected to acute blood loss (III)

Table II shows the individual values obtained in all the three groups. Means and standard deviations are represented on Fig. 2.

Discussion

In the first series of our experiments, about one fourth (4 ml/200 g body weight) of the circulating blood volume was removed. Thus, the blood loss suffered by the animals was doubtlessly sufficient to cause a maximal mobiliza-

tion of the animals' emergency mechanisms and thus to activate the numerous specific and nonspecific factors enhancing the secretion of aldosterone.

Hence, from the first part of our experiments it can only be concluded that the renin-angiotensin system is not the only factor to enhance aldosterone secretion.

In the other part of our experiments the incubation method allowed the degree of blood loss to be freely controlled. In this group not more than about 1/16 of the circulating blood volume was removed. The hypovolaemia thus induced was sufficient to stimulate aldosterone secretion.

On the basis of the results obtained in Series *B*, two possible explanations may arise. (i) The renin-angiotensin system plays no part in the physiological regulation of aldosterone secretion, (ii) The renin-angiotensin system is not the sole factor exerting an influence upon the rate of aldosterone production; this is supported by the observation that the increase in aldosterone secretion following acute haemorrhage occurs also after blocking or elimination of the renin-angiotensin system.

Summarizing the results, it was demonstrated that bilateral nephrectomy or ligation of the renal hilus fails to prevent the augmentation of aldosterone secretion *in vivo* and *in vitro*, that follows acute blood loss in rats. These findings indicate that the renin-angiotensin system is not necessary for the stimulation of aldosterone secretion.

This concept is supported by the observation of GANN and TRAVIS [12] that after thoracic caval constriction an increase in aldosterone secretion occurred also in nephrectomized dogs.

On the other hand, the observation of DAVIS *et al.*, which lead to the conclusion that the renin-angiotensin system constitutes the primary mechanism regulating aldosterone secretion, had been made in hypophysectomized dogs [9, 13]; the reliability of results obtained under such experimental conditions might be doubted.

Moreover, considerable doses of renin and angiotensin II, which produced a significant rise in blood pressure, had to be administered to cause an augmentation of aldosterone secretion [6, 20]. This points to a pharmacological effect of renin.

In view of the results presented here and reported by other authors, DAVIS' hypothesis, according to which renin is identical with the aldosterone-stimulating hormone and is regarded to be the primary mechanism responsible for the physiological regulation of aldosterone secretion, might be questioned. The renin-angiotensin system might actually enhance aldosterone secretion, but the proof of its exclusive role is lacking.

Considering our present knowledge in this field, it may be assumed that renin is responsible for increased aldosterone secretion in renal hypertension, but the renin-angiotensin system is not an essential mechanism

regulating the rate of aldosterone secretion under physiological conditions. Other factors, *e.g.* FARRELL's cerebral trophic hormone [11], are probably more concerned than renin in the physiological regulation of aldosterone secretion.

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János SÓLYOM, Zsuzsa KOTRA, Ákos SALAMON, József STURCZ
Budapesti Orvostudományi Egyetem Élettani Intézete, Budapest

CHANGES IN THYROIDAL COLD RESPONSE OF HEAT-ADAPTED RATS FOLLOWING BILATERAL LESIONS OF THE HABENULAR NUCLEI

By

B. MESS

INSTITUTE OF ANATOMY, MEDICAL UNIVERSITY, PÉCS

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The response to cold of the thyroid has been studied in heat-adapted albino rats following bilateral lesions of the habenular nuclei. Exposure to slight cold (14° C) produced an elevation of the T/S ratio in intact controls. This response was exaggerated in lesioned rats exposed to slight cold. No rise in T/S ratio occurred in rats exposed to severe cold (0—4° C) in intact controls or in rats bearing habenular lesions. The adrenal weights were higher in rats exposed to severe cold. The increase in adrenal weights was more significant in lesioned rats exposed to severe cold than in intact rats under the same circumstances. It has been concluded that the habenular nuclei play an important role in TSH-thyroid activation provoked by exposure to cold.

Introduction

Numerous experimental observations have indicated the important role of the habenular nuclei in the regulation of the TSH-thyroid system. As sequels of bilateral lesions of these nuclei, inhibition (i) of the goitrogenic effects of thiouracil [6]; (ii) of spontaneous goitre [6]; (iii) of the inactivating effects upon TSH activity of thyroxine administration [8]; (iv) of TSH release following thyroidectomy [7], have been registered. The blood TSH level is slightly elevated as early as 3—8 days after habenular lesion [9]. The goitre-blocking effect of habenular lesions has been confirmed by BOGDANOVE and BOGDANOVE [2, 3]. YAMADA [14] observed no blocking effect in rats bearing habenular lesions and fed with propylthiouracil during 19 days, but the applied dose of thiouracil was four times larger than in our experiments. Shortening the period of treatment to 9 days resulted in a similar goitre-blocking effect as observed by us. From our observation [6] that if the daily dosage of thiouracil reached 100 mg/kg body weight or more, the goitre-blocking effect of the lesion disappeared, predicted the findings of YAMADA, and it is only his interpretation of the effect which is different from ours. — YASUMURA and KNIGGE [15] found a supernormal elevation of the T/S ratio in thiouracil fed rats bearing habenular lesion. The elevation of the T/S ratio differed significantly in the thiouracil fed lesioned group from the ratio in thiouracil treated intact animals. The cited authors concluded from their results that "any thyroid regulatory role assumed by the habenular area may operate only during periods when the animal is subjected to changes which tend to overburden the pituitary TSH release mechanism."

The first observation in our own experiments was a characteristic disorder in thermoregulation. There was no change in the rise of oxygen consumption in cold environment (20° C), but in warm environment (35° C) oxygen consumption did not rise so as it does in intact animals (DONHOFFER *et al.* [4]). This effect has not been investigated further. BEATTIE and CHAMBERS [1] described that heat adapted rats bearing hypothalamic lesions respond to cold environment (17° C) with a significantly increased oxygen consumption and thyroidal ^{131}I uptake. The lesions demonstrated on the microphotograms of BEATTIE and CHAMBERS are localized so far dorsad as to be considered epithalamic rather than hypothalamic.

These latter results might suggest that the habenular nuclei could be involved in the response to cold of the TSH-thyroid system. — HARRIS [5] found that with relatively slight changes in environmental temperature (not colder than +6.5° C) it is the TSH-thyroid system that primarily responds, whereas severe cold (below +2° C) as a stressor activates the ACTH-adrenocortical system and consequently depresses the TSH-thyroid system. In the light of HARRIS' experiments it appeared interesting to examine, whether during exposure to cold the habenular nuclei have any regulatory effect on thyroid function, and, if so, whether this was the case in a low environmental temperature in general or only in relatively mild cold.

Methods

Experiments were performed on 81 male albino rats of 150 to 200 g body weight in two different series. The animals were kept on a standard diet containing 2 mg KI/kg food. The rats of both series had been adapted to 28 ± 1° C environment during 6 weeks, then bilateral habenular lesions were placed in one half of the animals. 8 days after the intervention the animals were divided into 4 groups.

Group A: Intact controls kept at 28° C temperature;

Group B: Intact controls exposed to 14° C environmental temperature in series I and to a temperature of 0—4° C in series II;

Group C: Animals bearing habenular lesions, kept at 28° C temperature;

Group D: Animals bearing habenular lesions, exposed to 14° C in series I, and to 0—4° C in series II.

The animals were kept in the cold environment for 10 days. On the last day of cold exposure all groups received subcutaneously 6 mg propylthiouracil and one hour later 10 μC ^{131}I, then another hour later the rats were exsanguinated by puncture of the abdominal aorta under light ether anaesthesia. One lobe of the thyroid was prepared and weighed with 0.2 mg accuracy. The T/S ratio was determined according to the original method of VANDERLAAN and GREER [12]. The other thyroid lobe, the hypophysis, the adrenals and the gonads were studied histologically, as well as serial sections of the brain for exact localization of the site and extension of the lesions.

Results

Series I. Results are summarized in Table I. The mean T/S ratio in the control rats kept at 28° C environmental temperature (Group A) was 25.18 (range: 19.4—32.2). This corresponds to the normal values given in the literature. No significant change was observed in the animals bearing habenular

Table I

T/S ratio and organ weights of normal rats and rats with habenular lesion, exposed to slight cold (+ 14° C)

Experimental group	Case No.	T/S ratio	Organ weights, mg	
			Thyroid	Pituitary
Group A Controls at 28° C	1	21.6	16	14
	2	23.6	20	15
	3	31.2	16	13
	4	19.4	13	10
	5	27.0	13	7
	6	32.2	15	11
	7	26.7	16	17
	8	23.3	15	11
	9	20.5	15	14
	10	26.4	14	11
Mean		25.18	15.3	12.2
Group B Controls at 14° C	11	27.2	16	13
	12	22.2	16	12
	13	18.4	17	14
	14	33.9	16	14
	15	28.9	16	13
	16	48.5	16	14
	17	33.0	19	14
	18	40.3	15	11
	19	37.6	16	14
Mean		32.22	16.3	13.2
Group C Rats with habenular lesion at 28° C	20	17.4	16	14
	21	20.0	14	14
	22	32.9	13	15
	23	31.8	16	14
	24	35.9	12	14
	25	28.4	14	13
	26	22.3	16	12
	27	28.8	15	12
	28	22.6	17	12
	29	33.8	12	10
	30	18.5	14	13
	31	33.6	13	10
Mean		27.33	14.3	12.7
Group D Rats with habenular lesion at 14° C	32	45.9	16	16
	33	32.5	10	12
	34	53.1	12	11
	35	73.1	16	10
	36	55.2	14	15
	37	30.0	16	14
	38	34.0	17	14
	39	56.4	16	18
	40	43.4	14	12
	41	31.4	16	12
	42	37.8	12	11
Mean		44.80	14.2	12.8

Table II

T/S ratio and organ weights of normal rats and rats with habenular lesion, exposed to severe cold (0 — 4° C)

Experimental group	Case No.	T/S ratio	Organ weights, mg		
			Thyroid	Pituitary	Adrenals
Group A Controls at 28° C	43	33.9	13.2	10.0	38.2
	44	32.5	14.0	11.4	46.7
	45	41.0	12.4	10.7	36.2
	46	38.6	9.6	11.0	46.8
	47	30.6	14.0	12.5	44.5
	48	22.4	14.4	11.1	35.5
	49	29.0	12.6	9.1	36.2
	50	25.8	12.2	10.0	28.5
Mean		31.7	12.8	10.7	39.1
Group B Controls at 0—4° C	51	32.3	20.0	10.9	44.9
	52	30.9	14.8	9.0	32.8
	53	27.2	15.6	10.0	39.1
	54	26.0	15.6	9.8	47.4
	55	—	18.0	12.0	44.6
	56	—	17.2	9.8	52.3
	57	—	15.2	9.0	41.3
	58	—	14.0	11.2	54.3
Mean		29.1	16.5	10.2	44.6
Group C Rats with habenular lesion at 28° C	59	34.7	12.4	7.3	44.7
	60	28.6	9.2	9.6	43.5
	61	22.2	8.8	8.6	43.3
	62	22.2	12.2	9.0	43.4
	63	28.3	10.4	8.6	38.2
	64	32.2	10.6	8.7	46.5
	65	30.0	12.4	9.3	40.5
	66	20.9	14.0	8.5	36.0
	67	19.4	13.0	8.8	37.2
	68	22.1	15.2	9.3	49.3
	69	22.5	12.0	8.5	36.1
	70	23.0	9.6	8.2	35.5
Mean		25.5	11.6	8.7	41.3
Group D Rats with habenular lesion at 0—4° C	71	49.2	14.8	5.5	52.9
	72	30.4	21.2	9.3	48.1
	73	25.6	15.4	9.2	58.2
	74	31.1	12.4	9.4	68.7
	75	33.5	13.2	9.2	58.0
	76	28.4	11.2	8.1	39.2
	77	32.7	17.0	7.6	49.2
	78	29.6	11.4	7.3	40.8
	79	26.1	12.0	8.6	54.7
	80	28.0	10.6	5.0	37.5
	81	16.8	14.4	7.0	49.0
Mean		30.1	13.9	7.8	50.6

lesions at the same environmental temperature (T/S ratio, mean 27.33; range, 17.4—33.8; Group C). The difference between the two groups was $t = 0.86$, not significant statistically. The intact animals exposed to 14° C (Group B) showed a slightly elevated T/S ratio (mean, 32.22; range, 18.4—48.5). The difference between the two intact groups (Group A and B) was near to statistical significance ($t = 2.15$). Rats with bilateral habenular lesions showed a marked increase of the T/S ratio following exposure to cold (mean, 44.80; range, 30.0—73.1). The difference between the two lesioned groups (Group C and D) was highly significant statistically ($t = 3.86$). The difference in T/S ratio between the two groups exposed to cold (Group B and D) was significant statistically ($t = 2.35$).

No significant difference was observed in thyroid and pituitary weights.

Series II. The results of this experiment are summarized in Table II. No change was observed in the T/S ratios between the two control groups (Group A = 31.7; Group B = 29.1), or between the two lesioned groups (Group C = 25.5; Group D = 30.1). The slight differences were not significant statistically.

The only appreciable change in organ weights was an increase in adrenal weight in the two groups exposed to cold. The increase was more marked in the rats with habenular lesion than in the controls exposed to cold. The difference between the two control groups was not significant ($t = 1.52$), while that between the two lesioned groups was significant ($t = 3.06$).

Discussion

Our experiments have confirmed the results of Beattie and Chambers [1], and of Harris [5]. — On the basis of previous experiments (Mess [6, 7, 8]) we initially supposed that the habenular nuclei are directly linked with the primary feedback loop regulating the activity of TSH-thyroid system (Szentágothai and Mess [11]). As no direct evidence could be presented in later experiments of the existence of a receptor system directly sensitive to the thyroxine blood level [10], and as the habenular mechanism appears to work only between narrow limits of the thyroxine blood level, our earlier hypothesis of a direct habenular feedback mechanism had to be abandoned. Recently, a new working hypothesis has been developed [10] according to which the habenular mechanism is not a part of the primary thyroxine feedback loop but a more remote part of the nervous mechanism integrating hormonal control with requirements arising from the environment. Although these experiments are far from being sufficient to elucidate the mechanism by which thermoregulation is coupled with the humoral feedback control in the TSH-thyroid circle, they clearly indicate that the two mechanisms

are not simply converging at the level of the anterior pituitary. Recent evidence [13] suggests the basic feedback-loop existing between anterior lobe trophic function and thyroid activity to be remarkably independent from the nervous mechanisms localized in the hypothalamus. The fact, however, that lesions of a restricted region of the epithalamus lead to quite specific — although among themselves paradoxically conflicting—alterations of sensitivity to changes in thyroxine blood level and at once characteristic disorders of response to cold, is a clear indication of a complex and remote purely nervous mechanism that integrates informations of relevance to both control mechanisms. Whether the descriptive term "sensitizing device" used by us [10] for the habenular nervous mechanism controlling TSH responses to changes in the thyroxine blood level is adequate in the light of these considerations, remains to be tested in further experiments. It is remarkable that the response to cold of the TSH regulation mechanism should so clearly be "switched off" below a certain environmental temperature. Whether this is caused primarily by the nervous mechanisms themselves or secondarily in consequence of the stress reaction that develops during exposure to severe cold, cannot be decided on the basis of these experiments. But there obviously cannot exist a direct and simple relation between the disinhibition of THS response to cold — in the mild range — and desensitization of the THS-thyroxine feedback circle, since both occur after bilateral lesions of the habenular nuclei.

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Béla Mess
Orvostudományi Egyetem Anatómiai Intézete, Pécs.

UNDERNUTRITION AND TEMPERATURE REGULATION IN ADULT RATS

By

T. Heim and J. Mestyán

DEPARTMENT OF PAEDIATRICS, MEDICAL UNIVERSITY, PÉCS

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1. In the course of undernutrition of adult rats a rapid initial and subsequent slow decline in basal O_2-consumption was observed. Colonic temperature did not change appreciably at the neutral thermal environment.

2. The metabolic response to hyperthermia decreased roughly parallel to the basal metabolic rate with the progress of undernutrition.

3. In the early phase of starvation the metabolic response to 20° C was percentually less than in the control period. With growing loss of weight, in association with an increasing fall in colonic temperature, the increase in heat production was relatively great.

4. Exposure to 5°—10° C caused a considerable increase in heat production and body temperature was maintained or even increased.

5. It is concluded that the regulated level of body temperature is reset in severe undernutrition.

Since Benedict's detailed studies the pathophysiological consequences of complete and partial starvation have been extensively investigated both in man and in animals [1, 2]. As a result of these investigations our knowledge about the effects of undernutrition upon a number of physiological functions and regulations has been considerably increased. Thus, similarly to nitrogen and mineral metabolism, the relationship between body size, body composition, O_2-consumption and body temperature of starving animals has been extensively investigated [3—6]. Among others, subnormal body temperature and liability to profound hypothermia are considered as the most obvious features of undernutrition.

Regarding the relationship between undernutrition and regulation of body temperature, the study of McCance and Mount on the growing pig, and the comprehensive clinical observations of Kerpel-Fronius and Varga on malnourished human infants, indicate unequivocally that thermal control is severely affected in starvation [7—9]. Of particular interest is McCance and Mount's finding that in severely undernourished growing pigs heat production sufficed to maintain the thermal equilibrium only at subnormal body temperatures. These observations were interpreted to suggest that the low body temperature is not so much due to an inability to increase heat production but rather the result of thermoregulatory changes. In an attempt to analyze this aspect of the problem, the present study was designed to examine in severely underfed adult rats the relationship between body temperature and basal metabolic rate on the one hand, and metabolic response to cold, on the other.

Methods

Twelve adult rats with a body weight of 200—300 g have been used. Each animal was kept separately in wire cages at a room temperature of 24°—27° C. The experiments were performed simultaneously on two or four animals and extended over a year. In the control period, free choice was offered of three foods each consisting of one third part of a standard mixture and of two thirds of starch casein and lard. Food consumption was measured daily, and caloric intake thus calculated. The average daily intake amounted to approximately 60—80 Cal. At the beginning of partial starvation, food intake was reduced to 22 Cal. and the protein content of the food to 2.8 per cent. On this regimen at the early stage of undernutrition the animals lost weight rapidly, but later loss of weight proceeded at a much slower rate. At this stage a further reduction of caloric and protein intake was usually necessary to maintain a progressive loss of weight until the animals died. Under these conditions survival time was 60 ± 16 days, and until death loss of weight amounted to about 50—55 per cent of the initial body weight.

O_2-consumption was measured in an airtight chamber immersed in a water bath, and in a few instances by a Noyous type diapherometer modified for small animals by SZEGVÁRI [10, 11]. Both apparatus permitted close observation of the animal so that errors due to movements and restlessness could be eliminated. O_2-consumption was estimated first at 29°—30° C (the neutral temperature of the normal rat), usually for three 15 min. periods. The rats were removed from the chamber at the third period for a few minutes, and colonic temperature was recorded immediately after removal. The animal was then exposed to 35° C. Since the air within the chamber was practically saturated with water vapour, exposure to 35° C was associated with a significant rise in body temperature. The metabolic response to 20° C and 5—10° C was usually examined one day after basal metabolic rate had been estimated. These observations were repeated weekly, i. e. 2—3 times before and in the course of undernutrition, usually between 5 and 12 o'clock p.m., until the death of the animal. Additionally, the animals were frequently exposed to 5° C for 1 hour, when, as a simple test of the ability to maintain body temperature, colonic temperature was recorded. The diapherometer permitted continuous recording of O_2-consumption. Using this method, body temperature was measured by means of a copper-constantan thermocouple inserted into the colon.

The changes and the relationship between the variables examined have been analyzed according to weight loss expressed as the percentage of the body weight prior to undernutrition. Thus, the period of undernutrition has been divided into five stages of progressively increasing weight loss. Fig. 1 represents the means and standard errors in ml O_2/dm^2 body surface/ hour for all estimations carried out on twelve animals at each stage of undernutrition. Body surface was calculated according to the formula $10 \sqrt[3]{kg^2}$.

Results

Fig. 1 represents basal metabolism and colonic temperature. It can be seen, that a 10—20 per cent loss of weight, which according to our appraisal, represents the first stage of undernutrition, was already associated with an appreciable fall in O_2-consumption at 30° C ($p < 0.001$). In the subsequent phases of weight loss no further decline in basal heat production could be observed until loss of weight had reached 40 per cent or more of the prefasting body weight. At the terminal stage very low basal metabolism rates occurred. At an environmental temperature of 30° C, colonic temperature remained practically unchanged throughout the whole period of undernutrition.

Fig. 2 illustrates changes in O_2-consumption and rise in body temperature at 35° C. O_2-consumption increased in hyperthermia and disappeared only after a weight loss of more than 50 per cent. Absolute heat production at 35° C showed, similarly to the basal metabolic rate, a progressive decline.

Attention must be drawn to the fact that in the more advanced phases of undernutrition the metabolic response to 35° C was smaller, although the same degree of hyperthermia was recorded.

The responses to 20° C and 5—10° C are shown by Fig. 3. Chemical thermoregulation was present in the cold throughout all stages of under-

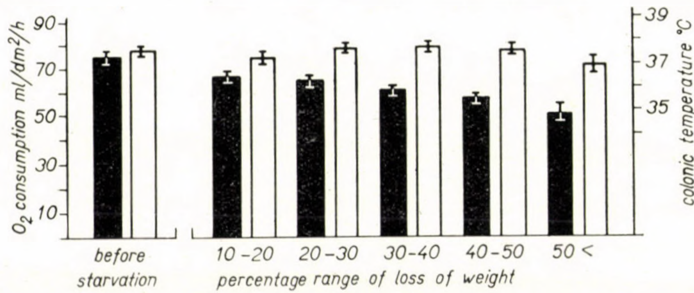

Fig. 1. O_2-consumption ■ and colonic temperature □ at the neutral thermal environment before and during starvation. The significance test calculated at a 5% level of confidence indicates that the basal metabolic rate at the first stage of undernutrition is significantly lower than the control level ($p < 0.001$)

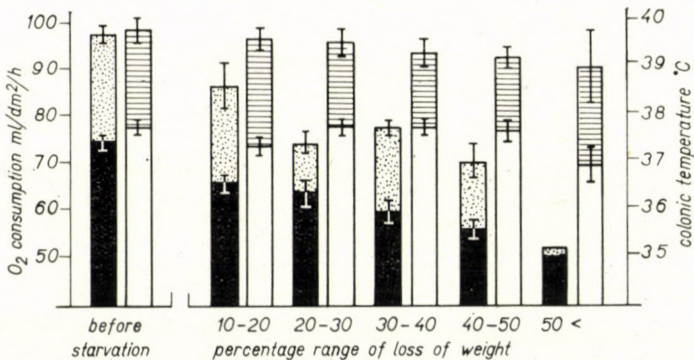

Fig. 2. O_2-consumption at 29° C ■ and 35° C ⬚. Colonic temperature at 29° C □ and 35° C ▤.

nutrition; a definite response was observed even when the loss exceeded 50 per cent of the initial body weight. The peak level attained, however, gradually declined with the severity of the nutritional state.

The relative increase in heat production at both ambient temperatures revealed some interesting findings regarding the ability to respond with increased heat production to cold. Fig. 4 demonstrates that in the beginning of undernutrition the percentual response, in terms of basal metabolic rate, was definitely less at 20° C than in the control period, but later, as fasting proceeded, a slight gradual increase could be observed (■) At an ambient

temperature of 5°—10° C, however, the relative increase in heat production was practically the same as in the normal state (▤).

If one divides the metabolic response to 5°—10° C into two components, as shown in Fig. 4, the increase in heat production at 20° C contributes less to the total response obtained at 5°—10° C in the starved animal.

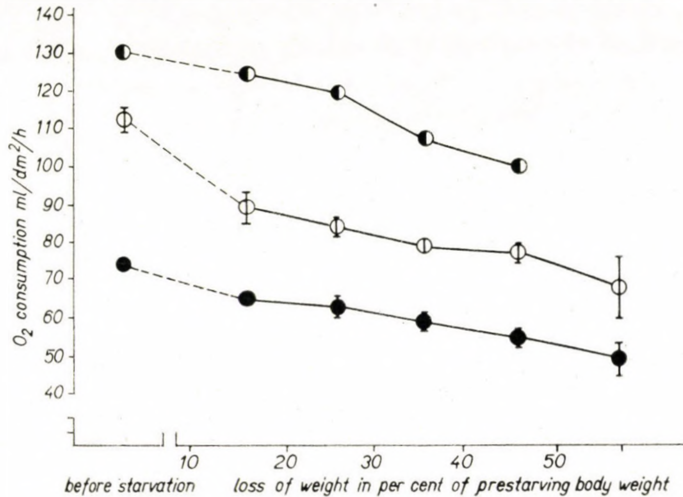

Fig. 3. O_2-consumption at an environmental temperature of 29° C ●–●, 20° C ○–○ and 5—10° C ◐–◐. In view of the few observations at 5°—10° C, the standard error was not calculated

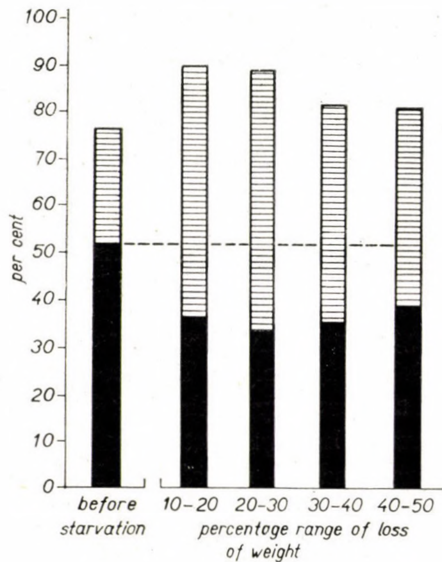

Fig. 4. Percentile increase in O_2-consumption in terms of basal metabolic rate at 20° C ■ and 5—10° C ▤, before and in the course of undernutrition

These observations suggested that in undernutrition not so much the failure of chemical thermoregulation but regulatory changes must be taken into account. Analysis of the colonic temperatures recorded at 30°, 20° and 5°—10° C (Fig. 5) revealed two features: *a)* at an ambient temperature of 20° C, body temperature fell and this fall increased with the severity of undernutrition; and *b)* when the animal had been transferred from 20° C to an ambient temperature of 5°—10° C, no further change or even a moderate rise in body temperature did occur. In the light of these observations the

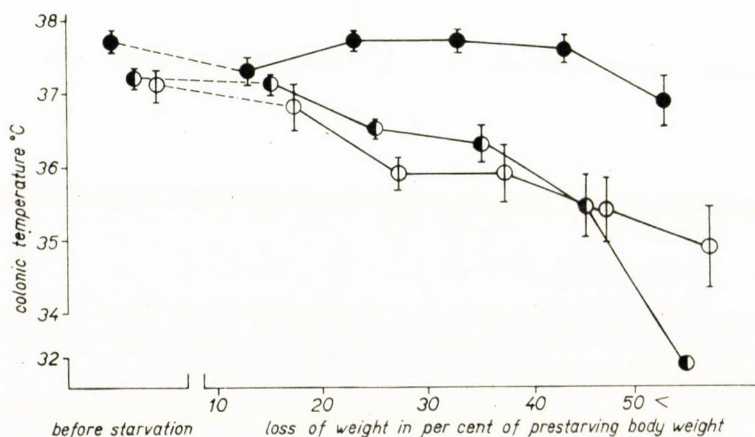

Fig. 5. Colonic temperature at the environmental temperatures of 29° C ●–●, 20° C ○–○ and 5°—10° C ◐–◐. Colonic temperature at 20° and 5—10° C was found to be significantly lower than at 29° C throughout the advanced stages of undernutrition. At a weight loss of 30—40 per cent, $p < 0.05$

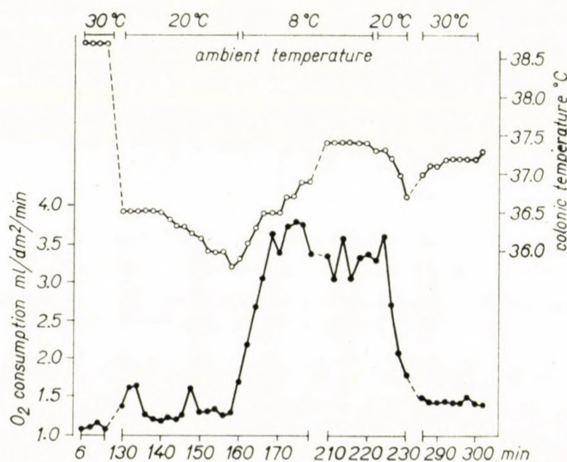

Fig. 6. O_2-consumption ●–● and colonic temperature ○–○ of an undernourished rat at different ambient temperatures. Body weight prior to starvation, 225 g; at the time of the experiment, 155 g. Weight loss, 31 per cent of the initial body weight

conclusion can be drawn that in the course of undernutrition thermal control, though at a progressively lower level, is well maintained in a cold environment.

How thermal equilibrium is achieved is demonstrated in Fig. 6 by an individual experiment. In this instance O_2-consumption and body temperature were continuously recorded. Exposure to 20° C caused a slight increase in O_2-consumption and a significant fall in colonic temperature. But lowering the ambient temperature to 8° C induced a tremendous rise in heat production, and body temperature, instead of falling further, rose by 0.7° C and was maintained at this higher level. Colonic temperature fell again when ambient temperature had been raised to 20° C.

Discussion

The conclusions to be drawn from the present findings raise some interesting problems regarding the regulations of energy metabolism and body temperature in fasting animals. Before discussing any aspects of the issue, it must be emphasized that our results are in full agreement with McCance and Mount's findings in growing pigs.

Basal metabolic rate

Since in the course of undernutrition body size and body composition change considerably, difficulties arise in the interpretation of O_2-consumption. However, recent data generally agree in that basal O_2-consumption decreases out of proportion to the decrease in body weight, surface area, and other parameters. The present findings in adult rats support the view that a real fall in basal metabolic rate occurs in the early stage of undernutrition. As to the mechanism of this early fall in minimal O_2-consumption, neither previous, nor the present studies provide definite information. Changes involving various hormonal or neural regulatory functions should be taken into consideration. Effects on the basal metabolic rate of quantitative or qualitative changes in food intake cannot be excluded entirely. That restriction of food intake might in itself contribute to the fall in basal metabolic rate is indicated by the observation [12] that the low basal metabolic rate found in undernourished infants suffering from pyloric stenosis recovered very soon when after operative treatment food consumption had been resumed.

Attention must be drawn to the observation that the rapid initial and subsequent slow decline in basal O_2-consumption was accompanied by normal colonic temperature. This finding confirms earlier observations indicating that the basal metabolic rate can be regulated at different levels independent of changes in body temperature.

No attempt has been made to examine a possible shift of the thermal neutral environment. Since colonic temperature did not differ from that of

the control period, and O_2-consumption remained at the minimal level, a significant shift beyond 30° C could be excluded.

Metabolic response to 35° C

It has been demonstrated by DONHOFFER *et al.* [13] that in rats the increase in O_2-consumption at an ambient temperature of 35° C is not the direct consequence of the rise in body temperature; the response can be abolished by thyroidectomy, hypophysectomy, as well as by certain hypothalamic and epithalamic lesions.

The results are conclusive. With the progress of undernutrition the metabolic response to hyperthermia decreased roughly parallel to the basal metabolic rate; at the terminal stage no response could be observed. It should be noted that the degree of hyperthermia at all stages of undernutrition was identical with that recorded prior to undernutrition. This fact lends support to the view that the increase in O_2-consumption associated with hyperthermia cannot be explained as the direct result of the rise in body temperature. Since the metabolic response to cold was found to be present after the hyperthermic increase in O_2-consumption had disappeared, the lack of the response to 35° C at a loss of weight surpassing 50 per cent is not the consequence of an overall inability to increase O_2-consumption.

Metabolic response to cold

The most interesting finding was the effect of undernutrition on the regulation of body temperature at ambient temperatures below thermal neutrality. In the early phase of starvation, the metabolic response to 20° C was percentually definitely less than in the control period. In spite of this difference, however, the fall in colonic temperature was not more marked than before starvation. This shows that neither the lower level of total heat production nor the smaller relative increase in O_2-consumption affected the control of body temperature. With increasing loss of weight, however, in association with an increasing fall in colonic temperature the increase in heat production was relatively more. Apparently, the greater fall in body temperature augmented the percentual increase in heat production in response to exposure to 20° C. One would be inclined to regard the hypothermia at 20° C as a manifestation of the deterioration of thermal control.

The fact that transfer from an ambient temperature of 20° C to 5—10° C was followed by a considerable increase in heat production, while body temperature, instead of falling further, was maintained or even increased, proved convincingly that the animal is well able to maintain its body temperature in a considerably colder environment. Obviously, the regulated level of body temperature had been reset as undernutrition proceeded.

In the early stage of undernutrition, when the basal metabolic rate was lower, and the metabolic response to 20° C was considerably less than that in the control period, body temperature was found to be within normal limits. This observation seems to indicate that changes in the control of energy metabolism and body temperature are not necessarily linked in undernutrition. At more advanced stages, when body temperature fell to subnormal levels, the response in heat production to 20° C tended to approach the control value. Comparing the percentual increase in O_2-consumption observed at 20° C and 5°—10° C, with the corresponding value recorded prior to undernutrition, shows that at 5°—10° C, in contrast to 20° C, no difference was observed.

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Tibor Heim, Gyula Mestyán
Orvostudományi Egyetem Gyermekklinikája, Pécs.

THE EFFECT OF SEPTAL LESIONS ON SIMPLE AND DELAYED CONDITIONING IN CATS

By

J. CZOPF and E. GRASTYÁN

INSTITUTE OF PHYSIOLOGY, MEDICAL UNIVERSITY, PÉCS

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The effect of lesions placed in the septal region of cats has been investigated with simple approach and avoidance conditional reflex as well as with a multiple choice delayed reaction test method.

No impairment of performance was found in the simple approach and avoidance situation irrespective of the site of the lesion. In connection with the multiple choice delayed reaction, the test animals could be divided into two groups. One displayed a transitory decline of performance, the other a permanent defect (the maximum delay by which the criterion of 90 per cent correct responses could be reached, having been only 5 seconds). A serious damage to the fornix column was found in the latter group.

The conclusion has been drawn that by lesioning the septal region it is the impairment of the hippocampus-fornix system, or the septo-hippocampal relay, which plays a role in the ensuing functional defects.

SPIEGEL, MILLER and OPPENHEIMER [8] were the first to report that lesions of the hippocampus-fornix system and the septal region were followed by marked outbursts of rage reactions. Subsequently, significant increase in both emotional reactivity and startle magnitude were found by BRADY and NAUTA [1] after lesions of the septal region in the rat. These important findings would suggest an important role of the septum in the transmission of neural impulses involved in determining affective behaviour. A possible contribution to the effects of the lesion of the hippocampus-fornix system could not, however, be ruled out in these experiments by finding that "the magnitude of the changes in the behaviour of the experimental septal animals appeared to be roughly commensurate with the extent of injury to the fornix column . . ." BRADY and NAUTA's observations were supported in general by KING [5], but contradicted by HARRISON and LYON [3]. In a recent study ELLEN and POWELL [2] have found that rats after septal lesions displayed a hyperexcitability in a positive reinforcement test (bar pressing reinforced with a fixed interval schedule).

The purpose of the present study was to investigate the effect of two different kinds of septal lesions (involving and sparing the fornix column) with the behavioural methods used formerly in a study of the effects of hippocampal lesions. It was expected that the role of the septal region might be separated in this way from the combined effects of lesions involving both septum and fornix.

Methods

14 adult cats of both sexes were used in the experiments. A simple alimentary conditional reflex was established in 3, a simple avoidance reflex in 4 animals. A more complicated delayed alimentary conditional reflex was elaborated in 11 cats (including 4 cats used for simple avoidance reflex). The simple alimentary conditional reflex consisted in a food-getting manipulation from a feeding device monitored from the outside by the experimenter and signalled by a conditional sound stimulus (10 c/s clicks). In the case of the avoidance reflex a painful electric shock administered by a metal grid fixed to the bottom of the apparatus was used which could be avoided in the five seconds following the conditional sound stimulus (1000 c/s tone) by jumping onto a small bench fixed to one of the side walls of the apparatus.

The delayed conditional alimentary reflex was established in an apparatus containing two or three feeding devices provided with separate sound and light sources, and a small cage fixed to the main apparatus in an equal distance from the feeding devices, in which the animal could be restrained for various time periods after the delivery of the conditional stimulus signalling the feeding device to be approached.

Delay was elaborated gradually from 0 to 1 min., generally in 5 steps (5, 15, 20, 30 and 60 seconds), the criterion being each time a 80 per cent correct performance. Generally, 20—25 trials were made in a session daily. Details of the conditioning methods have been published previously (Karmos and Grastyán [4]).

Lesions of the septal region were produced in the case of the delayed reaction after the criterion of 1 min. had been reached and in the case of simple reflexes when the conditional reactions had become automatic (constant latency time).

The lesions were produced electrolytically in a stereotaxic instrument, with the help of co-ordinates of the Jasper, Ajmone—Marsan atlas. Lesions were planned in all the cases to be symmetrical. The parameters used were, 4 mA, for 30 seconds.

In about half of the cases an attempt was made to confine the lesions more anteriorly, with the intention to spare the fornix column, in the other half to the more posterior region of the septum, with the intention to involve also the fornix. A sham operation was carried out in two cats (electrodes introduced up to the septum, without coagulation). The extent of the lesions was determined in Nissl stained sections.

Results

Simple conditioning. No significant change in the performance of simple alimentary as well as avoidance conditioning could be established in the septal animals, irrespective of the placement of the lesions. The conditional reaction appeared unimpaired immediately after recovery. On simple inspection even the impression was gained that sometimes performance of the reactions was quicker than in normal animals. This could not, however, be substantiated statistically.

Delayed conditioning (Table I). In marked contrast to the above mentioned findings, a serious impairment in the delayed conditional reflex situation was found in one group of the septal animals. The maximum delay by which the criterion could be reached was 5 seconds. By a delay of 10 seconds 40 per cent incorrect responses were obtained even after 9 weeks of retraining. Histological examination disclosed in all these cases a serious damage of the fornix column (Fig. 1).

Lesions placed more anteriorly were followed by a transitory decline of performance. After a retraining period of about 10 days the criterion of 1 minute delay could again be reached (Fig. 2).

The performance of the sham operated animals did not display any change (Fig. 3).

General observations. The definite impression was gained that emotional reactions of different character were easier to elicit in septal than in normal

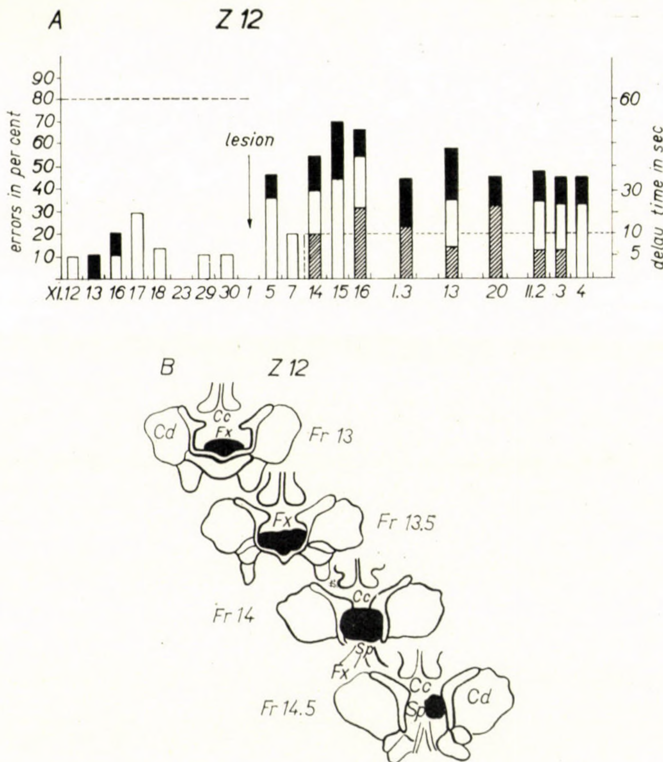

Fig. 1. Cat Z 12. A: Reconstruction of lesion after Nissl-stained sections. Abscissa: days. Ordinate left: per cent of incorrect responses. Ordinate right: time of delay in seconds. Broken line shows the actual delay on the corresponding experimental day. The entire height of the columns shows the total % of incorrect responses. Parts of columns (white, black, shaded) show the partial % of incorrect responses committed by the central (white), left (black), or right side (shaded) feeding device. Arrow: day of operation. B: Effect of septal lesion involving the fornix, in a triple choice delayed response situation. Maximum delay by which the criterion was reached, 5 seconds. 40—50% incorrect responses with 10 seconds delay.

animals. All the septal animals looked more vivid than the normals, they often displayed adulation of the experimenter, but agressivity manifested itself even so easily.

Startle reactions were conspicuously increased. The "following reaction" described by Brady and Nauta [1] occurred in a marked form in one case. It should, however, be mentioned that any of these general manifestations had been scored on a quantitative measure.

Table 1

Summary of data gained in 11 lesioned and in 1 sham operated case

Double line separates two groups of animals according to the anterior or posterior placement of the lesions, sparing and involving the fornix, respecitvely. Sp: Septum, Fx: Fornix, Cc: Corpus callosum, Cd: Nucleus caudatus, Thal: Thalamus

Before operation			Co-ordinates of lesion and histological control	After operation		
Animal	Average % of 10 sess. perform.	Time of delay	Anterior group	Average % of 10 sess. perform.	Maximum delay	Length of postop. observ.
Z 14	83.4	60 sec	Fr. 17.5 Not evaluated	75	60 sec	2 weeks
Z 23	82.7	60 sec	Fr. 16 Lesion in Sp. Fx only partially involved	86.9	60 sec	6 weeks
Z K 2	89.5	60 sec	Fr. 16.5 Lesion in Sp and Cc. Fx. (?)	88.5	60 sec	3 weeks
Z 25	92.5	60 sec	Fr. 16 Lesion in Sp dors., CC and Cd. Fx intact	93.7	60 sec	2 weeks
Z 24	98.1	60 sec	Fr. 16.5 Not evaluated	89.7	60 sec	10 days
Z 24	94.1	60 sec	Sham operation	97.7	60 sec	2 days
			Posterior group			
Z 8	93.5	60 sec	Fr. 14.5 Extensive lesion in Fx, Sp, small lesion in Thal.	87.6	30 sec	5 weeks
Z 9	95.7	60 sec	Fr. 14.5 Extensive lesion in Fx, Sp. Small lesion in Thal.	86.0	30 sec	5 weeks
Z 11	87.8	60 sec	Fr. 14.5 Large lesion in Sp. Cc and Cd partially involved	50.0	5 sec	6 weeks
Z 12	88.5	60 sec	Fr. 14 Extensive lesion in Fx and Sp. Small lesion in Thal. medialis	39.7	10 sec	9 weeks
Z 13	95.8	120 sec	Fr. 14.5 Large lesion in Fx and Sp.	55.4	10 sec	6 weeks
Z 2 S	92.2	60 sec	Fr. 14 Lesion in Fx ventr. and Sp. med. Caud.(?)	69.7	5 sec	6 weeks

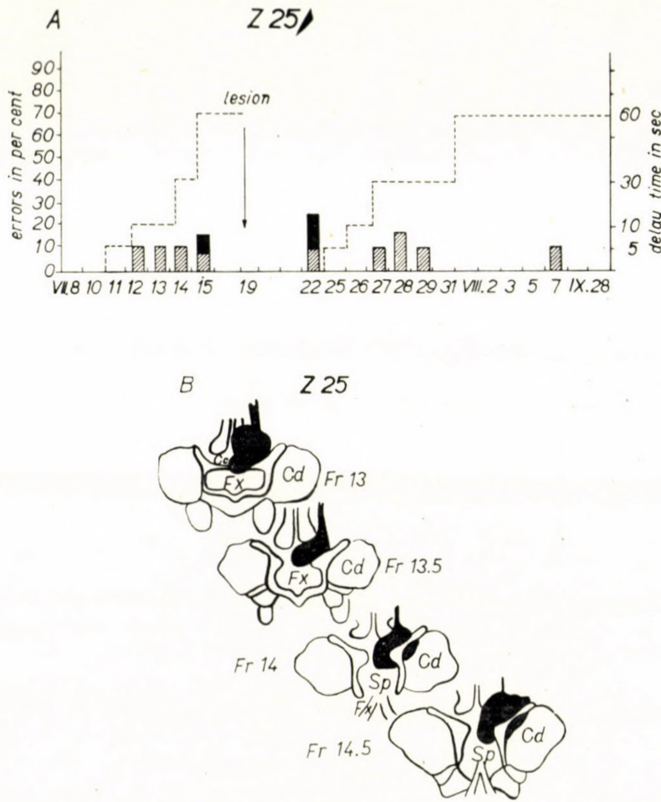

Fig. 2. Cat Z 25. A: Effect of septal lesion practically sparing the fornix in a double choice delayed response situation. Ten days after operation 90—100% correct performance was recorded with 1 min. delay. Black part of columns shows mistakes committed with the left, white part with the right, feeding device. B: Reconstruction of the lesion after Nissl-stained sections

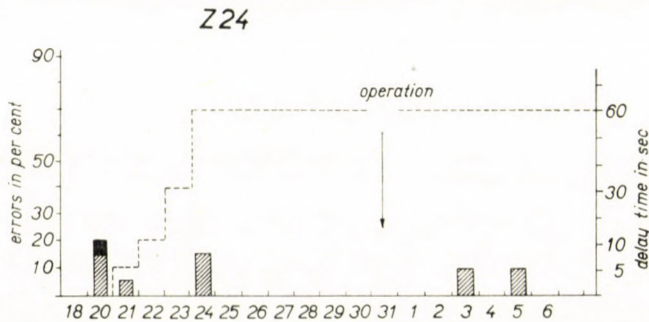

Fig. 3. Cat Z 24. Performance of a sham-operated animal in a double choice delayed response situation. No significant change in performance after operation

Discussion

The finding that a lesion of the septal region is not followed by any marked changes in simple conditional reflex performance would seem to support the conclusion of LYON and HARRISON [6] that " . . . generalizations concerning the role of these structures in affective behaviour be accepted with caution." Taking into consideration, however, the above described finding according to which a permanent functional impairment is disclosed in septal animals with a more sensitive behavioural testing (delayed conditioning) a reciprocal of the cited conclusion seems also warranted, namely that generalizations concerning the capacities of simple behavioural tests in reflecting complex functional impairments should be accepted with caution.

By the statement that lesions of the septal region were followed by serious defects in a delayed conditional reflex situation, the authors do not claim that the structural origin of the disturbance has been settled. The histological examination clearly showed that the magnitude of the functional impairment was roughly commensurate with the involvement of the fornix into the lesion. The same kind of disturbance was found in a former series of experiments after hippocampal lesions. Both these facts are in agreement with the conclusion of BRADY and NAUTA [1] and suggest that, essentially, the source of the behavioral changes should be looked for in the hippocampus-fornix system. Taking into account, however, that the posterior septal region constitutes an important relay station (PETSCHE *et al.* [7]; STUMPF *et al.* [9]) conveying impulses from lower structures to the hippocampus, it cannot be excluded that destruction of these parts of the septum and not exclusively the descending fornix is responsible for the irreversible functional defects.

As far as the mechanism of the changes is concerned, a similar conclusion as that derived from observations by hippocampal animals seems to hold true in this case, *viz.* that by destroying the connections of the hippocampus a mechanism controlling the hypothalamus and lower brain-stem disappears and consequently several functions of these structures are released. This assumption should also conform with the general behavioral observation that different kinds of affective manifestations are equally easy to elicit in septal animals. According to our own direct observations, a marked increase of the orientation reflex would be responsible for the poor performance in the delayed conditional situation. This interpretation seems to be applicable also to the changes (increased rate of bar pressing in fixed interval reinforcement schedule) recently described by ELLEN and POWELL [2].

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József Czopf, Endre Grastyán
Orvostudományi Egyetem Élettani Intézete, Pécs.

AN ELECTROPHYSIOLOGICAL STUDY OF CEREBELLO-HIPPOCAMPAL RELATIONSHIPS IN THE UNRESTRAINED CAT

By

V. V. Fanardjian and Hilda Donhoffer

INSTITUTE OF PHYSIOLOGY L. A. ORBELI, ARMENIAN ACADEMY OF SCIENCES, YEREVAN, USSR
AND INSTITUTE OF PHYSIOLOGY, UNIVERSITY MEDICAL SCHOOL, PÉCS

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Potentials elicited from the cerebellum have been examined with acoustic and visual stimuli, and with electrical stimulation of the sensorimotor cortex and the hippocampus in 12 cats with chronically implanted electrodes.

(1) The properties of the potentials elicited by natural stimuli were the same as those seen in acute experiments. Their amplitude, however, varied greatly with the animal's state of wakefulness.

(2) The essential properties of the cerebellar potentials elicited by hippocampal stimulation closely corresponded to those of the responses to natural stimuli. This fact was confirmed by interaction examinations.

(3) In no case could evoked potentials be recorded from the hippocampus on stimulating the cerebellum with single impulses, although the same stimuli invariably elicited responses from the sensorimotor and the association cortex. This means that, in contrast to the hippocampo-cerebellar relationship, no cerebello-hippocampal relationship could be demonstrated.

(4) Stimulation of the cerebellum with serial stimuli resulted in neocortical desynchronization. If the same stimulation parameters were used during hippocampal seizure, neocortical activity remained unaffected. In some cases it was possible to inhibit hippocampal seizures with strong stimulation associated with motor phenomena.

A considerable number of electrophysiological observations on cerebellar connections and functions have been made in the last two decades. The majority of the studies have involved acute preparations (anaesthetized animals, or encéphale isolé), and now the question might be raised how far these observations are valid for the chronic preparation.

On the other hand, the majority of investigations has dealt with peripheral as well as neocortical effects, and there are few data on cerebello-archicortical connections and function.

Methods

In 12 adult cats anaesthetized with sodium pentobarbital, steel needle electrodes were implanted in the ventral hippocampus, the dentate nucleus, the fastigial nucleus, and silver ball electrodes on the sensorimotor and association cortex and the cerebellar surface (Fig. 1). The deep electrodes were inserted according to Snider and Niemer's Atlas [22].

Coordinates of the hippocampus	A 7	L 12	V −4
Coordinates of the dentate nucleus	P 9	L 7	V −1
Coordinates of the fastigial nucleus	P 9	L 1	V 1

The experiments were begun after the animals had completely recovered.

Clicks were employed as acoustic stimuli, and flashes of light as visual ones. Electrical stimulation was provided by a square wave generator.

A Disa oscilloscope (Universal Indicator 51 B 00) and an 8-channel Schwarzer EEG were used for recording.

At the end of the experiment the animals were killed and their brains fixed in formol. The slides for histological examination were stained according to Nissl.

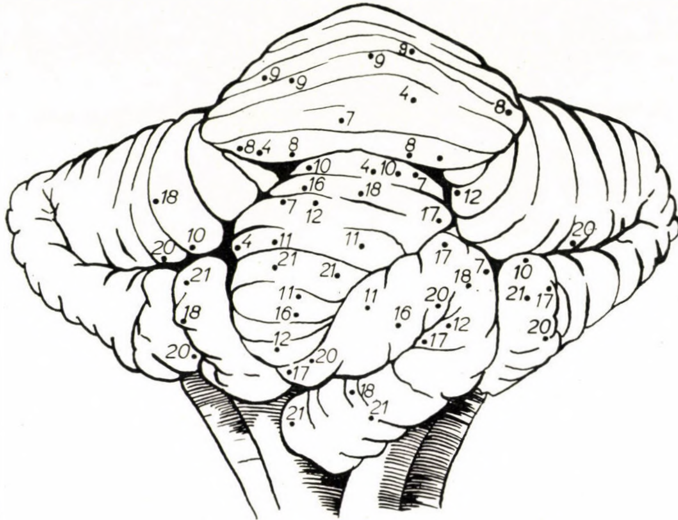

Fig. 1. Diagram showing the loci of the cerebellar surface examined. The numbers refer to the individual experimental animals

Results

Effects of auditory and visual stimuli

The general morphology of cerebellar evoked potentials elicited by natural (click, flicker) stimuli in the chronic preparation did not differ essentially from that in the acute preparation. In general it appeared in a polyphasic form with a dominant primary biphasic surface positive-negative sequence, the first component being more stable than the second one. The surface negative component consistently appeared in the case of clicks, it proved more unstable in the case of flickers. The first two dominant components were often followed by some oscillations of small amplitude.

The latency time of the click-evoked response was generally between 7 and 16 msec, that of the flicker-evoked response between 15 and 45 msec. Because of the great variability of the later components, depending on the alertness of the experimental animal, an analysis of the different components of the evoked potential was more difficult in the chronic than in the acute preparation. A marked decrease in amplitude or practical disappearance of the potentials were often observed during strong attention. The most stable potentials, similar to those in acute preparations, were recorded during superficial sleep. A decrease of amplitude or even the disappearance of poten-

tials was found in deep sleep. Potentials elicited by flicker proved highly unstable in every respect (Fig. 2).

In contrast to the acute experiment [15, 20, 21] in which both acoustic and visual evoked potentials had been localized to the pyramid, simplex,

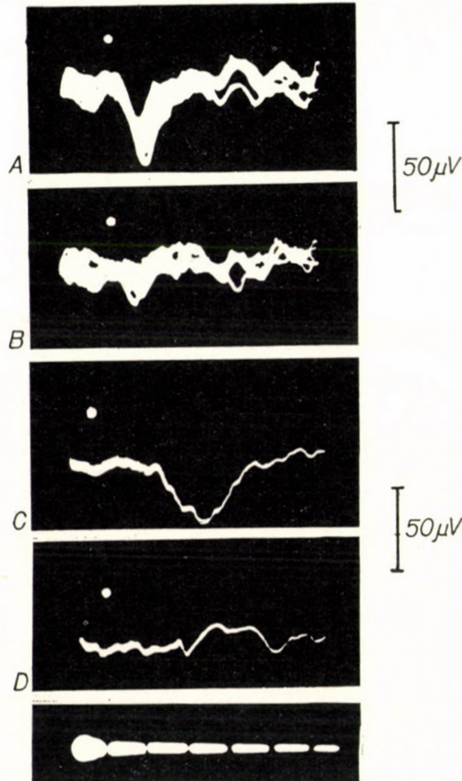

Fig. 2. Cerebellar responses to clicks in the waking animal (A), during sleep (B), to visual flashes during light sleep (C) and deep sleep (D). In cases A and B the bipolar leads were taken from the right central lobule, in cases C and D from the pyramid and uvula. The acoustic and visual stimuli are indicated by a dot over the tracings. Note that amplitude decreases with deepening sleep. Time, 20 msec

paramedian lobule, definite responses could be recorded from all the regions investigated in the chronic preparation (central lobule, ansiform lobule, uvula, cerebellar nuclei).

Stimulation of the motor cortex

On stimulating the motor cortex with single impulses, biphasic potentials with a latency of 2—15 msec appeared in every cerebellar point recorded. The only difference from the potentials elicited by natural stimuli was a more

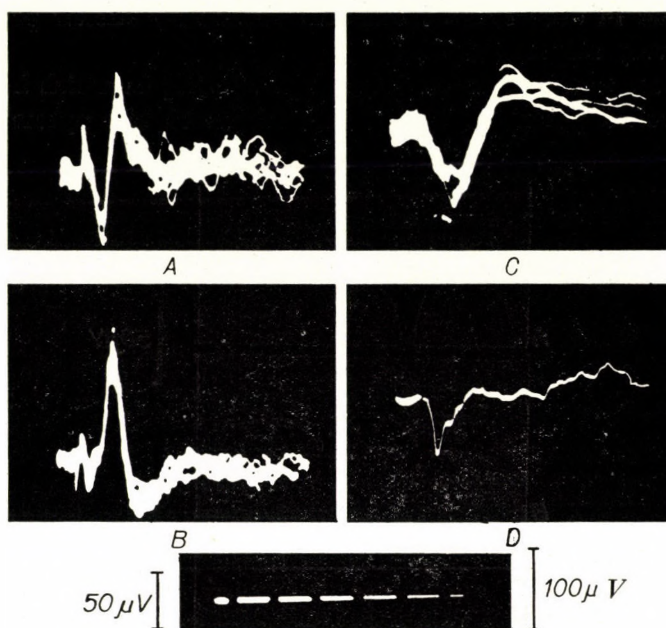

Fig. 3. Cerebellar potentials elicited by stimulating the motor cortex at a frequency of 1/sec. Bipolar leads.

	Stimulation		Leads
(A)	right motor cortex	—	left dentate nucleus
(B)	left motor cortex	—	right fastigial nucleus
(C)	right motor cortex	—	left cerebellar cortex
(D)	left motor cortex	—	left cerebellar cortex

For the cortical leads one of the poles was placed on the ansiform lobule, the other on the paramedian lobule. Note that the amplitude of the contralateral (C) response is higher than that of the ipsilateral (D). Time, 20 msec

stable amplitude. The amplitudes recorded from the contralateral side were generally larger, but exceptions also occurred (Fig. 3).

Stimulation of the hippocampus

A stable functional connection between hippocampus and cerebellum could be demonstrated with hippocampal stimulation. Single shocks elicited characteristic biphasic potentials in the cerebellum, with an early surface positive and later negative phase. The amplitudes of these potentials, although varying with the changes in awareness, proved more stable than any of the potentials described above. The lowest threshold of elicitation was found with an impulse duration of 0.03 msec. An oscillation of the amplitudes of the evoked potentials was observed at frequencies higher than 0.5/sec and 3/sec in the waking and in the sleeping animal, respectively. The average

amplitude decreased as the frequency was increased (Fig. 4). Higher frequencies often elicited seizures and it was remarkable that evoked potentials could be recorded even in the background of the hippocampal fits.

All the points seen in Fig. 1 responded to stimulation of both hippocampi. It should, however, be mentioned that stimulation of the two sides did not

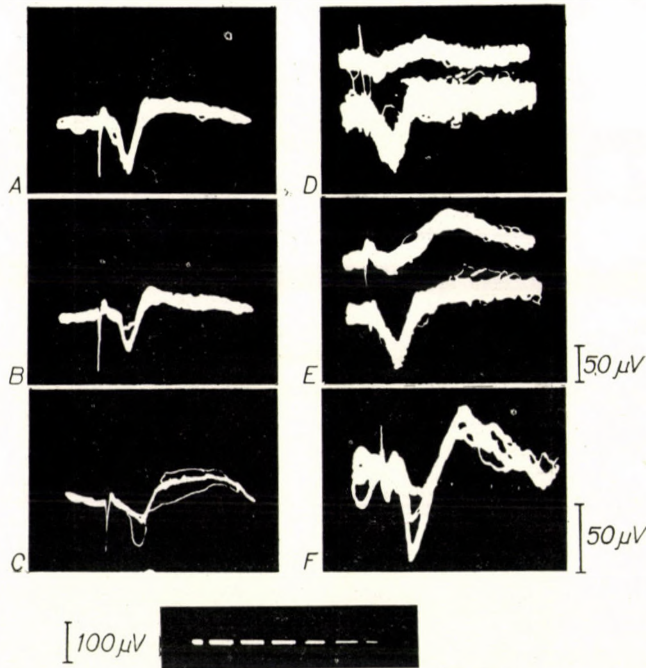

Fig. 4. Cerebellar responses elicited by electrical stimulation with a frequency of 1/sec of the left hippocampus and recorded with bipolar electrodes from the right fastigial nucleus [upper tracing (D)] the left dentate nucleus [lower tracing (D, E)] and the left pyramid [upper tracing (E)]. Stimulation of the left hippocampus with frequencies of 3/sec (A), 5/sec (B) and 8/sec (C). Note that with increasing frequency the amplitude decreases. (F) illustrates oscillation of the amplitudes of the potentials elicited by hippocampal stimulation. Time, 20 msec

have equal effects but invariably showed differences in amplitude and latency. As to laterality, no definite rule could be detected.

The latency time of the evoked potential elicited by hippocampal stimulation never exceeded 15 msec. The latency time recorded from the cerebellar subcortical nuclei was always 2—3 msec longer than that of the cerebellar cortical response.

Interaction

It is well-known from acute experiments [1, 2, 4, 5, 6, 13, 22] that test impulses are influenced by preceding conditioning impulses, the nature of

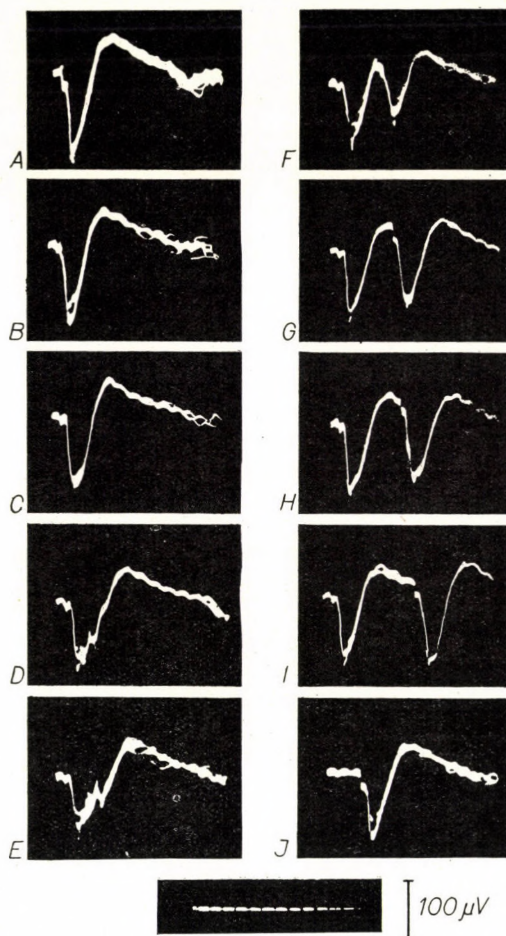

Fig. 5. The interaction of cerebellar potentials recorded with bipolar electrodes on the pyramid. Conditional stimulus, stimulation of the left hippocampus. Test stimulus, stimulation of the right hippocampus. Time elapsed between the two stimuli, in alphabetical order of the pictures 1, 10, 15, 30, 40, 60, 80, 100, 120 msec and response to test stimulus, alone. Time, 20 msec

the influence depending on the time elapsed between the two. This change is independent of whether the impulses belong to the same or different modalities. This assumption proved valid for the chronic experiment, too, including evoked potentials elicited by stimulation of the hippocampus.

In most of the present experiments, hippocampal stimulation was the conditioning stimulus, and as testing stimulus all the other possibilities described above were used (Figs 5, 6, 7, 8). Summation was found in the case of an interval of 1—15 msec and a depression of 15—80 msec.

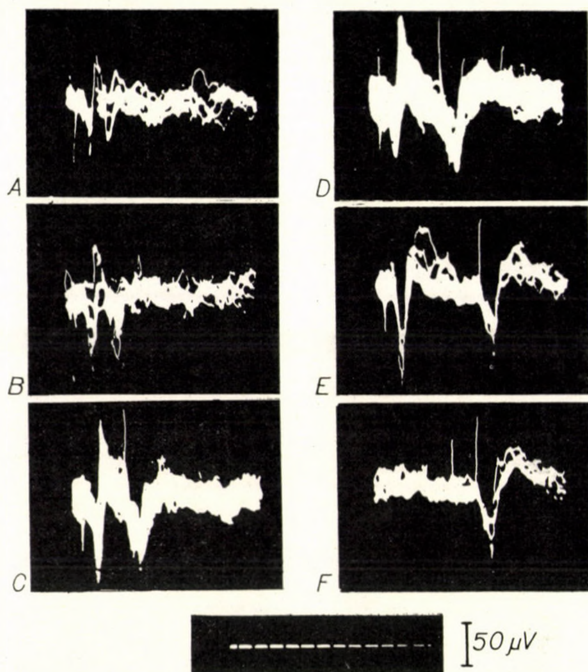

Fig. 6. The interaction of cerebellar potentials recorded with bipolar eletcrodes on the central lobule. Conditional stimulus, stimulation of left hippocampus. Test stimulus, stimulation of left motor cortex. Time elapsed between the two stimuli, in alphabetical order of the pictures 30, 40, 70, 80, 120 msec and response to test stimulus, alone. Time, 20 msec

A facilitation manifested itself if the conditioning stimulus had been applied to the motor cortex and the test stimulus to the hippocampus in the range of 20—30 msec (Fig. 9).

Effect of cerebellar stimulation

1. *Hippocampus*

In no case could potentials be recorded in the hippocampus by stimulating the cerebellar cortex or the cerebellar subcortical nuclei, at any parameters between physiological limits.

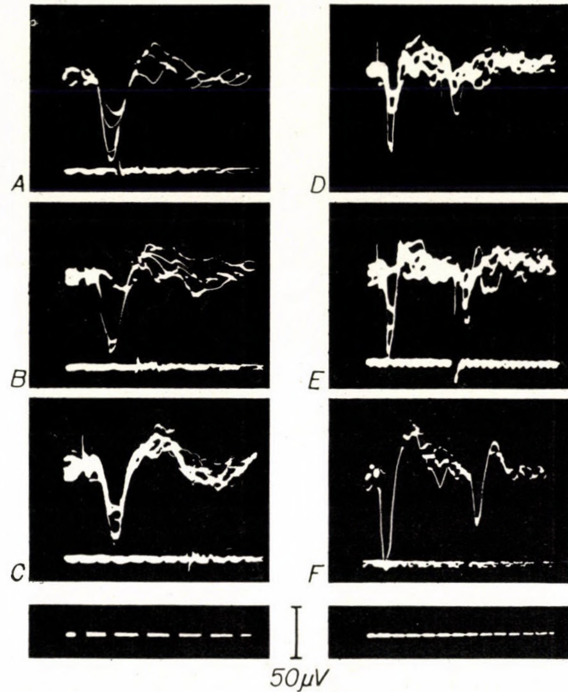

Fig. 7. The interaction of cerebellar potentials recorded with bipolar electrodes on the central lobule. Conditional stimulus, stimulation of left hippocampus. Test stimulus, click. Time elapsed between the two stimuli, in alphabetical order of the pictures, 20, 30, 60, 80, 100, 120 msec. Time, 20 msec

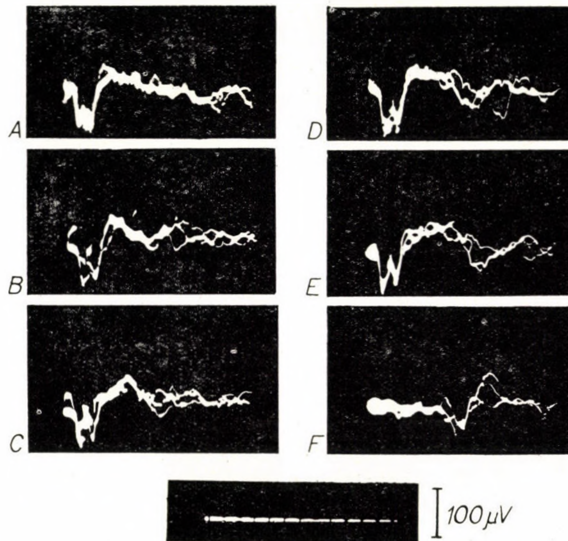

Fig. 8. The interaction of cerebellar potentials recorded with bipolar electrodes on the ansiform and paramedian lobules. Conditional stimulus, stimulation of right hippocampus. Test stimulus, flash of light. Time elapsed between the two stimuli, in alphabetical order of the pictures, 20, 40, 60, 80, 100 msec and response to test stimulus, alone. Time, 20 msec

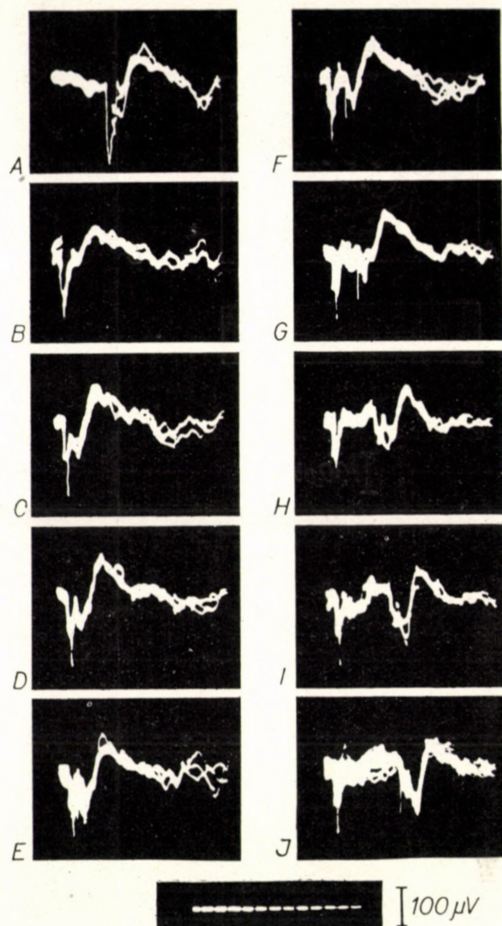

Fig. 9. The interaction of cerebellar potentials recorded with bipolar electrodes on the ansiform and paramedian lobules. Conditional stimulus, stimulation of left motor cortex. Test stimulus, stimulation of right hippocampus. Time elapsed between the two stimuli, in alphabetical order of the pictures, 1, 5, 10, 15, 20, 30, 40, 60, 80, 100 msec. In picture (A) the delay is longer. Time, 20 msec

2. *Motor cortex*

Both cortex and cerebellar subcortical nuclei produced definite evoked potentials in both sensorimotor regions, with the contralateral side generally showing higher amplitudes. Stimulation of the subcortical nuclei proved to be more effective (lower threshold) than that of the cortex.

3. *Association cortex*

Evoked potentials could be produced on the contralateral suprasylvian gyrus only by stimulating the dentate nucleus. Stimulation of the cerebellar

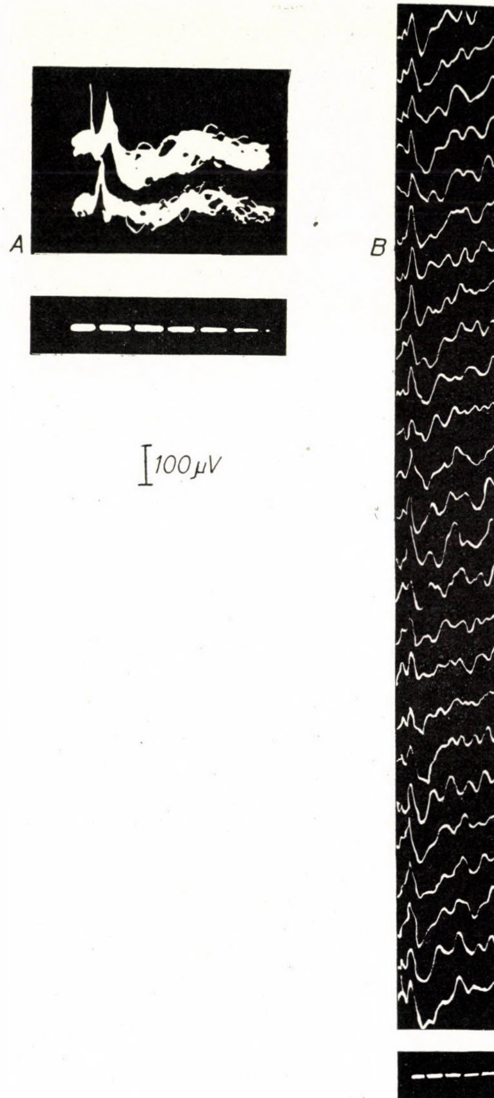

Fig. 10. The effect on the sensorimotor cortex of stimulation of the right dentate nucleus with single impulses. (A) upper tracing, left median suprasylvian gyrus, lower tracing, left posterior sygmoid gyrus. (B) serial picture of evoked potentials recorded from the association cortex. Note waxing and waning. Time, 20 msec

cortex was effective only in some cases of stimulation at higher intensities. Stimulation of the fastigial nucleus proved ineffective in most cases.

A definite recruitment [11, 12] was found on the contralateral neocortex (primarily association cortex) with stimulation of the dentate nucleus with frequencies of 6—10/sec. (Fig. 10).

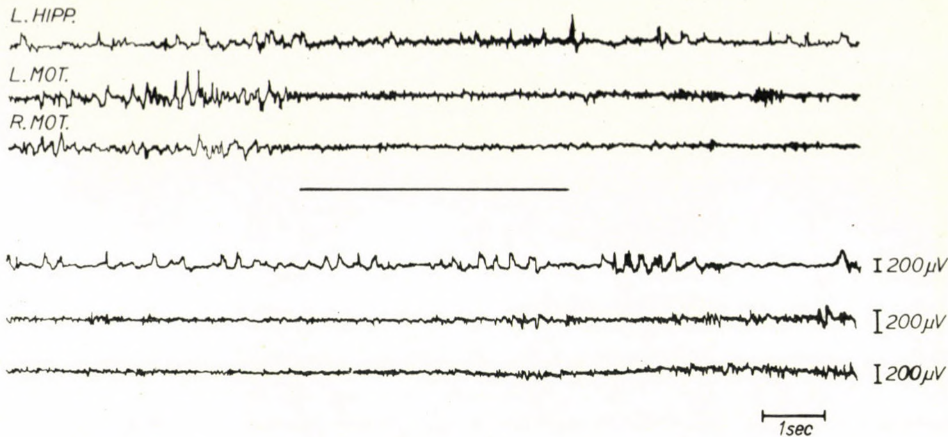

Fig. 11. The effect of stimulation of the cerebellum (pyramid) with serial stimuli (10 V, 0.1 msec, 100/sec) on the hippocampus and the motor cortex. The duration of stimulation is indicated by a horizontal line. Abbreviations, L. HIPP., left hippocampus; L. MOT., left motor cortex; R. MOT., right motor cortex.

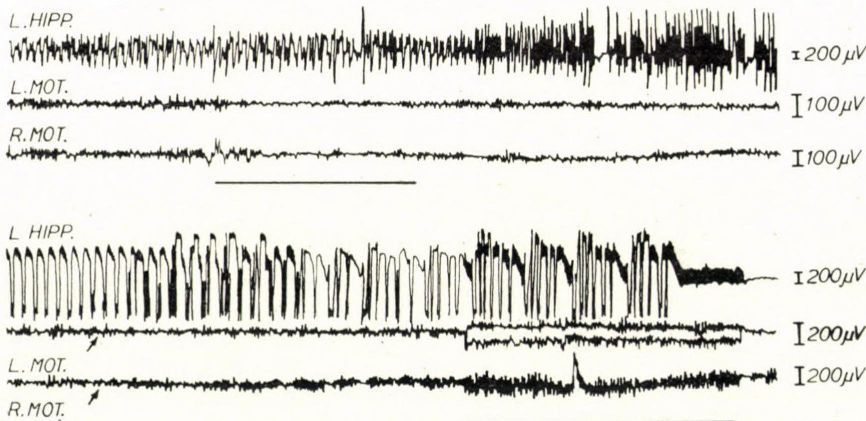

Fig. 12. Upper trace: Stimulation of cerebellum (pyramid; 10 V, 0.1 msec, 300/sec) fails to influence hippocampal seizure elicited by stimulation of right hippocampus. Lower trace: A hippocampal seizure may be interrupted by strong cerebellar stimulation (of the same locus; 50 V, 0.1 msec, 300/sec) associated with motor phenomena. Horizontal line indicates duration of stimulation. Abbreviations, L. HIPP., left hippocampus; L. MOT., left motor cortex; R. MOT., right motor cortex

Stimulation of the cerebellum with repetitive stimuli at rest and hippocampal seizure

1. *At rest*

Stimulation of the cerebellar cortex and the subcortical nuclei was followed by desynchronization in the motor cortex without overt somatic manifestation (Fig. 11).

2. *During hippocampal seizure*

a) Stimulation parameters producing desynchronization at rest failed to influence neocortical electrical activity (Fig. 12, upper trace).

b) Strong stimulation producing somatic manifestations sometimes stopped the hippocampal seizures (Fig. 12, lower trace).

Discussion

There used to be a considerable discrepancy between the different authors concerning the distribution of cerebellar evoked potentials. These controversies may be traced back to the different anaesthetics or preparations used in the acute studies. Even the physiological nature of the visual cerebellar evoked potential was questioned by several authors [3, 14, 16, 21]. This assumption was, however, disproved by FADIGA *et al.* [10], by demonstrating visual evoked potentials in different areas of the cerebellum (pyramid, simplex, crus I and II) even in curarized animals.

Considering that these potentials can consistently be recorded in the chronic animal, their physiological nature seems to be validated.

The fact that, unlike in the anaesthetized animal, in chronic preparations no strict localization of the evoked potentials could be found, does not seem to be unexceptional, a similar phenomenon occurring on the motor cortex.

Latency times in the acute and chronic preparations were generally found to be identical. It is well-known from acute experiments [21] that the amplitudes of evoked potentials decrease with deepening anaesthesia without a change in latency. Moreover, visual potentials were found to be more sensitive than acoustic ones [29]. Similar observations have been made during behavioral sleep in chronic experiments.

There are few data on the functional interdependence of the cerebellum and the archicortex. COXE and SNIDER [7] recorded evoked potentials on the cerebellum on stimulation of the caudate nucleus. The cerebellar evoked potential elicited by stimulation of the hippocampus, as reported in this study, is also suggestive of a functional connection between these two systems.

Taking into account that in these interaction studies the potentials elicited by stimulating the hippocampus behaved in the same manner as potentials of other modalities, the assumption seems justified that the same cerebellar elements are involved in both cases.

It should be emphasized that, in contradistinction to the reciprocal relationship between motor cortex and cerebellum [8, 9, 17], a definite one-way connection has been found between the hippocampus and the cerebellum. In view of the limited number of data at our disposal it would seem too early to analyze the functional significance of this one-way connection.

It has been established by IWATA and SNIDER [18] that in acute conditions hippocampal seizures can be stopped by cerebellar stimulation. This finding could not definitely be supported in the present study. It was only in a few cases that hippocampal seizures could be controlled with very strong stimulations producing unusual motor accompaniments.

The generator was designed and constructed by engineer L. KELLÉNYI, of the electronic laboratory of the Institute.

The authors' thanks are due to Mrs. I. SALAMON, of the Institute of Anatomy, for preparation of the slides, and to Mr. I. VADÁSZ, of the Institute of Physiology, for help in the documentation.

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V. V. FANARDJIAN (Yerevan), Hilda DONHOFFER
Orvostudományi Egyetem Élettani Intézete, Pécs.

ELECTROPHYSIOLOGICAL AND PHARMACOLOGICAL ANALYSIS OF THE EFFECT OF ADRENALINE AND NORADRENALINE ON THE INHIBITORY MECHANISM OF THE POSTERIOR ADDUCTOR IN LAMELLIBRANCHIATA

By

A. Puppi

INSTITUTE OF PHYSIOLOGY, MEDICAL UNIVERSITY, PÉCS

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1. Although pharmacological doses of adrenaline reduce the tone of the posterior adductor in *Lamellibranchiata*, the presence of this substance in molluscs has not been satisfactorily proved, so that, in the present state of our knowledge, there is no justification for the statement that adrenaline acts as inhibitor under physiological conditions.

High concentrations (1 mg/ml) of the drug inhibit, low concentrations (0.001 mg/ml) permanently increase, the bioelectric activity of the ganglia.

2. As regards its effect, noradrenaline is qualitatively equal and quantitatively inferior to adrenaline.

3. Dihydroergotoxin counteracts the inhibitory effect of adrenaline before, but fails to do so after, the transection of the connective. It is, therefore, presumed that the adrenergic effect gradually diminishes when the connection between the two ganglia is interrupted.

The fact that physiological concentrations of adrenaline increase bioelectric activity, while dihydroergotoxin decreases it, points to an antagonism between the two substances.

We have reported earlier [9] on experiments in which the ganglia of molluscs were treated with acetylcholine, 5-hydroxytryptamine, curare, nicotine, monoiodoacetic acid, and 2,4-dinitrophenol, and the effect of such treatments on the tone of the posterior adductor was studied. Results so obtained have induced us to analyze the catecholaminergic mechanism.

Data are scarce regarding the presence of adrenaline in molluscs, nor do we know the physiological significance of adrenaline on that scale of the philogenetic ladder. Several authors have reported on the presence of adrenaline and emphasized its physiological significance [1, 2, 5, 7, 10, 12], and even the presence of noradrenaline in *Octopus vulgaris* has been demonstrated [3].

Method

The tone of the posterior adductor muscle and the mechanism of its inhibition stand under the regulation of the cerebral and visceral ganglia. Myography was used for establishing the role of the individual elements, while a simultaneous registration of the bioelectric activity of the cerebral and visceral ganglia served for the analysis of neural activities.

We attached the posterior valve (separated from the anterior adductor) to the pen arm of a kymograph which recorded the processes occurring in the posterior adductor. Bipolar silver electrodes were used for recording the action potentials of the cerebral and viscera

ganglia by means of an ink-writing oscillograph. The activity of the cerebral ganglion was recorded from the cerebrovisceral connective, that of the visceral ganglion from the motor fibres running to the posterior adductor. The objection may arise that, with this arrangement, the effect of substances applied to the cerebral ganglion is impaired by eventual impulses conducted by the connective from the visceral to the cerebral ganglion, or that — if the substance is applied to the visceral ganglion — the nerves running to the muscle also contain fibres which pass to the posterior adductor without interruption. It should, however, be remembered that we are concerned here with deviations from the normal electrograms, further, that disturbances of the said kind are quite as likely to occur in the case of ganglionic leads. The amplifying frequencies varied from 0.2 to 75 Hz.

The chemically active agent (0.1 ml of 1 mg/ml, or 0.001 mg/ml concn. solutions per 10 g body weight), was absorbed on cotton swabs, and these were placed on the cerebral or the visceral ganglion. The circulation of the animals remained normal throughout the experiments. Since the visceral ganglion is near to the posterior adductor, swabs placed on the former structure exerted pressure on the latter.

A slide inductor (4 V) was employed for the reflex stimulation of the muscle. The stimulating electrodes were placed on the posterior margin of the mantle which abounds in nerve endings.

The myographic tracings were compared to control curves, while muscular changes were evaluated on the evidence of time characteristics.

Results

Myographic observations

Adrenaline. We applied this drug in a concentration of 0.001 mg/ml to the visceral ganglion (muscle) in the first group of experiments. On stimulation, the amplitude of contractions became 19 per cent less than in the controls.

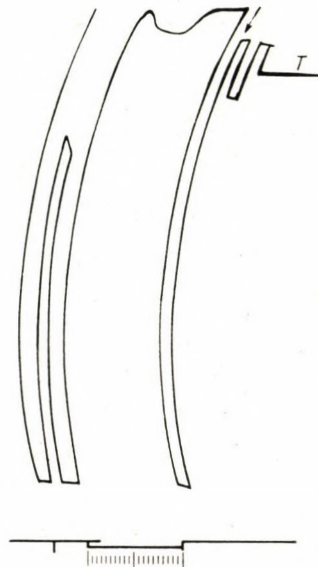

Fig. 1. Control myogram of posterior adductor (physiological saline applied to visceral ganglion). The first line (in all figures) indicates stimulation, the second indicates time (sec). The cylinder was stopped after stimulation. The arrow shows the division of the connective. The character "T" means the final level of tonicity. The cylinder was restarted for a short time after spontaneous contractions

Following stimulation for 20 min., the tone began to weaken normally, but relaxation terminated 76 per cent sooner than in the controls. The tone was likewise weaker after the division of the connective, in other words the level of tone multiplied with the time of tone-development gave a product which was higher than the corresponding value in the controls, indicating an intensification of the tetanic components (Fig. 2). (Fig. 1, control.)

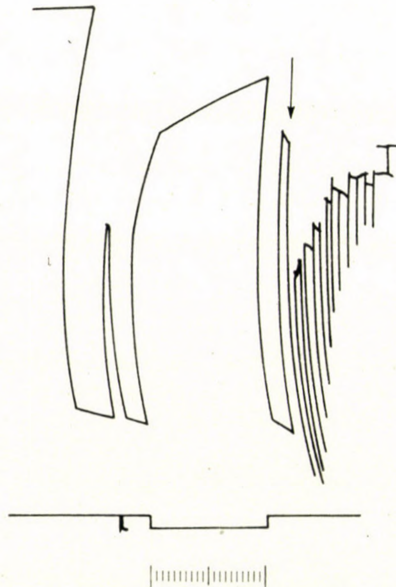

Fig. 2. Myogram of posterior adductor after application of adrenaline (concentr. 0.001 mg/ml) to the visceral ganglion

After transection adrenaline induced the appearance of spontaneous rhythmicity.

High concentrations of adrenaline (1 mg/ml) caused a still more pronounced inhibition. Following stimulation for 20 min., relaxation of the muscle began 51 per cent, and terminated 35 per cent, sooner than in the controls.

Inhibition by adrenaline of the tonicity was especially marked after the transection of the cerebrovisceral connective. This supports the theory that the inhibitory effect of adrenaline is proportional to the intensification of tonicity. After transection of the connective, the tone was 370 per cent weaker than in the untreated specimens, in other words, the level of tone in mm-s multiplied with the time of tone-development in minutes gave such a product.

Application of adrenaline to the cerebral ganglion produced the following changes in the posterior adductor.

After stimulation for 20 minutes with adrenaline at a concentration of 1 mg/ml, relaxation of the muscle began 103 per cent, and terminated 62 per cent, later than in the controls. This tendency did not change after division of the connective. Tonicity was 39 per cent stronger than normal. The results were less unequivocal at lower concentrations (0.001 mg/ml). Inhibition occurred in most cases. SALÁNKI [11] observed only inhibition at such concentrations. Generally speaking, adrenaline applied centrally affects the posterior adductor less intensively than if it is applied to the visceral ganglion, i.e. when it acts directly upon the muscle.

Noradrenaline. Application of this agent to the posterior neuromuscular apparatus at a concentration of 0.001 mg/ml for 20 minutes decreased the amplitude of concentrations markedly, while no such effect was produced at a concentration of 1 mg/ml. Both concentrations decreased the muscle's tonicity.

Relaxation — after stimulation for 20 min. at a concentration of 1 mg/ml began 98 per cent sooner, and terminated 36 per cent later, than in the controls. The corresponding figures at a concentration of 0.001 mg/ml were 97 and 51 per cent respectively.

It was only at a concentration of 1 mg/ml that a decrease in tonicity could be observed after the division of the connective, but even this effect was much less pronounced than that exerted by adrenaline. Low concentrations (0.001 mg/ml) of noradrenaline had no effect after transection of the connective. The effect of noradrenaline on the muscle elements of vertebrates is likewise weaker than that of adrenaline.

If noradrenaline was applied to the cerebral ganglia, relaxation began 98 per cent, and ceased 141 per cent, later than normally, a phenomenon indicative of an increase in tonicity of the posterior adductor, and pointing to a decrease in activity of the cerebral ganglia.

Tonicity increased by 112 per cent after transection of the connective, a phenomenon which shows that a preliminary inhibition of the cerebral ganglion increases the rate at which the tone is developing in the posterior adductor after the transection.

Dihydroergotoxin. Dihydroergotoxin *(SPOFA*, Prague) was applied in a solution of 0.1 per cent, for the further analysis of the supposed effect of adrenaline and noradrenaline.

Applied to the visceral ganglion, the drug decreased tonicity in the posterior adductor before, and failed to do so after, transection of the connective. Relaxation, following stimulation for 20 min., began 200 per cent (counting from 100 per cent), and ceased 14 per cent, sooner than in the controls. Relaxation mostly assumed the form of the pessimum reaction (gradual change of contractive effect into inhibition), and the second optimum failed to appear (renewed contractions). Applied to the cerebral ganglia, dihydroergotoxin produced no such marked changes.

Bioelectric observations

Adrenaline. There are numerous literary data to show that the effect of adrenaline in the neural elements is diphasic: it strengthens action potentials in the first, and weakens them in the second phase. These data refer, of course, to animals other than *Lamellibranchiata*.

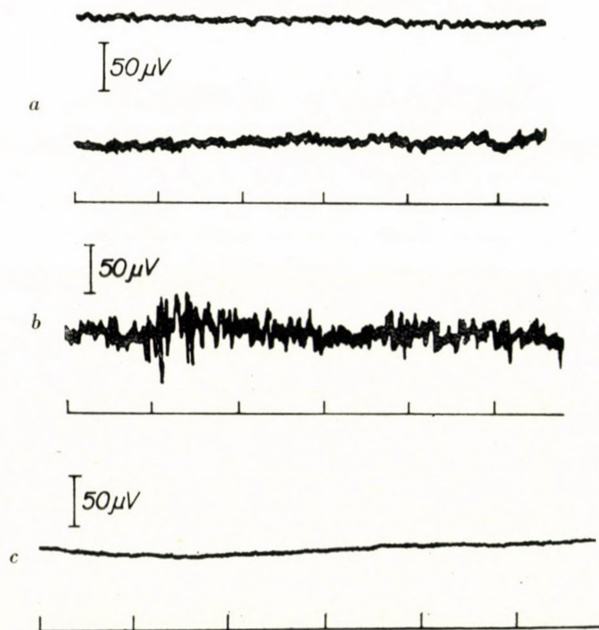

Fig. 3. Action potentials in the cerebral ganglion after adrenaline treatment: *a* = control; *b* = at a concentration of 0.001 mg/ml; *c* = at a concentration of 1 mg/ml

Adrenaline at a concentration of 0.001 mg/ml applied to the cerebral ganglion promptly intensified bioelectric activity in the cerebrovisceral connective (Fig. 3b, Fig. 3a control). The high oscillations remained unchanged for a long time.

Action potentials became likewise stronger in the fibres of the visceroefferent nerve (*viz.* the nerve running from the visceral ganglion to the adductor) synchronously with the impulse bursts of the connective, while there appeared the slow waves characteristic of the visceral ganglion. Thus, low concentrations of adrenaline reinforced impulse formation in both the cerebral ganglion and the visceroefferent nerve.

Application of adrenaline at low concentrations gave similar results.

High concentrations (1 mg/ml), whether applied to the cerebral or the visceral ganglion, inhibited biopotentials almost completely (Fig. 3c). It

follows that in molluscs adrenaline has either no diphasic effect or, else, that the first phase is very short on applying high concentrations and is over before oscillographic recording becomes operative.

Noradrenaline. The action potential in the connective showed a marked reduction after treatment of the cerebral ganglion with a concentration of 1 mg/ml (Fig. 4b, Fig. 4a control). This reduction was apparently accompanied by an inhibition of action potentials in the visceroefferent nerve, although

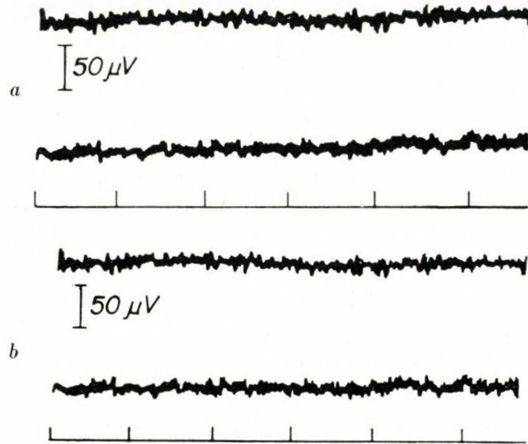

Fig. 4. Action potentials in cerebral (above) and visceral (below) ganglia after treatment with noradrenaline at a concentration of 1 mg/ml: *a* = control; *b* = applied to cerebral ganglion

the phenomenon was rather weak. Low concentrations of noradrenaline caused a lasting increase of activity.

Dihydroergotoxin and dihydroergotamine. Of these substances 0.1 per cent solutions were applied. The application of dihydroergotoxin to the cerebral ganglion lastingly decreased (with a delay of 70 to 80 sec.) the potentials of the connective in respect of both frequency and amplitude (Fig. 5b, Fig. 5a control). The decrease in the activity of the cerebral ganglion was accompanied by a slight decrease in impulse conduction in the visceroefferent nerve.

Application of dihydroergotoxin to the visceral ganglion gave similar results with the difference that the delay was longer in this case. It would follow that dihydroergotoxin diffuses into the nervous tissues at a slow rate (Fig. 5c).

Application of dihydroergotamine yielded similar, though less pronounced, results. An increase in the action potentials was observed in a few exceptional cases.

The fact that low (physiological) concentrations of adrenaline intensify, while dihydroergotoxin reduces, bioelectric activity, points to an antagonism between the two substances.

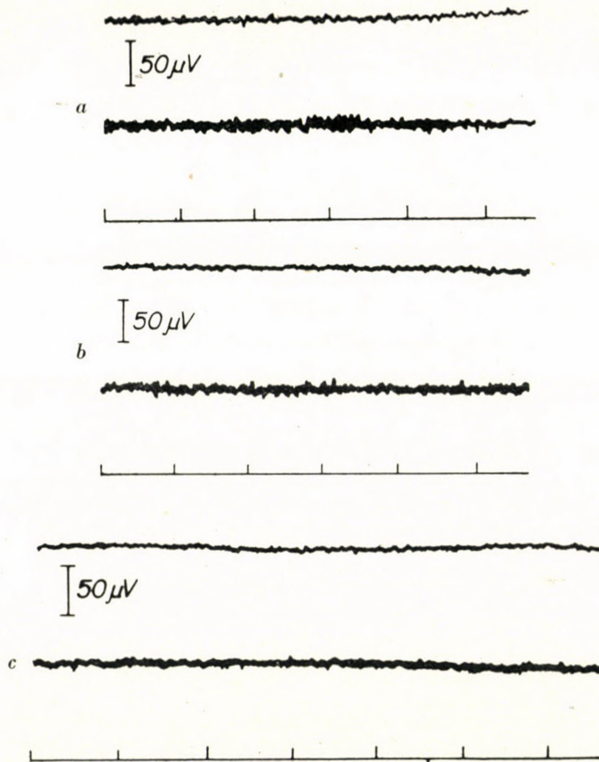

Fig. 5. Action potentials in cerebral (above) and visceral (below) ganglia after treatment with 0.1 per cent solution of dihydroergotoxin: a = control; b = applied to cerebral ganglion; c = applied to visceral ganglion

Discussion

Myographic analysis has shown that adrenaline, applied to the visceral ganglion, decreases the amplitude of contractions as well as tonicity in the posterior adductor, and that the intensity of this effect is proportional to the concentration of the drug. This in our opinion is due to that adrenaline at low concentrations, if applied to the visceral ganglion, decreases tonicity by acting directly on the muscle, whereas this effect is counteracted by the increased activity of the visceral ganglion. The activity of the visceral ganglion is inhibited at high concentrations, so that only the direct inhibitory effect remains in force.

The tonic component of the contractions is well pronounced; the afore-mentioned weakness of tonicity manifests itself through the rapidity

of relaxations and their non-tonic character. This would support the assumption of BÜLBRING and BURN [2] that the action of adrenaline consisted essentially in restoring the initial ionic asymmetry. If this assumption is correct, adrenaline is justifiedly regarded as a factor of tone inhibition. The theory is supported by the observation of SINGH [14] that low concentrations of adrenaline suffice to cause inhibition if the muscle is contracted, while higher concentrations are required to produce the same effect if the muscle is relaxed.

Rhythmicity (increase of irritability) after transection of the connective and intensification of phasic properties is presumably due to the same biochemical processes as are at play when adrenaline acts on tired muscles (via the sympathetic apparatus), a phenomenon which enabled GINETSINSKY and ORBELI to elucidate the adaptive-trophic activity of the autonomic nervous system.

ZHUKOV [15], in his work "Investigations concerning the tonicity of skeletal muscles", concludes as follows: "The working process, as also neurohumoral adaptive-trophic influences, may produce greater or lesser changes in the character of the activity of the apparatus. The tetanic apparatus may assume tonic properties, and the mobility of tonic elements may increase. It should be noted that such a reorganization is confined to certain limits, and that, in this process, the diapason of the tetanic elements differs from that of the tonic elements."

Similar tonotrophic effect of catecholamines was established by LEBEDINSKAJA and STRELITSOVA [6].

It is, of course, undeniable that literary data refer to adrenaline as a substance affecting tonicity only in respect of the skeletal muscles of higher animals. Our observations in molluscs would allow the conclusion that adrenaline produces an opposite effect at this low phylogenetic level.

Although it is evident that pharmacological doses of adrenaline inhibit the tonicity of the posterior adductor in *Lamellibranchiata*, it would not be justified to affirm that the drug is responsible for tone inhibition under physiological conditions, since its presence in molluscs has not been proved satisfactorily.

As regards the differences between adrenaline and noradrenaline, these substances are similar in chemical composition as also in respect of their physiological effect. We have seen that they influence muscular processes in the *Lamellibranchiata* similarly from a qualitative point of view, and that the action of noradrenaline is just somewhat weaker. Catecholamines behave, thus, identically in *Lamellibranchiata* and in higher animals.

Dihydroergotoxin caused a pronounced inhibition of tonicity as long as the connective remained intact. It is, on the other hand, known that catecholamines likewise decrease tonicity, and that they do so even after the

division of the connective. In contradistinction to dihydroergotoxin, adrenaline acts more vigorously after the transection, a phenomenon presumably due to that adrenaline affects tonicity, and dihydroergotoxin inhibits tetanism in the first place.

It is noteworthy that when — after the transection of the connective — the tetanic properties gradually disappear, the marked inhibitory action of dihydroergotoxin likewise disappears. It would follow that the adrenergic effect gradually diminishes after the cerebral and visceral ganglia have been disconnected. Provided this is true, the tonicity of the posterior adductor observed after division of the connective is partly due to a quantitative diminution of the catecholamines at play there. This assumption will have to be verified by biochemical investigations now in progress.

The present experiments were carried out in the Physiological Institute of the Zhdanov University, Leningrad.

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András Puppi
Orvostudományi Egyetem Élettani Intézete, Pécs.

THE IMPORTANCE OF CASE SELECTION IN CARIES-SALIVA STUDIES

By

D. HATTYASY, I. SZABÓ and K. TÓTH

DEPARTMENT OF DENTAL AND ORAL DISEASES, UNIVERSITY SZEGED

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In 1961, the authors could demonstrate significant differences in salivary pH and buffer capacity between selected young individuals free from caries and young subjects with multiple caries.

One year later both groups were re-examined, together with the examination of a group composed of young, caries-free females. The tests were made on three consecutive days, after fasting and after the midday meal, for oxygen consumption, acid production, oral pH and buffer capacity of the saliva.

As regards oxygen consumptions, the fasting values were closely similar in the caries-free and caries-resistant groups, whereas significantly higher values were obtained in the caries-active group. As to the values after lunch in the caries-free group amounted to the half of the fasting one, in the caries group they showed a slight decrease (the caries-free females could not be tested in the afternoon).

Acid production and oxygen consumption did not always run parallel. The pH of the culture medium varied from pH 4.70 to pH 3.70 in the tests. Acid production was as a rule higher in the caries group than in the caries-free and caries-resistant groups.

Salivary pH. Owing to the lower pH values for the caries-free subjects, the difference between the caries-free and caries groups was small (pH 0.25), the values for the caries-resistant groups were between the two. The afternoon values of the good and bad teeth groups were somewhat increased.

In a previous paper [3] we have reported on significant differences in pH and buffer capacity of the morning saliva between caries-free and caries-active young males. These tests were performed during the last days of May, 1961.

In accordance with our plans, we re-examined both groups during the first days of March, 1962.

In the caries-free group two members who had left the area had to be replaced from the large group we had originally selected the subjects from. In this group No. 14 turned caries-active (2 approximal caries), the teeth of the other 4 subjects remained intact. In addition, we examined a group of young female subjects free of caries, as controls facilitating comparison.

In the members of the caries group, 3 or more new caries were found.

In the experimental groups the saliva studies were done twice daily, mostly on three successive days, before breakfast, and after lunch. In the control (caries resistant) group only the morning values could be determined.

The following tests were made.

1. Oral salivary pH.
2. Salivary buffer capacity *in vitro*.
3. Salivary oxygen consumption (by the Warburg technique).

4. Salivary acid production (by SNYDER's method and pH determination).

The methods employed have been described in detail [3].

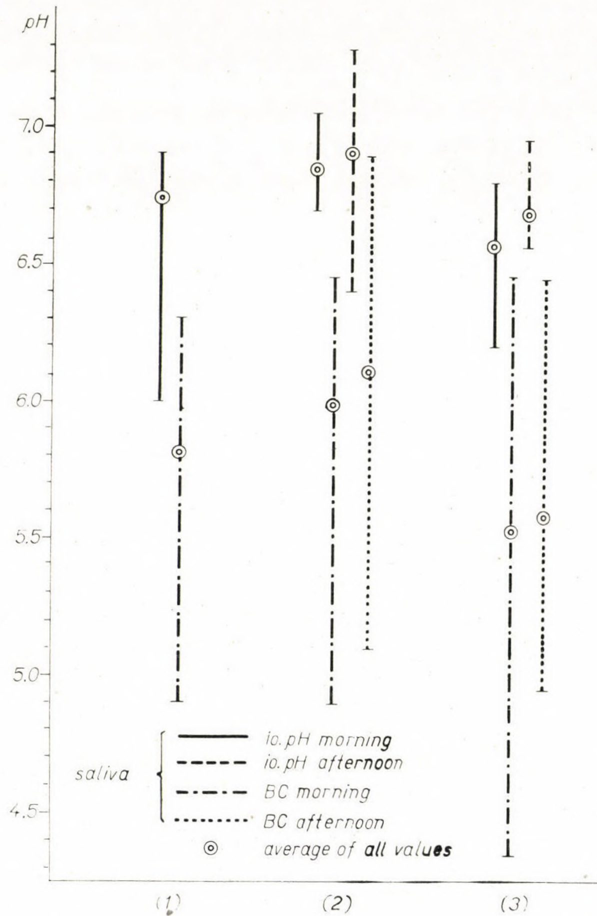

Fig. 1. Relation of intraorally determined saliva-pH and saliva buffering capacity (BC). 1962 early March values. (1) Averages of six young women control cases — caries free. (2) Averages of five selected caries free cases. (3) Averages of five selected multiplex caries cases

Results

1. The data for oral salivary pH and buffer capacity are presented in Table I (a, b and c), with the means for each group shown in Fig. 1. The morning values were similar in the caries-free and caries-resistant groups, The latter yielded somewhat lower figures.

Table I/a

Selected caries-free cases

Saliva pH and buffer capacity (B) values

Case No.	Age yrs.	Date 1962.	pH		BC	
			m.°	a.+	m.	a.
1	14	3.12.	6.80	6.70	5.35	5.60
		3.14.	6.75	6.96	5.06	5.10
		3.15.	6.90	6.80	4.90	5.10
		average	6.81	6.79	5.10	5.27
2	15	3. 12.	6.80	6.40	6.35	5.60
		3. 14.	6.85	6.85	6.45	6.25
		3. 15.	6.85	7.00	6.20	6.90
		average	6.83	6.75	6.33	6.23
3	10	3. 14.	6.80	7.05	5.85	6.20
		3. 15.	7.05	7.10	6.45	6.50
		3. 19.	6.85	7.00	6.20	6.90
		average	6.90	7.05	6.17	6.53
10	13	3. 12.	6.85	6.80	6.35	6.20
		3. 14.	7.00	6.90	6.30	6.50
		3. 15.	6.95	7.30	6.25	6.80
		average	6.93	7.00	6.30	6.50
14	15	3. 12.	6.75	6.90	6.30	5.70
		3. 14.	6.70	6.90	5.90	6.30
		3. 15.	6.80	7.15	6.00	6.20
		average	6.75	6.98	6.06	6.06
all 5 cases average			*6.85*	*6.91*	*5.99*	*6.11*
all 5 cases max. value			7.05	7.30	6.45	6.90
all 5 cases max. value			6.70	6.40	4.90	5.10

Abbr. here and in following tables:
○ = morning (before breakfast)
\+ = afternoon (after early dinner shortly)

The values for the caries-free group were significantly different from those for the caries group. (See below.)

2. Oxygen consumption at 10, 30, 60 and 120 minutes in the three groups are shown in Table II (a, b, c).

In Fig. 3 the log changes of oxygen consumption are plotted against time. Significant are the high values found at 30 and 60 minutes in the caries

Table I/b

Selected multiplex caries cases

Saliva pH and BC values

Case No.	Age yrs.	Date 1962.	pH		BC	
			m.	a.	m.	a.
4	14	3. 6.	6.60	6.80	5.05	5.10
		3. 7.	6.65	6.80	4.60	5.25
		3. 8.	6.65	6.70	5.80	6.40
		average	6.63	6.76	5.15	5.58
11	14	3. 6.	6.50	6.70	6.00	5.06
		3. 7.	6.80	6.75	5.55	5.90
		3. 8.	6.70	6.60	6.00	5.35
		average	6.66	6.68	5.85	5.43
15	14	3. 6.	6.45	6.70	5.86	6.25
		3. 19.	6.40	—	—	—
		3. 20.	6.50	—	5.70	—
		average	6.45	6.70	5.78	6.25
18	14	3. 6.	6.60	6.95	5.15	6.45
		3. 7.	6.70	6.80	5.80	5.65
		3. 8.	6.80	6.50	6.45	5.05
		average	6.70	6.75	5.80	5.71
19	13	3. 6.	6.20	6.60	4.35	4.95
		3. 7.	6.75	—	5.15	—
		3. 8.	6.50	6.65	5.60	5.52
		average	6.48	6.62	5.03	5.23
all 5 cases average			*6.58*	*6.70*	*5.52*	*5.58*
all 5 cases max. value			6.80	6.95	6.45	6.45
all 5 cases min. value			6.20	6.50	4.35	4.95

group, as well as the marked decrease of the afternoon values in the caries-free group.

In the caries-resistant group, oxygen consumption was considerably lagging, like in the caries-free group, whereas in the morning values of the caries group the increase was faster.

3. The data for acid production are presented in Table III (a, b and c). In the caries-free group, in addition to the colorimetric (Snyder) value, the actual pH was also determined. As to the colorimetric Snyder values, one cross (+, predominantly green) was found to correspond to pH 4.3 to 4.6;

Table I/c

Young vomen group, all caries-free

Saliva pH and BC values

Case No.	Age yrs.	Date 1962.	pH m.	BC m.
1	20	2. 23.	6.75	6.30
		2. 26.	6.90	5.75
		2. 27.	6.60	5.10
		average	6.75	5.71
2	24	2. 23.	6.80	5.50
		2. 24.	6.90	5.75
		2. 26.	6.60	5.10
		average	6.76	5.45
3	26	2. 23.	6.75	5.30
		2. 24.	6.75	6.10
		2. 26.	6.50	4.90
		average	6.66	5.43
4	25	2. 23.	6.60	5.90
		2. 24.	6.60	6.25
		2. 26.	6.50	6.10
		average	6.56	6.08
5	33	2. 23.	6.90	6.10
		2. 24.	6.80	6.15
		2. 26.	6.90	6.10
		average	6.86	6.11
6	26	2. 23.	6.90	6.10
		2. 26.	6.90	6.10
		2. 27.	6.85	6.30
		average	6.88	6.16
all 5 cases average			*6.74*	*5.82*
all 5 cases max. value			6.90	6.30
all 5 cases min. value			6.50	5.10

two crosses (++, yellow, yellowish-green) to pH 4.3—4.1; three crosses (+++, yellowish green) to pH 3.9—3.3; four crosses (++++, yellow) to pH values lower than 3.3 (Table IV).

Table II/a

Selected caries-free cases

Saliva oxygen consumption — morning values. Values in mm³

Case No.	Age yrs	Date: 1962.	Minutes			
			10	30	60	120
1	14	3. 12.	7.4	52.8	89.9	169.7
		3. 14.	24.1	65.7	92.7	197.5
		3. 15.	20.0	49.4	91.4	185.0
		4. 2.	24.1	70.5	118.7	233.5
		4. 3.	27.8	46.4	76.9	190.2
		average	20.6	56.8	93.9	193.2
2	15	3. 12.	12.6	25.3	57.9	125.0
		3. 14.	13.7	22.1	40.0	135.6
		3. 15.	12.0	17.6	61.2	174.5
		4. 2.	19.0	26.4	40.0	69.4
		4. 3.	5.3	22.1	35.8	90.4
		average	12.5	22.7	47.0	119.0
3	10	3. 14.	27.2	84.1	145.1	303.5
		3. 15.	12.4	20.6	69.2	226.8
		3. 19.	15.8	39.9	69.7	177.2
		4. 2.	5.0	16.6	24.8	51.2
		4. 3.	12.4	23.1	45.3	142.6
		average	14.5	36.8	70.8	180.2
10	13	3. 12.	15.7	37.9	75.9	203.7
		3. 14.	27.1	87.4	154.6	275.4
		3. 15.	10.5	17.5	61.0	191.0
		4. 2.	6.2	24.4	41.0	110.6
		4. 3.	26.1	36.7	87.3	217.7
		average	17.1	40.7	83.9	199.6
14	15	3. 12.	65.4	166.7	274.4	525.1
		3. 14.	27.5	94.5	137.1	168.8
		3. 15.	28.9	63.4	181.2	361.5
		4. 2.	18.0	62.2	109.9	198.8
		4. 3.	46.6	59.4	94.5	202.3
		average	37.2	89.2	159.4	291.3
all 5 cases average			*20.4*	*49.2*	*91.0*	*196.6*
all 5 cases max. value			65.4	166.7	274.4	525.1
all 5 cases min. value			5.0	16.6	24.8	51.2

Table II/b

Selected multiplex caries cases

Saliva oxygen consumption — morning values. Values in mm³

Case No.	Age yrs.	Date: 1962.	Minutes			
			10	30	60	120
4	14	3. 6.	8.3	35.2	68.6	119.6
		3. 7.	9.1	37.9	74.2	133.6
		3. 8.	19.5	73.3	173.4	261.6
		average	12.3	48.8	105.2	171.6
11	14	3. 6.	28.4	75.7	147.0	260.0
		3. 7.	49.7	168.9	288.5	451.5
		3. 8.	22.1	84.6	232.0	331.1
		average	33.4	109.7	222.5	347.5
15	14	3. 6.	17.4	53.6	103.1	179.8
		3. 19.	18.3	52.2	100.2	143.7
		3. 20.	30.6	93.2	186.3	335.9
		average	22.1	66.3	129.8	219.8
18	14	3. 7.	45.5	119.6	181.3	287.4
		3. 8.	28.9	58.8	272.2	519.5
		3. 19.	14.8	41.2	78.3	211.8
		average	29.7	73.2	177.2	339.5
19	13	3. 6.	6.8	24.6	40.4	71.7
		3. 7.	18.6	46.4	77.9	120.6
		3. 8.	14.9	48.0	102.7	160.2
		average	13.4	39.7	73.7	117.5
all 5 cases average			*22.1*	*67.5*	*141.7*	*239.2*
all 5 cases max. value			49.7	168.9	288.5	519.5
all 5 cases min. value			6.8	24.6	40.4	71.7

Discussion

The results obtained have indicated that characteristic results can be ensured by a suitable selection of caries-free and caries-affected subjects from a large material. Estimation of oral salivary pH seems to be more reliable than examination of the saliva *in vitro*. Owing to the selection of cases, the values within one group are closely similar, whereas the means of the two experimental groups (caries-free and multiple caries) differ from one another significantly.

Table II/c

Young women group, all caries-free

Saliva oxygen consumption — morning values. Values in mm³

Case No.	Age yrs.	Date: 1962.	Minutes 10	30	60	120
1	20	2. 23.	24.9	41.8	66.2	197.2
		2. 26.	19.5	40.8	80.6	223.5
		2. 27.	17.5	62.4	108.3	209.7
		average	20.6	48.3	85.0	210.1
2	24	2. 23.	30.1	57.0	97.7	221.3
		2. 24.	3.2	6.4	16.9	28.4
		2. 26.	20.0	31.6	56.8	162.0
		average	17.7	31.7	57.1	137.2
3	26	2. 23.	25.6	57.4	101.4	240.1
		2. 24.	9.1	23.1	58.6	153.6
		2. 26.	28.8	44.5	100.6	231.0
		average	21.2	41.7	86.9	208.2
4	25	2. 23.	47.9	138.8	248.9	505.6
		2. 24.	11.3	32.2	69.6	165.0
		2. 26.	49.6	147.0	249.6	501.0
		average	36.2	106.0	189.3	390.5
5	33	2. 23.	11.3	16.4	18.2	41.9
		2. 24.	4.8	10.2	32.9	92.4
		2. 26.	2.0	10.4	18.0	53.7
		average	6.0	12.3	23.0	62.7
6	26	2. 23.	31.8	81.7	153.8	324.7
		2. 24.	23.5	50.8	114.8	263.4
		2. 27.	14.5	44.2	69.3	164.0
		average	23.2	58.9	112.6	250.7
all 6 cases average			*20.8*	*50.8*	*92.3*	*209.9*
all 6 cases max. value			49.6	147.0	249.6	505.6
all 6 cases min. value			2.0	6.4	16.9	28.4

In the present investigations we have undertaken to reproduce our earlier studies, on the one hand, and to supplement them with the afternoon values, on the other.

We have also made comparative studies of a group composed of caries-resistant subjects and of the caries-free group.

Table II/d

Selected caries-free cases

Saliva oxygen consumption — afternoon values. Values in mm^3

Case No.	Age yrs.	Date: 1962.	Minutes			
			10	30	60	120
1	14	3. 12.	21.3	56.6	95.5	166.0
		3. 14.	12.0	24.1	42.7	109.5
		3. 15.	13.0	18.4	42.6	86.2
		average	15.4	33.0	60.2	120.5
2	15	3. 12.	6.4	18.0	25.3	55.7
		3. 14.	6.8	13.7	26.4	42.1
		3. 19.	5.0	10.7	29.8	90.7
		average	6.0	14.1	27.1	62.8
3	10	3. 14.	11.5	28.9	65.9	174.8
		3. 15.	6.6	16.5	38.8	73.5
		3. 19.	7.4	13.0	31.6	77.4
		average	8.5	19.4	45.4	108.5
10	13	3. 12.	8.3	16.6	34.7	75.0
		3. 14.	13.2	32.3	61.0	139.2
		3. 15.	3.5	15.7	34.1	101.2
		average	8.3	21.5	43.2	105.1
14	15	3. 12.	7.0	31.4	71.4	112.3
		3. 14.	12.4	27.2	56.3	146.8
		3. 15.	28.3	64.3	152.8	239.8
		average	15.9	40.9	93.5	166.3
all 5 cases average			*10.8*	*25.7*	*54.0*	*112.6*
all 5 cases max. value			28.3	64.3	152.8	239.8
all 5 cases min. value			3.5	10.7	25.3	42.1

A comparison of the last year results with the present ones has shown very little differences in the pH and buffer capacity values of the caries-free group, whereas in the caries group buffer capacity was significantly (mean: 1.54), the oral pH somewhat (mean: 0.2), higher.

This finding indicates that early in March of this year the carious saliva contained more inorganic substances than last May. It may be assumed that the difference was due to a seasonal change similar to that observed by one of us [2] a few years ago regarding the F content of saliva. A comparison of the individual values in the caries group obtained in two years (Table VI)

Table II/e

Selected multiplex caries cases

Saliva oxygen consumption — afternoon values. Values in mm^3

Case No.	Age yrs.	Date: 1962.	Minutes			
			10	30	60	120
4	14	3. 6.	24.1	40.8	86.3	168.8
		3. 7.	18.6	25.4	68.4	156.1
		3. 8.	17.7	42.3	86.3	178.5
		average	20.1	36.1	80.3	167.8
11	14	3. 6.	17.9	24.3	59.1	121.5
		3. 7.	23.1	55.3	158.8	402.2
		3. 8.	15.8	37.5	83.4	220.6
		average	18.9	39.0	100.1	248.1
15	14	3. 6.	31.3	60.2	126.2	203.8
		3. 20.	33.9	133.8	207.5	460.5
		average	32.6	97.0	166.8	332.1
18	14	3. 6.	25.3	53.2	136.7	278.4
		3. 7.	24.3	39.1	92.0	250.1
		3. 8.	4.9	10.7	26.4	75.9
		average	18.1	34.3	85.0	201.4
19	13	3. 6.	15.8	24.5	68.0	141.6
		3. 8.	22.6	58.3	109.6	166.2
		average	19.2	41.4	88.8	153.9
all 5 cases average			*21.8*	*49.6*	*104.2*	*220.6*
all 5 cases max. value			33.9	133.8	207.5	460.5
all 5 *cases* min. value			4.9	10.7	26.4	75.9

reveals that all five cases behaved similarly. The example of case No. 15 makes it clear that buffer capacity may increase without an increase in salivary pH, but, like in the caries-free group, higher buffer capacity values were mostly associated with higher pH values. One of the 5 individuals of the caries-free group (Table I, No. 14) has developed caries. We did not exclude him from the study, having already had to replace two other members in that group. This subject last year had a somewhat higher pH value (7.05), and approximately the same buffer capacity value as this year.

Last year, when we determined the pH changes *in vitro*, Case No. 14 yielded the greatest difference between the 60 minute values measured orally

Table III

Acid-production of the saliva

(With the mod. Snyder-method)
a) Selected caries-free group (boys)

Case No.	Age yrs.	First determ. (3. 12. 1962)		Second determ. (3. 14. 1962)		Third determ. (3. 15. 1962)	
		24 hrs.	48 hrs.	24 hrs.	48 hrs.	24 hrs.	48 hrs.
1	14 m. *a)*	0	0	0	+	0	+
	b)	0	0	0	+	0	+
	a. *a)*	0	+	0	++	0	++
	b)	0	+	0	++	0	++
2	15 m. *a)*	0	+	0	+	0	+
	b)	0	+	0	+	0	+
	a. *a)*	0	++	0	+	0	+
	b)	0	++	0	+	0	+
3	10 m. *a)*	0	0	0	+	0	0
	b)	0	0	0	+	0	0
	a. *a)*	0	0	0	0	0	0
	b)	0	0	0	0	0	0
10	13 m. *a)*	0	++	0	+	0	0
	b)	0	++	0	+	0	0
	a. *a)*	0	0	0	0	0	+
	b)	0	0	0	0	0	+
14	15 m. *a)*	0	+	0	++	0	0
	b)	0	+	0	++	0	0
	a. *a)*	0	0	0	+	0	++
	b)	0	0	0	+	0	++
Average of crosses		0	0.7	0	1.0	0	0.8

and *in vitro* (diff.: 0.60 pH, Table VII). Both now and last year, this subject yielded high initial oxygen consumption values, indicating the presence of oxygen-consuming bacteria, and this value increased steeply, reaching a record height at 120 minutes (Table II a, No. 14). Retrospectively, the phenomenon seems to have been a sign of caries-activity.

As to the relationship between pH and buffer capacity, in Table I a, b, c and Fig. 2, the pH was plotted against buffer capacity. Single parts of the different groups are overlapping, but show a clearly discernible tendency in the means. Thus for example the mean morning values of the caries-resistant group are closely similar to those for the caries-free group and are different from the afternoon values of the caries group. The changes from the morning means of the caries group (left, below) to the afternoon means of the caries-

Table III

Acid-production of the saliva

(With the mod. Snyder-method)
b) Selected multiplex-caries-cases (boys)

Case No.	Age yrs.	First determ. (3. 6. 1962)		Second determ. (3. 7. 1962)		Third determ. (3. 8. 1962)	
		24 hrs.	48 hrs.	24 hrs.	48 hrs.	24 hrs.	48 hrs.
4	14 m. *a)*	0	+	+	+	+	+++
	b)	0	+	+	+	+	+++
	a. *a)*	0	+	+	+	0	+++
	b)	0	+	+	+	0	+++
11	14 m. *a)*	0	++	+	+++	+	+++
	b)	0	++	+	+++	+	+++
	a. *a)*	0	+++	0	+++	0	++
	b)	0	+++	0	+++	0	++
15	14 m. *a)*	0	+++	0	0	0	++
	b)	0	+++	0	0	0	++
	a. *a)*	0	++++	0	+++	—	—
	b)	0	++++	0	+++	—	—
18	14 m. *a)*	0	++	0	+	0	+
	b)	0	++	0	+	0	+
	a. *a)*	0	+++	0	+++	0	++
	b)	0	+++	0	+++	0	++
19	13 m. *a)*	0	+++	+	+++	0	++
	b)	0	+++	+	+++	0	++
	a. *a)*	0	+++	—	—	0	+
	b)	0	+++	—	—	0	+
Average of crosses		0	2.5	0.4	2.0	0.2	2.1

free group (right, above) are continuous and thus indicative of a wide range of diurnal and individual variations.

This leads us to the problem of afternoon values, where a tendency "to be more caries-free" seemed to exist. Here the values deviate to the right and upward, with smaller variations in the caries group, and a wider range of variations in the caries-free group. This means that the pH is higher and buffer capacity is greater in the afternoon than in the morning. We are planning to investigate this problem in a large selected material.

The variations of the morning pH were small in both caries-free groups (Table I a and c), and greater in the caries group. In all three groups the buffer capacity varied over a wide range, but there were three individuals in the caries-resistant group showing closely similar buffer capacity values

Table III

Acid-production of the saliva

(With the mod. Snyder-method)
c) Young women group, all caries-free
(only morning values)

Case No.	Age yrs.	First determ. (2. 23. 1962)		Second determ. (2. 24. 1962)		Third determ. (2. 26. 1962)	
		24 hrs.	48 hrs.	24 hrs.	48 hrs.	24 hrs.	48 hrs.
1	20 *a)*	0	0	0	0	0	+
	b)	0	0	0	0	0	+
2	24 *a)*	0	0	0	0	0	0
	b)	0	0	0	0	0	0
3	26 *a)*	+	++	0	+	0	+
	b)	+	++	0	+	0	+
4	25 *a)*	0	0	0	0	0	0
	b)	0	0	0	0	0	0
5	33 *a)*	0	++	0	++	0	++
	b)	0	+++	0	++	0	++
6	26 *a)*	0	+	0	+	0	+
	b)	0	+	0	+	0	+
Aver. of crosses		0.1	0.9	0	0.7	0	0.8

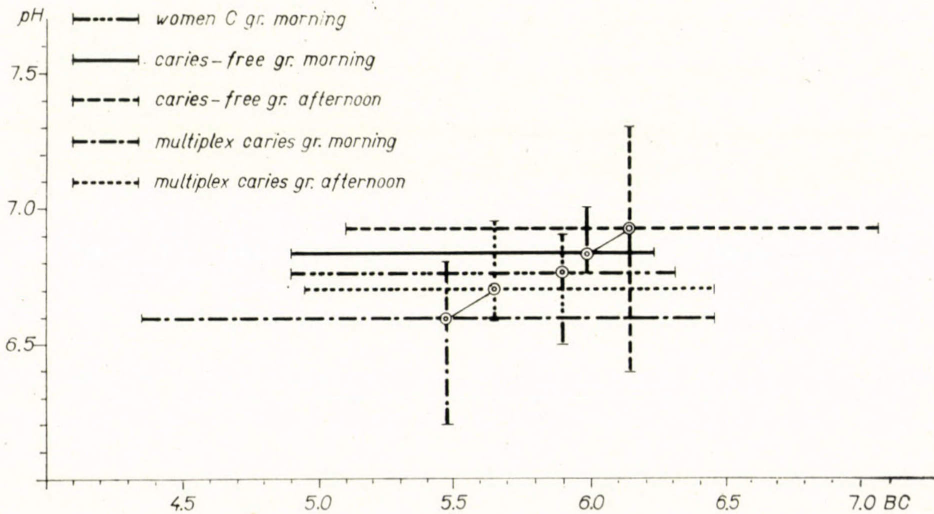

Fig. 2. Saliva pH and buffering capacity (BC). 1962 early March values. Group-averages (·) and maximal and minimal values

Table IV

Saliva acid production

Caries-free group (boys)

Comparative determination of the pH and colour-change with the mod. Snyder-method (only morning values)

Case No.	Age yrs.	Colour				pH			
		first determ. (2. 4. 1962)		second determ. (3. 4. 1962)		first determ. (2. 4. 1962)		second determ. (3. 4. 1962)	
		24 hrs.	48 hrs.	24 hrs.	48 hrs.	24 hrs.	48 hrs.	24 hrs.	48 hrs.
1	14 *a)*	+	+	+	+	4.45	4.40	4.40	4.30
	b)	+	++	+	++	4.45	4.20	4.45	4.20
2	15 *a)*	+	+	++	+++	4.60	4.30	4.20	3.90
	b)	+	+++	++	+++	4.60	3.90	4.10	3.70
3	10 *a)*	0	0	0	0	4.85	4.80	4.80	4.75
	b)	0	0	0	+	4.85	4.85	8.80	4.70
10	13 *a)*	0	0	0	+	4.85	4.80	4.75	4.45
	b)	0	+	0	+	4.85	4.50	4.60	4.45
14	15 *a)*	+	+	+	++	4.45	4.40	4.45	4.20
	b)	+	++	+	++	4.45	4.35	4.30	4.25
Average of crosses viz. pH-values		0.6	1.1	0.8	1.6	4.64	4.45	4.48	4.29

Table V

Saliva pH and BC values of caries-free and multiplex caries-groups

Comparison between 1961 and 1962 data. Group average morning values.

	Caries-free group			Multiplex caries group		
	1961	1962	Diff. 1961–1962	1961	1962	Diff 1961–1962
pH	6.915	6.830	—0.085	6.375	6.580	+0.205
BC	6.075	5.980	—0.095	3.990	5.520	+1.530
Diff. pH—BC	—0.840	—0.850	—0.010	—2.385	—1.060	+1.325

Table VI

Selected multiplex caries group : pH and BC data

Comparison 1961—1962

No.	Sex	Age	1961* pH	1962** pH	pH diff. 1961–1962	1961+ BC	1962++ BC	BC diff. 1961–1962
4	♂	13—14	6.55	6.63	+0.08	3.60	5.15	+1.55
11	♂	13—14	6.37	6.66	+0.29	4.38	5.85	+1.47
15	♂	13—14	6.45	6.45	0.00	4.78	5.78	+1.00
18	♂	13—14	6.37	6.70	+0.33	4.02	5.80	+1.78
19	♂	12—13	6.13	6.48	+0.35	3.18	5.03	+1.85
Average			6.37	6.58	+0.21	3.99	5.52	+1.53

* Averages of 2 following days intraoral pH-determinations (3 parallels); late May 1961, morning values.

** Averages of 3 following days intraoral pH-determinations (3 parallels); early March 1962, morning values.

\+ As*, only in vitro BC-determinations.

++ As**, only in vitro BC-determinations.

on three consecutive mornings (Cases No. 4, 5 and 6, Table I, c). Mechanisms and significance of this phenomenon are unclear; they might perhaps indicate a regularity or stability of saliva secretion.

The 6 resistant female subjects selected at random represented two groups as regards salivary pH and buffer capacity. In cases 4, 5 and 6 the buffer values were similar and the pH values slightly lower than the afternoon values in the caries-free group. The other 3 resistant subjects yielded different results. Thus, the means calculated from all the 6 cases may be misleading; for this reason do we stress the importance of separate and extensive individual studies. There was a difference in the after-lunch values of the pH and the buffer capacity between the caries-free and caries-active groups. The means of the caries-free group were somewhat higher (0.1 pH), but the values varied over a wider range. An opposite phenomenon was observed in the caries-active group, in which the morning values of pH and buffer capacity showed wider ranges of variation. In this group, too, like in the caries-free group, the means were somewhat higher (Fig. 2).

Salivary O_2 consumption. All the data are included in Table II, a, b, c. Last year we at first determined the 60-minute values only; subsequently, and in the present studies in order to study the dynamism of the process, we determined the 10, 30 and 120-minute values as well. In Fig. 3 the log values for the three groups have been plotted against time. The morning means of the caries-free group and the afternoon means of the caries group were at about the same level. The afternoon values of the caries-free group

were lower at all four points of time, while the 30 and 60-minute means of the caries group were higher.

It would be difficult to interpret this phenomenon at the present stage of our investigations. Among the possible factors influencing it are the dif-

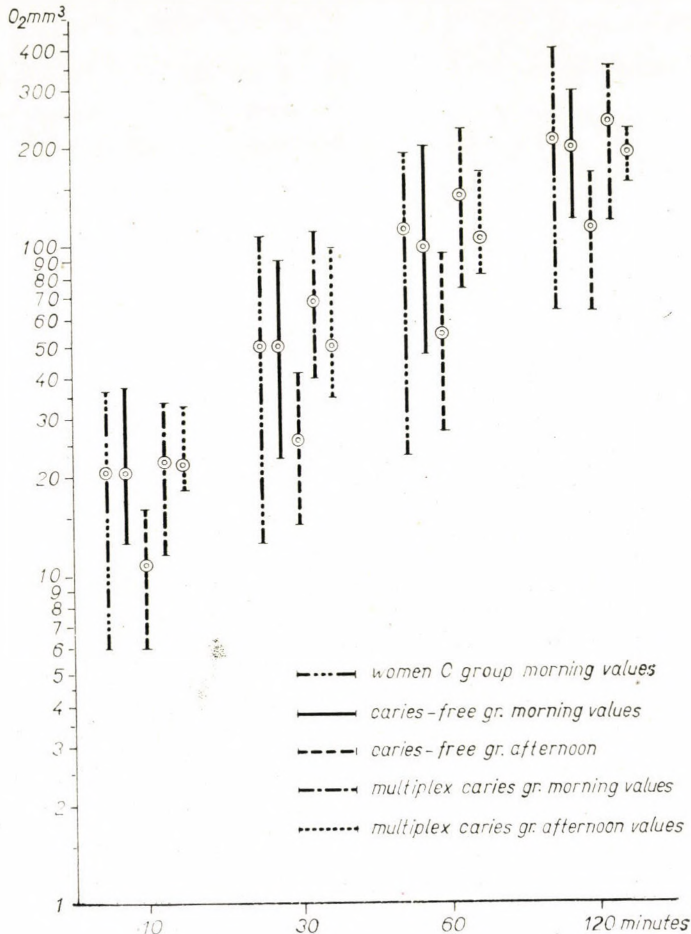

Fig. 3. Saliva oxygen consumption. 1962 early March values. Three days' group averages (·) and maximal and minimal values

ferences in the nature of bacteria in the different groups, differences in their number, differences in the saliva as a "culture medium", and the potential presence of stimulators or inhibitors of bacterial growth. From our data no conclusions can be drawn as to the responsible factor. Most likely is the presence of a factor inhibiting bacterial growth, but a difference in the O_2 requirement of the bacteria (OCR) might also exist (Fig. 4).

Such factors have been described by several authors. In our case it may indirectly be surmised that one or several factors inhibiting bacterial growth are present. (The ratio of the 10′ O_2 consumption vs. the 30′, 60′ or 120′ values (OCR) shows the increment of O_2 consumption.) As regards morning values, in the caries group the OCR was higher in all three cases,

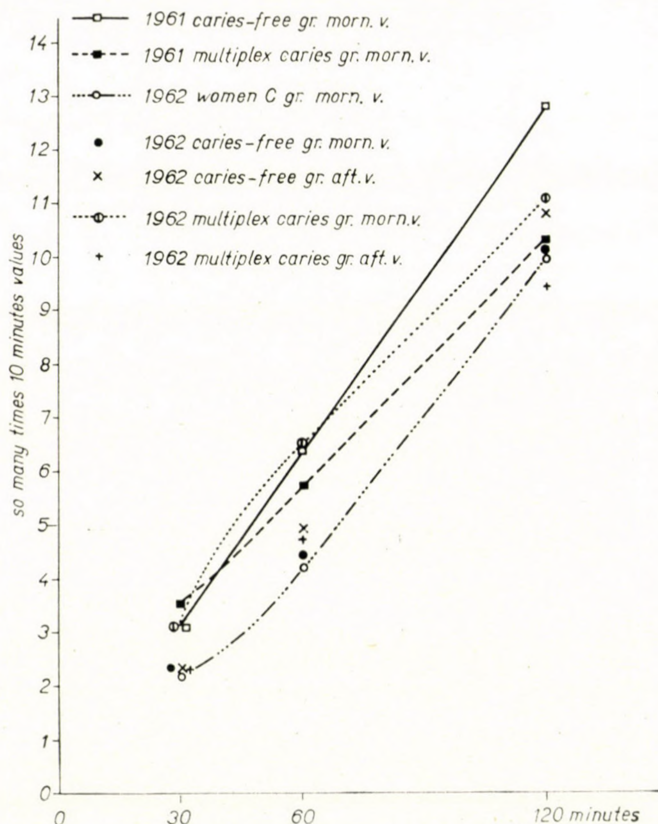

Fig. 4. Comparison of oxygen consumption rates of salivas of different groups. 1962 early March 3 days average and maximal and minimal values

than in the caries-free and caries-resistant groups. On the other hand, the afternoon values of the caries-free group showed a tendency to increase, while those of the caries group tended to decrease.

A tendency to an increase of the OCR is detectable in the afternoon values of the caries-free group, too, but this is associated from the beginning with a low oxygen consumption, which might mean a low bacterial count. If we compare the 1961 and 1962 OCR values (Fig. 5), the above mentioned "inhibitory" effect is found in the 1962 caries-free, the caries-resistant and

the afternoon values of the caries-active groups. It is not clear why this effect was lacking in the morning values of the 1961 caries-free group.

As determined by SNYDER's method, salivary acid production was high in the caries group (Table III, Table IV). This, however, cannot serve as a reliable caries index in individual cases. For example, cases No. 1, No. 2 of

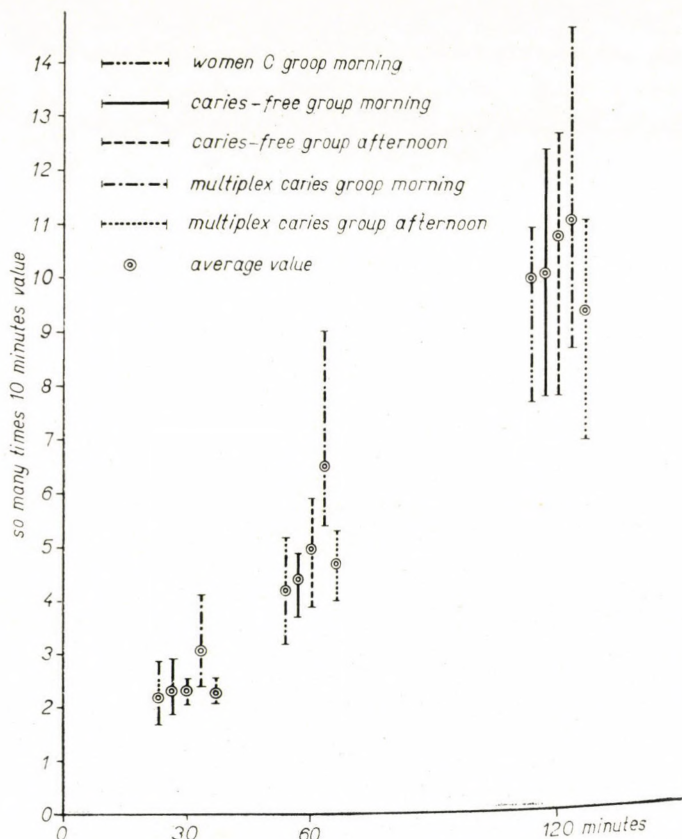

Fig. 5. Saliva oxygen consumption average values. Comparison between 1961 late May and 1962 early March values

Table IV, and No. 3 and No. 6 of Table III were caries-free and produced much acid but did not show the differences in pH and buffer capacity indicated in Table I and Table II.

On the other hand, the caries-active cases No. 4 and No. 15 (Table III, b) yielded extremely low values in one or more tests. For this reason several determinations should be made at different points of time. The indicator colour often varies; this is another difficulty of colorimetric evaluation. We therefore suggest the use of a fluid medium and determination of the pH parallel with the colorimetric estimation (Table IV).

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Dezső Hattyasy, Imre Szabó, Károly Tóth
Orvostudományi Egyetem Fog- és Szájbeteg Klinikája, Szeged.

Pharmacologia

EFFECT OF PROTAMINE SULPHATE ON THE TRANSMISSION PROCESSES IN PERIPHERAL SYMPATHETIC GANGLIA

By

E. MINKER and M. KOLTAI

INSTITUTE OF PHARMACOLOGY, MEDICAL UNIVERSITY, SZEGED

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The effect of protamine sulphate having polycationic properties on the transmission processes of the cat's superior cervical ganglion has been investigated. It has been observed that — under certain conditions — the compound even by itself inhibits synaptic transmission and increases synergetically the effect of other ganglionic blocking substances. The effect of the material examined is antagonized by potassium salts. Trimethylpolyanthinium iodide, a polypeptide with polycationic character, also behaves like protamine sulphate. The fact that the above compounds have different chemical structures and only one common characteristic, *i.e.* their polycationic property, suggests that they inhibit — in consequence of their charges rather than because of their configuration — the transmission processes as they are deposited at the receptor areas in the interneuronal synapses of the superior cervical ganglion.

Introduction

In the course of earlier investigations concerning synaptic transmission it was observed that heparin, a polyanion, influenced in a particular manner the susceptibility to ganglionic blocking agents of the synaptic structures [12]. In cats pretreated with heparin about one half hour after administration of the drug, ganglionic blocking substances, even in larger quantities than the usual doses, cannot permanently prevent synaptic transmission, and, after a blockade lasting not more than a few minutes, the synapses show a constantly increasing transmission of preganglionic stimuli. At first, only the size of the contractions increases and later on — when their amplitude reaches the original size — their duration also begins to increase.

The heparin molecule represents a considerable negative charge and exerts its coagulation-inhibiting effect owing to this charge. It was assumed that if the above described ganglionic effect of heparin is related to the negativ charge of the molecule then the polycations with the opposite charge ought to support the effect of ganglionic blocking agents. In the course of the experiments it has been found that protamine sulphate (PS), which has polycationic properties, on the one hand increases synergetically the effect of other ganglionic blocking agents and, on the other, prevents even by itself the synaptic function.

Methods

The experiments were carried out on cats anaesthetized by a mixture of chloralose and urethane. The investigated substances were administered through a cannula inserted either into the femoral vein or, for intraarterial administration, into the lingual artery. At the beginning of the experiments the superior cervical ganglion and its pre- and postganglionic fibres were isolated, carefully preserving the blood supply of the ganglion, and the common carotid artery and its side-branches were ligated above the ganglion.

For stimulation square-wave stimuli 0,5 Volt at a frequency of 15/sec were used.

It should be noted that the effect of PS was also investigated *in situ.* After preparing the superior cervical ganglion and the pre- and postganglionic fibres of the cervical sympathetic trunk, into the slit formed by the deep muscles of the neck surrounding the ganglion PS was administered as an organ bath. Following development of the blocking effect, part of the PS was sucked off and the slit was washed with isotonic NaCl solution.

In the course of the experiments, the pre- and postganglionic fibres of the cervical sympathetic trunk were stimulated. The synaptic function was indicated by the movements of the nictitating membrane; these were recorded electronically by means of THURÁNSZKY and BALASSY's apparatus [14]. During the experiments the blood pressure of the femoral artery was continuously recorded by means of an electromanometer.

Results

After intraarterial administration a 10 mg dose of PS *(Hoffmann—La Roche*, Basel), isotonized and warmed to body temperature, completely prevented transmission in the interneuronal synapses of the superior cervical

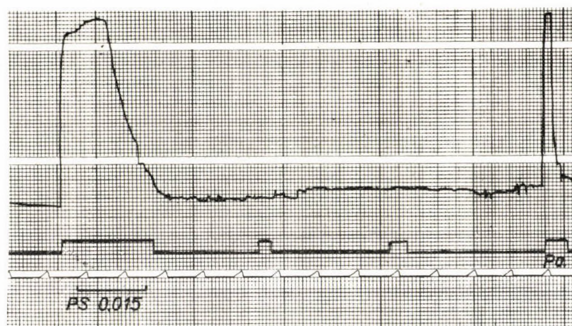

Fig. 1. Contractions of the nictitating membrane after PS administration. Marks: On the base line: preganglionic stimulation. Below: injcetion of drug. Po: postganglionic stimulation. Time trace: 1 minute

ganglion. The continuous contraction of the nictitating membrane elicited by electrical stimulation of the preganglionic fibres of the cervical sympathetic trunk ceased parallel to the injection of PS; at the same time, the effect of postganglionic stimulation was unchanged (Fig. 1).

This phenomenon could not be elicited by the intravenous administration of a 1 per cent solution of PS.

Since earlier we have found that the effect of ganglionic blocking substances can be compensated by potassium salts [8, 9], now it was attempted to pre-

vent the PS-induced ganglionic blockade by potassium. After transmission had been inhibited by intraarterial PS administration, intravenous infusion of a 2 per cent solution of KSCN was started and following the infusion of 0.04—0.05 g/kg preganglionic stimulation was performed every two minutes. The con-

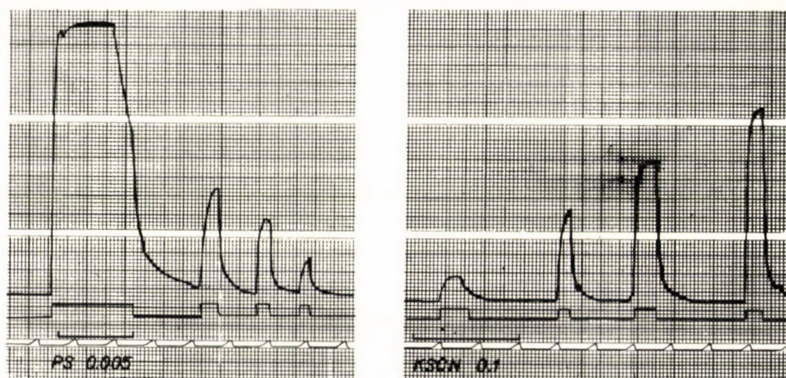

Fig. 2. Contractions of the nictitating membrane after intraarterial PS administration followed after 23 minutes by intravenous KSCN infusion. The transmission inhibited by PS is restored under the effect of KSCN. Notations as in Fig. 1

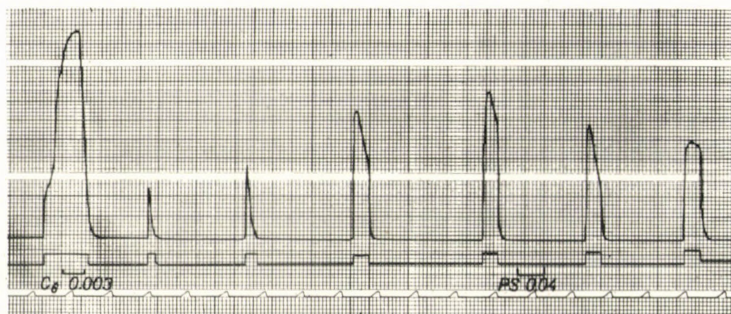

Fig. 3. Male cat, 3.30 kg. Transmission temporarily blocked by 0,003 g of intravenous hexamethonium. Subsequently the synapses can be stimulated periodically and preganglionic stimulation results in gradually increasing contractions of the nictitating membrane. Following the intravenous injection of 0.04 g of PS, the size of the contractions elicited by preganglionic stimulation decreases, showing the reduced transmission of the synapses and the increasing effect of the ganglionic blocking agent. Notations as in Fig. 1

tractions of the nictitating membrane were steeply increasing in size, pointing to a rapid enhancement of synaptic transmission (Fig. 2).

PS injected intravenously enhanced the effect of other ganglionic blocking substances, such as hexamethonium, d-tubocurarine, trimethaphane. This effect was mainly observed after a dose of the ganglionic blocking agents

causing transitional paralysis. If the injection of PS was started just when the effect of the ganglionic blocking compound was disappearing, after a PS dose of 0.015—0.02 g/kg the nictitating membrane contractions elicited by preganglionic stimulation began to diminish (Fig. 3).

The combined effect of ganglionic blocking agents and PS was similarly antagonized by potassium salts.

The ganglionic effect of PS was investigated also *in situ*. If the PS was injected into the slit prepared around the superior cervical ganglion, the

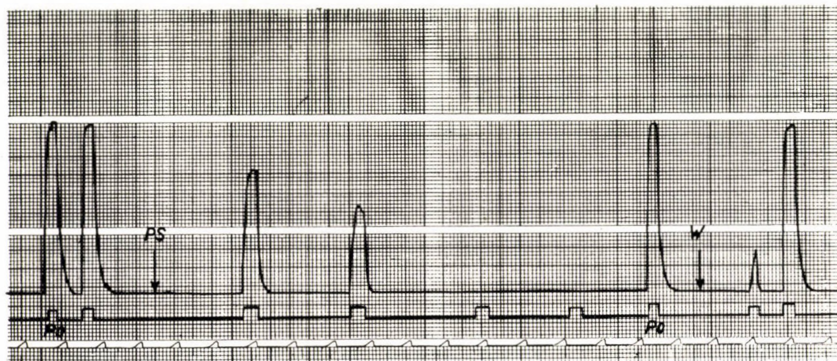

Fig. 4. Effect of PS in situ. PO: postganglionic stimulation. W: wash. For the experimental conditions, see in the text. Notations as in Fig. 1. Time trace: 1 minute

size of the nictitating membrane contractions slowly decreased and then the contractions ceased (Fig. 4). When PS has been sucked off and the environment of the ganglion was repeatedly washed with isotonic NaCl solution, synaptic transmission was resumed. Repeated application of PS caused a further paralysis. Preganglionic stimulation then became uneffective, whereas postganglionic stimulation resulted in a contraction of the membrane; this excluded the possibility of a local anaesthetic action. Otherwise, PS has no local anaesthetic effect, because dropped into the guinea pig's eye it does not prevent the corneal reflex. Under conditions *in situ*, too, the synergism between PS and the other ganglionic blocking agents, *e.g.* hexamethonium, could be demonstrated. Concerning the experiments *in situ*, it should be mentioned that the blocking effect of PS was not so consistent as after intra-arterial administration.

The effect of PS on blood pressure was most pronounced. In this respect, heparin behaves in a similar manner [17]. There is, however, an essential difference between the effect of the two substances on blood pressure. While on the administration of heparin the fall of blood pressure ensues gradually and after a certain period, doses of 0.015 g/kg of PS already cause a sudden and sometimes lethal hypotension, particularly following pre-treatment with

such a small dose (0.001 g/kg) of hexamethonium which induces only a temporary fall of blood pressure (Fig. 5).

Experiments carried out on rats suggested that the synergism between hexamethonium and PS was concerning not only their ganglionic effect but also their toxicity.

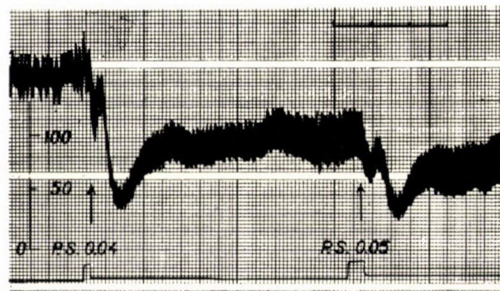

Fig. 5. Male cat, 3.30 kg. Effect of intravenous PS on blood pressure, after a small dose of hexamethonium. In the 10th and 16th min. following the intravenous injection of 0.003 g of hexamethonium, PS was administered in doses of 0.04 g and 0.05 g, respectively. On the base line: injection of PS. Time trace: 30 sec

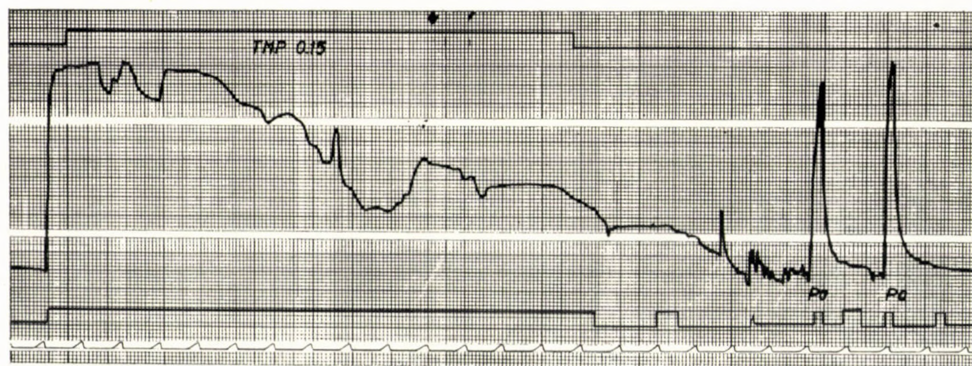

Fig. 6. Contractions of the nictitating membrane after the intraarterial injection of TMP. Parallel to the administration of TMP, the effect of preganglionic stimulation gradually decreases and then ceases, whereas postganglionic stimulation (PO) remains effective. Above: administration of TMP. Below: electrical stimulation. Time trace: 1 minute

A polypeptide, trimethylpolyanthinium iodide (TMP) prepared by Kovács and Kótai [5], also having polycationic properties but a chemical structure different from that of PS, showed a similar behaviour. Jancsó [3, 4] has shown that TMP, and polyanthinium derivatives in general, possess coagulation and inflammation inhibiting properties, and that their effect can be antagonized by heparin. The molecular weight of TMP is about 15 000. After intraarterial administration TMP produces a slowly developing ganglionic

effect characterized by the fact that preganglionic stimulation at the 5- to 10-fold of the original potential cannot induce contractions of the nictitating membrane, whereas postganglionic stimulation is invariably effective (Fig. 6). TMP given intravenously enhances the effect of other ganglionic blocking agents.

Discussion

Knowledge concerning the pharmacological behaviour of PS is scarce and mostly recent. In addition to its heparin-antagonizing effect, PS by itself prevents blood coagulation [2, 7], evokes eosinophilia in rats [14, 15], and, under conditions *in vitro*, if added to guinea pig brain slices, it inhibits the increase in their oxygen consumption induced by electrical pulses [6].

The discussed polycationic effects of PS and TMP, particularly if they are considered in comparison to the behaviour of the polyanionic heparin, cannot be evaluated from the point of view of practical pharmacology. They ought primarily be studied to establish the further properties of the transmission mechanism. Their reactions seem to show that PS and TMP gain access from the circulation to the surface of the ganglionic cells and the synaptic structures playing a role in transmission. Furthermore, our experiments have show that PS and TMP possessing a positive charge promote the effect of the true ganglionic blocking substances and, administered intraarterially by themselves, also have a transmission inhibiting character. Considering the different chemical structure and the common polycationic property of PS and TMP, they may produce their effect not by their chemical structure (*e.g.* competition), but owing to their charge. Taking into account the effect of the polyanionic heparin, it may be supposed that the synaptic function is influenced by substances which — owing to their considerable electric charge — exert an electrostatic effect upon the bioelectric phenomena taking place on the surface of the synaptic membranes.

We have dealt with the question, what kind of blocking type can be produced by PS, and how its effect develops. In earlier investigations it has been observed that preganglionic electrical stimulation of the superior cervical ganglion and its chemical excitation elicited by the intraarterial injection of different drugs do not run parallel after treatment with ganglionic blocking substances [10, 11]. It has been found namely that some ganglionic blocking agents administered in doses inhibiting the effect of preganglionic stimulation decreased only slightly the effect of acetylcholine injected — close to the ganglion intraarterially —, and did not prevent, or even increased, the exciting effect of phenyl-choline-ether bromide. On the other hand, certain doses of other types of ganglionic blocking substances did not inhibit synaptic transmission, and at the same time completely prevented the effect of acetylcholine and

phenyl-choline-ether injected into the ganglionic circulation. Large doses of these compounds could block the transmission, as well. To the first type belong hexamethonium, tetraethylammonium, d-tubocurarine and azamethonium, and to the second, sulpha-methyl-thiazole [13] and 2-β-(2-[4,5,3',4',-tetramethoxy-stilbenyl]-ethyl)-trimethyl-ammonium-methyl-sulphate, the compound synthesized by BRUCKNER, KOVÁCS and SZÁSZ [1]. PS may be ranged into the second group, *viz.* before it inhibits synaptic transmission, it abolishes the exciting effect of phenyl-choline-ether injected in a dose of 10 μg into the ganglionic artery. According to our previous suggestions, we assume that the PS molecules are deposited primarily on the free surfaces of the ganglionic cells and only when their concentration has increased they penetrate into the intersynaptic space and, bound to the receptor apparatus of the synaptic surfaces, inhibit the depolarization processes.

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Emil MINKER, Mátyás KOLTAI
Orvostudományi Egyetem Gyógyszertani Intézete, Szeged.

ANTICOAGULANT AND ANTIPHLOGISTIC PROPERTIES OF PHLOGODYM (NEODYMIUM PYROCATECHOL DISULPHONATE)

By

I. A. Oyvin,* V. P. Baluda, S. M. Shegel, O. Y. Tokarev, E. A. Venglinskaya and E. G. Yagodkina

DEPARTMENT OF PATHOLOGICAL PHYSIOLOGY, KUBAN MEDICAL INSTITUTE, KRASNODAR, USSR

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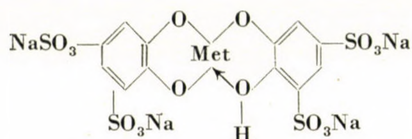
1. Phlogodym (Neodymium pyrocatechol disulphonate) administered intravenously in doses of 35 mg/kg 30 minutes before the influence of phlogistic irritants (xylene, heat) upon the rabbit's ear skin, completely prevents the development of inflammation (oedema formation, leucocytic emigration).

2. Intravenous injection of Phlogodym in the same dose causes in the rabbit a delay of blood clotting (clotting time, recalcification time, prothrombin time, and thrombin time are increased, tolerance to heparin diminished). Fibrinogen concentration changes insignificantly, but its heat resistance is lowered, fibrinogen B appears and blood fibrinolytic activity increases.

3. The anticoagulant properties of Phlogodym may be explained by disturbances of thromboplastin generation leading to inhibition of thrombin formation. Phlogodym probably influences the conversion of fibrinogen to fibrin. Apparently, Phlogodym has a direct influence on the blood clotting factors since it disturbs blood clotting both *in vitro* and *in vivo.*

4. The antiphlogistic effect of Phlogodym is not directly connected with its anticoagulant properties. It may perhaps be explained by a direct influence on the blood vessels and blood flow, since after Phlogodym administration the ear skin temperature is lowered, the skin capillary diameter diminishes and the time of radiophosphate disappearance from the skin is enhanced.

Jancsó has recently synthesized new earth metal (lanthanum, neodymium, praseodymium, samarium) complexes of pyrocatechol sodium disulphonate in which the metal atom is attached to the oxygen atoms of two phenolic residues



These complexes are tolerated well by rats and rabbits and possess anticoagulant and antiphlogistic properties [1]. The mode of action of these compounds upon the inflammation and coagulation system has scarcely been investigated. It seemed therefore interesting to study the properties of Phlogodym (Neodymium pyrocatechol disulphonate).**

* Present address: Department of Pathological Physiology, Medical Radiology Institute, Obninsk, USSR.

** Supplied by the *Chemical Works Gedeon Richter, Ltd.*, Budapest.

Methods

Albino and pigmented rabbits weighing 2.0 to 2.9 kg were used in the experiments.

Inflammation was produced by painting the rabbit's ear with 0.1 ml of xylene or by applying on it for 1 minute a copper disc, 15 mm in diameter, maintained by circulating water at a temperature of 54 $\pm$ 0.05° C.

The development of inflammatory oedema on the ear was investigated by the radiometric method of JUHLIN [2]. The intensity (E) of oedema, which is proportional to the degree of beta ray absorption, was calculated by the equation $E = \lg \frac{N_0}{N_I}$, where N_0 is the observed count above the normal ear and N_I — the count above the inflamed ear. The experiment continued for one hour during which 6 counts of 5 minutes duration each were made at 5 minute intervals. The details of the method have been published by VENGLINSKAYA [3].

Pieces of ear skin were cut out 1 hour after the application of xylene and heat, and studied histologically (paraffin embedding, haematoxylin-eosin staining). The intensity of leucocytic emigration was investigated by counting the leucocytes in sections of skin by the method of VENGLINSKAYA [4].

The ear skin temperature was recorded by means of a Biotherm electric thermometer.

The diameter of the ear skin blood capillaries was measured by an ocular micrometer attached to an apparatus for direct observation of the capillaries, after painting with paraffin oil the ear skin.

Ear skin blood flow was studied by the radiometric method [5]. Indicator doses of $Na_2H^{32}PO_4$ were added to saline and 0.1 ml of this solution was injected into the ear skin. Radioactivity was measured by means of a G. M. tube with a thin end-window situated 2 cm above the wheal formed by the radiophosphate injection. Measurements were taken during 1 minute and repeated at 1 minute intervals over a period of 25—90 minutes.

$Na_2H^{32}PO_4$ disappearance half time (an index of skin blood flow) was calculated on the basis of experimental curves (with the number of counts per minute on the ordinate and the time in minutes on the abscissa).

Blood clotting time was measured by the method of Lee and White.

The other determinations carried out were, calcium clotting time [6]; tolerance to heparin [7]; prothrombin time [8]; thrombin generation test [9]; fibrinolytic activity [10] with 3 hours incubation of fibrin at 37° C; fibrinogen [11]; fibrinogen B [12, 13]; heat resistance of fibrinogen [14].

Phlogodym was injected into the marginal ear vein, in doses of 35 mg/kg.

Student's t test was used for statistical analysis. The differences between results were considered significant with the $P < 0.05$.

Results

Changes of ear thickness after xylene and heat influence determined in normal rabbits and those pretreated with Phlogodym are summarized in Tables I and II.

Table I

Effect of Phlogodym on the development of oedema of the rabbit ear painted with xylene

Time (minutes) after xylene application						
0	5—10	15—20	25—30	35—40	45—50	55—60
Control group (10)						
0.00	0.03 $\pm$ 0.02	0.09 $\pm$ 0.02	0.14 $\pm$ 0.02	0.15 $\pm$ 0.02	0.16 $\pm$ 0.02	0.18 $\pm$ 0.02
Experimental group (10)						
0.00	0.01 $\pm$ 0.01	0.02 $\pm$ 0.01	0.02 $\pm$ 0.01	0.02 $\pm$ 0.01	0.01 $\pm$ 0.01	0.01 $\pm$ 0.01

Control group application of xylene. Experimental group: application of xylene 30 minutes after the intravenous injection of Phlogodym. Values: mean beta-ray absorption, with standard error of the mean after figures in parenthesis show the number of rabbits.

Table II

Effect of Phlogodym on the development of thermal oedema of the rabbit ear

Time (minutes) after burn						
0	5—10	15—20	25—30	35—40	45—50	55—60
Control group (5)						
0.00	0.03 ± 0.01	0.11 ± 0.01	0.18 ± 0.02	0.20 ± 0.01	0.23 ± 0.02	0.23 ± 0.01
Experimental group (5)						
00.0	0.04 ± 0.02	0.03 ± 0.02	0.01 ± 0.02	—0.06 ± 0.04	—0.04 ± 0.07	—0.01 ± 0.04

Control group: burn only. Experimental group: burn 30 minutes after the intravenous injection of Phlogodym. Values: as in Table I.

As seen from the Tables I and II, a statistically significant increase of ear thickness was evident 15—20 minutes after the application of xylene or heat. The oedema increased during 45—60 minutes. Phlogodym completely prevented development of the exudation caused by xylene or burn. In some experiments with heat injury the thickness of the ear diminished, as shown by the minus sign before the mean values of beta-ray absorption. This also explains the high standard error in these experiments. Histologically there were no changes to be seen in the ear skin treated with heat or xylene after the application of Phlogodym.

Phlogodym completely prevented the leucocytic emigration into the skin irritated by xylene or burn. The average leucocyte count per cu. mm. of ear skin 1 hour after the application of xylene was 539 ± 8, in rabbits pretreated with Phlogodym, 125 ± 1, while in normal rabbits 129 ± 5. One hour after the thermal injury the leucocyte count in the inflamed ear skin was 464 ± 46 per cu. mm, while in rabbits pretreated with Phlogodym, 126 ± 6 per cu. mm.

The influence of Phlogodym upon blood flow in the ear skin was studied 30 minutes after the intravenous administration of 35 mg/kg of the drug and the rate of $Na_2H^{32}PO_4$ removal from the ear skin, the skin temperature, and the diameter of the ear skin capillaries was determined. The results obtained in 10 rabbits are shown in Table III.

The data in Table III show an enhancement of radio-phosphate absorption, a lowering of the skin temperature and a diminution of the capillary diameter. These changes were significant statistically with confidence limits surpassing 99.9 per cent.

Table III

Phlogodym influence upon the blood flow in the rabbit ear skin

Statistical indexes	Half time of $Na_2HP^{32}O_4$ removal (minutes)	Temperature	Diameter of the capillary
	Before administration		
M	13.4	30.9	3.28
m±	1.1	1.0	0.8
	After administration		
M	35.8	24.1	24.5
m±	7.3	1.4	0.9
P	0.001	0.001	0.001

The effect of intravenously administered 35 mg/kg Phlogodym upon the blood clotting system and blood fibrinolytic activity is summarized in Table IV.

Significant changes in blood clotting appeared 10 minutes after the injection of Phlogodym. Blood clotting time and calcium clotting time increased more than 7 times; prothrombin time, 4 times; thrombin time, to the double; and tolerance to heparin diminished 5 times. Fibrinogen concentration changed insignificantly but its heat resistance was lowered (the temperature at which precipitation occurred diminished, the time until incipient precipitation was reduced). Blood fibrinolytic activity was slightly enhanced.

Thirty minutes after the injection of Phlogodym, fibrinogen B appeared in the blood and blood fibrinolytic activity increased more than twice. After 4—8 hours all the indexes investigated were still changed, but there was a tendency to return to normal. However, even 12 hours after Phlogodym administration normalization was not yet complete.

Discussion

Phlogodym administered intravenously completely inhibited the development of the early manifestations of inflammation (exudation, leucocytic emigration) caused by moderate phlogistic irritation. At the same time, Phlogodym showed marked anticoagulant properties, causing a delay of clotting, changes of fibrinogen quality, and an enhancement of fibrinolytic activity.

According to Soulier and Wieilland [15], neodymium, and particularly its 3-sulphoisonicotinic salt, is affecting the prothrombin, proconvertin complex and Stuart's factor in the blood and all this results in an inhibition of thromboplastin generation and thus a delay of blood clotting. Neodymium added to blood *in vitro* neutralizes the same factors whose synthesis is inhibited

Table IV

Phlogodym influence on blood clotting system and blood fibrinolytic activity

Clotting factors	Statistical indexes	Time after Phlogodym injection					
		0	10 min.	30 min.	4 hours	8 hours	12 hours
Clotting time (minutes)	M	4	30	30	21	14	10
	m±	0.14	0.0	0.0	1.8	1.3	0.7
	P		<0.001	<0.001	<0.001	<0.001	<0.001
Calcium clotting time (seconds)	M	80	580	580	420	260	170
	m±	2.4	34.4	24.1	17.7	20.1	11.6
	P		<0.001	<0.001	<0.001	<0.001	<0.001
Tolerance to heparin (seconds)	M	160	830	890	590	410	250
	m ±	2.5	28.3	6.2	28.3	28.3	13.5
	P		<0.001	<0.001	<0.001	<0.001	<0.001
Prothrombin time (seconds)	M	10	42	50	30	22	14
	m±	0.1	4.9	2.8	2.2	1.5	0.8
	P		<0.001	<0.001	<0.001	<0.001	<0.001
Thrombin time (seconds)	M	20	51	52	33	23	22
	m±	0.1	2.0	2.2	1.4	1.2	0.8
	P		<0.001	<0.001	<0.001	<0.001	<0.05
Fibrinolytic activity (%)	M	8	11	20	26	20	17
	m±	0.4	0.2	1.6	1.6	1.8	1.4
	P		< 0.001	<0.001	<0.001	<0.001	<0.001
Fibrinogen (mg%)	M	420	470	470	490	480	440
	m±	9.8	11.7	11.2	10.7	10.6	9.8
	P		<0.01	<0.01	<0.001	<0.001	<0.2
Fibrinogen B	M	0	0	+++	+++	+++	++
Heat resistance of fibrinogen *a)* Temp. (0°) at which precipitation occurred	M	51.7	51.0	50.7	50.6	50.8	51.3
	m±	0.01	0.01	0.08	0.08	0.09	0.06
	P		<0.001	<0.001	<0.001	<0.001	<0.001
b) Time until incipient precipitation (seconds)	M	170	100	70	60	80	130
	m±	2.0	6.3	7.7	7.6	9.8	5.7
	P		<0.001	<0.001	<0.001	<0.001	<0.001

by coumarine and its derivatives *in vivo.* Jancsó [16] found that after the intravenous administration of 75 mg/kg of Phlogodym the prothrombin time of rabbit blood increased from 7 to 66 seconds; after 4 hours it was still 20 seconds.

Our results have confirmed that intravenously administered Phlogodym disturbs thromboplastin generation, as a result of which thrombin formation is inhibited. For example, thrombin generation in normal rabbit plasma

takes 3—4 minutes, while 10 minutes after the intravenous administration of 35 mg/kg Phlogodym there is no thrombin formation.

The experiments made with rabbit's blood *in vitro* have shown that 0.25 mg per ml of plasma of Phlogodym delayed calcium clotting time 3—4 times, prothrombin time 1.5 times, thrombin time — more than 10 fold and lowered the tolerance to heparin to a half. Thrombin generation was markedly disturbed.

The analogy of the disturbances of blood clotting caused by Phlogodym *in vitro* and *in vivo* suggests that the compound may act directly upon the clotting factors.

Apparently, Phlogodym also influences the conversion of fibrinogen to fibrin, since in the presence of the drug the properties of fibrinogen undergo a change (lowering of heat stability, fibrinogen B appearance) and the fibrin clot formed is friable and lacking its usual threadlike structure.

In previous investigations [17, 18, 19], we have shown that the early signs of inflammation (derangement of vascular permeability, exudation and leucocytic emigration) do not depend on the blood clotting system. Intravascular blood clotting and thrombosis are the most prominent links in the pathogenesis of hyperergic inflammation. Therefore, the antiphlogistic influence of Phlogodym cannot be explained by its anticoagulant properties. Perhaps under our experimental conditions the development was inhibited by the direct influence of Phlogodym upon the ear vessels and ear skin blood flow. In fact, intravenously administered Phlogodym caused a lowering of the ear skin temperature and a diminution of the skin capillary diameter. The delay of radiophosphate removal also showed a reduction of the blood flow. Phlogodym thus may exert a direct "packing" effect on the small vessel walls [16] and thus reduce the tissues' reactivity to phlogistic irritants.

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J. A. Oyvin, V. P. Baluda, S. M. Shegel, O. Y. Tokarev, E. A. Venglinskaya, E. G. Yagodkina

Department of Pathological Physiology, Kuban Medical Institute, Krasnodar, USSR.

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РЕЗЮМЕ

ГЕТЕРОГЕННАЯ МЕЧЕНОСТЬ ЦИТОПЛАЗМАТИЧЕСКИХ РИБОНУКЛЕИНОВЫХ КИСЛОТ ПОДЖЕЛУДОЧНОЙ ЖЕЛЕЗЫ ГОЛУБЕЙ

М. СЕКЕЙ, Э. ГАЛ, и Б. ЛОВАШ

В бесклеточной системе, полученной из поджелудочной железы голубей, цитоплазматические рибонуклеиновые кислоты гетерогенно маркировались при инкубации в присутствии ортофосфата, содержащего ^{32}P.

Наличие ядерной фракции не было необходимым для того, чтобы меченый фосфор включился во фракции цитоплазматических рибонуклеиновых кислот.

Удельная радиоактивность рибонуклеиновых кислот клеточной фракции, содержащей большие гранулы, была больше, чем специфическая активность рибонуклеиновых кислот, изолированных из микросом.

Включение меченого фосфора в РНК наблюдалось и в том случае, если большие гранулы инкубировались безо всех остальных клеточных фракций.

Надо полагать, что большие гранулы могут играть значительную роль в обмене (веществ) рибонуклеиновых кислот панкреатических клеток.

ИЗМЕНЕНИЕ СОДЕРЖАНИЯ НУКЛЕИНОВЫХ КИСЛОТ В ДЕНЕРВИРОВАННОЙ ПОДЧЕЛЮСТНОЙ ЖЕЛЕЗЕ СОБАК

Й. МОЛНАР, А. ТИДИ, и К. ЛИШШАК

Авторами изучалось изменение содержания нуклеиновых кислот и общего количества азота (N) выделенной слюны в денервированной правой подчелюстной железе (glandula submaxillaris) собак.

В первую очередь изменилось содержание РНК, относительное и абсолютное количества которого показывали выраженное снижение в течение 2—3 недель после денервации.

Относительные (перечисленные на 100 г свежей ткани) величины содержания ДНК повышались, но абсолютные значения ДНК-содержания за такой короткий промежуток времени однозначно не менялись.

После удаления ganglion cervicale superius не наблюдалось существенное изменение в содержании нуклеиновых кислот.

Изменения, вызываемые перерезкой chorda tympani, в значительной степени увеличивались удалением ganglion cervicale superius.

Количество выделяющейся под действием пилокарпина слюны снижалось после денервации, но отношение выделяющего общего количества азота на 1 мг ткани железы показывало повышающуюся тенденцию.

ДЕЙСТВИЕ ХРОНИЧЕСКОЙ НАГРУЗКИ НА ОКСИТОЦИЧЕСКУЮ И АНТИДИУРЕТИЧЕСКУЮ АКТИВНОСТИ ГИПОФИЗА У КРЫС

К. ФЕНДЛЕР, Г. ТЕЛЕГДИ и З. ЭНДРЭЦИ

Белые крысы-самки были заставлены плавать до полного изнурения ежедневно в течение 29 дней. Подопытные животные были разделены на группы и подвергались воздействию по группам через каждые 3—4 дня. У животных исследовалось изменение окситоцической и антидиуретической активностей мозгового придатка.

Авторы установили, что окситоцическая активность гипофиза подопытных крыс показывает не выраженное снижение на 6—8-й дни эксперимента, но на 12-й и 15-й дни уже, наоборот, наблюдалось большой степени повышение указанной активности. Максимум 4—7-кратного повышения окситоцической активности достигался на 18-й день опыта, а на 22—29-й дни наблюдалось постепенное снижение активности, но уровень последней за время опыта не снижался, до исходных величин.

При аналогичных экспериментальных условиях авторы обнаруживали повышение антидиуретической активности мозгового придатка подопытных крыс.

ИССЛЕДОВАНИЕ ЗНАЧЕНИЯ СИСТЕМЫ РЕНИН-АНГИОТЕНСИНА В РЕГУЛЯЦИИ ПРОДУКЦИИ АЛЬДОСТЕРОНА

Я. ШОЙОМ, Ж. КОТРА, А. ШАЛАМОН и И. ШТУРЦ

Авторы исследовали роль системы ренин-ангиотенсина при повышении синтеза альдостерона после обескровливания. Образование альдостерона надпочечниками измерялось в опытах in vitro и in vivo после наложения лигатуры на гилюс почек или после нефрэктомии. Было установлено, что повышение продукции альдостерона после кровотечения наблюдается также и при отсутствии системы ренин-ангиотенсина.

ИЗМЕНЕНИЯ ОТВЕТНОЙ РЕАКЦИИ ЩИТОВИДНОЙ ЖЕЛЕЗЫ ПОД ВЛИЯНИЕМ ХОЛОДА ПОСЛЕ БИЛАТЕРАЛЬНОГО ПОВРЕЖДЕНИЯ ЯДЕР habenulae У КРЫС, ПРЕДВАРИТЕЛЬНО АДАПТИРОВАННЫХ К ТЕПЛОЙ СРЕДЕ

Б. МЕШШ

Автор исследовал изменения ответной реакции щитовидной железы, появляющиеся под влиянием холодной окружающей среды после билатерального повреждения ядер habenulae у белых крыс, предварительно адаптированных к теплой среде. Умеренный холод (+14° С) вызывал повышение коэффициента T/S у контрольных животных. Этот эффект повышался у животных с поврежденными ядрами habenulae, подвергнутых умеренному холоду. Однако, при действии сильного холода коэффициент T/S не повышался ни у контрольных ни у животных с поврежденными ядрами. Вес надпочечников повышался у группы, подвергнутой сильному холоду. Повышение веса надпочечников было у подверженных сильному холоду животных, поврежденными ядрами значительнее, чем у животных интактной группы при аналогичных условиях. Из полученных результатов автор приходит к выводу, что ядра habenulae играют важную роль в деле и активации системы TSH-щитовидная железа, под влиянием холодной температуры окружающей среды.

НЕДОСТАТОЧНОЕ ПИТАНИЕ И ТЕРМОРЕГУЛЯЦИЯ У ВЗРОСЛЫХ КРЫС

Т. ХЕИМ и И. МЕШТЪЯН

При частичном голодании снижение потребления кислорода у взрослых крыс вначале протекало быстрее, а затем замедленнее. Ректальная температура в нейтральной окружающей среде существенно не изменилась. По мере продолжительности голодания гипертермическая реакция обмена веществ снижалась прибл. параллельно основному обмену веществ.

В ранней стадии голодания при температуре среды в 20° С повышение обмена веществ было сравнительно меньшим, чем в контрольном периоде. Повышение теплопродукции, наблюдаемое в холодной среде параллельно с прогрессирующим уменьшением веса тела, было сравнительно большим, чем до голодания.

При температуре среды в 5—10° С измерялось дальнейшее значительное повышение потребления кислорода, причем температура тела не была меньше температуры, измеряемой при температуре среды в 20° С, и иногда даже превышала эти величины.

На основании полученных результатов авторы пришли к заключению, что при процессе голодания происходят изменения регуляции температуры тела.

ИЗУЧЕНИЕ ДЕЙСТВИЯ СЕПТАЛЬНОЙ ДЕСТРУКЦИИ У КОШЕК В ОПЫТАХ С ПРОСТЫМИ УСЛОВНЫМИ РЕФЛЕКСАМИ И СО СВОБОДНЫМ ВЫБОРОМ ОТВЕТА

Й. ЦОПФ и Э. ГРАШТЯН

Изучалось влияние деструкции септальной области у кошек при помощи простых пищевых и оборонительных условных рефлексов и с помощью свободного выбора ответа.

При простых условно-рефлекторных ситуациях, независимо от точной локализации поражения, не удавалось обнаружить функциональных выпадений. По результатам опытов с отставлением, животных можно разделить на две группы. У первой группы наблюдались проходящие, а у второй — постоянные функциональные выпадения (при максимальном отставлении на 5 сек в случае 90%-ного правильного ответа). У второй группы гистологически удалось выявить тяжелое поражение свода.

На основе своих наблюдений авторы пришли к выводу, что за функциональные дефекты, образующиеся при раздражении септальной области, можно считать ответственными поврежденные гипокампально-форникальную, или септо-гипокампальную переключительные системы.

ЭЛЕКТРОФИЗИОЛОГИЧЕСКОЕ ИССЛЕДОВАНИЕ СВЯЗИ МЕЖДУ МОЗЖЕЧКОМ И АММОНОВЫМ РОГОМ НА КОШКЕ

В. В. ФАНАРДЖЯН и Х. ДОНХОФФЕР

Авторы исследовали на 12 кошках с хронически имплантированными электродами мозжечковые потенциалы, вызванные слуховыми и зрительными раздражениями и электрическим раздражением двигательной коры и гиппокампа. Установлено, что

1. Свойства потенциалов, вызванных естественными раздражениями такие же как при острых экспериментальных условиях, но их амплитуда в хроническом препарате сильно колебается в зависимости от степени бодрости и внимания.

2. Существенные свойства мозжечковых потенциалов, вызванных раздражением гиппококампа, совпадают с ответом, полученным на естественные раздражения. Этот факт подтверждается также исследованиями интеракции.

При раздражении мозжечка ни в одном случае не удалось зарегистрировать потенциалы, вызванные в гиппокампе, хотя в двигательной и ассоциационной областях коры получался ответ на каждое раздражение. Таким образом, в противоположность связи между гиппокампом и мозжечком, не удалось выявить обратной связи.

Раздражение мозжечка серийными раздражениями вызвало в коре головного мозга десинхронизацию. Проведение раздражения во время гиппокампального приступа параметрами, вызывающими десинхронизацию, не влияло на функцию коры. В отдельных случаях при помощи раздражения, вызывающего весьма сильные двигательные эффекты, удалось прекратить гиппокампальный приступ.

ЭЛЕКТРОФИЗИОЛОГИЧЕСКИЙ И ФАРМАКОЛОГИЧЕСКИЙ АНАЛИЗ РОЛИ АДРЕНАЛИНА И НОРАДРЕНАЛИНА В МЕХАНИЗМЕ ТОРМОЖЕНИЯ ЗАДНЕГО АДДУКТОРА У ПЛАСТИНЧАТОЖАБЕРНЫХ

(Lamellibranchiatae)

А. ПУППИ

Невзирая на то, что адреналин в фармакологических дозах оказывает выраженное действие на нервные и мышечные процессы пластинчатожаберных и снижает тонус заднего аддуктора на основании нынешних знаний все же нельзя утверждать, что адреналин в физиологических условиях играет роль тормозящего медиатора, так как пока нет достоверных данных о наличии адреналина у них.

Адреналин в большой концентрации (1 мг/мл) тормозит, а в низкой концентрации (1,001 мг/мл) на длительное время повышает биоэлектрическую активность ганглиев.

Действие норадреналина качественно одинаково с действием адреналина, и оказывается только количественно менее эффективным.

Опытами было доказано, что дигидроэрготоксин до перерезки коннэктива выключает тормозящее действие адреналина на тонус аддуктора. Так как это действие дигидроэрготоксина после перерезки коннэктива больше не проявляется, можно предполагать что адренергический эффект при прекращении связи между двумя ганглиями постепенно снижается.

Ввиду того, что адреналин в физиологической концентрации повышает биоэлектрическую активность, а дигидроэрготоксин снижает ее, можно предполагать, что между двумя веществами существует антагонизм.

ИССЛЕДОВАНИЯ СЛЮНЫ ЗДОРОВЫХ ЛЮДЕЙ И ПАЦИЕНТОВ С КАРИОЗОМ

Д. ХАТТЯШИ, И. САБО и К. ТОТ

Авторы исследовали pH слюны, буферную ёмкость, потребление кислорода и продукцию кислоты слюны в течение трех дней утром после обеда у групп больных кариозом и здоровых в этом отношении людей. За последнее время исследовалась (только по утрам) также контрольная группа, состоящая из здоровых в отношении кариоза женщин-цыганок.

При сравнении результатов исследований, проводившихся в течение двух лет в отношении величины pH наблюдались незначительные расхождения. Значение буферной емкости изменялась в большей мере. В потреблении кислорода через 10, 30 и 120 мин. обнаруживались отклонения. Результаты исследования группы цыганок были весьма сходны с величинами, полученными у группы здоровых в отношении кариоза лиц. По-видимому существует фактор (или несколько факторов) задерживающий рост бактерий. Этот фактор проявляется в различных группах по-разному. Возможно также, что изменения наступают в зависимости от времени года, однако, для выяснения этого вопроса необходимы еще дальнейшие исследования.

Буферная ёмкость не зависит от величины pH, и в отдельных случаях она может оказаться весьма постоянной величиной.

ДЕЙСТВИЕ ПРОТАМИНСУЛЬФАТА НА ТРАНСМИССИОННЫЕ ПРОЦЕССЫ В ПЕРИФЕРИЧЕСКИХ СИМПАТИЧЕСКИХ ГАНГЛИЯХ

Э. МИНКЕР и М. КОЛТАИ

Авторы изучали действие протаминсульфата, обладающего свойствами поликатиона, на процессы передачи возбуждения в симпатических ганглиях. Эксперименты проводились на ganglion cervicale superius кошек.

В своих опытах авторы установили, что указанное соединение уже само тормозит симпатическую передачу возбуждения при определенных условиях и синергически повышает действие других ганглионных ингибиторов. Действие исследуемого вещества, тормозящего трансмиссию, антагонизируются солями калия.

Подобно протаминсульфату ведет себя и триметилполиантиниумйодид (который также обладает свойствами поликатионов), представляющий собой полипептид с молекулярным весом 15 000.

Так как названные соединения имеют различную химическую структуру и общими между ними являются только их поликатионические свойства, то авторы считают, что эти вещества, присоединяясь к рецепторным структурам, тормозят процессы передачи возбуждения в интернейрональных синапсах ganglion cervicale superius скорее благодаря своим зарядам, чем своим пространственно-структурными особенностями.

АНТИКОАГУЛЯНТНЫЕ И ПРОТИВОВОСПАЛИТЕЛЬНЫЕ СВОЙСТВА ФЛОГОДИМА

И. А. ОЙВИН, В. П. БАЛУДА, С. М. ШЕГЕЛЬ, О, Ю. ТОКАРЕВ, Е. А. ВЕНГЛИНСКАЯ и Е. Г. ЯГОДКИНА

Флогодим (Neodymium pyrocatechol disulphonate) при внутривенном введении (35 мг/кг) кроликам за 30 минут до воздействия на кожу флогогенных раздражителей (ксилол, ожог) полностью предотвращает развитие воспаления (отек, эмиграция лейкоцитов). Внутривенное введение флогодима в той же дозе резко удлиняет время свертывания крови, время рекальцификации, протромбиновое и тромбиновое время, уменьшает толерантность плазмы к гепарину. Концентрация фибриногена в плазме меняется незначительно, но уменьшается термоустойчивость фибриногена и появляется фибриноген В; фибринолитическая активность крови возрастает. Антикоагулянтное действие флогодима объясняется нарушением процесса образования тромбопластина, ведущим к торможению образования тромбина. По-видимому, флогодим оказывает также влияние на процесс превращения фибриногена в фибрин. Нарушение свертываемости крови наблюдается и при добавлении флогодима к крови вне организма, что свидетельствует о его непосредственном действии на факторы свертывания крови. Противовоспалительное действие флогодима не зависит от его антикоагулянтных свойств и обусловлено, по-видимому непосредственным влиянием на кровеносные сосуды, т. к. в результате внутривенного введения флогодима снижается температура кожи уха, уменьшается диаметр магистральных капилляров и замедляется скорость удаления внутрикожно введенного $Na_22^9HP^{32}O$.

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Biophysica

THE CROSS STRIATION OF THE INSECT FLIGHT MUSCLE AT DIFFERENT SARCOMERE LENGTHS

By

N. GARAMVÖLGYI, J. KERNER and M. CSER-SCHULTZ
INSTITUTE OF BIOPHYSICS, MEDICAL UNIVERSITY, PÉCS

(Received April 18, 1963)

Insect flight muscle fibrils of different striation patterns have been investigated by interference microscopy. Some changes of the cross striation, particularly in the range of extreme stretch, could not be explained on the basis of the sliding model. This contradiction has been supported by photometric measurements of electron micrographs.

The insect flight muscle fibrils represent a highly suitable material for general ultrastructure research of the striated muscle, due to their large diameters and sarcomere lengths, and allows work using the various kinds of light microscope and the electron microscope. The cross-striation pattern of the insect flight muscle is, apart from some specific features similar to that of vertebrate muscle.

Of the pertaining literature we only refer to HANSON's observation [1] that the organization of the longitudinal structure of the flight muscle is based on the sliding mechanism, described originally for the rabbit psoas muscle [2—5]. ERNST and BENEDECZKY [6] and ERNST [7] criticized the sliding model by comparing the scheme assumed by HUXLEY and HANSON with the quantitative values measured in the figures of HUXLEY's papers.

In this paper we shall present some observations on the flight muscle of the bee. Certain points of the results seemed to be contradictory to the sliding model.

Materials and methods

Fibrils of the indirect flight muscle of the bee were isolated according to the method used in our Institute [8], by disintegrating the fibres by needles on a slide, in a drop of Pringle's solution. In these preparations fibrils of different striation patterns are regularly present.

1. We used the interference microscope of BAKER [9, 10]. The specimen was illuminated by a mercury vapour lamp, for photography and measurements we applied a 5460 Å green filter. A 100 × double focus, water immersion objective was used. We chose areas in the preparations, in which there was only one fibril, the ground was free of traces of sarcoplasmic components and, apart from mitochondria, nothing could evoke a disturbing optical effect. The optical path difference (o. p. d.) was measured with BAKER's "half-shade eyepiece" [9], a compensator of rotating analyzer. For this purpose the fibrils had to be oriented transversally, in order to set a long part of the fibril on the half-shade limit and compensate the stripes of as many sarcomeres as possible. Three, or five, readings were made of the ground area and

of each the stripes of the cross striation. The optical path difference of the single structural elements was calculated according to the equation,

$$\Delta = \frac{2 \cdot \varphi \cdot \lambda}{360}$$

where Δ means the o. p. d., λ the wawelength of the illuminating light, φ the difference between analyzer-values of the reference area and the specimen, expressed in degrees *(i.e.* the half of the phase retardation Φ).

The sarcomere length, the diameter and length of the single bands were measured on photomicrographs at a final magnification of 3000.

2. Together with these measurements we made electron micrographs at low magnification for the purpose of photometric evaluation. On dried specimens on slides a formvar membrane and additionally a reinforcing thick collodion membrane was prepared. This membrane was floated on a water surface together with the specimen thus separated from the slide which then was mounted on a second slide and dried. The grids in suitable areas of the preparation were examined under a phase contrast microscope. This was followed by a second floating and mounting on a new slide, reversing the membrane. Finally, the reinforcing collodion membrane was removed by isoamylic acetate.

3. The electron micrographs made at a direct magnification of 2000 were measured by a "Schnellfotometer" of Zeiss in steps of 0.1 mm, and in this way the curve with the different bands on the micrograph, the pattern of the sarcomere could be reconstructed in the form of a diagram.

The photometric curves were constructed similarly on the basis of interference microscopic pictures. The photometric records of light micrographs are considered semiquantitative, serving only to show the direction of the differences in optical density. The discrepancies between the values obtained by photometry and by measuring the o. p. d. may be explained by the fact that the slit-width and the size of the corresponding structure are in the same order of magnitude. Therefore, particularly structures of narrow dimensions *(e.g.* Z-lines, or short I-bands) present themselves as optically more, or less, dense than measured by determining their phase retardation.

Results

Interference microscopy [11]

In the examined material the average diameter of the fibrils was about 3 μ, the sarcomere lengths varied from 3 to about 9 μ. In this range we may discern four basic types of striation pattern.

Type (i). Sarcomere length, 3 μ. Z-lines and A-bands (sometimes M-lines in their middle) present, but no I-bands. This state is frequent in the insect flight muscle. We designated it as "unstretched". It is doubtful, however, whether this type represents the contracted, or the resting, state (Fig. 1a, b).

The values of the o. p. d. are, Z-line, $\sim 0.15\ \lambda$,
A-band, $\sim 0.1\ \lambda$.

Type (ii), "stretched". Sarcomere length, 3.5—5.5 μ. The distinguishable structural elements are, Z-line, I-band, A-band. Average length of A-band, 3 μ; its o. p. d., 0.1 λ. Width of Z-line, $\sim 0.5\ \mu$; o. p. d., 0.1 λ. The length of the I-band varies with the sarcomere length, its o. p. d. cannot be determined exactly, because it nearly coincides with the accuracy of the instrument (φ — 2°,

which means an o. p. d. of about 0.01 λ). In contrast to type (i), the o. p. d. of the Z-lines is not higher, than that of the A-band, but equal, or something lower (Fig. 2a, b).

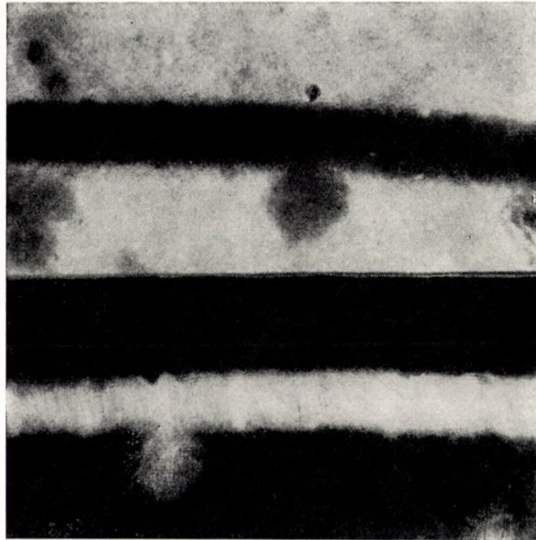


Fig. 1. a, Fibril of Type (i) in positive and negative contrast. Interference microscope, 100 × 10

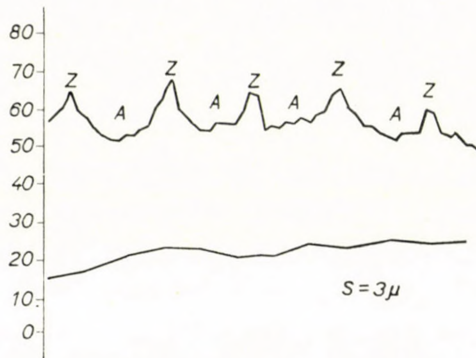


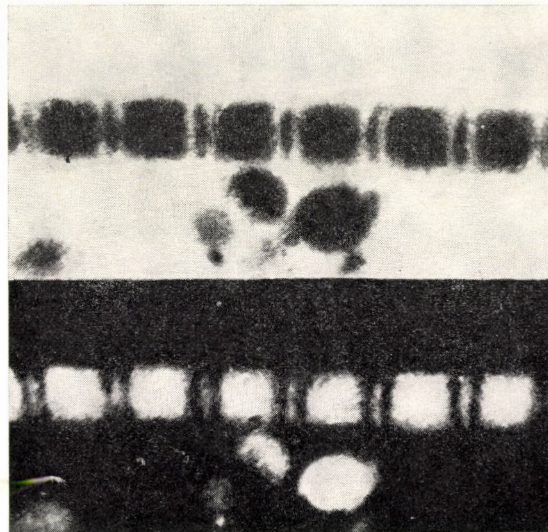
Fig. 1. b, Photometric curve of the same fibril

The characteristic features of this range are, that the A-band length does not change, the A-band seems to be entirely homogeneous, and that no H-band can be detected.

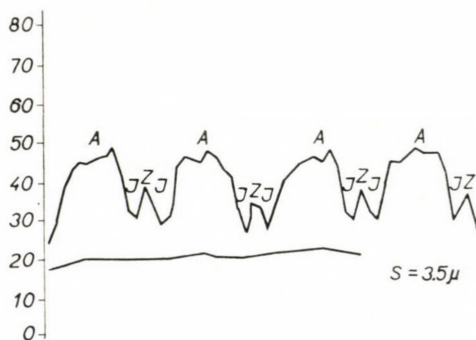
Type (iii), "highly stretched". This type is very rare. Sarcomere length, about 6—7 μ. The sharp borders between the A- and I-bands vanish and

a third, intermediate band appears, which we have termed E ("Extra")-band, following the nomenclature of ROLLETT [12]. No H-band is visible (Fig. 3a, b).

Type (iv) "extremely stretched", frequently seen in our preparations. The longest sarcomeres here have a length of 8—9 μ. In the A-band a zone of



a

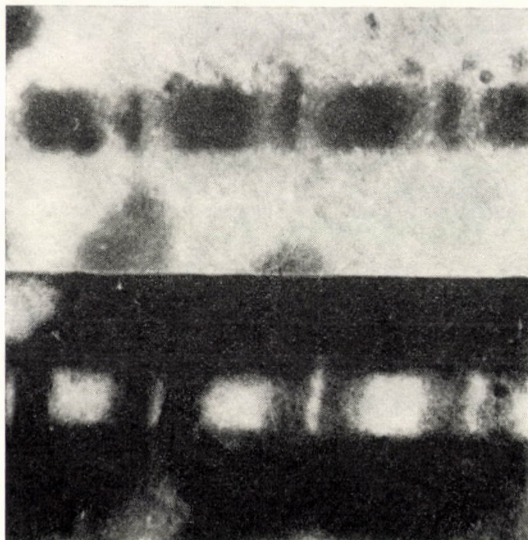


b

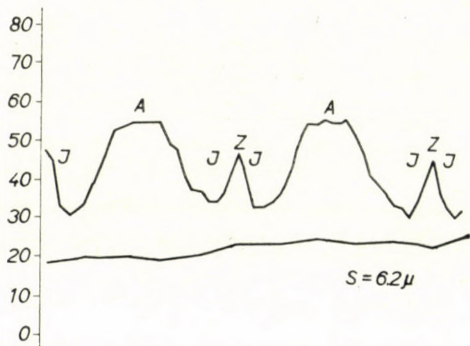
Fig. 2. a, b. Fibril of Type (ii). See Fig. 1

decreased density appears between two very dense lines which is divided by a dense line. Altogether 5 stripes can be seen. A similar splitting of the A-band due to chemical or mechanical treatment has been described by ERNST, BÉLECZKI and NAGY [8]. In the present case, however, the zone of the decreased density is due to stretching, and it is not certain that the mechanism is

the same as after chemical treatment. Among the five lines the two dense zones can perhaps be identified with the two halves of the A-band (which is called S-band by SJÖSTRAND and ANDERSON-CEDERGREEN [13] and "overlap-



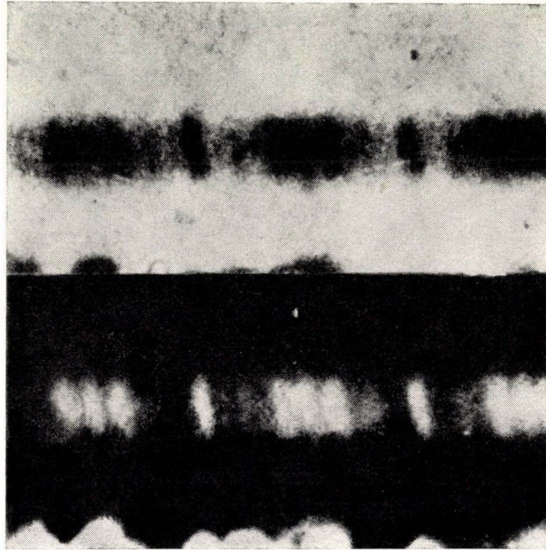
a



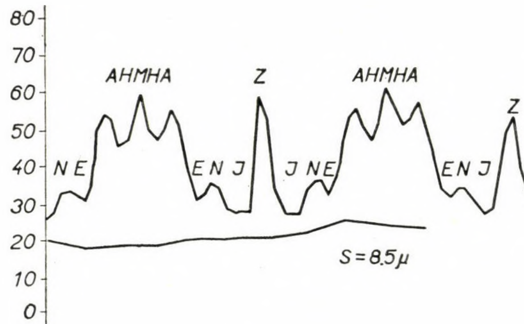
b

Fig. 3. a, b. Fibril of Type (iii). See Fig. 1

zone" by CARLSEN *et al.* [14]), the middle light stripe with the H-band, and the line visible in the middle of the H-band with the M-line. The E-bands persist. O. p. d. about 0.1 λ, for Z-line, M-line and A-band; about 0.04 λ, for E- and H-band. The o. p. d. of the I-bands could not be determined (Fig. 4a, b).



a



b

Fig. 4. a, b. Fibril of Type (iv). See Fig. 1

Electronmicroscopic investigations [15]

Type (i) fibrils could not be studied, being untransparent in their entire length. Pictures taken of Type (ii) fibrils did not show particular features (Fig. 5a, b).

The figures of Type (iii) fibrils show the existence of the intermediate E-band more clearly, than the light microscopic ones and show the lack of the H-band (lack of decreased density) in the middle of the sarcomere.

Fibrils of Type (iv) are similar to their light microscopic appearance, with the only exception that a sharp limiting line perhaps an N-line is visible between the I- and E-bands.

H-band and M-line inside A-band.

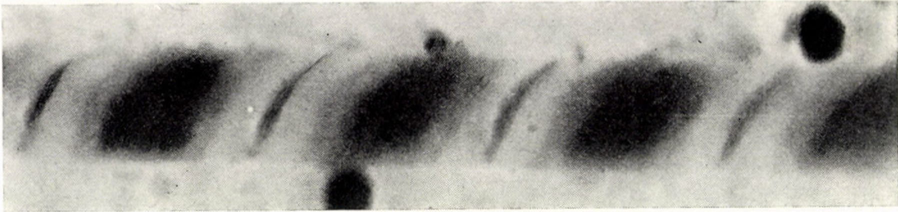


Fig. 5. a, Low magnification electron micrograph of an intact fibril of Type (iii). Magnification, × 6000.

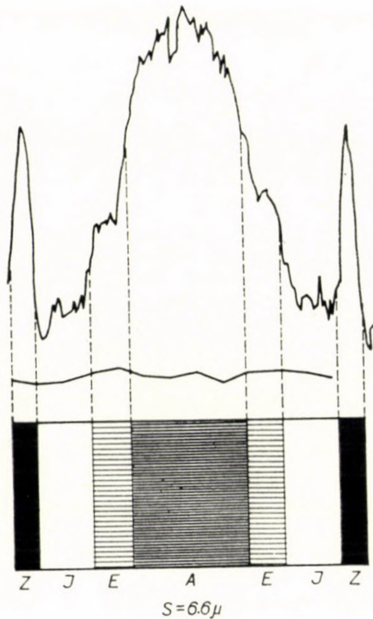


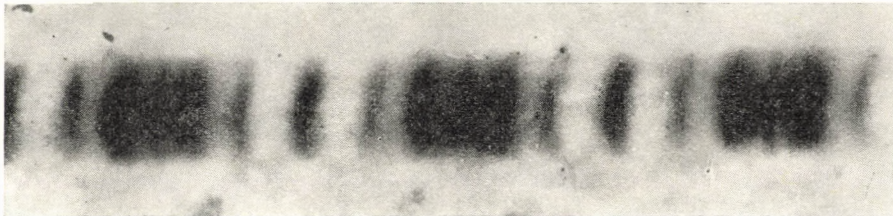
Fig. 5. b. Photometric curve of the same fibril and diagram of a sarcomere. See in text

Discussion*

The sliding model of muscle contraction based on the phase contrast and electronmicroscopic studies of HUXLEY and HANSON was later supported by their interference microscopic findings [16]. They examined psoas muscle in

*The interference microscopical data showing for the I-bands nearly the same "optical density" as for the "ground", will be dealt with in a later paper (by ERNST).

a certain range of sarcomere length, *i.e.* fibrils at resting length and moderately stretched fibrils. The cross striation of this range corresponds to the striation pattern designated as Type (ii) in this paper. Outside this range there are two questions of particular interest and according to our opinion they are substantial with respect to the possible structural arrangement of the sarcomere.



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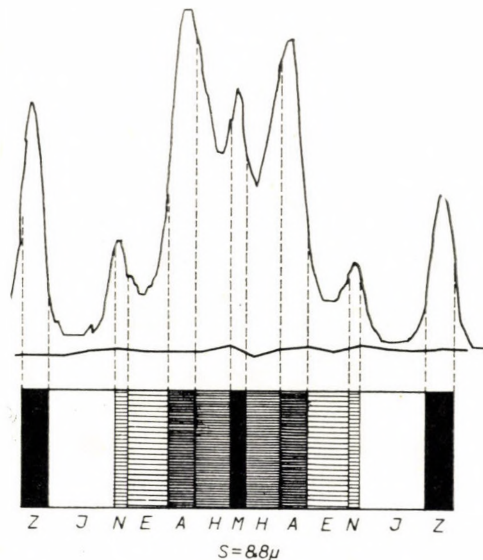

b

Fig. 6. a, b. Electron micrograph and photometric curve of a Type (iv) fibril. See Fig. 5. and text

1. One of them is the mechanism of the disappearance of the I-bands with contraction and the simultaneous formation of the contraction bands (C_z-bands according to HODGE [17]). The results concerning the Type (i) and the (ii) fibrils show the optical density of the Z-line to increase about 50 per cent when no I-band is visible. This can be interpreted as an uptake of substances by the Z-line from another band, as supposed in older concepts and more recently by HODGE [17, 18]. This assumption has been supported by a study

of one of us (N. G.) [19],* according to which the primary (thick) filaments obviously cross the Z-line without interruption if I-bands are absent, moreover they are considerably more thick and dense inside the Z-line than outside it.

2. The second very attractive question arisen in connection of our work is the possible organization of the longitudinal structure in the case of strong passive stretch. Our pictures show some structural details which seem to be hardly compatible with the sliding-model. The contradicting facts are as follows.

a) There are no H-bands in Type (ii) fibrils. Although the A-band length remains constant in this range and the length of the I-band changes (in accordance with the sliding model), there is no sign of a certain zone, of which the secondary filaments would have been withdrawn.

b) In Type (iii) fibrils no H-bands are apparent, although the withdrawal of the thin filaments was complete and an intermediate band (the E-band) appeared. The o. p. d. of the E-band is much higher than that of the I-band. This means that the E-band material cannot originate from the I, but only of the A-band. If the I-band material may draw out a substance denser than itself, there is a contradiction to the supposition that the two sets of filaments would be separated from each other when their overlap has ceased, and that no tension would be exerted by the thin filaments on thick ones (see Ernst and Benedéczky [6]).

c) The H-band appears only in Type (iv) fibrils, where the constant presence of the E-bands shows that an overlap can no longer exist. Therefore, the A-band proper can hardly be interpreted as an overlap-zone and the H-band as a zone without overlap.

It was unexpected that the E-bands should lie in the same position where Buchthal *et al.* [9] described a gap between the primary and secondary filaments and where more recently Sjöstrand [20] described filaments bridging that gap. In our specimens no gap, but a material of higher density appeared between the A- and I-bands. We cannot explain this difference, except that the gap was found by both authors in ultrathin sections, whereas our pictures showing dense intermediate bands were taken of intact fibrils. The two photometric curves presenting extremely stretched sarcomeres, recorded from a photomicrograph and an electron micrograph respectively, show a remarkable similar form. The native state of the fibril on the photomicrograph is doubtless.

The observed sarcomere lengths are of course far from the physiological range of the insect flight muscle, but changes of striation in these long sarcomeres may perhaps supply valuable information concerning the principal arrangement of the sarcomere's longitudinal structure.

* Done in the Laboratoire de Synthèse Atomique du CNRS, Ivry-sur-Seine, France and the senior author wishes to express his thanks for the possibilities rendered by Professor C. Magnan and Miss N. Carasso.

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Miklós Garamvölgyi,
János Kerner,
Mária Cser-Schultz,

Orvostudományi Egyetem Biofizikai Intézete, Pécs

Biochemia

PYRUVATE METABOLISM IN LIVER MITOCHONDRIA

By

T. KÖNIG, I. MAROSVÁRI and A. LIPCSEY

INSTITUTE OF BIOCHEMISTRY, MEDICAL UNIVERSITY, BUDAPEST

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Pyruvate metabolism has been studied in liver mitochondria under different conditions. Under aerobic conditions, utilization of pyruvate in liver mitochondria is a rapid process. The accompanying oxygen consumption is relatively low but continues to proceed at a considerable rate after the pyruvate has been exhausted. Part of the pyruvate is converted into acetoacetate, a greater part of the rest is oxidized to CO_2 and H_2O. Upon addition of dicarboxylic acids, the production of acetoacetate diminishes and an increased amount of acetyl-CoA enters the citric acid cycle via citrate. As long as a considerable amount of pyruvate is present in the reaction mixture, the oxygen consumption and CO_2 production is low as compared to the utilization of pyruvate. This excess of pyruvate utilization is only partly accounted for by the accumulation of acetoacetate, citrate and α-oxoglutarate. PEP and lactate are not accumulated during the utilization of pyruvate. Anaerobic utilization of pyruvate is negligible. Measurement of the oxygen consumption led to the conclusion that, even in the presence of 0.008 *M* malonate, pyruvate is not completely converted into acetoacetate, but part of it continues to enter the citric acid cycle.

Our results suggest that in liver mitochondria a great part of the pyruvate is first converted into dicarboxylic acid by way of CO_2 fixation and enters the citric acid cycle in this form. The different metabolic pathways of pyruvate and the probable mechanism and significance of dicarboxylic acid synthesis from pyruvate $+ CO_2$ in mitochondria are discussed.

Abbreviations used: PEP: phosphoenolpyruvate, ATP: adenosine triphosphate, EDTA: ethylenediamine tetraacetate, Acetyl-CoA: acetyl coenzyme A, ATPase: adenosine triphosphatase, DNP: 2,4-dinitrophenol.

The reaction mechanisms of the citric acid cycle are well known. Less information is available about the regulatory mechanisms controlling the different metabolic pathways of pyruvate and the effect of its transformations on the functioning of the citric acid cycle in different tissues. In the present work, these problems were studied on liver mitochondria. Although experimental conditions in a homogenate are closer to physiological ones than is a preparation of mitochondria, the latter was preferred in view of the advantages presented by a simplified system, such as the lower concentration of endogenous substrates, lower endogenous respiration, and the absence of some complicating factors present in homogenates (see below).

Hepatic pyruvate matabolism has widely been investigated. These studies, however, are not unequivocal as the different experiments were made on different tissue preparations and under different conditions. EVANS [1],

KREBS and EGGLESTON [2] observed a considerable accumulation of citric acid cycle intermediates in minced liver. As in their experiments pyruvate utilization was not inhibited by malonate, an accumulation of succinate took place and pyruvate utilization was increased by bicarbonate, the conclusion was drawn that in the liver dicarboxylic acids are synthesized from pyruvate by way of CO_2 fixation [2]. However, LEHNINGER [3] in washed rat liver suspensions, RECKNAGEL and POTTER [4] in rat liver homogenate, COPENHAVER and LARDY [5] in rat liver mitochondria, found a quantitative conversion of pyruvate into acetoacetate in the presence of malonate. Citrate synthesis and a diminished acetoacetate production was observed upon the addition of dicarboxylic acids [3, 4]. In the experiments of BANDURSKI and LIPMANN [6] with rat and guinea pig liver mitochondria, catalytic amounts of succinate increased only slightly the oxygen consumption and PEP production observed in the presence of pyruvate. From this the authors came to the conclusion that the minute amount of bicarbonate present in their system was sufficient to convert pyruvate into dicarboxylic acid. JUDAH [7] on the basis of the oxygen consumption of rat liver mitochondria in the presence of dicarboxylic acids, suggested that pyruvate is completely oxidized in the citric acid cycle, whereas McCANN [8] observed acetoacetate production in rat liver mitochondria even in the presence of dicarboxylic acids. The interpretation of the results obtained with different tissue preparations is complicated by the extramitochondrial location of some citric acid cycle enzymes. Thus, according to LOWENSTEIN [9], the extramitochondrial enzymes of the citric acid cycle can convert a considerable amount of acetyl-CoA into α-oxoglutarate. Malic dehydrogenase [10] as well as L-glutamate-oxalacetate transaminase [11] (an auxiliary enzyme of the citric acid cycle), also exist both in extra- and intramitochondrial form.

Our results suggest that in rat liver mitochondria — also under conditions *in vitro* — a significant part of pyruvate is first converted into dicarboxylic acid by way of CO_2 fixation and enters as such the citric acid cycle. Some of the results presented here have been published in an abstracted form [12, 13, 14, 15].

Materials and methods

Male Wistar rats of 150—200 g weight were used in the experiments. The rats were fed a standard diet *ad libitum*. Before the experiments the animals were fasted for 12 hours.

All chemicals used were of analytical grade. The glass distilled water was boiled before use.

Mitochondria were prepared according to the method of SCHNEIDER [16], all manipulations being carried out at 0°. After decapitation, the animals were exsanguinated, the organs used in the experiment were removed immediately, chilled and homogenized in a Potter—Elvehjem-type all glass homogenizer. A 15 per cent homogenate in 0.25 *M* sucrose solution containing 0.002 *M* EDTA was prepared. Differential centrifugation was carried out in the superspeed head of an MSE Major refrigerated centrifuge. After removing the nuclei by centrifugation at 700 *g* for 10 min, the supernatant was centrifuged for 15 min at 8500 *g*. The fluffy layer was removed carefully and the mitochondrial sediment was washed twice

with 0.25 *M* sucrose (containing no EDTA) and centrifuged as above. Finally, the mitochondrial preparation was suspended in isotonic sucrose solution. The standard reaction mixture used for the metabolic studies contained in 3 ml; 30 μmoles of K-phosphate buffer, pH 7.5, 9 μmoles of $MgCl_2$, 6 μmoles of ATP, 750 μmoles of sucrose.

The substrates were dissolved immediately before use (in the concentrations given in the tables) and were added from the side arm of the Warburg vessel to the reaction mixture after a preincubation period of 7 min. (Pyruvate was used as the Na-, fumarate as the K-salt.) Oxygen consumption was measured by the Warburg technique at 37° C, in air as the gas phase and 90 strokes per min. CO_2 was absorbed by 0.1 ml 20 per cent KOH in the central well. In the experiments where CO_2 production was measured, the direct method of Warburg and Dixon [17] was applied, and corrections were made for the retention of CO_2. In the anaerobic experiments, the Warburg vessels were gassed with nitrogen, the substrates were added to the main compartment and the mitochondrial preparation was placed in the side arm. Determinations of the metabolites were made after deproteinization of the reaction mixture with freshly prepared trichloroacetic acid (final concentration, 3 per cent) or, for the assay of lactate, with 2 per cent tungstate.

Pyruvate was determined by the method of Friedemann and Haugen [18] or Koepsell and Sharpe [19]. The method of the latter authors was employed also for the determination of α-oxoglutarate. PEP was hydrolyzed according to Reynard *et al.* [20] and assayed as pyruvate by the method of Friedemann and Haugen [18]. Citrate was determined according to Schneider *et al.* [21], acetoacetate according to Walker [22], and lactate according to Hullin and Nable [23]. Nitrogen content was measured by the micro Kjeldahl technique.

Results

The time course of pyruvate utilization and oxygen consumption was studied in the presence and in the absence of fumarate.

The results of such an experiment are shown in Table I. For comparison, data obtained with kidney mitochondria prepared in the same way as those of the liver, are also included.

In liver mitochondria, metabolism of pyruvate was rapid, accompanied by a relatively low oxygen consumption. The O/pyruvate ratio increased in time, and considerable oxygen consumption could be observed even after the total exhaustion of pyruvate (about 120 min). Oxygen consumption was somewhat increased by fumarate whereas the utilization of pyruvate was diminished. The results with kidney mitochondria were different. Here pyruvate utilization was accompanied by increased oxygen consumption and both pyruvate utilization and oxygen consumption were catalytically enhanced by fumarate. The increase in oxygen consumption was greater than that of pyruvate utilization. Thus, the entrance of pyruvate into the citric acid cycle was enhanced and its oxydation in the cycle improved. According to Bartley [24a, 24b], in kidney preparations pyruvate is completely oxidized only at low concentration and in the presence of a sufficient amount of dicarboxylic acid or bicarbonate. Otherwise pyruvate is converted largely into acetate. This has been confirmed by our experiment, where the O/pyruvate ratio was less than 5. In liver mitochondria the O/pyruvate ratio — calculated on the basis of the 240 min values — was 3 (or 3.4 if fumarate was also present). It may thus be supposed that part of the pyruvate was not oxidized to CO_2 and H_2O. As in the liver, a conversion of pyruvate to acetoacetate may occur, the rate of acetoacetate production was determined in our system.

Table I

Time course of pyruvate utilization and oxygen consumption in liver and kidney mitochondria

The substrate was placed in the main compartment of the Warburg vessel and pyruvate utilization was calculated at different time intervals as related to a 0 min value obtained after 10 min preincubation. Measurement of oxygen consumption was started after the same preincubation period

Liver				
Substrate	Pyruvate, 25 μmoles		Pyruvate, 25 μmoles+ Fumarate, 1 μmole	
Time, min	oxygen uptake μatom	pyruvate utilization μmole	oxygen uptake μatom	pyruvate utilization μmole
20	5.5	4.9	8.3	4.4
40	13.8	10.1	17.6	10.5
80	31.6	21.2	35.5	18.4
120	48.0	24.9	52.0	24.7
160	60.7	—	64.0	—
240	77.0	—	87.0	—

Nitrogen content of mitochondria: 1490 μg per reaction vessel.

$$\frac{\mu\text{atom oxygen consumption}}{\mu\text{mole pyruvate utilization}} = 3.1 \qquad \frac{\mu\text{atom oxygen consumption}}{\mu\text{mole pyruvate utilization}} = 3.4$$

Kidney				
Substrate	Pyruvate, 25 μmoles		Pyruvate, 25 μmoles+ Fumarate, 1 μmole	
Time, min	oxygen uptake μatom	pyruvate utilization μmole	oxygen uptake μatom	pyruvate utilization μmole
20	5.0	1.7	14.9	4.5
40	10.6	4.5	33.2	10.2
80	22.6	9.8	68.5	19.5

Nitrogen content of mitochondria, 1070 μg per reaction vessel.

In the experiment presented in Table II, the endogenous respiration of the mitochondria was negligible. The proportionality of oxygen consumption and substrate concentration was the proof of the oxygen consumed being in fact utilized for the oxydation of pyruvate. A substantial amount of acetoacetate was also formed from the pyruvate. It was remarkable that at higher concentrations relatively less pyruvate was converted into acetoacetate. At the same time the O/pyruvate ratio was independent of substrate concentration.

Table II

Oxydation of pyruvate and production of acetoacetate in liver mitochondria

Time of incubation: 240 min

	Substrate		
	25 μmoles	18.75 μmoles	12.5 μmoles
	pyruvate		
Oxygen consumption μatoms	80.5*	60.0*	42.3
Acetoacetate production μmoles	3.57	3.17	2.65**
μatom oxygen consumption / μmole pyruvate utilization	3.2	3.2	3.4
corrected ratio μatom oxygen consumption / μmole pyruvate utilization ***	4.1	4.3	5.2

* In the presence of 25 and 18.75 μmoles of substrate, a slight oxygen consumption was still observed after 240 min of incubation.

** Determination of acetoacetate was performed at 60 min, pyruvate was exhausted at 40 min.

*** For explanation, see text.

Considering that for the formation of one mole of acetoacetate 2 moles of pyruvate and 2 atoms of oxygen are utilized, the corrected value of O/pyruvate can be calculated, *i.e.* the ratio giving the number of O atoms consumed per moles of pyruvate utilized in reactions other than acetoacetate formation. This corrected ratio is high in the case of low pyruvate concentrations (in the presence of 12.5 μmoles of pyruvate it roughly corresponds to the theoretical value of 5) and low when the pyruvate concentration is high. Considering that oxygen consumption was detectable even after 240 min, after longer incubation this ratio might have been higher. There is, however, a possibility that during the long incubation period the mitochondria had been damaged and lost some of their coenzymes. It is improbable that pyruvate should have been transformed by other side reactions. The above experiment suggests that most of the pyruvate which had not been converted into acetoacetate was oxidized to CO_2 and H_2O.

From Table I it can be seen that as long as pyruvate concentration was high in the reaction mixture, the O/pyruvate ratio was low. On the other hand, the experiment in Table II proves that the pyruvate not transformed into acetoacetate is completely oxidized. Thus, it can be assumed that first some intermediates are being accumulated which undergo oxidation in the later stages of incubation. In order to check this possibility, in the next experiments the synthesis of citrate and α-oxoglutarate was followed, parallel to the determination of oxygen consumption, pyruvate utilization and acetoacetate production. The results of such an experiment are shown in Table III.

Table III

Accumulation of citrate, α-oxoglutarate and acetoacetate from pyruvate in liver mitochondria
Nitrogen content of mitochondria: 1710 μg per reaction vessel. Time of incubation: 30 min

	Pyruvate 25 μmoles	Pyruvate 25 μmoles + Fumarate 1 μmole
Oxygen uptake μatoms	12.8	14.3
Pyruvate utilization μmoles	12.2	11.4
Citrate accumulation μmoles*	0.80	1.71
α-Oxoglutarate accumulation μmoles*	0.24	0.14
Acetoacetate production μmoles	1.59	0.61
μatom oxygen uptake / μmole pyruvate utilization	1.0	1.3
"rest" oxygen μatoms**	8.3	—
"rest" pyruvate μmoles**	7.0	—
"rest" oxygen μatoms ** / "rest" pyruvate μmoles	1.2	—

* 0 min values were subtracted from the amount found in the medium at the given time.
** For explanation, see text.

In this experiment "rest" pyruvate means that part of the pyruvate utilized which was not accounted for by the formation of acetoacetate, citrate and α-oxoglutarate. The oxygen consumption accompanying the utilization of this "rest" pyruvate is called "rest" O. "Rest" O/pyruvate ratio means the ratio of "rest" oxygen consumption to "rest" pyruvate utilization. The calculations have been made on the basis of the stoichiometric equations represented in Table IV.

The results in Table III confirm the fact that oxygen consumption is only slightly increased by fumarate (see also Table I). Fumarate exerts a slight inhibition on the utilization of pyruvate. This inhibition was observed in every experiment and its extent increased with the amount of fumarate added. Experiments with labelled fumarate would be necessary to decide whether we are dealing here with a real inhibition or rather with an apparent decrease of pyruvate disappearance caused by a partial conversion of fumarate into pyruvate. In agreement with literary data, acetoacetate formation is decreased, and citrate synthesis increased, by fumarate [25]. Some α-oxoglutarate is also formed.

As oxygen consumption is increased and pyruvate utilization is decreased by fumarate, the O/pyruvate ratio is increased. The "rest" O/pyruvate ratio cannot be calculated in this way, as the amount of oxygen utilized for the oxidation of fumarate is unknown. It is interesting that the "rest" pyruvate

Table IV

Amount and ratio of oxygen uptake and CO_2 production accompanying the formation of different metabolites from pyruvate

	Oxygen needed	CO_2 produced	Oxygen atom / pyruvate mole	CO_2 mole / pyruvate mole
2 moles pyruvate → 1 mole acetoacetate	2	2	1	1
2 moles pyruvate → 1 mole β-hydroxybutyrate	1	2	0.5	1
2 moles pyruvate → 1 mole citrate	1	0	0.5	—
2 moles pyruvate → 1 mole α-oxoglutarate	2	1	1	0.5
2 moles pyruvate → 1 mole succinate	3	2	1.5	1
2 moles pyruvate → 1 mole fumarate or 1 mole malate	4	2	2	1 } via citrate
2 moles pyruvate → 1 mole oxalacetate	5	2	2.5	1 } via citrate
1 mole pyruvate → 1 mole malate or 1 mole fumarate	—1	—1	—	— } via CO_2 fixation
1 mole pyruvate → 1 mole oxalacetate	0	—1	—	— } via CO_2 fixation

should have amounted to as much as 60 per cent of the total pyruvate utilized. In this experiment for 1 mole of "rest" pyruvate 1.2 atoms of oxygen were utilized, even less than the oxygen needed for the formation of succinate from pyruvate. Considering the data of KREBS, that in liver preparations during the utilization of pyruvate the citric acid cycle intermediates subsequent to α-oxoglutarate are also accumulated [2], and the well-known fact that the metabolism of succinate is very rapid, it follows that in our experiments part of the pyruvate was probably metabolized further than the succinate stage, *via* acetyl-CoA. The low "rest" O/pyruvate ratio obtained in balance experiments may be explained by assuming that part of the "rest" pyruvate was metabolized by another pathway, or, else, that pyruvate, or a metabolic product of it, behaved as a H acceptor.

One possible reaction of pyruvate is its conversion into lactate. However, under aerobic conditions no formation of lactate was detected.

Another possibility is the conversion of pyruvate into β-hydroxybutyrate. KULKA *et al.* [26] and KREBS *et al.* [27] revealed the capability of acetoacetate to behave as a H acceptor. In the experiment described below it was proved that, if at all, only a small part of the pyruvate disappearance might be accounted for by β-hydroxybutyrate formation.

Particles of liver and kidney [28, 29] as well as liver mitochondria [6] are known to form PEP from citric acid cycle intermediates and from pyruvate.

In our system, however, no PEP was detected. From this it does not follow that no PEP had been formed, there being a possibility that PEP had been reconverted into pyruvate at a rate equal to its formation, as a result of the combined action of the pyruvate-ADP-transphosphorylase contaminating the mitochondrial preparation and of the mitochondrial ATPase [30]. In a whole homogenate PEP can further be metabolized by the glycolytic enzymes.

A further possibility is the conversion of pyruvate into dicarboxylic acids by way of CO_2 fixation. With respect to this mechanism an indirect conclusion can be drawn from the extent of CO_2 production.

In the two experiments shown in Table V, pyruvate utilization, oxygen consumption and CO_2 production were measured. The CO_2 production corresponding to the utilization of 1 mole of pyruvate was 0.7 in the first experiment and 0.8 in the second. This CO_2 production did not even account for the formation of succinate from pyruvate, although, as already mentioned, part of the pyruvate is very probably metabolized in the citric acid cycle *via* acetyl-CoA further than the succinate stage. The results of this experiment excluded the importance of β-hydroxybutyrate formation in the fast utilization of pyruvate, as the conversion of pyruvate to β-hydroxybutyrate, too, would give a CO_2/pyruvate ratio of 1. Thus, the most probable assumption remains the one proposed above, *i.e.* that a considerable part of the pyruvate is converted into dicarboxylic acids by way of CO_2 fixation.

This assumption has been confirmed by experiments performed in the presence of malonate. Malonate, in a concentration below 0.01 *M*, has an inhibiting effect on succinodehydrogenase [31]. Thus, if there was no conversion of pyruvate into dicarboxylic acids, and the system did not contain larger

Table V

CO_2 production during pyruvate utilization

Experiment 1. Nitrogen content of mitochondria: 1670 μg per reaction vessel. Time of incubation: 40 min.

	Oxygen consumption μatom	CO_2 production μmole	Pyruvate disappearance μmole
25 μmoles pyruvate	13.9	8.8	12.9
25 μmoles pyruvate + 1 μmole fumarate	16.7	10.0	12.3

Experiment 2. Nitrogen content of mitochondria: 2300 μg per reaction vessel. Time of incubation: 40 min.

	Oxygen consumption μatom	CO_2 production μmole	Pyruvate disappearance μmole
25 μmoles pyruvate	22.7	17.7	21.1

amounts of dicarboxylic acids, in the presence of malonate a complete conversion of pyruvate into acetoacetate should have been expected. It can be seen in Table VI that 0.008 *M* malonate had no such effect. In this experiment endogenous respiration was completely blocked by the malonate, proving that the mitochondria contained practically no endogenous dicarboxylic acids.

Table VI

Effect of malonate on pyruvate oxydation in liver mitochondria

Time of incubation: 240 min.

	25 μmoles pyruvate	25 μmoles pyruvate + 25 μmoles malonate	12.5 μmoles pyruvate	12.5 μmoles pyruvate + 25 μmoles malonate
Oxygen consumption μatoms	74.0 *	43.5 *	40.8 *	21.4

* Oxygen consumption detectable even after 240 min incubation.

Determinations made after the cessation of oxygen uptake showed that pyruvate was completely utilized by the mitochondria. In this experiment no direct determination of acetoacetate was made (as the method of Walker cannot be used in the presence of malonate), but the rate of oxygen consumption sufficed to show that in the presence of malonate the conversion of pyruvate into acetoacetate was not complete and part of the pyruvate was partially oxidized in the citric acid cycle, in good agreement with EVANS' data [1] obtained in pigeon liver homogenates. This observation also confirms that part of the pyruvate had first been converted into dicarboxylic acids by way of CO_2 fixation. The results of LEHNINGER [3], RECKNAGEL and POTTER [4], COPENHAVER and LARDY [5], which are at variance with our and EVANS' findings [1], could perhaps be explained by the difference that the liver preparations of the former authors did not form dicarboxylic acids from pyruvate.

Another mechanism which might explain the relatively low oxygen consumption accompanying pyruvate utilization would be the anaerobic metabolism of part of the pyruvate. According to KREBS *et al.* [27] acetyl-CoA is formed from pyruvate even under anaerobic conditions in liver homogenates. Their assumption is based on the observation that under anaerobic conditions β-hydroxybutyrate and acetoacetate are still formed from pyruvate. We have studied the liver mitochondria as to their ability to utilize pyruvate under anaerobic conditions, but under our experimental conditions pyruvate disappearance was very slight. Thus, in liver mitochondria, the results of KREBS *et al.* were confirmed in the sense that — even if only to a little extent — pyruvate can be utilized anaerobically. The rate of this utilization is, however, far less than that of the "rest" pyruvate observed in our experiments.

Discussion

Our balance experiments revealed that about 60 per cent of the pyruvate utilized cannot be accounted for by the oxygen consumption and by the accumulation of the metabolites determined by us. The question arises, by which intermediary metabolic pathways this so called "rest" pyruvate is oxidized. That eventually it is completely oxidized was shown unequivocally in the experiment in Table II. However, the "rest" O/pyruvate ratios obtained and the CO_2/pyruvate values of less than 1 did not even allow for the formation of succinate from pyruvate. On the other hand, the experiments of KREBS and EGGLESTON [2] suggest that part of the pyruvate is metabolized *via* acetyl-CoA in the citric acid cycle to form succinate and further metabolites. The relatively low oxygen consumption and CO_2 production accompanying the utilization of pyruvate are suggestive of a synthesis of dicarboxylic acids. Such a reaction needs the fixation of 1 mole CO_2 per mole pyruvate and in the case of malate formation 2 H atoms per mole are also taken up. Thus, by this mechanism the oxidation of another part of the pyruvate *via* acetyl-CoA beyond the succinate stage could also be accounted for. Other possible mechanisms which would explain the low oxygen consumption accompanying the utilization of pyruvate were excluded, *i.e.* the formation of β-hydroxybutyrate and PEP. The probability of dicarboxylic acid synthesis is supported by the experiments performed in the presence of malonate, since in that case during the utilization of pyruvate oxygen consumption was considerably higher than that necessary for the quantitative conversion of pyruvate into acetoacetate. A further indirect proof of this hypothesis is the observation that uncoupling concentrations of DNP inhibit the utilization of pyruvate and the oxygen consumption, but are enhancing acetoacetate formation from pyruvate [32].

PEP formation can also be excluded, as no accumulation of PEP was observed in our system. It is still a possibility that transamination might play a part in the disappearance of pyruvate. By transamination α-oxodicarboxylic acids would be formed in an amount equivalent to the disappeared pyruvate. The following observations make it, however, very improbable that under our conditions transamination should have played an important role in the utilization of the "rest" pyruvate: *a)* mitochondria contain only a negligible amount of amino acids able to participate in transamination reactions; *b)* only a small amount of α-oxoglutarate accumulated during the experiment; *c)* the oxydation of α-oxoglutarate in the citric acid cycle would have been accompanied by a higher oxygen consumption than that of pyruvate [15].

On the basis of all these considerations, we have to interpret our results by suggesting that part of the "rest" pyruvate is first transformed into malate by way of CO_2 fixation and reduction, *i.e.* it enters the citric acid cycle as a dicarboxylic acid.

The idea that pyruvate can be metabolized by CO_2 fixation and that oxalacetate may act as a H acceptor has been mentioned by Krebs and Eggleston [2]. Since this first observation several papers were dealing with the process of dicarboxylic acid synthesis from pyruvate by way of CO_2 fixation occurring in animal tissues [31]. The question arises, by which metabolic pathway the dicarboxylic acid is synthesized and what is the physiological significance of this synthesis. For a time the malic enzyme was considered responsible for the synthesis of dicarboxylic acids, but it was shown then that in the liver of birds this enzyme is localized exclusively in the supernatant fraction [33, 34, 35, 36]. However, no information being available as to the localization of the malic enzyme in mammalian liver, the possibility cannot be excluded of this enzyme taking part in the dicarboxylic acid synthesis occurring in mitochondria. Recently, Utter and Keech [37] have isolated from bird and beef liver an enzyme which forms oxalacetate from pyruvate by CO_2 uptake, utilizing the energy of ATP. This enzyme is located in the mitochondria. There are several reasons to suggest that it is this pathway that leads to the formation of dicarboxylic acid in isolated liver mitochondria, *viz. a)* the location of the enzyme in mitochondria; *b)* its high activity at low bicarbonate concentrations; *c)* the inhibitory effect of uncoupling concentrations of DNP upon dicarboxylic acid synthesis [38, 7, 39, 32].

Dicarboxylic acid synthesis in mitochondria is supposed to take part — by way of PEP formation — in cytoplasmic gluconeogenesis [33, 40, 41]. Oxalacetate, by its effect upon succinodehydrogenase, functions as a regulator of the citric acid cycle [42] and it also controls the utilization of acetyl-CoA. Studies on the incorporation of ^{14}C-compounds into glutamic acid *in vivo* led also to the conclusion that, especially in the liver, a significant part of the pyruvate is metabolized *via* oxalacetate [43, 44, 45]. These observations, too, point to the physiological significance of dicarboxylic acid synthesis.

Our results suggest that the formation of dicarboxylic acids from pyruvate by CO_2 fixation occurs to a considerable extent also in isolated mitochondria. However, the significance of extramitochondrial enzymes in dicarboxylic acid synthesis and in the functioning of the citric acid cycle, and the way of interaction between extra- and intramitochondrial enzymes in the cell are problems still unsolved.

Note added in proof. After this paper had been submitted for publication, an article of R. J. Haslam and H. A. Krebs appeared [Biochem J. **86**, 432 (1963)] part of which was dealing with similar aspects of pyruvate metabolism in rat-liver homogenates. Our results on a mitochondrial system concerning the importance of the carboxylation of pyruvate are in good agreement with those found in the homogenate system by the above authors.

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Tamás König,
István Marosvári,
Attila Lipcsey

Budapesti Orvostudományi Egyetem
Biokémiai Intézet, Budapest.

COMPARATIVE GEL DIFFUSION ANALYSIS OF NATIVE AND CHROMIUM-LABELLED OVALBUMIN

By

MÁRIA KÁVAI and GABRIELLA BÁTORY

INSTITUTE OF PATHOPHYSIOLOGY, MEDICAL UNIVERSITY, DEBRECEN, AND INSTITUTE FOR SEROBACTERIOLOGICAL PRODUCTION AND RESEARCH "HUMAN", BUDAPEST

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The properties of chromium-labelled ovalbumin and of native ovalbumin are described as determined by immuno-diffusion and immuno-electrophoretic analysis. It has been shown that labelling with chromium at alkaline reaction does not influence the size, antigenicity and electrophoretic mobility of the protein molecules.

Labelling with natural or radioactive chromium has been found suitable for following-up the fate of antigens injected into the organism. Only minute quantities of chromium namely occur in the organism [1], while the half-life of ^{51}Cr is favourable and its radiation effect is slight [2].

In previous reports it has been shown that as far as electrophoretic mobility and antigenicity (as tested by quantitative precipitation) are concerned, the chromovalbumin prepared by our method (in the following Cr—OA) had properties similar to those of native ovalbumin [3, 4]. In the present paper we shall report on immune diffusion and immune electrophoretic studies from the results of which it has been concluded that Cr—OA does not differ from normal ovalbumin (in the following, N—OA) either in the character of the antigen determinants, or in molecular size.

Materials and methods

1. *Antigens :* Solutions of four times recrystallized N—OA, homogeneous by paper electrophoresis [5], and of ovalbumin labelled with chromium at a ratio of 1 : 25 [3] were used.

2. *Immune serum :* This was serum from rabbits immunized with native ovalbumin (in the following IS).

3. *Linear single immuno-diffusion method* [6, 7]: The immune serum diluted 1 : 2.8 with physiological NaCl solution and mixed with a 1 per cent solution of agar in physiological NaCl was measured into precipitation tubes 3 mm in diameter. The antigen solutions were diluted serially in steps of 1 : 2 and the single dilutions were layered in the liquid state over the mixture. The tubes were sealed with wax, kept at 22° C, and the distance of the front of the precipitation lines from the interface was measured at intervals of 24 to 48 hours. This distance (h) is directly proportional to the square root of the time (t) between the adding of the antigenic solution and the time of reading. The migration rate of the front (designated k) is expressed by the $h \cdot t^{-1/2}$ ratio, the direction tangent of the straight line. If, at a constant antibody level, the value k is plotted against the log of antigen concentration, a straight line results, expressed by the equation $k = mx + b$, where x is the log of antigen concentration, and m and b are constants characteristic of the reacting system. The value of m depends also on the diffusion coefficient, and thus also on the size of the molecule [8, 9]. To compare the m values, we determined an equation from the data of measurements on the basis of the principle of the least squares [10].

4. *Comparative single immuno-diffusion method :* The technique of OUDIN [11] was employed. The reagents were mixed with 4 per cent melted agar, then so placed on a square plate that the two antigen solutions to be compared should be in direct contact with each other, as well as with the immune serum. The method has the advantage over the procedures of similar purpose that the concentration relations of the reagents at the interface are constant and thus the reactions indicative of identity or relationship can reliably be evaluated. The fronts of the precipitation lines migrate according to the laws of single diffusion, and therefore from the site of the precipitation lines conclusions may be drawn as to the concentration relations.

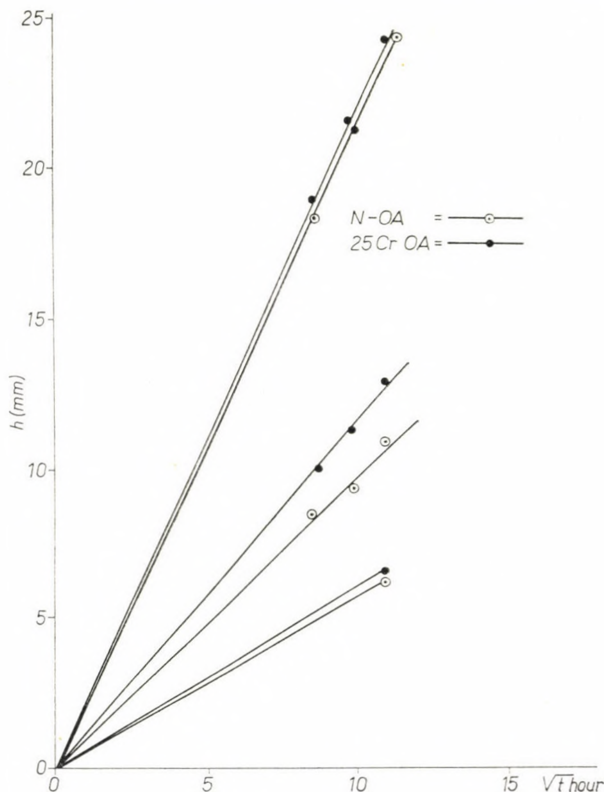

Fig. 1. Relationship between the distance of the front of the precipitate from the interface (*i*), and the square root of the reaction time (*t*), in the case of the antigens of N—OA and Cr—OA The migration rate of the single antigens is expressed by $h \cdot t^{-\frac{1}{2}}$

5. *Qualitative immuno-electrophoresis :* The method of GRABAR and WILLIAMS [12] as modified by WUNDERLY [13] was used. Filterpaper wetted with the antigen was applied to the agar plate. Veronal buffer of 0.05 ionic strength and pH 8.2 was used. The immune serum mixed with agar was placed beside the strip of agar containing the electrophoretically separated antigens.

6. *Quantitative immuno-electrophoresis* was carried out according to BACKHAUSZ [14]. With this method the evaluation of the results is based on the fact that the antigens separated in agar by electrophoresis react with the corresponding antibodies according to the laws of single immuno-diffusion, and therefore from the rate of migration of the maximums of the precipitation arcs, conclusions can be drawn as to the concentration of the single antigens.

7. *Absorption immuno-electrophoresis :* The principle of BÁTORY's method [15] is that the solution presumably containing a maximal number of antigens or antigen determinants is subjected to electrophoresis in agar gel by the usual method. Between the immune serum-

agar mixture and the electrophoretically separated antigens is placed the absorbing solution presumably containing a smaller number of antigens or less of the determinants, mixed with agar. Parallel with the line of contact, precipitation lines appear resulting from the reaction between immune serum and the antigens of the absorbing layer. If there is a lack of antigen in the absorbing solution, as compared with the electrophoresed solution, semicircular precipitates can be find in suitable positions. If the electrophoresed solution contains no determinants different from those contained in the absorbing solution, no semicircular precipitates will be formed. In our experiments we subjected the N—OA solution to electrophoresis and used the 1 : 10 dilution of Cr—OA as the absorbing layer.

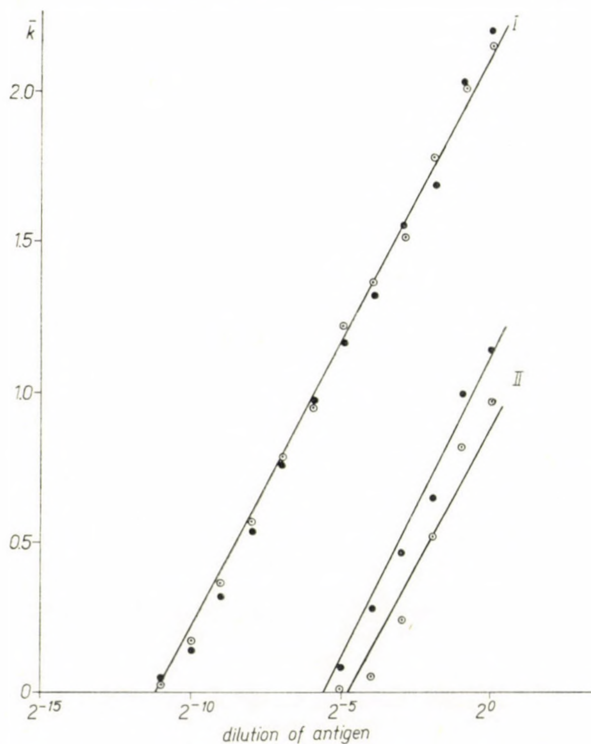

Fig. 2. Correlation between the k values obtained at different antigen concentrations and the log values of antigen concentration in the case of the antigens of N—OA and Cr—OA. The direction tangents of the straight lines obtained, the m values are characteristic of the molecular size of the corresponding antigens

Results

By the single linear immuno-diffusion method, 3 antigens were demonstrable in both the N—OA and the Cr—OA solutions. The component present at the lowest concentration was observable only at the third, 120-hour reading (Fig. 1).

If we analyze the change of k values in the function of the 2-base logarithm of antigen concentration (Fig. 2), the straight lines of the component present at the highest concentration were practically identical in the N—OA and the Cr—OA solutions.

The somewhat different behaviour of the second components was due to differences in the concentration of the corresponding antigen. The slope of the straight line was practically the same as in the previous case. These investigations revealed nothing as to the third component, because owing to the low concentration of this protein its precipitation band soon disappeared on diluting the solution.

The antigenicity of the three components of N—OA and Cr—OA was the same (Fig. 3). No spur formation indicative of a difference in determinants could be observed. The concentrations of components I and III proved to be

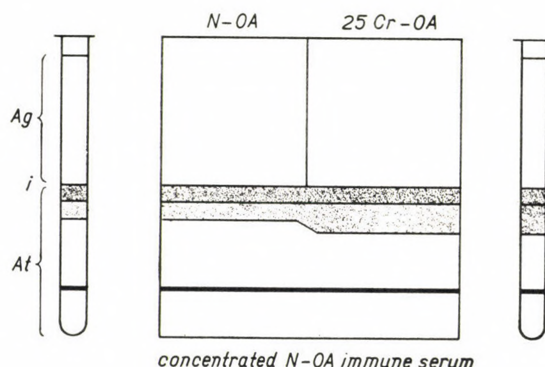

Fig. 3. Simple gel diffusion for the comparison of the antigens of N—OA and Cr—OA solutions. On the two sides of the plate are represented Oudin tubes of similar composition (i = interface, the line of contact between Ag and At). The pattern shows the identity of the three components. The precipitation zone in the middle indicates a difference in the concentration of the corresponding antigen between the two solutions

identical. Component II was present at a higher concentration in Cr—OA than in N—OA; the second precipitation bands of the two systems diverge at the point of junction.

As determined by qualitative immuno-electrophoresis (Fig. 4), the component present at the highest concentration in both OA and Cr—OA showed albumin motility. The two minor antigen components migrated at a lower velocity than albumin; owing to their low concentrations, their precipitation arcs developed in the antigen excess and were therefore pale. The three components of N—OA and Cr—OA showed the same motilities.

Quantitative immuno-electrophoresis showed the quantity of the two minor antigens to amount to about 2 to 3 per cent of the total protein.

The results of the absorption immuno-electrophoresis have shown that the antigenic components of N—OA and Cr—OA were identical; no characteristic precipitation arcs appeared in the absorbing layer.

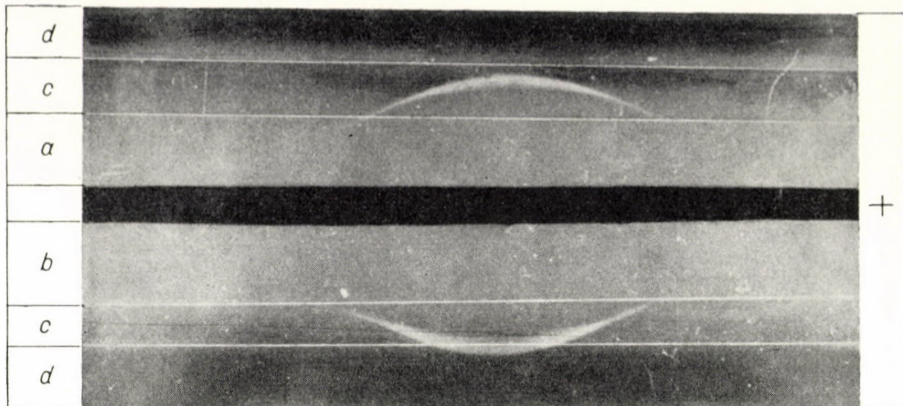

Fig. 4. Immuno-electrophoretic patterns of N—OA and Cr—OA (Grabar and Williams' method). The two antigens have the same electric motility

Discussion

The above results have fully confirmed our assumption that, if carried out adequately, labelling with chromium [3] does not change the original properties of the protein. The practically identical slope of the straight lines obtained by single linear immuno-diffusion proves that treatment caused no demonstrable changes either in the number of the antigen-antibody systems, or in the size of the N—OA molecule and the contaminating antigen molecule present at higher concentration. The identity of the slopes in the two systems indicates that no change has taken place in the number of the antigen determinants of the reaction, either.

The results of the comparative immuno-diffusion and the absorption immuno-electrophoretic studies show that chromium treatment caused no qualitative changes in the antigen determinants.

While with free and paper electrophoresis both N—OA and Cr—OA were shown to consist of one single component, the immunochemical studies demonstrated 3 antigens of different motility. The minor components amount to about 2 to 3 per cent of the total protein content. They are apparently conalbumin and ovomucoid contaminations [12], not separated from ovalbumin. The contaminating antigens, like the principal component, ovalbumin, underwent no change in immunochemical properties and motility following labelling with chromium.

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Mária Kávai, Gabriella Bátory
Orvostudományi Egyetem Kórélettani Intézete, Debrecen
és Humán Oltóanyagtermelő és Kutató Intézet, Budapest.

STUDIES ON THE ANTIGENIC PROPERTIES AND CHEMICAL STRUCTURE OF IRRADIATED PROTEIN

By

S. Damjanovich, Mária Kávai and L. Kesztyüs

INSTITUTE OF PATHOPHYSIOLOGY, MEDICAL UNIVERSITY, DEBRECEN

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Antigenicity of ovalbumin solutions at various concentration levels, as well as their SH-group content have been investigated before and after X-ray irradiation. With the doses used, no qualitative alteration has been found in antigenicity. The quantitative changes observed have been explained by alterations in the number of SH-determinant groups, a suggestion corroborated by the results of SH-group determination. The conclusion has been drawn that, in irradiation-induced damages of the protein molecule, the SH-groups exhibit the greatest sensitivity from both the biological and radiochemical point of view.

In view of the important part played by proteins in biological processes many authors have studied the radiation-induced alterations of both native and crystalline proteins. The problem of structural degradation, as well as the changes in the antigenic properties, of irradiated proteins have been investigated in detail. Little attention has, however, been paid to the question of when an alteration in the protein structure can be considered biologically significant.

The immunologic activity of irradiation-altered proteins has been investigated by Fricke *et al.* and, most recently, by Luzzio. These authors demonstrated a marked diminution in the serologic activity of irradiated crystalline ovalbumin. With a dosage of 10^6 *r*, this change took place before any alteration had occurred in the physical properties of the protein, *e.g.* in its solubility [1—5].

Luzzio, measuring the serologic activity of in vitro irradiated serum γ-globulin made an attempt to obtain information as to the degree of radiation injury in the total-body irradiated human patient [5].

Our present experiments deal with the correlation between the immunochemical and radiochemical damages of ovalbumin irradiated *in vitro*.

Methods

Irradiation : The experimental specimens were subjected to irradiation according to the method already described [6]. A *Siemens*-Stabilivolt apparatus was used without filter but with an inherent filtration by an aluminium tube 2.5 mm thick. The total dose was 50 000 *r*, the dose rate, 1665 *r*/min. Both volume and layer thickness of the irradiated solutions were

kept strictly constant. Dosage was controlled by means of a *Fricke*-type dosimeter [7]. Since denaturation of irradiated protein is prompt even at a temperature of 4° C, all experiments were performed with protein irradiated on the same day [8].

Antigen : Four times crystallized ovalbumin, prepared in this laboratory [9], was used at two concentration levels. (i) 4.8 per cent untreated and irradiated ovalbumin (in the following, native ovalbumin = NOA and irradiated ovalbumin = ROA), as well as (ii) 0.5 per cent NOA and 0.5 per cent ROA. All antigen solutions were made in a phosphate buffer at pH 5.4.

Antibody : Antiovalbumin-antibodies were obtained from the serum of rabbits immunized with each of the antigens described above (4.8 per cent NOA—IS and ROA—IS, as well as 0.5 per cent NOA—IS and ROA—IS; IS = immune-sera).

Immune reactions : Linear gel diffusion as well as comparative linear gel diffusion were made, according to the method of Oudin [10, 11]. For quantitative precipitation the procedure of Kabat and Mayer [12] was used. Inhibition of the determinant groups in the antigen, *i.e.* inactivation of the SH-groups present in NOA and ROA antigens, was brought about by p-chloromercuribenzoate (PCMB), monoiodoacetic acid and $CdCl_2 \cdot 2.5\ H_2O$, respectively; monoiodoacetic acid proved the most suitable inhibitory substance. One mole of ovalbumin was incubated with 10 moles of monoiodoacetic acid in Tris-buffer at pH 7.0 for 30 minutes. Thereafter, the excess of monoiodoacetic acid was removed by dialysis against physiological saline. Prior to the antigen-antibody reaction the protein content of the sample was determined.

Determination of SH-groups : The total amount of SH groups in native and irradiated proteins was determined with PCMB, and the amount of the so-called superficial SH-groups, with nitroprusside sodium, by the spectrophotometric method of Brückman and Wertheimer [14,].

Spectrophotometry : UV absorption curves between 240 and 320 mμ of NOA and ROA were measured by means of a *Unicam* SP-500 spectrophotometer using 1 cm quartz cells and the buffer solution as blank.

Results and discussion

The first part of our experiments was devoted to the study of the structural changes in ovalbumin after irradiation. As well-known, the absorption curve between 240 and 320 mμ of proteins shows, after indirect irradiation with 10^4 to 10^5 *r*, a dose-depending elevation which runs roughly parallel to the abscissa. The height of the elevation, *i.e.* the degree of radiation injury, is in inverse ratio to the concentration, provided that dilute solutions and a constant dosage have been used [6, 7, 17]. The absorption curve of 4.8 per cent protein solutions fails to show any alteration, while 0.5 per cent solutions exhibit, after irradiation, an UV absorption higher than that of the controls (Fig. 1). Prior to the spectrophotometric measurements both protein solutions were diluted with buffer to give a final protein concentration of 0.05 per cent. It was assumed, on the basis of our previous findings, that in both cases of the ovalbumin molecule the structural parts stabilized by non-covalent bonds (secondary and tertiary structures) were injured. In addition to absorption spectrophotometry, this assumption has been substantiated by investigations into sedimentation and electrophoretic homogeneity [6].

Investigating the amino acid composition of proteins irradiated with high doses, Kumta *et al.* [18] found the sulphur-containing amino acids to be the most susceptible to irradiation. Since protein SH-groups, too, are involved in the formation of the secondary and tertiary structures, an attempt was

made to establish the degree of radiation injury by determining the changes in the amount of SH-groups.

The result of a SH-group determination depends on the method used. The value for the so called total SH-groups is obtained when using the PCMB procedure of BOYER [16], while the method with nitroprusside sodium only

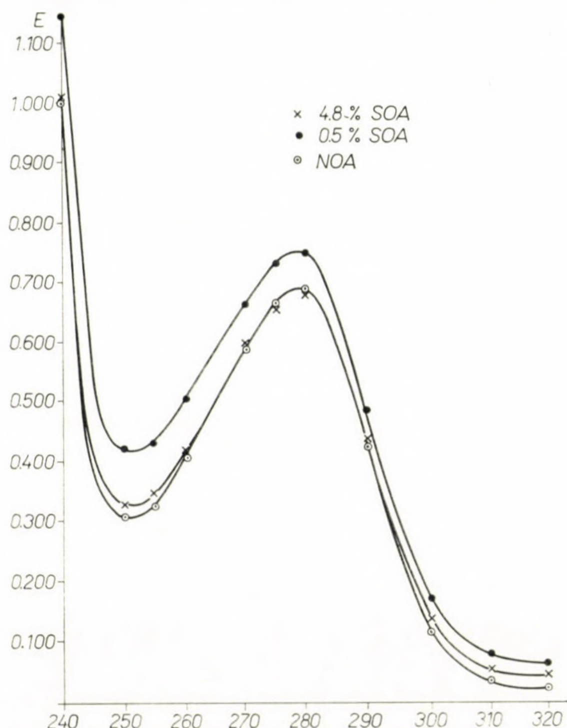

Fig. 1. Absorption curve of NOA (native ovalbumin) and ROA (irradiated ovalbumin) between 240 and 320 mμ. The changes due to irradiation with 50,000 *r* depend on the concentration used. The absorption of 4.8 per cent ROA differs but slightly from the control, while 0.5 per cent ROA shows marked post-radiation alterations

gives the amount of the superficial, so-called unmasked SH-groups. The masked SH-groups, not being readily attacked by the reagent, are situated presumably at some sterically sheltered sites of the molecule or, else, they form some kind of a weak non-covalent bond [19, 20].

On the basis of such considerations, determination of SH-groups was made by two different methods. The experiments with PCMB after irradiation with 50,000 *r* failed to show a change in the amount of the SH-groups of ovalbumin either with a 0.5 per cent protein solution or with a ten-times more concentrated one (Figs. 2 and 3). Table I shows the results of SH-group determi-

nations made with nitroprusside sodium before and after irradiation with 50,000 *r*. The intensity of the nitroprusside reaction given by a control solution containing 0.05 per cent protein — $E_{1\,cm}^{0.05\,\%}$ at 520 mμ — was taken for 100 per cent and the colour developed with proteins irradiated in more dilute or more concentrated solutions was compared with that value. In the case of 4.8 per cent protein the nitroprusside test revealed a 10 to 30 per cent post-radiation

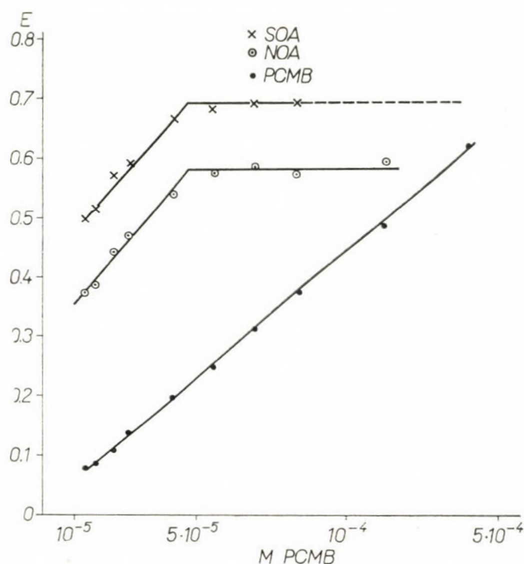

Fig. 2. Determination with PCMB of SH-groups in 0.5 per cent NOA before and after irradiation with 50,000 *r*. Breaking-points of both the middle and upper curves lie at about identical PCMB concentrations, *i.e.* irradiation failed to alter the number of SH-groups. All points except those in the calibration curve (bottom line) represent the average of five experiments

increase in the amount of SH-groups. When, however, an 0.5 per cent ovalbumin solution was used, the number of SH-groups failed to show any change when checked by nitroprusside sodium.

Table I

Determination of SH-groups with nitroprusside sodium

Number of experiments	0.5 per cent control ovalbumin	0.5 per cent irradiated ovalbumin	4.8 per cent control ovalbumin	4.8 per cent irradiated ovalbumin
1.	100	105	100	121
2.	100	98.2	100	130
3.	100	102	100	137
4.	100	95	100	110
5.	100	99	100	130

An attempt was made at following these subtle structural alterations by immunochemical methods. Rabbits were immunized with irradiated and control proteins, respectively. The antibodies obtained afforded a possibility to investigate the effect of irradiation on the specificity of the antigen. The

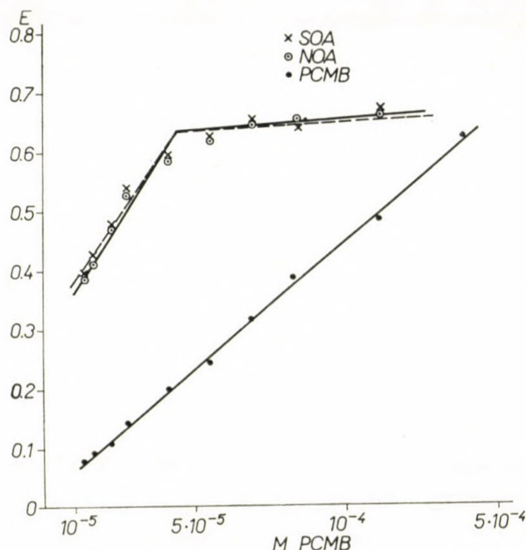

Fig. 3. Determination with PCMB of the number of SH-groups in 4.8 per cent NOA before and after irradiation with 50,000 *r*. No change in the number of SH groups

identical angles of inclination of the straight lines obtained after simple linear gel diffusion ($\text{tg}\ a_{ROA} = \text{tg}\ a_{NOA} = 0.203$) prove that the irradiation of NOA in 4.8 per cent solution failed to cause any observable alteration in the number of antigen-antibody systems or in the size of the NOA molecule. Similarly,

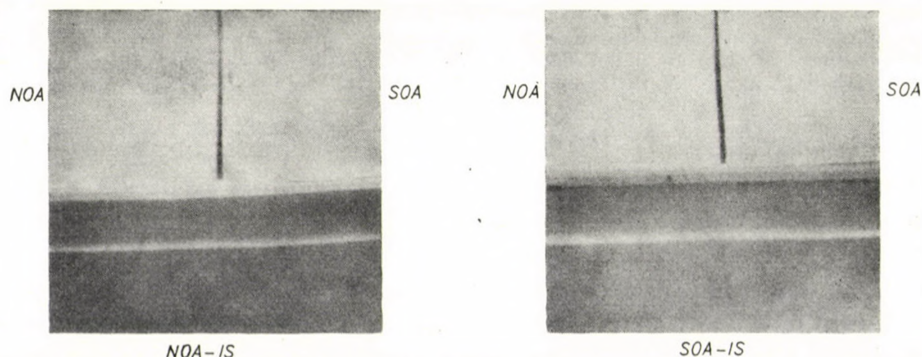

Fig. 4. Comparative gel diffusion analysis on glass-plate after 48 hours of the reaction. The lower part of the plate is occupied by 4.8 per cent NOA-IS and ROA-IS, respectively. The two antigens to be compared are placed above the immune sera, at the upper quarter of the plate

comparative gel diffusion revealed no such qualitative change in the antigen determinants, which could have been attributed to irradiation (Fig. 4).

After our failure in finding qualitative differences, the investigations were extended to the quantitative aspects of the problem. As seen from Fig. 5, precipitation performed with 4.8 per cent NOA in ROA—IS showed 8 to 12 per cent less antibodies in eight of the immune sera. The constant a in the

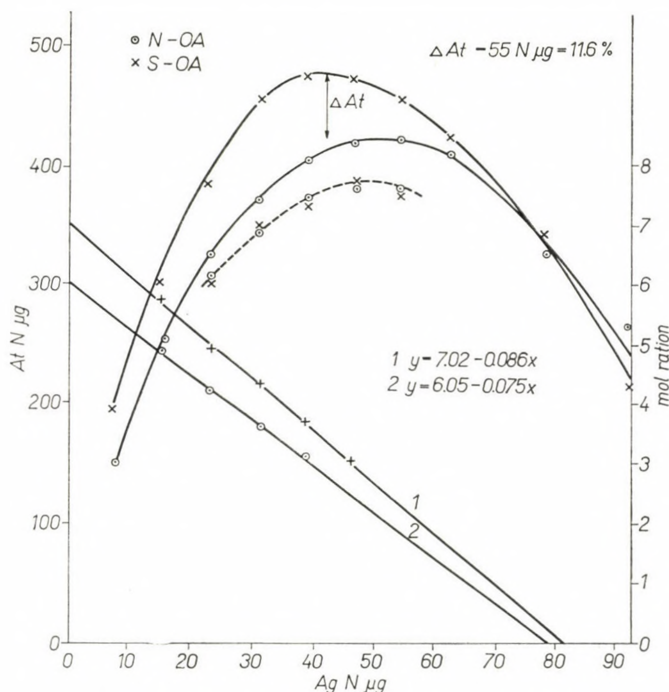

Fig. 5. Quantitative precipitation of 4.8 per cent ROA-IS with 4.8 per cent NOA and ROA, as well as with monoiodoacetic acid-treated NOA and ROA (—). The antibody content as well as the molar ratio of antigen to antibody are expressed as a function of the antigens. The corresponding equations are also shown. Δ *At* means the difference in antibody content obtained with ROA and NOA, the former being taken for 100 per cent and the difference being expressed in percentile terms

equation characterizing the antigen-antibody reaction was smaller than in the case of precipitation with homologous 4.8 per cent ROA. Both irradiated and native 4.8 per cent antigens reacted similarly with ROA—IS (Fig. 6), the content in traceable antibodies having been the same. The difference found in constant a ($\Delta a = 0.30$) is indicative of a slight decrease in the combinatory capacity of the antigen following irradiation. In contrast, 0.5 per cent ROA showed an antibody content similar to NOA both in ROA—IS (Fig. 7) and NOA—IS (Fig. 8). The only difference was in the decreased combinatory

capacity of ROA with both immune sera, as well as in the diminished solubility of the precipitate in the antigen-preponderance zone.

Immunologically, the SH-groups are determinant groups as well; immunologic methods have revealed that, during the antigen-antibody reaction, part of the antibodies produced against the antigenic protein becomes attached to SH-groups [21]. In the light of our investigations into the chemical structure, an attempt was made to establish by means of cysteine-hapten inhibition,

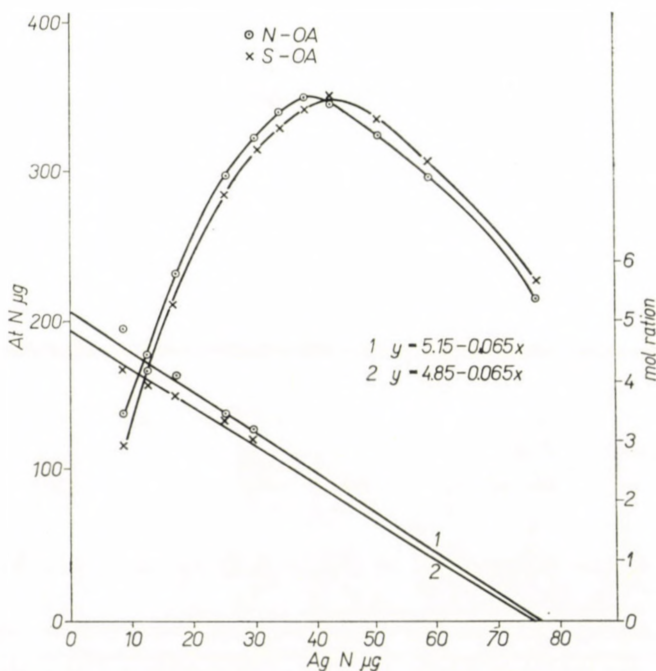

Fig. 6. Quantitative precipitation of 4.8 per cent NOA-IS with 4.8 per cent NOA and ROA. Antigenic functions of both the antibody content and the molar ratio are shown together with the corresponding equations

whether the excess amount of precipitate, given by 4.8 per cent ROA—IS with 4.8 per cent ROA, against that obtained with NOA, was due to an increase in the number of ROA—SH determinant groups. Even though the investigations of KULYBERG [13] as well as our own observations showed that cysteine did not cause a deterioration of antibodies, its potent inhibitory effect on the antigen-antibody reaction is not entirely due to hapten inhibition. Thus, this latter procedure is not suitable to answer the question raised in the previous paragraph.

Another possibility was to fix the determinant groups. Treatment of antigens with SH-reagents led to the disappearance of the difference in the

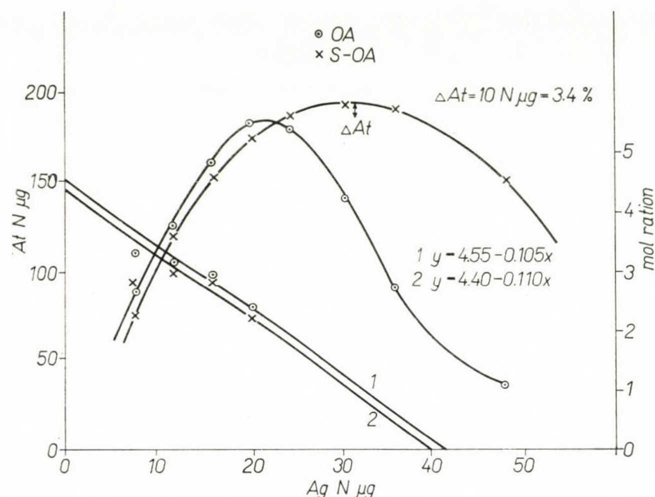

Fig. 7. Quantitative precipitation of 0.5 per cent ROA-IS with 0.5 per cent NOA and ROA. For explanation, see Fig. 5

amounts of precipitate obtained with ROA and NOA, in their reaction with ROA—IS (Fig. 5).

The results of our physicochemical and immunochemical investigations can be explained as follows. Depending on the protein concentration used, irradiation evokes such changes in the secondary and tertiary structures of

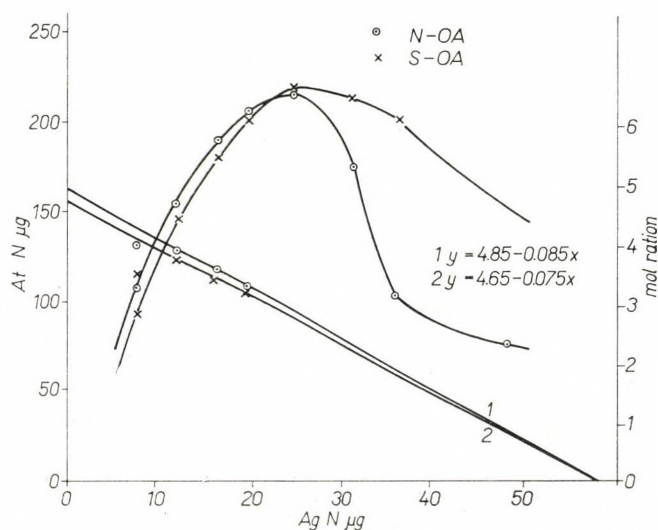

Fig. 8. Quantitative precipitation of 0.5 per cent NOA-IS with NOA and ROA. For explanation, see Fig. 6

ovalbumin as to cause the liberation of SH-groups originally masked either by steric structural peculiarities, or by weak bonds. An interesting finding concerning this mechanism is the observation that an agent with predominantly oxidizing properties, such as irradiation, can so alter the protein structure that the reducing power of 4.8 per cent ovalbumin is enhanced. In more dilute solutions, *i.e.* under the effect of more marked radiation injuries, it is the oxidizing effect which becomes manifest, without, however, influencing either the antibody-determinant properties of the SH-groups or their determination with PCMB, in the case of used dose and concentration. In solutions of higher concentration, the masked groups, which are biologically internal or latent determinant groupings, undergo such post-irradiation changes that they gain real antigen determinant properties.

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Sándor Damjanovich, Mária Kávai, Loránd Kesztyüs
Orvostudományi Egyetem Kórélettani Intézete, Debrecen.

ISOLATION OF THE CLOTTABLE PROTEIN FROM THE SECRETION OF THE RAT'S SEMINAL VESICLE

By

S. Mányai

INSTITUTE OF MEDICAL CHEMISTRY, MEDICAL UNIVERSITY, BUDAPEST

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The paper electrophoresis of the secretion of the seminal vesicles of rats, mice and guinea-pigs in the presence of urea leads to the separation of different protein components. The presence of a highly basic protein fraction was demonstrated in the case of each species. Characteristic changes in the protein pattern have been found following the enzymic coagulation of the secretions by the vesiculase of the coagulating glands: the highly basic protein fraction was precipitated. The same clottable protein of the secretion of the rats seminal vesicles has been isolated both by DEAE cellulose column chromatography, and by precipitation with glycerol. The isolated basic protein was found to be homogeneous as judged by paper electrophoresis at different pH values.

Protein synthesis in the seminal vesicles of the rat and the influence of testosterone treatment on this process was studied in previous investigations [28, 29, 30]. In the course of this work a highly labelled protein fraction was found associated with the nucleic acid fractions isolated from vesicular mince *in vitro* incubated with radioactive labelled amino acids. The specific radioactivity values of this protein fraction were about 50 times higher than those of other proteins of the same gland. The possibility of the identity of this protein fraction and the proteins (or their precursors) secreted by the gland arises. A detailed study of the proteins of the vesicular secretion was therefore undertaken.

As in the case of other rodents a copulatory plug is formed out of the secretion of the seminal vesicles of the rat following its depletion from the gland: the fluid clots instantaneously due to the effect of the vesiculase produced by the coagulatory gland [23, 38, 14, 21, 22, 4, 7, 42, 18, 26, 27, 31]. Gotterer and Williams-Ashman [9, 10] described in detail the mechanism of the clotting effect of vesiculase: an approximately 30 fold purification of the enzyme was achieved by these authors, and it was established that the effect depends on the presence of bivalent cations, and the clotting mechanism does not resemble the mode of action of thrombin.

The protein components of the secretion of the seminal vesicles are much less known. A protein component similar to fibrinogen and present in the secretion of the seminal vesicles of the guinea-pig is mentioned by Landwehr [18]. The occurrence of a histone-like protein in the same secretion of the rat was described by Walker [42]. Sixty per cent of the proteins of human

vesicular secretion consist of proteoses, *i.e.* material which would pass through a cellulose membrane impermeable to serum proteins [16]. Immunoelectrophoretic properties of the secreted proteins were studied by GRANT and EVERALL [11] to establish the role of the various accessory sexual glands in the production of the individual protein components of the human seminal plasma. Data concerning the electrophoretic mobilities of the proteins of the vesicular secretion of the pigs were presented by BOURSNELL *et al.* [1]. The electrophoretic investigation of human [17, 15, 36, 37, 32, 13, 41, 35, 25] and mammalian seminal plasma [19, 20, 40, 41, 2, 3] was the subject of a series of communications. Although varying from species to species the main bulk of seminal plasma consists of the vesicular secretion, the secretions of this organ and of the remaining accessory glands are mixed during ejaculation. This being so results gained by the investigation of the final seminal plasma must not be referred to the vesicular secretion directly.

The secretion of the seminal vesicles of the rat and guinea-pig was fractionated by 60 per cent acetone and dried by the pure reagent in the experiments of GOTTERER and co-workers [9, 10], the 0.15 *M* NaCl extract of the dry powder obtained by this procedure served as the substrate in the investigations of the mode of action of the vesiculase. Three different protein fractions were found in this extract by paper electrophoretic separation. The isolation and the properties of the individual proteins are not mentioned.

The present paper describes the studies on the proteins secreted by the seminal vesicles of rats, guinea-pigs and mice. Changes in the protein composition following the clotting of the secretion by vesiculase was studied. The isolation of a highly basic protein is described. This protein clots rapidly in the presence of vesiculase.

Methods

Adult male white rats of 180—300 g, guinea-pigs of 600—700 g and mice of 18—20 g body weight were used in the experiments. The animals were sacrificed by decapitation and in order to obtain the secretion of the seminal vesicles the glands of laparotomized animals were punctured with a thick hypodermic needle. The highly viscous secretion was diluted — if not stated otherwise — with 2 to 3 volumes of a mixture containing 0.15 *M* NaCl + + 0.01 *M* borate buffer solution (pH 8.07) and was spun with 20,000 $\times$ *g* for 20 mins. in a MSE "17 High Speed Refrigerated" centrifuge. The supernatant obtained by this procedure was a slightly yellowish water clear liquid and called the "soluble fraction". It contained nearly 60 per cent of the total protein content of the secretion.

Paper electrophoresis of the soluble fraction and of the proteins isolated from this fraction was performed on Schleicher—Schuell's 2043 *b* or Whatman 3 MM paper, in a phosphate-borax buffer solution at pH 8.6, $\mu = 0.05$ [8]. The buffer solution usually contained 4 *M* urea (in some cases 8 *M* urea, resp. none). The electrophoresis was continued for 16 to 20 hours at 6.2 V per cm potential gradient, with a current of 20 to 26 mA. The position of the individual proteins on the electropherograms was indicated with the aid of bromophenolblue. Two different methods were employed for the quantitative evaluation of paper electropherograms.

a) The paper stained with bromophenolblue was cut transversely in strips of 4 mm, the bound dye was eluted from these strips with a 0.5 *N* NaOH solution containing 50 per cent of ethanol and was estimated photometrically in an Uvifot photometer at 578 nm wave length.

b) The dried unstained paper was cut into strips as described, each piece of paper was extracted individually by rinsing in 4 ml of a solution containing 0.02 per cent K-Na-tartarate and $CuSO_4$ and 2 per cent Na_2CO_3 dissolved in 0.1 *N* NaOH for 10 minutes. 0.4 ml of Folin's reagent was given to the tubes without centrifuging the paper strips and the protein content was determined [24]. Usually 1 to 4 mg of protein was present on the paper electropherograms and especially when electrophoresis was run in presence of urea this facilitated the subsequent elution of proteins from the paper, it was possible to recover and determine this amount (distributed on 25 to 30 strips of paper) with an error not exceeding $\pm$ 2 per cent.

The percentual distribution of the protein components of the soluble fraction based on methods *a)* and *b)* did not coincide; 1 mg of the highly basic protein fraction (determined by the Lowry method) was able to bind 1.6 times more of the dye compared to the other protein components. The Lowry protein estimation method was used in the overwhelming majority of experiments (cf. *b*).

In order to separate the undiluted total secretion of the seminal vesicles by DEAE cellulose column chromatography, columns of 0.8 $\times$ 32 cm size prepared from 2 g of DEAE cellulose powder were adopted. The cellulose columns were washed with 20 ml of 0.1 *N* NaOH, followed by washings with 75 ml of 0.01 *M* borate buffer (pH 8.2). The same buffer solution was used in the elution procedure as well. The effluent was collected in fractions of 2.4 ml each. The protein content of the fractions was determined according to the method of Lowry *et al.* [24].

The 10,000 $\times$ *g* supernatant (15 mins.) prepared by 0.15 *M* NaCl from the 10 per cent homogenate of pooled coagulatory glands of several rats served as source of vesiculase enzyme activity. Same preparations were obtained from pooled organs of mice and guinea-pigs, resp.

All the applied chemicals (*Reanal*, Budapest) were of analytical grade. Glycerol used in the isolation of the basic protein fraction was a product of *Merck*, Darmstadt. A Whatman DE 50 preparation of DEAE cellulose powder was used.

Experimental results

A) *The paper electrophoretic assay of the soluble fraction of the secretion of the seminal vesicle*

i) *Experiments with rats*

The secretion of the seminal vesicles was diluted with a 0.15 *M* NaCl + + 0.01 *M* borate buffer solution (pH 8.07) and was centrifuged with 20,000 $\times$ *g* for 20 minutes. The supernatant was used for further investigations. This soluble fraction when submitted to paper electrophoresis in a phosphate-borax buffer solution, which contained 4 *M* of urea (pH 8.6) could be separated into 5 protein fractions (Fig. 1). One of these fractions containing 52 to 60 per cent of the total protein (Fraction *N*) remained in the vicinity of the starting line just slightly shifted towards the positive pole. The presence of two further fractions (A_1, A_2) containing not more than 5 to 10 per cent of the total protein content moved in the same direction. The most characteristic and most striking protein fraction of the secretion of the seminal vesicles is nevertheless the highly basic protein fraction (*B*), comprising 35 to 40 per cent of the soluble protein which migrated towards the negative pole under the same conditions. It was observed in numerous cases that the basic protein fraction appeared in the form of two adjacent components (B_1, B_2) which could be revealed sepa-

rately both by the bromophenolblue staining technique and protein estimations following direct elution of the paper electropherograms.

Essentially the same components could be detected, if paper electrophoresis was carried out in phosphate-borax buffer solutions in the absence of urea (Fig. 2). Quantitative evaluation was, however, more difficult and more uncertain in this case due to the circumstance that the separation of the individual protein components was poor, they were found close to each other, and near to the starting line.

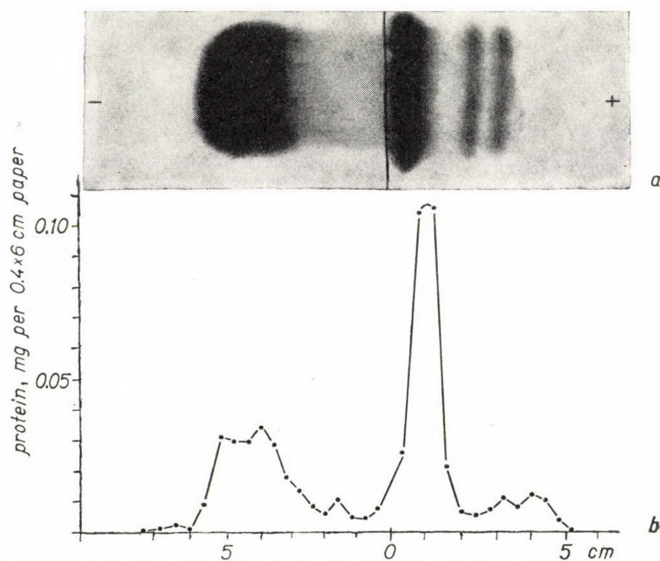


Fig. 1. Protein components in the soluble fraction of the vesicular secretion of rats separated by paper electrophoresis

a) The paper electropherogram of the soluble fraction in phosphate-borax buffer solution containing 4 *M* urea, pH 8.6, $\mu = 0.05$, 6.4 V/cm, 26 mA, 18 hours, stained with bromophenolblue. *b)* Quantitative evaluation of the same, but unstained electropherogram run parallel with *a)*. The evaluation was based on protein determinations

The percentual distribution of the individual proteins separated in a phosphate-borax buffer solution containing 4 *M* urea, refers only to the soluble fraction, and as mentioned earlier, represents only 60 per cent of the total protein content. Forty per cent of the protein was precipitated on diluting the secretion with 0.15 *M* NaCl solution. The amount of the precipitate did not change if the diluting medium contained 1 to 10 m*M* of EDTA. On the other hand if the secretion of the seminal vesicles was collected by dilution with 4 *M* urea solution, the amount of the precipitated protein is lowered to 20 per cent. The basic protein component appears in higher quantities on the electropherograms of the soluble fraction obtained by this procedure, an

indication, that the solubility of the basic protein component is particularly poor in 0.15 M NaCl and is precipitated comparatively easier than the remaining proteins of the secretion. At the same time the presence of urea is primarily essential in keeping the basic protein fraction in solution. A highly viscous,

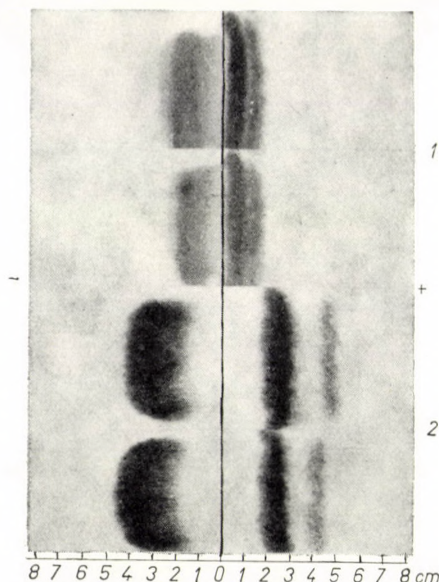


Fig. 2. The effect of urea on the electrophoretic mobilities of the protein components of the seminal secretion of rats

Characteristics of the paper electrophoretic assay: *1.* Phosphate-borax buffer solution, pH 8.6, $\mu = 0.05$; *2.* 4 M urea plus phosphate-borax buffer solution, pH 8.6, $\mu = 0.05$, 6.4 V/cm, 17 mA, 16 hours, stained with bromophenolblue

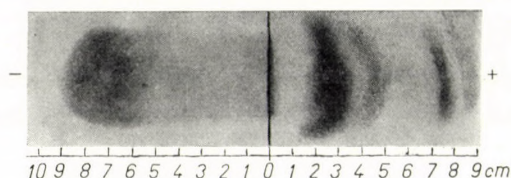


Fig. 3. Paper electropherograms of the vesicular secretion of rats tapped by 8 M urea

Characteristics of the paper electrophoretic assay: Phosphate-borax buffer solution containing 8 M urea, pH 8.9, $\mu = 0.05$, 6.4 V/cm, 24 mA, 18 hours, stained with bromophenolblue

opalescent solution was obtained on collecting the secretion of the seminal vesicles with 8 M urea in the course of the tapping of the gland; no material could be sedimented from this solution by centrifuging for 1 hour at 20,000 $\times$ g. Paper electrophoresis of the same solution carried out in phosphate-borax buffer solution containing 8 M urea resulted in the appearance of more than 5 fractions. The quantitative evaluation of this pattern was impossible as part

of the protein subjected to paper electrophoresis (approximately 15 per cent) was adsorbed in an irreversible way at the starting line (Fig. 3). Reduction of the urea concentration of the solution caused the formation of a precipitate. This was dissolved in 8 *M* urea, and then submitted to paper electrophoresis in a phosphate-borax buffer solution containing 4 *M* urea. The precipitate appeared to consist mainly of the basic protein component.

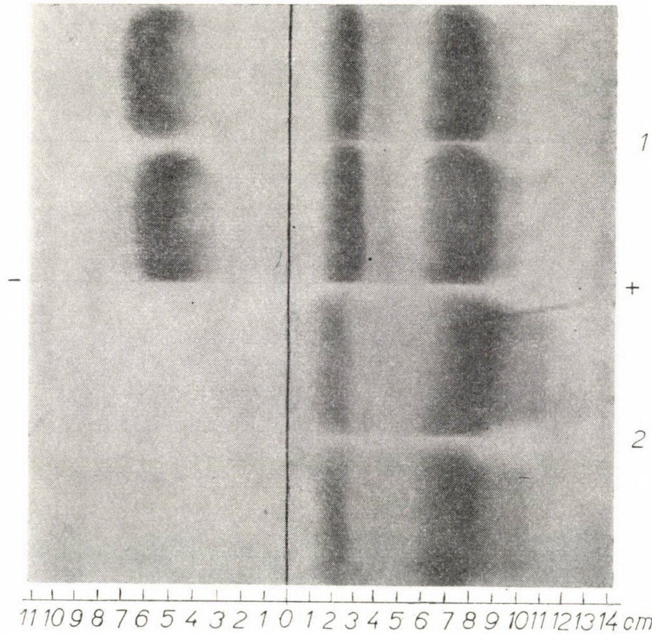


Fig. 4. The effect of enzymic clotting on the protein components of the vesicular secretion of guinea-pigs

Characteristics of the paper electrophoretic assay: Phosphate-borax buffer solution containing 4 *M* urea, pH 8.6, $\mu = 0.05$, 6.4 V/cm, 25 mA, 17 hours, stained with bromophenolblue.

1. The soluble fraction of the vesicular secretion of guinea-pigs;

2. The supernatant of the same fraction after clotting with homologous vesiculase and subsequent centrifugation

ii) *Experiments with guinea-pig and mice*

The paper electrophoretic patterns of the soluble fraction obtained from the secretion of the seminal vesicles of guinea-pigs and mice are presented on Figs. 4 and 5. In accordance with experiments with rats, the presence of the highly basic protein fraction moving towards the cathode at pH 8.6, could be demonstrated in both species. Furthermore 4 protein components clearly separated from each other could be revealed in addition to the basic protein, both in the secretion of the seminal vesicles of guinea-pigs and mice.

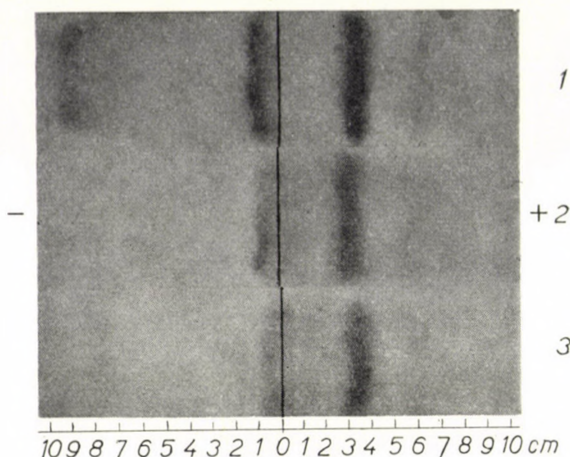


Fig. 5. The effect of enzymic clotting on the protein components of the vesicular secretion of mice

Characteristics of the paper electrophoretic assay: Phosphate-borax buffer solution containing 4 *M* urea, pH 8.6, $\mu = 0.05$, 6.4 V/cm, 28 mA, 17.5 hours, stained with bromophenolblue.

1. The soluble fraction of the vesicular secretion of mice;

2. The supernatant of the same fraction after clotting with heterologous (rat) vesiculase, and subsequent centrifugation;

3. The supernatant of the same fraction *(1)* after clotting with homologous vesiculase, and subsequent centrifugation

B) *The effect of the vesiculase enzyme on the protein components of the soluble fraction of the secretion of the seminal vesicles*

The soluble fraction of the secretion of the seminal vesicles of rats, guinea-pigs and mice is clotted due to the effect of the vesiculase enzyme produced by the coagulatory gland. The clotting process is connected with the formation of a gel and takes place in the course of 10 to 20 seconds even at room temperature, provided that the protein concentration of the soluble fraction is higher than 10 mg per ml. If the protein content was lower than this value the formation of a precipitate consisting of large clots or a milky turbulence was only observed. The gelatinous vesicular secretion clotted by vesiculase is changing into a crude precipitate on standing at room temperature for 10 minutes. Stirring with a glass rod had a similar effect. The paper electropherogram of the clear supernatant obtained after removal the clot compared to the pattern of the original native soluble fraction is demonstrated on Fig. 6. The most striking difference to be observed is the absence of the characteristic basic protein in the supernatant of the clotted secretion of the seminal vesicles.

The protein component characterized by its displacement towards the cathode at pH 8.6 was equally absent from the electrophoretic patterns of the

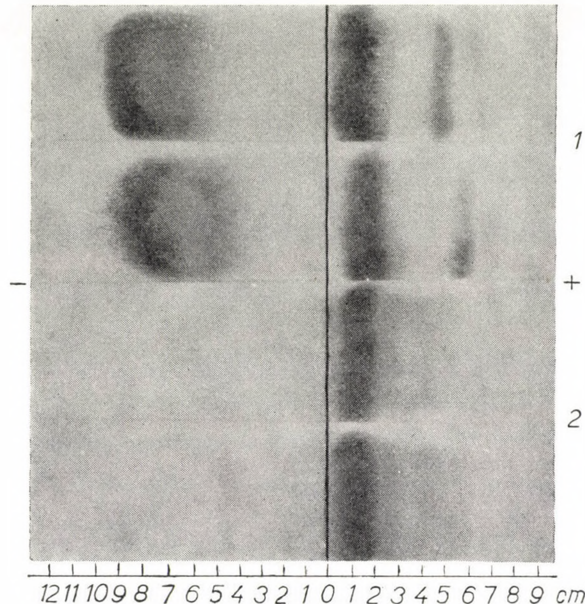


Fig. 6. The effect of enzymic clotting on the protein components of the vesicular secretion of rats

Characteristics of the paper electrophoretic assay: Phosphate-borax buffer solution containing 4 *M* urea, pH 8.6, $\mu = 0.05$, 6.4 V/cm, 24 mA, 16 hours 15 mins., stained with bromophenolblue.

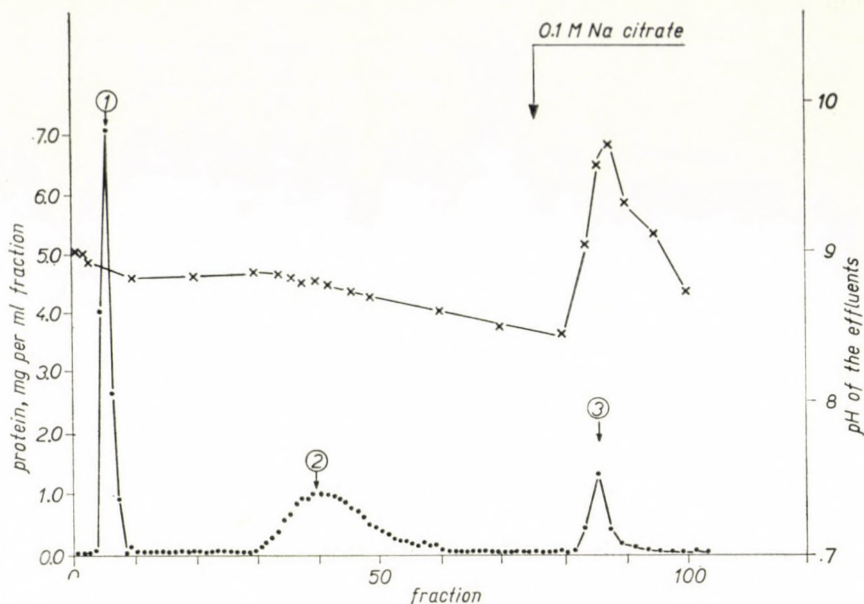
1. The soluble fraction of the vesicular secretion of rats;

2. The supernatant of the same fraction after clotting with homologous vesiculase, and subsequent centrifugation

vesiculase-treated secretions originating from the vesicular organs of rats, guinea-pigs and mice. It was irrelevant in this respect whether the soluble protein fraction was clotted by the homologous vesiculase or by a heterologous one. All this supports the assumption that it is just the highly basic protein component of the seminal vesicles of these rodents that is clotted by the vesiculase enzyme. In order to prove this fact in a more clear-cut way the complete and undiluted secretion of the seminal vesicles of the rat was subjected to preparative chromatography on a DEAE cellulose column. The basic protein component was subsequently isolated and its clotting ability was examined.

C) *DEAE cellulose column chromatography of the total secretion of the seminal vesicles of the rat*

The seminal vesicles of rats were punctured using a dry syringe, the total secretion obtained by this procedure was applied to a DEAE cellulose column (0.8 × 32 cm). The column was eluted with 0.01 *M* borate buffer



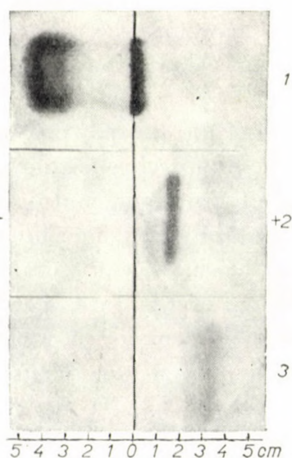


Fig. 7. DEAE cellulose column chromatography of the total, undiluted vesicular secretion of rats

The elution was carried out with 0.01 *M* borate buffer solution (pH 8.2), the latter being substituted by 0.1 *M* sodium citrate solution (pH 8.3) from fraction 76. Fractions of 2.2 ml volume were collected. Characteristics of the paper electrophoretic assay of several, individual fractions: Phosphate-borax buffer solution containing 4 *M* urea, pH 8.6, $\mu = 0.05$, 6.4 V/cm, 21 mA, 15 hours 20 mins., stained with bromophenolblue.

1. Fraction 6 (highly turbid, part of the proteins was adsorbed on the paper at the start line);

2. Fraction 40;

3. Fraction 86

solution (pH 8.2) followed by 0.1 *M* sodium citrate. Three main protein components (basic, neutral, and acid components) were separated (Fig. 7). The DEAE cellulose column was washed before use first with 0.1 *N* NaOH, then with 0.01 *M* borate buffer solution and it was found to be a prerequisite for good separation of the protein components to keep the pH of the effluent between pH values 8.5 to 9.0 at the moment when the secretion was given to the top of the column. As evidenced on Fig. 7 the basic protein passes the column without being bound to it under these conditions. The effluent containing the basic proteins is a milky and turbid one, some times it contains a precipitate, it has a high viscosity, threads are drawn from its droplets on falling down at the bottom of the chromatographic column.

The second protein fraction eluted from the DEAE cellulose column with 0.01 *M* borate buffer (pH 8.2) was always homogeneous in paper electrophoretic assays carried out in 4 *M* urea + phosphate-borax buffer solution ($\mu = 0.05$, pH 8.6) and proved to be identical with the neutral protein component (*N*) formerly mentioned.

The subsequent elution of the DEAE cellulose column performed with 0.1 *M* sodium citrate raised the pH value of the effluent nearly to 10, owing to the participation of OH^--ions of DEAE cellulose which were exchanged in this process, the acid protein fraction was eluted meanwhile. A variable part of the protein was denaturated on the surface of the cellulose and could be eluted only with 0.1 *N* NaOH. Therefore the proportion of the different protein fractions in the total secretion of the seminal vesicles could not be determined by this method.

Addition of vesiculase (1.2 mg protein per ml) to the various fractions obtained by DEAE cellulose column chromatography caused the immediate formation of a precipitate in all fractions containing the basic protein, these mixtures were clotted after standing for some minutes. The neutral and acid protein fractions did not react with the clotting enzyme.

The basic protein fraction of the total vesicular secretion, which ran through the column without being bound to it was subjected to paper electrophoresis under the same conditions as usual, *i.e.* in a phosphate-borax buffer solution containing 4 *M* urea ($\mu = 0.05$, pH 8.6). Two proteins moving towards the cathode could be distinguished near to each other on the patterns in every case (Fig. 7).

D) *The isolation of the basic protein component of the secretion of the seminal vesicles of the rat*

The following procedure was adopted both for further purification of the basic fraction of the complete secretion of the seminal vesicles after DEAE cellulose column chromatography and for the isolation of the clottable protein

of the soluble fraction of the vesicular secretion. The procedure is based on the chance observation, that the basic protein component is precipitated with 20 per cent glycerol (final concentration) at the isoelectric point, pH 9.7. This observation served as the main principle of the isolation. It was possible to purify the basic fraction from contaminating proteins by repeated washings with borate buffer solution (pH 9.7) and reprecipitations at the isoelectric point respectively. According to our experience the efficiency of isolation depends on the isoelectric reprecipitation step. The course of isolation is shown in the following scheme:

Soluble fraction of the secretion of the seminal vesicles, or basic fraction obtained by the DEAE cellulose column chromatography
+ equal volume of 0.1 *M* borate buffer, pH 9.7
+ glycerol, 20 per cent (v/v), final concentration

Kept at 0° C for 15 to 60 minutes, followed by centrifugation

Supernatant discarded,	precipitate washed twice with 0.1 *M* borate buffer solution (pH 9.7), then dissolved with 1 ml of 0.1 *N* NaOH, and reprecipitated with 4 volumes of 0.1 *M* borate buffer solution (pH 9.7). The precipitate was washed further with various solvents as follows: 0.1 *M* borate buffer solution (pH 9.7), twice, 0.4 *N* $HClO_4$, twice, ethanol, twice, ether, twice.

The final, washed precipitate was dried by mild heating under an infrared lamp. (In some cases washings with $HClO_4$, ethanol, and ether were omitted and the precipitate was not dried.)

The basic protein isolated either in the form of an ether dried powder, or as a precipitate was soluble in 8 *M* urea, or 0.5 to 1.0 *M* citric acid up to 5 to 10 per cent protein content. 2 *M* urea was already sufficient to make a 0.1 to 1 per cent solution. The protein solutions appeared homogeneous by paper electrophoresis in phosphate-borax buffer solutions (μ=0.05) containing 4 *M* urea at pH 8.6 or 10.8, resp. (Fig. 8). In the former case the protein migrated towards the cathode, in the latter case towards the anode. The amount of protein contamination did not exceed 1 per cent of the total protein contents of the solutions.

The basic proteins isolated from the secretion of the seminal vesicles of the rat dissolved in urea-containing solutions (protein concentration: 10 mg

per ml; urea concentration: 2 to 4 *M*) is clotted by the vesiculase enzyme (1.8 mg protein per ml) within a few seconds at room temperature. On the other hand the protein fraction remaining in solution after the glycerol treatment, and containing all the soluble proteins of the vesicular secretion is not clottable by the vesiculase. It was established in control experiments that glycerol did not affect the activity of vesiculase at concentrations applied in these experiments.

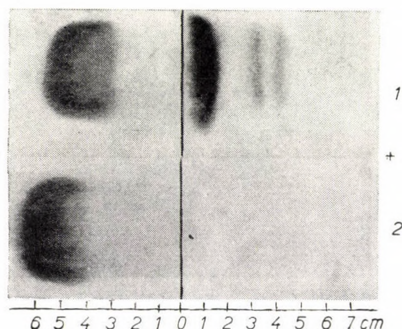


Fig. 8. Paper electropherogram of the basic protein component isolated from the vesicular secretion of rats

Characteristics of the paper electrophoretic assay: Phosphate-borax buffer solution containing 4 *M* urea, pH 8.6, $\mu = 0.05$, 6.4 V/cm, 24 mA, 15.5 hours, stained with bromophenolblue.

1. The soluble fraction of the vesicular secretion of rats;
2. The basic protein component isolated from the same fraction and dissolved in 8 *M* urea

Discussion

The experiments presented in the previous sections established the fact that the secretions of the seminal vesicles of the rat, mouse and guinea-pig are equally characterized by the presence of a highly basic protein fraction. The same protein fraction is clotted by the vesiculase enzyme produced by the coagulatory gland. This assumption was supported by two different observations. First, the basic component migrating towards the cathode at pH 8.6 was not detectable in any of the paper electrophoretic patterns, if the paper electrophoretic assay was carried out after having clotted the secretions. On the other hand the isolated basic protein was clottable by the vesiculase, while the remaining part of the secretion, containing the neutral and acid protein components only, did not react with the clotting enzyme. This experimental finding is regarded as a direct evidence for the role of the basic protein in the clotting mechanism.

The secretion of the seminal vesicles, the protein content of which is about 27 per cent is an extremely unstable system. It was described in the

early work of WALKER [42], that the seminal vesicle secretion of the guinea-pig which is a solution with low viscosity in the living animal, stiffens to a rigid mass in the gland within a few minutes *post mortem*. According to our own observations 5 to 10 minutes were necessary for this process in rats. It was possible to puncture the seminal vesicles of the rats, using a dry syringe, in deep ether narcosis or immediately after the decapitation of the animals. A highly viscous solution with yellowish colour was obtained by this procedure, the material remained in liquid state for 5 to 10 minutes and then it stiffened to a gel. Stirring or shaking of the secretion raised the rate of gel formation.

A precipitate was formed if the secretions of the seminal vesicles of rats were diluted with neutral or slightly alkaline solutions (0.15 to 3.0 *M* NaCl, sucrose; 0.1 to 1.0 *M* sodium phosphate buffer solution, pH 7.4; glycerol). This precipitate consisted mainly of the basic protein component. Depending on the degree of dilution only a slight if any precipitate is formed if dilution is carried out with 4 to 8 *M* urea or 0.1 to 1.0 *M* citric acid solutions. Secretions diluted this way were also highly viscous and opalescent. The isolated clottable protein could be dissolved only in concentrated citric acid or urea solution.

The question emerges: do the protein fractions separated by paper electrophoresis in solutions containing 4 *M* urea reflect the physiological conditions? The separation of the individual protein components was poor if urea was omitted, its presence improved the efficiency of the procedure in direct proportion to its concentration (2 to 8 *M*). However, essentially the same components are separated in absence of urea, though the separation was poor. It has been described that urea enhances the separation of α_2-macroglobulin of blood serum [34], and the separation of β resp. γ casein in an electrophoretic assay on starch gel [43, 33, 12]. Acid induced transformation products of insulin were separated by paper electrophoresis in the presence of 7 *M* urea by SUNDBY [39], while the chromatography of insulin, resp. glucagon on Amberlite IRC 50 columns using phosphate buffer solutions containing 7 *M* urea was reported by COLE [5, 6].

In our experiments, DEAE cellulose chromatography of the vesicular secretion (in absence of urea) yielded fractions which could be identified with those obtained by paper electrophoresis in urea-containing solutions. It seems likely that the pattern of protein components is not an artefact.

Acknowledgement

The author wishes to express his indebtedness to Prof. F. B. STRAUB for his interest and critical remarks in the course of the present study. Part of this work was carried out during the course of the rebuilding of our Institute in the laboratories of the Experimental Research Department of the Medical University, Budapest. Grateful acknowledgement is due to Dr. A. G. B. KOVÁCH, head of the Department for his kind hospitality. Thanks are due to Mrs. K. OROSZ for skilful and conscientious assistance.

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Sándor Mányai

Budapesti Orvostudományi Egyetem, Orvosi Vegytani Intézet, Budapest.

Physiologia

ÜBER DIE REGULATION DES KREISLAUFS IN DER LEBER

Von

A. Fischer und L. Takács

mit der technischen Assistenz von A. Karai und V. Vajda

II. MEDIZINISCHE UNIVERSITÄTSKLINIK, MEDIZINISCHE UNIVERSITÄT, BUDAPEST

(Eingegangen am 19. Februar 1963)

In 21 Hundeversuchen wurde die Regulation des Kreislaufs in der Leber untersucht. Nach Abklemmung der Leberarterie hat die gesamte Leberdurchblutung abgenommen, die Pfortader hat den Ausfall der arteriellen Durchblutung nicht kompensiert.

Bei Ablenkung des Pfortaderblutes in die Femoralvene ist die arterielle Durchblutung der Leber in jedem Fall deutlich angestiegen, manchmal auf das Doppelte des Ausgangswertes. Die Erweiterung der Leberarterie ist der wichtigste Faktor in der Regulation des Leberkreislaufs.

Bei direkter Darreichung in die Leberarterie bewirkten Adrenalin und Acetylcholin die Erweiterung der Leberarterie, während nach Noradrenalin Gefäßverengung erfolgte.

Versuche, die reflektorische Erweiterung der Leberarterie bei Pfortadershunt durch Atropin bzw. Dibenzylamin zu verhindern, führten zu keinem positiven Erfolg, auch die Versuche durch Procain etwaige Barorezeptoren ausschalten, waren erfolglos.

Das Problem des Mechanismus der Regulation des arteriellen Kreislaufs der Leber wird diskutiert.

Bei parallelen Bestimmungen des arteriellen und portalen Kreislaufs der Leber konnten wir [1, 2, 3] einen deutlichen Zusammenhang zwischen der Größe des arteriellen Anteils und der gesamten Leberdurchblutung feststellen; bei Abnahme des portalen Anteils blieb die arterielle Durchblutung unverändert oder erhöhte sich auch im absoluten Maßstab. Ferner wurde beobachtet, daß sich bei geringgradiger Hypoxie der arterielle Anteil erhöht, während hochgradige Hypoxie eine starke Abnahme der arteriellen Durchblutung zur Folge hat. Diese Befunde weisen darauf hin, daß der arterielle Anteil des Leberkreislaufs einer besonderen Regulation unterliegt. Die Versuche, über welche hier berichtet werden soll, verfolgten das Ziel, den Mechanismus dieser Regulation aufzuklären.

Methodik

Die Versuche haben wir an 21 Hunden durchgeführt. Die Bestimmung der gesamten Leberdurchblutung (EHBF) erfolgte mit Hilfe des Bromsulfaleinverfahrens nach vorausgegangener Katheterisierung einer Lebervene unter Röntgenkontrolle. Die arterielle Durchblutung wurde mit Hilfe eines Rotameters bestimmt, das am Ende des Versuches an demselben Tier kalibriert wurde. Nähere Einzelheiten der Methode sind aus unseren früheren Veröffentlichungen [1—3] ersichtlich. Die Sauerstoffbestimmung im Blut erfolgte mit Hilfe des Kippschen »Haemoreflektors«.

Versuchsergebnisse

1. *Die Frage der gegenseitigen Kompensierung der Pfortader und der Leberarterie*

Die Reaktion des einen Kreislauffaktors auf die plötzliche Unterbindung des anderen Faktors bedeutet zweifellos den extremen Fall der Regulationsaufgabe der Lebergefäße. Aus diesen Versuchen erwarteten wir die Antwort auf die Frage, ob beide Faktoren des Leberkreislaufs sich gegenseitig kompen-

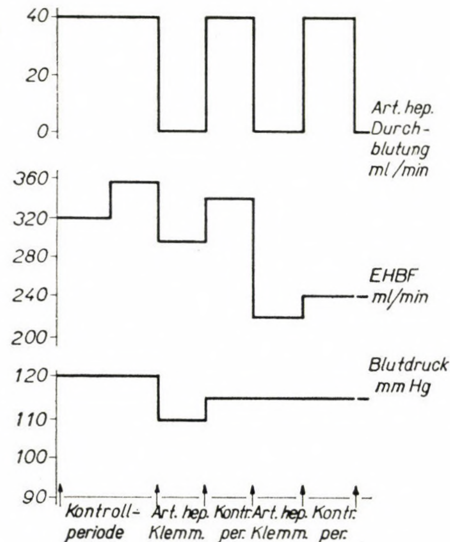

Abb. 1. Änderung der gesamten Leberdurchblutung (EHBF), der arteriellen Durchblutung der Leber und des Blutdrucks nach Abklemmung der Leberarterie

sieren können, ob es daher Regulationsmechanismen im Dienste der Aufrechterhaltung einer ausreichenden Leberdurchblutung gibt.

a) *Unterbindung der Leberarterie.* In 4 Versuchen haben wir nach einigen Vorperioden die Leberarterie abgeklemmt und den arteriellen Kreislauf für eine oder mehrere Versuchsperioden ausgeschaltet. Sodann wurde die Klemme entfernt und die Kreislaufsgrößen nach Wiederherstellung der arteriellen Durchblutung weiter bestimmt. Die Ergebnisse der Versuche zeigt Abb. 1 und Tab. I.

Wie aus Abb. 1 und Tab. I hervorgeht, ist nach Abklemmung der Leberarterie die gesamte Leberdurchblutung in jedem Fall gesunken, ungefähr um soviel, wie der weggefallene arterielle Beitrag ausmachte. Daraus folgt, daß die Pfortader den Ausfall der arteriellen Durchblutung nicht kompensiert hat, es erfolgte keine Erhöhung der portalen Durchblutung.

b) *Shunt des Pfortaderblutes.* Um das Verhalten der arteriellen Durchblutung der Leber bei Verschluß der Pfortader zu prüfen, haben wir 13 Ver-

Tabelle I

Hund Nr.	EHBF ml/Min.	Art. Durchblutg. ml/Min.	Blutdruck	Eingriff
203				
13 kg	230	60	100	—
	240	42	90	—
	210	0	90	Leberarterie abgeklemmt
	330	42	90	Klemme gelöst
204				
16.5 kg	400	70	135	—
	250	0	115	Leberarterie abgeklemmt
	350	0	110	Leberarterie abgeklemmt
	290	60	110	Klemme gelöst
205				
10 kg	315	42	120	—
	360	42	120	—
	295	0	110	Leberarterie abgeklemmt
	340	42	115	Klemme gelöst
	198	0	115	Leberarterie abgekl.
	205	42	115	Klemme gelöst
206				
14 kg	430	128	110	—
	490	108	110	—
	375	0	110	Leberarterie abgeklemmt
	230	108	90	Klemme gelöst

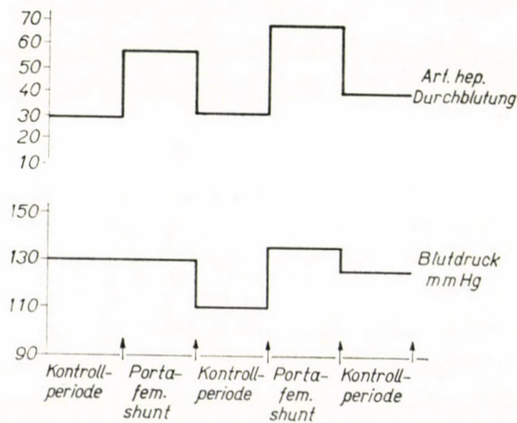

Abb. 2. Änderung der arteriellen Durchblutung der Leber und des Blutdrucks nach portofemoralem Shunt

Tabelle II

Hund Nr.	EHBF ml/Min.	Art. Durchblutung ml/Min.	Blutdruck	Eingriff
209		63	100	—
16 kg				
		96	105	Pfortadershunt
		73	110	—
		96	110	Pfortadershunt
210				
15 kg		115	95	—
		135	90	Pfortadershunt
		115	85	—
211				
10 kg	210	58	85	—
	132	75	115	Pfortadershunt
	136	45	85	—
	68	66	90	Pfortadershunt
213				
19 kg		33	130	—
		59	130	Pfortadershunt
		32	110	—
		68	135	Pfortadershunt
		37	125	—
214				
17 kg		38	85	Pfortadershunt
		28	75	—
		39	80	Pfortadershunt
		26	70	—
216				
14.5 kg		78	100	—
		93	100	Pfortadershunt
		68	100	—
		93	100	Pfortadershunt
		68	100	—
		93	100	Pfortadershunt
		68	100	—

suche durchgeführt. Nach Laparotomie wurde die Pfortader durchtrennt und in beide Enden eine breite T-Kanüle eingeführt. Der senkrechte Schenkel der Kanüle wurde mit Hilfe eines Polyäthylenschlauches mit der Femoralvene verbunden. Durch abwechselnde Abklemmung der beiden Verbindungswege konnte dann entweder die normale Pfortaderdurchblutung hergestellt, oder das Pfortaderblut von der Leber abgelenkt werden. Die arterielle Durchblutung wurde während der ganzen Versuchsdauer mit dem Rotameter gemessen. Die Ergebnisse dieser Versuche fassen Abb. 2 und Tab. II zusammen.

Wie aus Abb. 2 und Tab. II sowie den anderen später zu besprechenden Versuchen hervorgeht, ist die arterielle Durchblutung bei Ablenkung des Pfort-

aderblutes von der Leber in jedem Fall deutlich angestiegen, in manchen Fällen auf das Doppelte des Ausgangswertes. Der Anstieg begann 10—20 Sekunden nach Ablenkung des Pfortaderblutes von der Leber und hielt sich während der Dauer des Shunts meist auf derselben Höhe, um nach Wiederherstellung des normalen Pfortaderkreislaufs in jedem Fall auf den Ausgangswert zurückzukehren. In einigen Fällen haben wir neben der arteriellen Durchblutung auch die Gesamtdurchblutung der Leber bestimmt und fanden, daß in den Perioden des Pfortadershunts die Werte stets stark abnahmen, wenn

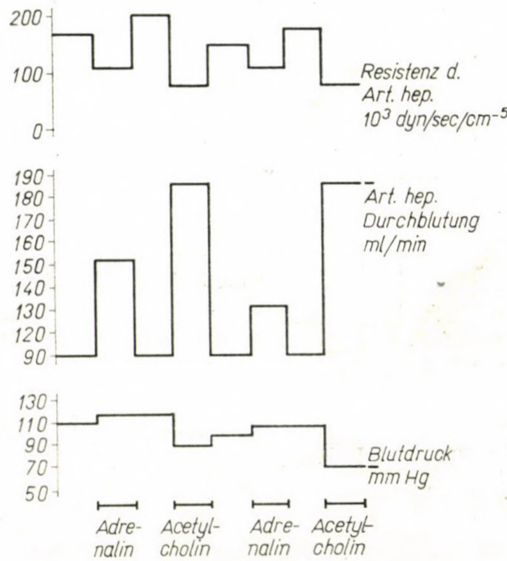

Abb. 3. Änderung der arteriellen Durchblutung und Resistenz der Leberarterie sowie des Blutdrucks nach direkter Applikation von Adrenalin und Acetylcholin

auch, wie z. B. in der 2. Periode bei Hund 211, die Werte immer noch höher waren, als die arterielle Durchblutung, was vielleicht darauf zurückzuführen ist, daß die Leber nicht allein aus der A. hepatica sondern auch aus anderen Quellen (Zwerchfellarterien) arterielles Blut erhält.

2. *Pharmakologische Prüfung der Regulation der Leberarterie*

Aus den bisherigen Versuchen geht eindeutig hervor, daß die portale Durchblutung nach Abklemmung der Leberarterie nicht erhöht wird, die Leberarterie jedoch nach Ablenkung des Pfortaderblutes in jedem Fall mit Erhöhung der Durchblutung antwortet. Die Pfortaderdurchblutung richtet sich daher nicht nach den Bedürfnissen der Leber, während die Leberarterie sich offenbar den jeweiligen Bedürfnissen der Leber anzupassen vermag. Vom physiologischen Standpunkt aus ist daher der Mechanismus der vermehrten arteriellen Durchblutung von besonderem Interesse.

In einer früheren Mitteilung [1] haben wir über die Wirkung von intravenös verabreichtem Adrenalin, Noradrenalin, Acetylcholin und Dibenzylamin berichtet. Nach 30—80 μg/Min. Adrenalin kam es zwar in allen Fällen zu einer deutlichen Erhöhung der arteriellen Durchblutung der Leber, gleichzeitig war aber auch der Blutdruck erhöht und die berechnete Resistenz der Leberarterie ist eher angestiegen. Nach Verabreichung von 30—50 μg/Min. Noradrenalin war die arterielle Durchblutung ebenfalls erhöht, ebenso auch der Blutdruck und die berechnete Resistenz der Leberarterie. Nach Injektion von 60—120 μg/Min. Acetylcholin hat die arterielle Durchblutung in allen

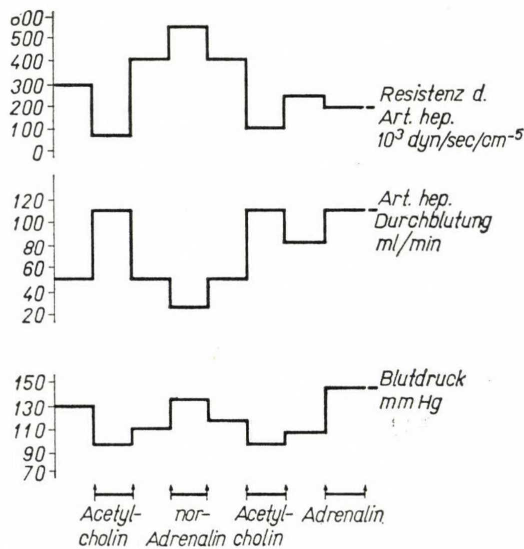

Abb. 4. Änderung der arteriellen Durchblutung und Resistenz der Leberarterie sowie des Blutdrucks nach direkter Applikation von Acetylcholin, Noradrenalin und Adrenalin

Fällen abgenommen, ebenso auch der Blutdruck, und die Resistenz der Leberarterie ist angestiegen. Aus diesen Versuchen ging daher hervor, daß nach intravenöser Verabreichung der vasoaktiven Mittel in jedem Fall eine Konstriktion der Leberarterie eintrat und die erhöhte Durchblutung nach Adrenalin bzw. Noradrenalin lediglich die Folge des erhöhten Blutdrucks war.

Die Deutung dieser Versuche darf jedoch die von GRAYSON [4] beschriebene Autoregulation des Leberkreislaufs auf Änderungen des systematischen Blutdrucks nicht unberücksichtigt lassen. Änderungen des Blutdrucks führen reflektorisch zu vasomotorischen Änderungen der Leberarterie. Falls wir daher die direkte Wirkung vasoaktiver Stoffe auf die Leberarterie untersuchen wollen, müssen wir Schwankungen des systemischen Blutdrucks vermeiden. Zu diesem Zwecke haben wir in weiteren Versuchen die Wirkstoffe direkt in

Tabelle III

Hund Nr.	Art. hep. Durchblutung ml/Min.	Art. hep. Resistenz 10^{-3}	Blutdruck mm Hg	Eingriff
207 18.5 kg	54	320	130	—
	111	87	95	5 μg/Min Acetylcholin
	50	350	130	—
	35	540	140	3 μg/Min. Noradrenalin
	50	340	130	—
	111	120	100	7.5 μg/Min. Acetylcholin
	80	210	125	—
	111	170	140	3 μg/Min. Adrenalin
208 18.5 kg	90	166	110	—
	155	104	120	6 μg/Min. Adrenalin
	90	180	120	—
	180	67	90	10 μg/Min. Acetylcholin
	90	133	110	—
	135	110	110	6 μg/Min. Adrenalin
	90	160	110	—
	180	51	70	10 μg/Min. Acetylcholin

die Leberarterie, über das Rotameter verabreicht. Die Ergebnisse zeigen Tab. III und Abb. 3 und 4.

Wie aus Tabelle III und Abb. 3 und 4 hervorgeht, konnten auf diese Weise größere Schwankungen des Blutdrucks vermieden werden. Aus den Versuchen geht eindeutig hervor, daß sowohl Adrenalin wie auch Acetylcholin bei direkter Injektion in die Leberarterie Zunahme des arteriellen Blutstroms, Abnahme der Resistenz, daher Vasodilatation der Leberarterie hervorrufen unabhängig von den Schwankungen des Blutdrucks. Dagegen hat die direkte einmalige Injektion von Noradrenalin, wie erwartet, verringerte arterielle Durchblutung und Vasokonstriktion der Leberarterie zur Folge gehabt. In weiteren 4 Versuchen hat Acetylcholin die Durchblutung der Leberarterie stets erhört, während Adrenalin in Dosen von 2—6 μg/Min. die Durchblutung der Leberarterie in 3 Fällen erhöht, in 1 Fall erniedrigt hat. Über Vasokonstriktion der Leberarterie nach direkter Verabreichung von 10 μg Adrenalin haben GREEN und Mitarb. (13) berichtet. Offenbar hängt die Wirkung auch von der individuellen Empfindlichkeit der Versuchstiere ab.

Aus den bisherigen Untersuchungen ging hervor, daß die wichtigste Regulation des Leberkreislaufs, die Erweiterung der Leberarterie bzw. die Erhöhung der arteriellen Durchblutung, sowohl durch Acetylcholin wie durch

Tabelle IV

Hund Nr.	Art. Durchblutung ml/Min.	Blutdruck mm Hg	Kreislauf	Behandlung
215 12 kg	30	120	Normal	—
	40	110	Shunt	—
	25	110	Normal	—
	35	110	Shunt	—
	27	110	Normal	—
	35	110	Shunt	—
	32	110	Normal	1 mg Atropin i.v.
	35	110	Shunt	—
	27	110	Normal	—
217 20 kg	132	100	Normal	—
	132	110	Shunt	—
	120	110	Normal	—
	132	110	Shunt	—
	108	105	Normal	—
	132	120	Shunt	—
	87	90	Normal	100 mg Dibenzylamin i.v.
	108	100	Shunt	—
	87	85	Normal	—
	108	90	Shunt	—
	87	100	Normal	2 mg Atropin i.v.
	120	100	Shunt	—
	73	100	Normal	—
222 12 kg	60	110	Normal	—
	66	120	Shunt	—
	60	120	Normal	—
	73	115	Shunt	—
	60	110	Normal	—
	73	110	Shunt	—
	73	105	Normal	1 mg Atropin in die Leberarterie
	73	120	Shunt	—
	60	110	Normal	—
	66	120	Shunt	—
	54	120	Normal	—
223 14 kg	96	110	Normal	—
	120	110	Shunt	—
	90	120	Normal	—
	117	120	Shunt	—
	96	120	Normal	1 mg Atropin in die Leberarterie
	125	130	Shunt	—
	84	130	Normal	—
	117	120	Shunt	—
	72	120	Normal	1 mg Atropin i.v.
	117	110	Shunt	—
	72	110	Normal	—
	84	90	Normal	5 mg Atropin i.v.
	96	90	Shunt	—
	84	90	Normal	—

Adrenalin bewirkt werden kann. Es erhob sich daher die Frage, ob die kompensatorische Erweiterung der Leberarterie beim portofemoralen Shunt auf adrenergischem oder auf cholinergischem Wege zustandekommt. In Anbetracht der bekannten Schwierigkeiten in der Deutung von Denervationsversuchen haben wir beschlossen, diese Frage auf pharmakologischem Wege, mittels Blockade der Nervenendigungen durch Dibenzylamin bzw. Atropin zu prüfen. Wir haben daher die Shuntversuche (Vgl. Tab. II) unter Einwirkung der erwähnten Mittel wiederholt. Die Ergebnisse geben Tab. IV und Abb. 5 wieder.

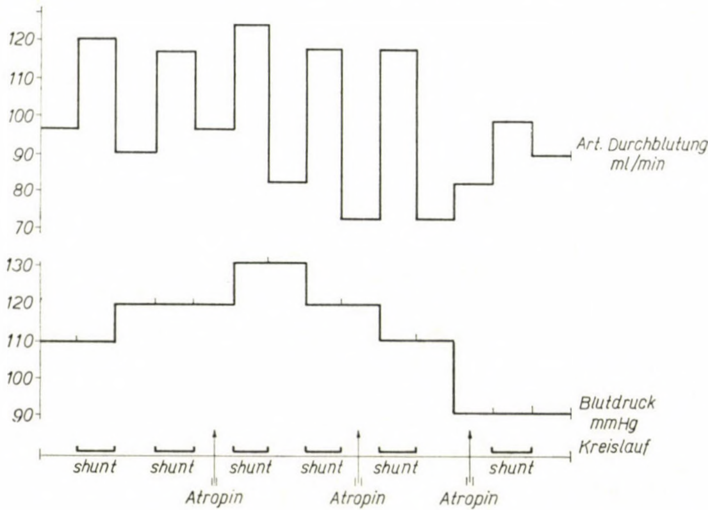

Abb. 5. Wirkung von Atropin auf die Erweiterung der Leberarterie bei Shunt

Wie aus Tab. IV ersichtlich, hat die Darreichung selbst hoher Dosen von Atropin die bei Pfortadershunt eintretende Erhöhung der arteriellen Strömung der Leber nicht verhindert; auch die direkte Applikation des Atropins in die Leberarterie durch das Rotameter erwies sich als wirkungslos.

Eindeutig wirkungslos zur Unterdrückung der reflektorischen Erweiterung der Leberarterie erwies sich auch das Dibenzylamin (Tab. IV und V).

In Versuch 221 haben wir festgestellt, daß einige Perioden nach Verabreichung des Dibenzylamins intravenös verabreichtes Adrenalin den Blutdruck nicht erhöhte, die sympathikolytische Wirkung des Dibenzylamins war daher zweifellos nachweisbar.

Es erhob sich noch die Frage, ob die reflektorische Erweiterung der Leberarterie durch Vermittlung von Barorezeptoren erfolgt, welche durch die Druckverminderung im Pfortaderbereich erregt werden, die infolge des Shunts eintritt. In der Annahme, daß diese hypothetischen Rezeptoren durch Procain gelähmt werden, haben wir in den Versuchen 218 und 221 verhältnismässig hohe Dosen von Procain teils durch das Rotameter direkt in die Leberarterie,

Tabelle V

Hund Nr.	Art. Durchblutung ml/Min.	Blutdruck	Kreislauf	Behandlung
218				
18 kg	100	130	Normal	—
	138	135	Shunt	—
	92	135	Normal	—
	138	140	Shunt	—
	92	120	Normal	2 ml 2% Procain in die Leberarterie
	138	130	Shunt	—
	125	130	Normal	—
	138	130	Shunt	—
	112	120	Normal	100 mg Dibenzylamin i.v.
	130	120	Shunt	—
	112	110	Normal	—
221				
13 kg	81	110	Normal	—
	106	110	Shunt	—
	81	110	Normal	—
	106	110	Shunt	—
	87	125	Normal	100 mg Dibenzylamin i.v.
	106	125	Shunt	—
	81	130	Normal	2 ml 2% Procain in die Leberarterie
	87	120	Shunt	—
	60	130	Normal	—
	81	130	Shunt	—
	66	130	Normal	30 μg Adrenalin i.v. + 2 ml Procain i. portal
	87	130	Shunt	—
	60	130	Normal	—

teils direkt in die Pfortader injiziert und danach die Wirkung auf die arterielle Durchblutung der Leber bei normaler Zirkulation und nach Pfortadershunt untersucht (Tab. V). Wie aus Tab. V und Abb. 6 ersichtlich, hatte das Procain, wie immer auch appliziert, die beim Shunt eintretende Erweiterung der Leberarterie nicht verhindert.

Besprechung

Die Regulation der beiden Faktoren des Leberkreislaufs im Sinne einer gegenseitigen Kompensierung wurde bereits 1932 von SCHWIEGK [5] angenommen. Mit Hilfe der Reinschen Thermostromuhr hat er eine Zunahme der arteriellen Durchblutung um 50—100% bei Pfortaderdrosselung gefunden. Andererseits fand er bei Zunahme der Pfortaderdurchblutung infolge Verdauungsarbeit eine Abnahme der arteriellen Durchblutung, während die Zunahme der arteriellen Durchblutung nach Dehydrocholsäure eine Abnahme der Pfortaderdurchblutung zur Folge hatte. Auf die Fehlerquellen der Thermostromuhr wurde wiederholt hingewiesen. In einigen Untersuchungen [1] fanden wir

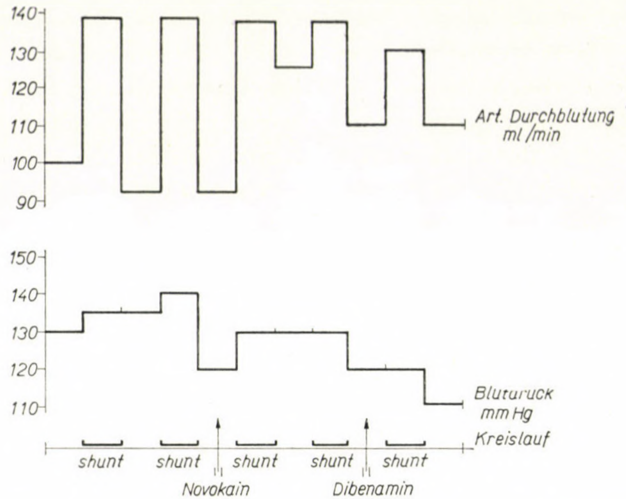

Abb. 6. Wirkung von Procain und Dibenzylamin auf die Erweiterung der Leberarterie bei Shunt

nach Dehydrocholsäure die arterielle Durchblutung meist verringert. Unsere eingangs geschilderten Versuche zeigten, daß nach Abklemmung der Leberarterie die portale Durchblutung nicht erhöht wird, die Pfortader vermag daher den Ausfall der arteriellen Durchblutung nicht zu kompensieren. Es war von vornherein nicht zu erwarten, daß die Pfortaderdurchblutung sich den Bedürfnissen der Leber anzupassen vermag; wir haben [6] bereits früher darauf hingewiesen, daß das portale Blut keine besondere »Aufgabe« in der Leber zu erfüllen hat. Die Anpassung an die wechselnden Bedürfnisse der Leber erfolgt, ebenso wie bei allen anderen Organen, mit Hilfe des arteriellen Kreislaufs, im gegebenen Fall mit Hilfe der Leberarterie. Unsere eingangs geschilderte Versuchsserie beweist eindeutig, daß bei Verringerung bzw. Abschluß der portalen Durchblutung die arterielle Durchblutung reflektorisch erhöht wird. Nach Abschluß unserer Versuche erschien die Arbeit von SCHENK, McDONALD und DRAPANAS [7]: Sie bestimmten mit Hilfe eines elektromagnetischen Strömungsmessers die arterielle und portale Durchblutung von 9 Patienten, bei welchen eine Laparotomie durchgeführt worden war; nach Abklemmung der Pfortader beobachteten sie in jedem Fall eine Zunahme der arteriellen Durchblutung um 42—85%. Es ist bekannt [8], daß nach Anlegung einer end-to-side portakavalen Fistel die gesamte Leberdurchblutung etwa 50% des Normalwertes beträgt; das bedeutet, da es sich ja ausschließlich um arterielles Blut handeln kann, eine erhebliche Zunahme der arteriellen Durchblutung, die normalerweise weniger als 20% beträgt. In einer früheren Mitteilung [1] haben wir bereits gezeigt, daß die Abnahme der portalen Durchblutung infolge Operationsschock die Erhöhung des arteriellen Anteils der Leberdurchblutung zur Folge hat. Es handelt sich offenbar um eine außerordentlich wichtige

Regulation, da, wie seit langem bekannt, [9] das Aufrichten allein bereits eine Abnahme der Pfortaderdurchblutung um 40% bewirkt. Auch Arbeitsleistung führt zu starken Abnahmen der portalen Durchblutung: so fanden BISHOP und Mitarb. [10] nach Arbeit am Ergometer bei liegenden Personen eine Abnahme auf 43% des Ruhewertes; WADE und Mitarb. [11] fanden nach »leichten Körperübungen« eine Abnahme um etwa 300 ml/Min.

In Anbetracht der großen Empfindlichkeit der Leber auf Hypoxie [12] ist daher die kompensatorische Regulation der arteriellen Durchblutung auf die unter physiologischen und pathologischen Bedingungen eintretenden Schwankungen der Pfortaderdurchblutung von vitaler Bedeutung. Es war daher von großem Interesse, den näheren Mechanismus dieser Regulation kennen zu lernen. In erster Reihe mußte die Frage geklärt werden, auf welche Weise Konstriktion und Dilatation der Leberarterie bewirkt werden.

Die Wirkung gefäßartiger Stoffe auf die Leber wurde von verschiedenen Autoren, so auch von uns [1] untersucht. Meist wurde dabei die Wirkung intravenös verabreichter Wirkstoffe untersucht und in manchen Fällen widersprechende Resultate gefunden. Das hat — wie bereits erwähnt — seinen Grund wahrscheinlich darin, daß die Autoregulation des Leberkreislaufs auf Änderungen des systematischen Blutdrucks [4] nicht berücksichtigt wurde. Einwandfreie Resultate erhielten wir durch direkte Verabreichung der Wirkstoffe in die Leberarterie, wobei der systematische Blutdruck nicht oder wenig beeinflußt wurde. Dabei ergab sich, daß Noradrenalin die Leberarterie eindeutig verengt, während sowohl Adrenalin, wie Acetylcholin die Leberarterie erweitern, die arterielle Durchblutung erhöhen, die Resistenz herabsetzen. Die für die Regulation grundlegend wichtige Erweiterung der Leberarterie kann daher sowohl von Adrenalin wie auch von Acetylcholin bewirkt werden.

Unsere Versuche zur Klärung der reflektorischen Erweiterung der Leberarterie bei Pfortadershunt haben kein eindeutiges Ergebnis gebracht. Weder Atropin, noch Dibenzylamin haben die reflektorische Gefäßerweiterung verhindert. Auch der Versuch, durch Procain etwaige Barorezeptoren zu lähmen, welche beim Zustandekommen eines solchen Reflexes eine Rolle spielen könnten, ist mißlungen. Auf Grund unserer Versuche können wir lediglich so viel mit Bestimmtheit aussagen, daß bei direkter Applikation ein deutlicher Antagonismus zwischen Adrenalin und Noradrenalin besteht; Adrenalin erweitert, Noradrenalin verengt die Leberarterie. Es wäre durchaus denkbar, daß Dibenzylamin im Zeitpunkt des Shunts die adrenalinempfindlichen Rezeptoren in der Leberarterie nicht zu lähmen vermochte; in diesem Fall wäre Adrenalin für die Erweiterung der Leberarterie verantwortlich. Nach der jetzt gültigen Auffassung wird die Sekretion von Adrenalin und Noradrenalin durch hypothalamische Zentren getrennt reguliert; diese Zentren werden durch Senkung des Blutdrucks erregt, doch ist es unbekannt, welche Faktoren zu Adrenalin-, und welche zu Noradrenalinsekretion führen.

Von SCHENK und Mitarb. [7] wird die Möglichkeit diskutiert, daß die Erhöhung der arteriellen Durchblutung bei Pfortadershunt die Folge des Wegfallens der portalen Durchblutung sein könnte: Es bestünde vielleicht eine Art von Kompetition zwischen den beiden Kreislauffaktoren um den verfügbaren Gefäßraum innerhalb der Leber. So einleuchtend dieser Gedanke auf den ersten Augenblick auch erscheint, erweist er sich bei näherer Prüfung als unwahrscheinlich. Der Blutdruck in den Sinusoiden beträgt nach BALFOUR und Mitarb. [12] um 4—5 mm Hg weniger als der portale Druck, also etwa 2—5 mm Hg. Die Resistenz der Sinusoide, in welchen sich sowohl das arterielle wie das Pfortaderblut mischen, ist daher im Verhältnis zur Höhe des arteriellen Druckes verschwindend gering und die geringe Drucksenkung nach Ausfall des Pfortaderblutes kann daher den beträchtlichen Anstieg der arteriellen Durchblutung kaum erklären.

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Anton FISCHER, Lajos TAKÁCS
Budapesti Orvostudományi Egyetem II. sz. Belklinikája, Budapest.

THE ROLE OF THE MESO-DIENCEPHALIC ACTIVATING SYSTEM IN THE EEG AROUSAL REACTION AND CONDITIONED REFLEX ACTIVITY

By

E. ENDRŐCZI, L. KORÁNYI, K. LISSÁK and G. HARTMAN

INSTITUTE OF PHYSIOLOGY, MEDICAL UNIVERSITY, PÉCS

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It has been demonstrated in chronic cat and rat experiments that following lesions to the basal septum, medial forebrain bundle, and the ventral and medial areas of the globus pallidus (this lesion affects the inferior thalamic peduncle) both the EEG arousal reactions and the previously conditioned avoidance reflex are undergoing changes. Stimulation of the basal septum enhances the habituation of the EEG arousal reaction resulting from environmental stimuli as well as from stimulation of the mesencephalic reticular formation. Some properties of the habituation of the EEG arousal reaction and the dishabituation caused by differentiating new stimuli are discussed.

On the basis of the neuroanatomical connexions it is surmised that the meso-diencephalic activating system acts circuitwise; through its connexions it takes part in the maintenance of rhinencephalic and cortical activation, as well as in evoking the attention reaction.

The disturbance in meso-diencephalic activation prevents the decoding of the information content of environmental stimuli and through this impairs the organization of self-preservative functions, which is reflected by the cessation of feeding and avoidance conditioned reflex activity.

The non-specific diffuse activation system of the brain stem plays a leading role in the organization of the behavioural reaction and the EEG arousal reaction to environmental stimuli (MORUZZI and MAGOUN [24], LINDSLEY, BOWDEN and MAGOUN [25]). The mesencephalic reticular formation, as well as the non-specific thalamic nuclei contribute to the development of the states of sleeping and wakefulness, in the orienting reaction and the organization of conditioned reflex activity. These phenomena are manifestations of the complex interaction of brain stem, and forebrain structures and the neocortex. (For detailed surveys, see MORRELL [26, 27], ROITBAK [38], LIVANOV [23], ANOKHIN [1], KREINDLER [19], GASTAUT [16].)

The view according to which the subcortical structures would represent a mosaic-like organization of certain somatic, visceral or neuroendocrine processes is being replaced by an extremely dynamic concept, placing the biological unity of the environment and living organisms, in the fore. In studies of the behavioural processes it has been recognized that electrical stimulation of the mesencephalic and diencephalic areas facilitating the conditioned reflex reaction evokes the dominant conditioned reflex reaction in a given environmental situation (ENDRŐCZI, LISSÁK, YANG and MEDGYESI, 1958, 1959). The

conditioned reflex evoked by the stimulation of subcortical structures corresponds to a facilitating activity exerted on the function of the neocortex, that in its form of appearance is similar to the driving force playing a role in the behavioural processes taking place under natural conditions. Although both the motivation and its driving force component represent a fundamental problem of experimental psychology, at present we have scarce information as to which structures are involved in these phenomena and what connexions they have with other neurophysiological events. In previous investigations we have shown that the spontaneous goal-directed intersignal motor activity observed in the early phase of conditioned reflex activity might be considered a somatic manifestation of motivation. As the conditioned reflex develops, the spontaneous intersignal motor activity diminishes as the result of the discriminative internal inhibition, which can be facilitated just as well as it can be inhibited by electrical stimulation of various points of the diencephalon (ENDRŐCZI and LISSÁK [10], LISSÁK, 1962). The present paper deals with the role of the non-specific mesencephalic and diencephalic activating system in the organization of the EEG arousal reaction and avoidance conditioned reflex activity, as well as the neuroanatomical structures serving the above phenomena and self-preservation.

Methods

The observations have been made in 37 cats and 40 albino rats. In the cats 7 to 14 cortical or deep electrodes were implanted into various areas of the brain under pentobarbital anaesthesia, using a stereotaxic apparatus. The surgical techniques and the pertaining details have been described (ENDRŐCZI and LISSÁK, 1962, LISSÁK and ENDRŐCZI, 1962, ENDRŐCZI, LISSÁK and HARTMAN, 1963). Electrical activity was recorded by means of an 8-channel EEG apparatus. During the observation period the animals were kept in an electrically insulated soundproof chamber. For stimulation, a square pulse generator was used, making it possible to change independently the frequency, duration and intensity of the impulse. Subcortical areas were lesioned by anodal electrocoagulation with stereotaxic apparatus, at 3 mA for 7 sec. Two bells of different pitch, but of the same sound intensity, as well as an impulse generator connected with an amplifier and a loudspeaker served as the sources of auditory stimuli.

Considering that following lesion to subcortical structures most animals ceased to carry out self-preserving activities (feeding, washing, *etc.*), in some cases survival did not exceed 5 to 7 days after operation. These animals were subjected to study 3 to 7 days after the operation, and the results were compared with those obtained in animals with implanted electrodes, but without electrocoagulation, experimented on at the same time. This has made it possible to eliminate the influence of eventual post-anaesthesic effects on the evaluation of the results.

In rats avoidance conditioned reflex was set up, as described in detail earlier (KORÁNYI, ENDRŐCZI and LISSÁK, 1964). The animals were kept in a cage, the floor of which was a metal grid 30 × 40 cm in size. As an unconditioned stimulus, an electric shock was administered through the grid. This stimulus was paired with the sound stimulus of a bell presented for 10 seconds. When the animal performed the conditioned reflex with 100 per cent precision and the shortest latency, the subcortical areas were electrocoagulated. The conditioned reaction was the jumping up on to a bench 10 cm from the floor of the cage. Just as in the case of cats, certain diencephalic lesions shortened survival, because of a loss of self-preserving activities. The data for experimental days, survival and general behaviour are shown in Table I.

At the end of the experiments the brain of the animals was perfused with 40 per cent formalin, then the electrocoagulated sites as well as electrode placement were checked histologically (GUZMAN—FLORES *et al.*, 1958).

Results

EEG arousal reaction to sound and its habituation in normal cats

In these experiments sound of a bell or a sound stimulus of 700 cps were presented for 5 or 10 seconds, at intervals of 2 to 3 minutes. The EEG and behavioural arousal reaction in response to the first sound stimulus was

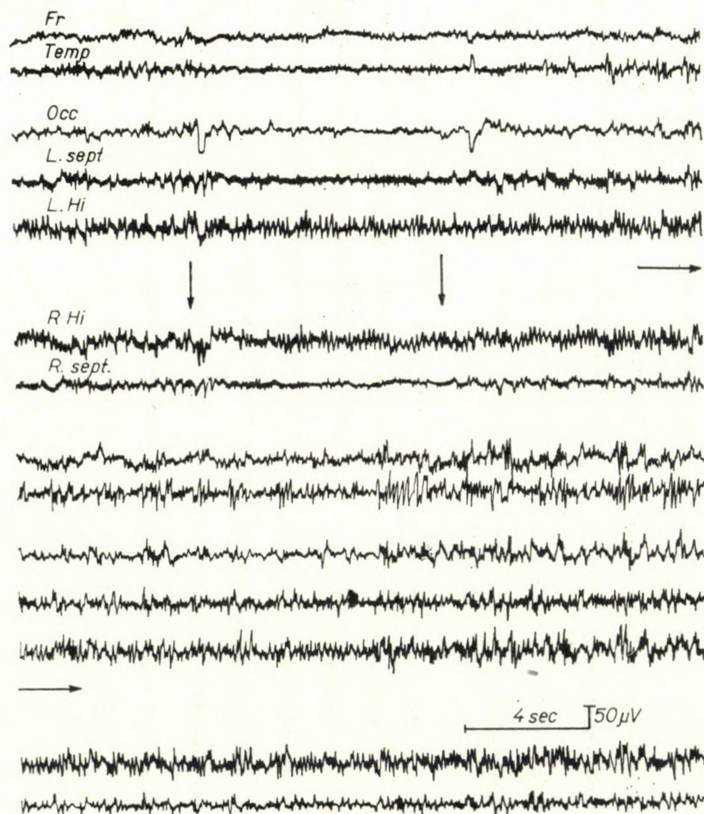

Fig. 1. EEG arousal reaction evoked by the first ringing of a bell, in a cat. Recordings from the frontal, temporal and occipital cortex, the left and right septum, and dorsal hippocampus. Desynchronization in neocortex and septum, appearance of theta activity in hippocampus. In both neocortical and septal electrical activity, initial desynchronization is followed by high-amplitude slow activity, "sensory after-discharge"

characterized by a desynchronization in the neocortex, septum and reticular formation, while in the dorsal hippocampus marked 5 c/sec theta activity was observable. After having presented the sound stimulus 6 to 8 times, high amplitude 4 to 6 c/sec waves appeared first in the septal, then in the neocortical electrical activity. These were considered sensory after-discharges. The appearance of the slow activity resulted at the same time in a diminution

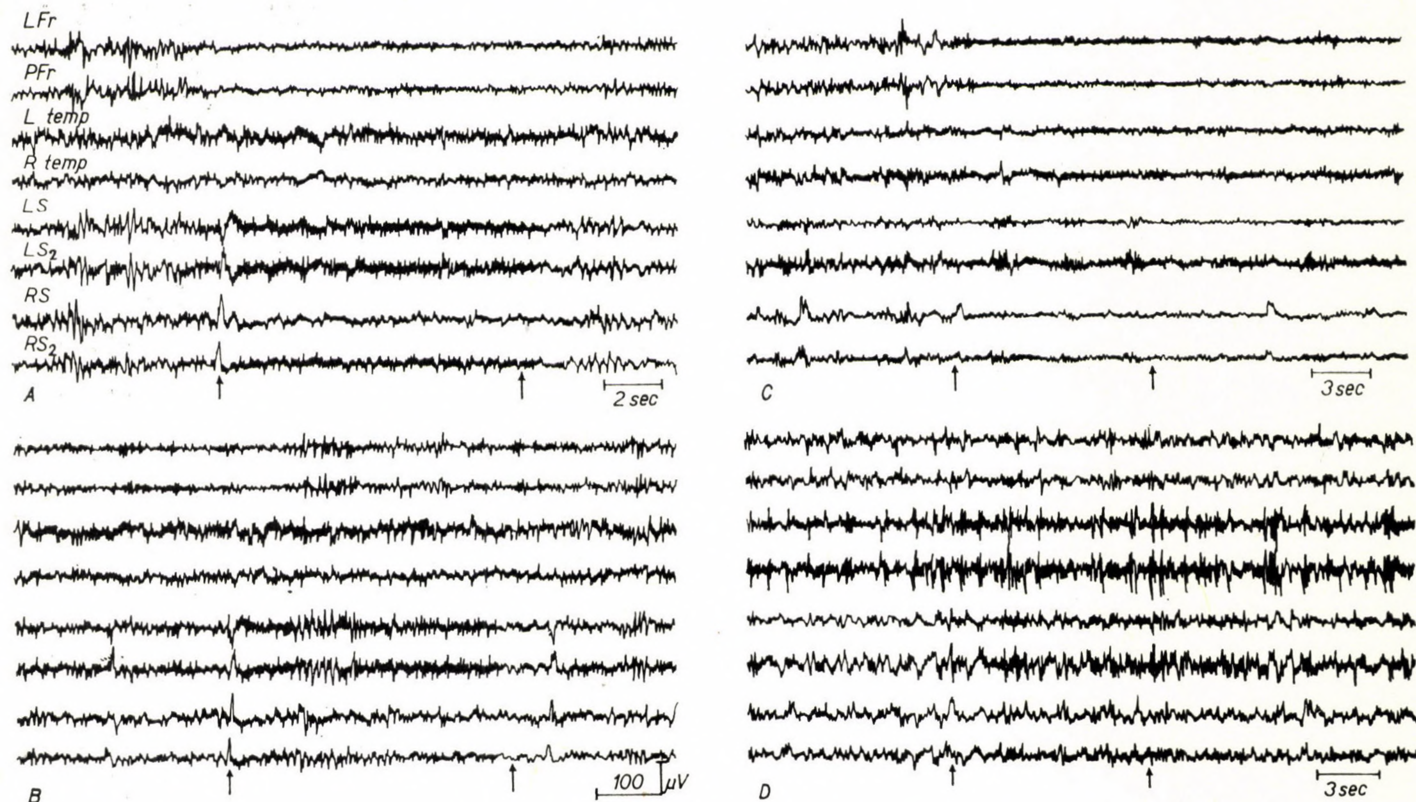

Fig. 2. Changes in electrical activity in the course of habituation. Recordings from the frontal and temporal cortex, two bipolar leads from the right and left septum. *A:* EEG arousal reaction in response to the first presentation of the sound stimulus. *B:* high-amplitude 4 c/sec slow waves appear in the septum during initial habituation. *C:* following the presentation of the differential sound stimulus, the original bell sound again evokes the EEG arousal reaction. *D:* period of complete habituation after the 80th presentation of the sound stimulus; temporary increase of the high-amplitude slow activity of septum and temporal cortex

of hippocampal theta activity. In most animals, after the 20th to 30th presentation of the sound stimulus, the intersignal resting electrical activity was characterized by a pattern of high-amplitude, 6 to 8 c/sec spindle formation.

Habituation was relative in character, considering that the presentation of some new, formerly not presented differential sound stimulus evoking EEG arousal caused dishabituation of the previous basic sound stimulus. The duration of the dishabituation elicited with the same differential sound stimulus became gradually shorter, but a new differential sound stimulus again caused dishabituation of the test sound stimulus. In the experiments no complete habituation could be attained by presenting 350 sound stimuli over a period of 4 days. Dishabituation was observable in that period, too, in the animals interest was aroused by some unusual sound of a formerly not applied pitch. However, in this late phase of habituation stimuli with a closely similar pitch and intensity no longer produced dishabituation, and even the effect by which they evoked the EEG arousal reaction was merely temporary and short-lived (Fig. 1, Fig. 2).

The effect of short sound stimuli (clicks) on the EEG arousal reaction

In response to the presentation of clicks at 2-second intervals after an initial EEG arousal and behavioural reaction the animals showed resting EEG activity. The electrical response to the click could clearly be recorded from the neocortex, septum, and the area of the reticular formation. The amplitude of the responses to clicks presented at 2-second intervals over a period of 3 hours changed merely temporarily, but no habituation developed. Considering that the resting EEG activity was characterized by irregular waves of high amplitude and by 6 to 8 c/sec spindle formation, the response to the click was difficult to observe. During the initial 15 to 20 seconds of the EEG arousal reaction to the ringing of the bell, at a time a marked desynchronization was present, the

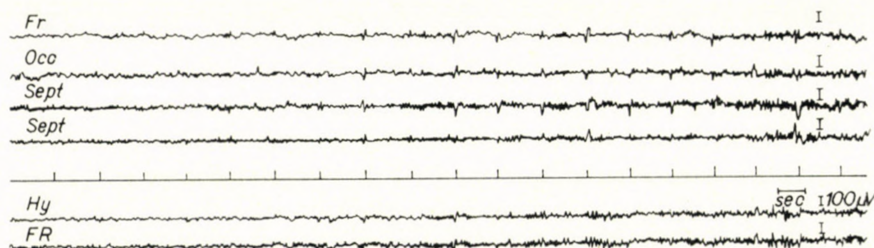

Fig. 3. Increment of the electrical response to clicks presented at 2-second intervals during and after the attention reaction evoked by sound. Frontal and temporal cortical, bilateral basal septal, bipolar posterior hypothalamic, and reticular formation leads. Broken signal in center indicates presentation of clicks. In the initial phase of the attention reaction the electrical response to clicks is hardly visible, later it increases gradually

electrical response to click was absent, but later it manifested itself with a more marked amplitude 20 to 25 seconds after the presentation of the sound stimulus evoking the attention reaction. This phenomenon was also observed on the presentation of other environmental stimuli evoking the attention reaction. In our 4 experimental animals the amplitude of the response to clicks varied in size, with the maximum ranging from 120 to 150 μV. As far as the single areas of the brain are concerned, the most marked responses were recorded from the septum, mesencephalic reticular formation and neocortex, while no change in activity was recorded from the posterior and medial hypothalamic areas (Fig. 3).

The effect of the electrical stimulation of septum and mesencephalic reticular formation on the habituation of the sound-evoked EEG arousal reaction

From the point of view of electrode placement, the responses to the electrical stimulation of the septum may be divided into two groups. By stimulating the rostral and dorsal septal area above the anterior commissure mainly the fibres of the descending fornix were affected. In the basal septum, under the anterior commissure, the anterior space perforated and Broca's diagonal bundle was stimulated.

The stimulation of the basal septum at 3 to 12 c/sec, 0.2 to 3.0 V and 0.1 to 1.0 msec evoked first an orienting reaction accompanied by an EEG arousal reaction. If stimulation was continued for 20 to 60 seconds, the desynchronized cerebral cortical activity was replaced by the appearance of 4 to 6 c/sec slow waves. If in a naive animal the basal septum was stimulated electrically and at the same time a sound of a bell was presented, after the EEG arousal reaction to the first sound stimuli rapid habituation developed. Cortical activity was characterized by slow waves and spindle formation. In this period sound stimuli similar in intensity but different in pitch produced a complete EEG arousal reaction.

Stimulation of the reticular formation at 2.0 V, 25 to 100 c/sec, 0.1 to 1.0 msec, produced a marked EEG arousal reaction, which was not habituated after 15 to 25 presentations. In the period following the EEG arousal reaction evoked by the stimulation of the reticular formation, dishabituation of the formerly habituated sound stimulus occurred, as observed previously in connexion with the dishabituating effect of the differential sound stimulus.

Stimulation of the septum for 2 minutes with 25 c/sec, 2.0 V, 0.1 msec, followed within 5 to 10 seconds by electrical stimulation of the reticular formation for 15 seconds with 2.0 V, 100 c/sec, 0.1 msec, enhanced the habituation of the EEG arousal reaction evoked from the brain stem. After 6 to 8 presentations of the two consecutive electrical stimulations the EEG arousal reaction elicited by the reticular formation ceased to appear. Under such condi-

tions the animal was lying relaxed and showed no behavioural arousal reaction. Habituation of the EEG arousal reaction on stimulation of the reticular formation was relative in character; an increase in intensity to 2.5 V, or a change in frequency from 100 to 25 c/sec evoked the EEG arousal reaction. Dishabituation resulted when an attention reaction was evoked with some environmental stimulus. Subsequently, the previously habituated stimulation of the reticular formation became temporarily effective, during 1 to 3 stimulations.

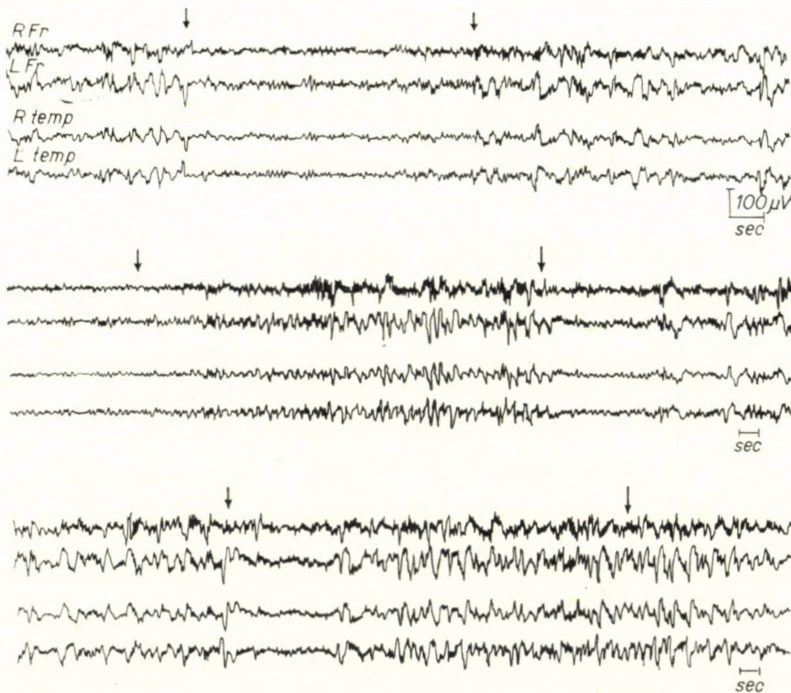

Fig. 4. Changes in the electrical activity of frontal and temporal cortex during stimulation of basal septum. A: EEG reaction to a non-habituated bell. B: high-amplitude slow activity in response to stimulation (12 c/sec) of basal septal area. C: After three consecutive associations of the non-habituated sound stimulus and electrical stimulation of the basal septum, presentation of the sound by itself causes merely an initial desynchronization. The stimuli were presented between the two arrows

At low and high frequencies alike, stimulation of the rostral and dorsal septum induced EEG desynchronization. Stimulation with low frequency and different intensities continued for minutes evoked no high-amplitude slow activity in the neocortex and did not influence the habituation of environmental stimuli.

Like in the case of the basal septum, stimulation of the preoptic region and medial forebrain bundle evoked EEG synchronization. The medial forebrain bundle was stimulated bilaterally and bipolarly at the level of the optic chiasma, in the *zona incerta* (Fig. 4).

The EEG and behavioural arousal reactions following destruction of the basal septum, medial forebrain bundle, and the inferior thalamic peduncle

After extensive destruction of the basal septum below anterior commissure, neocortical activity was characterized by high-amplitude slow waves, often accompanied by 6 to 8 c/sec spindle activity. The animals were somnolent,

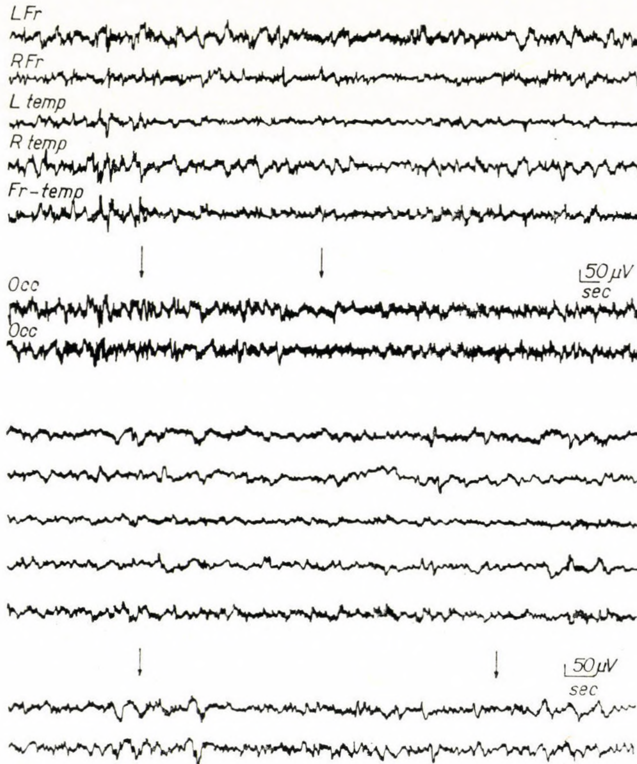

Fig. 5. Absence of EEG arousal reaction to sound or to the painful stimulation of the hind limb after lesioning the basal septum (upper, *A*), and the inferior thalamic peduncle (below, *B*), respectively. In the case of *A* ringing of a bell, in the case of *B*, painful stimulation was applied. Presentation of stimuli occurred in the period between the two arrows.

but their locomotor activities were coordinated. They did not feed, nor did they respond to environmental stimuli (visual, auditory, tactile), and the behavioural and EEG arousal reactions were absent. No EEG arousal reaction resulted from painful electrical stimulation of the hind limb. Although the animals responded to pain by pulling away the limb and altering posture, they did not escape and there was no neocortical arousal reaction. Electrical stimulation of the mesencephalic reticular formation at 2.0 to 4.0 V, 100 c/sec, 1.0 msec, for 15 to 30 sec evoked no EEG arousal reaction, although during

stimulation the animals showed somatomotor reaction, turning their head and trunk.

During the 3 to 5 days following destruction of the medial forebrain bundle the animals were somnolent; their behaviour was in some cases the same as that seen after lesion to the mesencephalic reticular formation. No desynchronization in cortical activity resulted either on environmental stimuli, or on stimulation of the reticular formation. The basic activity was characterized by irregular slow waves, with single spikes appearing at intervals. Stimulation of

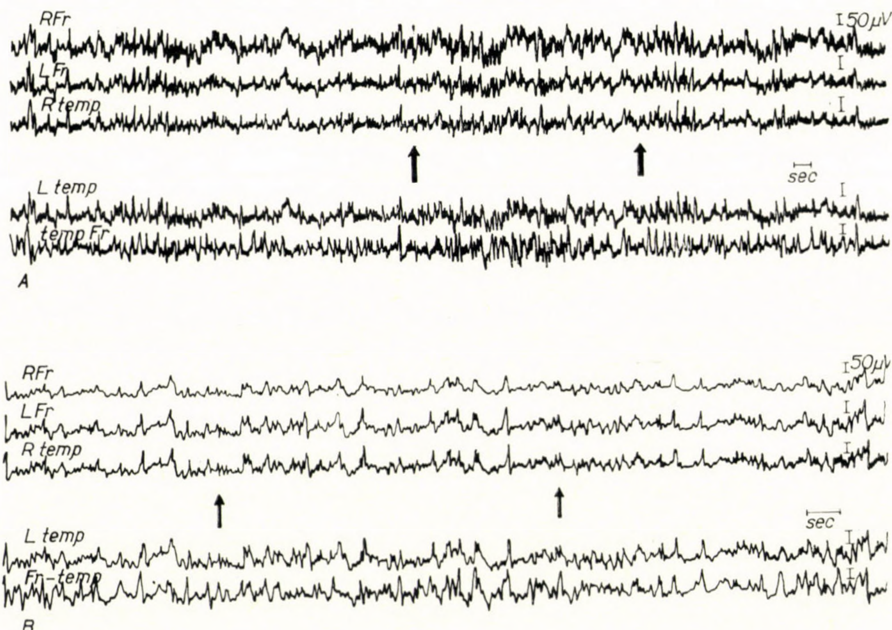

Fig. 6. Neocortical electrical activity following lesion to medial forebrain bundle (*A*) and thalamic intralaminar nuclei (*B*). In the period indicated by the two arrows the hind limb was painfully stimulated

the reticular formation resulted in a turning of head and trunk, but neither somatomotor hyperactivity, nor behavioural arousal reaction could be observed (Fig. 5, Fig. 6).

The lesion to the inferior thalamic peduncle affected also the ventral and medial parts of the globus pallidus. The animals continued to be somnolent for 2 to 3 days after the operation, spontaneous locomotor activity was absent. The animals did not respond with EEG and behavioural arousal reaction either to pain or to auditory stimulation. In 3 of the 7 animals tested, marked catatonic type of locomotor hyperactivity of variable intensity developed on the 3rd day after operation. These animals were walking around in the cage continually for days, did not try to avoid obstacles, but apparently wanted

to pass through them. They paid no attention to their environment and showed no feeding and self-preserving reactions. Unlike in the somnolent ones, in these animals constant desynchronized EEG activity could be observed, replaced at intervals by irregular slow waves. Although in these animals the lesions were probably different from those in the other four, histology revealed no information that could have explained the differences. In all of the animals the sublenticular area, the ventro-medial part of the globus pallidus and the inferior thalamic peduncle were lesioned (Fig. 7, Fig. 8).

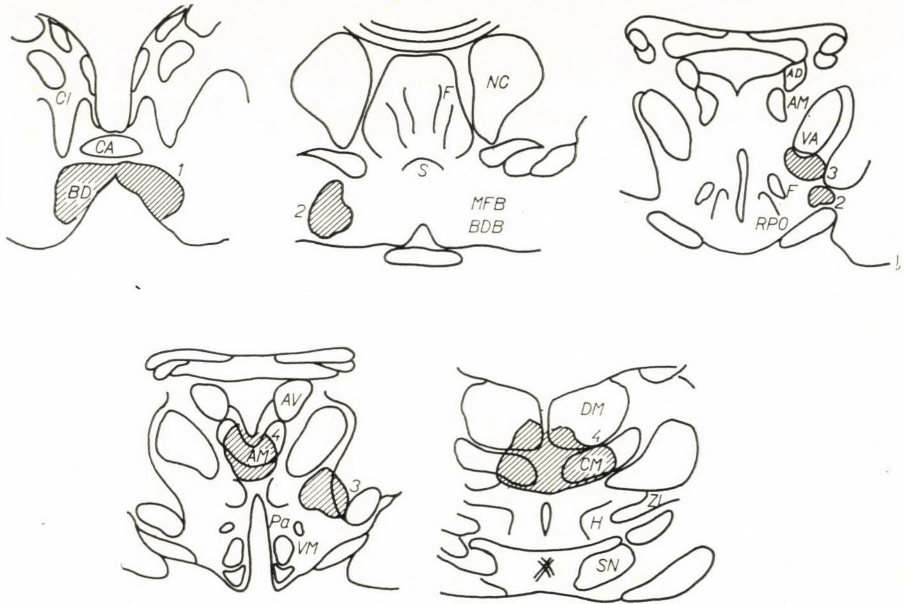

Fig. 7. Schematic representation of the bilateral lesions produced in different parts of the diencephalon. 1. basal septum; 2. medial forebrain bundle; 3. inferior thalamic peduncle; 4. intralaminar thalamic nuclei. Abbreviations: AD: anterodorsal thalamus; AM: antero-medial thalamus; AV: antero-ventral thalamic nuclei; BDB: Broca's diagonal bundle; CA: anterior commissure; CI: capsula interna; F: fornix; CM: centromedian group of thalamic nuclei; DM: dorsomedial group of thalamic nuclei; H: Forel's field; MFB: medial forebrain bundle; NC: caudate nucleus; Pa: paraventricular area; RPO: preoptic region; S: septum, SN: substantia nígra; VA: ventral anterior group of thalamic nuclei; VM: ventral medial group of thalamic nuclei; ZI: zona incerta

No change could be demonstrated in the EEG and behavioural arousal reaction evoked by sound 3 to 7 days following lesion to the caudate nucleus, dorsal or lateral globus pallidus, and partial destruction of the internal capsule. The animals began feeding two days after operation, some displayed a temporary locomotor hyperactivity. Likewise, no change in the EEG and behavioural arousal reaction resulted from lesioning the supracommissural rostral septum. These extensive lesions destroyed bilaterally the descending fornix and the subcallosal area. The site of the lesions and their locations are shown in Fig. 7.

Following lesion to the basal septum 3 animals began to show slow improvement of the EEG arousal reaction from the 4th postoperative day onward. At first, desynchronization of neocortical activity occurred during one or two presentations of the auditory stimulus. Next day, both the duration and frequency of appearance of desynchronization increased. On the 7th day the auditory stimulus evoked a marked orienting reaction, manifested in an EEG arousal reaction lasting 20 to 60 sec. Histology showed that in these animals the medial subcallosal area below the anterior commissure was intact, electrocoagulation had affected the area of the anterior perforated space only. In these cases spontaneous locomotor activity and feeding, too, were restored when the EEG arousal reaction had appeared.

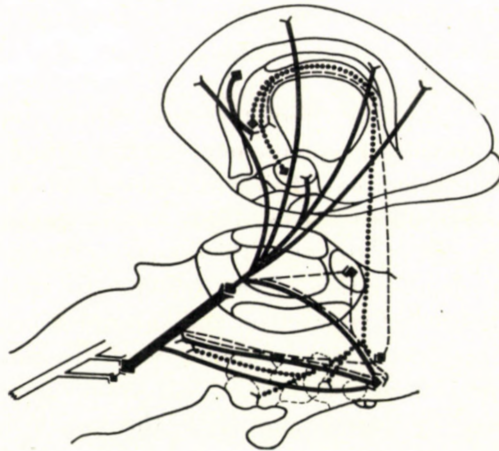

Fig. 8. Diagrammatic representation of the meso-diencephalic circuit and its rhinencephalic connexions with the nonspecific activating system. Solid line: activating, broken line: inhibitory connexions

Effect of the destruction of basal septal area, medial forebrain bundle and thalamo-septal connexions on the avoidance conditioned reflex activity in the rat

Bilateral destruction of the rostral septum, caudate nucleus, ventral posterior thalamus and nuclei of the amygdala had no influence on the previously established avoidance conditioned reflex activity; the animals continued to perform the reflex on the second postoperative day already. The period of latency was somewhat increased following lesions to the rostral septum, but the period normalized in a day or two. Following destruction of the basal septum, medial forebrain bundle and the ventral and medial parts of the globus pallidus, avoidance reflex activity ceased in every animal. The animals did not perform the earlier established reflex in response to either the conditioned stimulus or the unconditioned painful stimulus. Pain sense remained un-

changed, considering that the animals were escaping by non-goal-directed leaps when electric shock was administered.

Food uptake ceased after the lesions had been inflicted. As a result, postoperative survival was short. Marked somnolence and catatonic postural reactions could only be observed following lesion to the medial forebrain bundle. Other lesions caused no disturbances of locomotor coordination.

Discussion

Some neuroanatomical aspects of the observations

The ventral part of the central tegmental gray matter and the Tsai's ventral tegmental area send direct efferent fibres to the forebrain structures, partly through the medial forebrain bundle, partly through the bundle of the inferior thalamic peduncle. This efferent system constitutes part of the diencephalic projection of the mesencephalic non-specific diffuse activating system, considering that the intralaminar thalamic nuclei similarly send efferents to the basal forebrain structures (NAUTA, 1958, VALENSTEIN and NAUTA, 1959, TREMBLEY and SUTIN, 1962, GUILLERY, 1959, CRAGG, 1961). Following removal of the neocortex, the degeneration of the non-specific thalamic nuclei is not complete. For this, division of the ventral and anterior branches of the internal capsule is needed. These connexions, running ventrally and rostrally, do not represent cortical projections; they establish connexions first of all with the various forebrain structures and with the basal diencephalic nuclei (OLSZEWSKI, 1954). The results of experiments on fibre-degeneration indicate that the non-specific groups of thalamic nuclei constitute the origin of a direct subcortical projection which ends partly in the strio-pallidal system and partly in the different hypothalamic nuclei. The fibres of the inferior thalamic peduncle in the sublenticular area send projections partly to the medial forebrain bundle which takes its origin in the basal septum, and partly to different areas of the rhinencephalon (PAPEZ, 1958, GLOOR, 1960).

The anterior perforated space below the anterior commissure receives ample afferentation partly from the rhinencephalon through the descending fornix and stria terminalis, and partly from the olfactory and orbito-frontal cortex. The role of the septal nuclei in the generation of the synchronized theta activity of the archicortex has been shown recently (PETSCHE and STUMPFF, 1960, 1961). The medial forebrain bundle originates in the basal septal area, runs through the area of the substantia innominata of the lateral hypothalamic area and establishes direct septo-tegmental connexions (NAUTA, 1956). The same fibre system supplies ample afferent connexions to the medial hypothalamic nuclei from the tegmentum. The diffuse lateral hypo-

thalamic nuclei embedded in the medial forebrain bundle may be considered to be the embedded group of nuclei of this fibre system (NAUTA, 1956, PAPEZ, 1958).

In the present investigations electrocoagulations were performed in three areas having ample neuroanatomical connexions with one another. The lesions inhibited the EEG and behavioural arousal reaction and the self-preserving activities of the animals. The dorsal connexion originates in the tegmentum and the non-specific thalamic nuclei and runs to the basal forebrain structures (inferior thalamic peduncle), the second is the medial forebrain bundle running ventrally and laterally. The third point of destruction, that proved to be effective, affected the area in the basal septum, which corresponds to the rostralmost point of closure of the formerly mentioned fibre networks. This mesodiencephalic system is circuit-like, and on the basis of its functional properties, in the first place, may be outlined as follows: tegmentum → non-specific thalamic nuclei → subthalamic connexions → medial forebrain bundle → tegmentum. While the basal septum is the most rostral point of this circuit, many posterior closures of this circuit must arise at the subthalamic and posterior hypothalamic levels. It should also be taken into consideration that both the ventral and the dorsal tegmental-forebrain projections ensure reciprocal connexions, thus, the activation cannot be considered unidirectional·

The EEG arousal reaction following lesion to the mesodiencephalic activating system

On severing the subthalamic-septal connexions, and lesioning the basal septum or the medial forebrain bundle, the behavioural and electrophysiological arousal reactions cease. Although most of the animals showed no serious impairment of wakefulness, they were somnolent and neutral to environmental stimuli. They stopped feeding and the absence of self-preserving reactions led to death. Cats recovering slowly after the diencephalic lesions, as far as the behavioural reactions to environmental stimuli and the reactions of self-preservation are concerned, showed a gradual return of the EEG arousal reaction. The cessation of the EEG arousal reaction following lesion to the rostral diencephalic structures indicates that the activity of the non-specific diffuse activating system of the brain stem described by MAGOUN and MORUZZI requires the activation of the diencephalic and basal forebrain structures. This activation takes part not only in the development of the EEG desynchronization evoked by the stimulus, but also in the maintenance of the electrophysiological manifestations of the attentive reaction elicited by the environmental signal. The ample connexions of the meso-diencephalic system with the rhinencephalon imply that the latter structure might take part in the maintenance of the orientation and EEG arousal reactions. Destruction of

the rostral septum, which interrupts both the fornical and terminal strial connexions, produced no change either in the EEG arousal reaction, or in conditioned reflex activity. Similar observations have been reported by BRADY (1957), BRADY, SCHREINER, GELLER and KLING (1954), BRADY and NAUTA (1953, 1955), although the removal of limbic structures results in changes in the emotional behaviour. The meso-diencephalic activating circuit described here is under both facilitatory and inhibitory influences from the limbic structures through ample reciprocal connexions, and its effect manifests itself in the most fundamental organization of higher nervous activity. On the basis of these considerations, in its main characteristic features the meso-diencephalic activating system can be distinguished from the brainstem-limbic activating circuit suggested by NAUTA. The latter system takes part in the organization of the differentiated behavioural and emotional reactions (NAUTA, 1958).

In earlier studies, the electrical and chemical stimulation of the basal septum and antero-lateral hypothalamus indicated an inhibition of the tegmental and posterior hypothalamic activities through the medial forebrain bundle. This inhibition manifests itself in electrophysiological phenomena (STERMAN and CLEMENTE, 1962, HERNANDEZ-PÉON, 1962), in conditioned behavioural reaction (ENDRŐCZI and LISSÁK, 1962, LISSÁK and ENDRŐCZI, 1962), and in neuroendocrine relations (ENDRŐCZI and LISSÁK, 1963). In the present experiments an acceleration has been observed in the habituation of the EEG arousal reaction under the effect of environmental stimuli and stimulation of the reticular formation. In the habituated environment the animals showed superficial sleep, a phenomenon explained by HERNANDEZ-PÉON (1962) as well as STERMAN and CLEMENTE (1962) by assuming the existence of a sleep centre in the forebrain. This explanation has to be rejected in view of the phenomenon being one of increased habituation.

In our earlier experiments stimulation of the basal septum and medial forebrain bundle caused an inhibition of the spontaneous goal-directed motor activity, together with an increase of discriminative activity (ENDRŐCZI and LISSÁK, 1962, LISSÁK and ENRŐCZI, 1962). This phenomenon is closely correlated with the general statement that with the development of the conditioned reflex the driving force of motivation gradually diminishes owing to the discriminative internal inhibition (in the Pavlovian terminology). In the present experiments the role of the meso-diencephalic activating system in the development of the EEG arousal reaction and in the organization of the attention reaction was examined; essentially, the observations included the discrimination of environmental stimuli. The destruction of the meso-diencephalic activating circuit results not only in an impairment of complex vegetative processes and of the self-preservation reactions associated with them, but an inability of the animals to perceive and encode the information content of environmental stimuli. In this sense, meso-diencephalic activating system has a

decisive role in the maintenance of the central excitatory state evoked by external and internal environmental stimuli and producing under physiological conditions the EEG arousal reaction and the attention reaction.

The habituation on sound stimuli of the EEG arousal reaction has been investigated by numerous authors; and it has been pointed out that the phenomenon was highly frequency specific and involved a discriminative process (SHARPLESS and JASPER, 1956, JOUVET and HERNANDEZ-PÉON, 1957, SEGUNDO, ROIG and SOMMER-SMITH, 1959, *etc.*). The waxing and waning periods observed in the course of habituation are based most probably on changes in the excitatory state of the meso-diencephalic activating system influenced by internal environmental stimuli, the nature of which requires further investigations. In the course of the restricted number of reticular formation stimulations applied by us the EEG arousal reaction was not habituated. SEGUNDO *et al.* (1956, 1959) on repeated stimulation of the mesencephalic reticular formation observed a gradual elevation of the threshold of the EEG and behavioural arousal reactions both during the individual experiments and throughout the entire experimental period. As indicated by our observations, among others, the cause of the phenomenon cannot be sought in technical circumstances (electrode polarization, tissue reaction around the tip of the electrodes). The mesencephalic reticular formation reaction associated with stimulation of the basal septum soon changed into habituation, but it could be evoked again under the same experimental conditions, by applying formerly not presented environmental stimuli. The excitatory state of the meso-diencephalic activating circuit may be inhibited by two mechanisms, partly by the inhibitory activity of the cortex and the extradiencephalic structures, and partly by the self-inhibition within the circuit. It is not yet clear which of the two mechanisms is involved in the habituation and the inhibition of the discriminative activity accompanying the attention reaction. This role of the corticofugal connexions has been recognized by some observers while the self-inhibition of the meso-diencephalic activating circuit is still a hypothesis.

Inhibition of meso-diencephalic activation by a surgical intervention not only eliminates the effect of environmental stimuli, but also inhibits the organization of the fundamental reactions of self-preservation. The neuroanatomical connexions have shed light on the interpretation of feeding, as well as the EEG and behavioural arousal reactions to environmental stimuli. A lesion in the lateral hypothalamic area destroys the formerly claimed "feeding center". The detailed investigations of MORGANE (1961) have already suggested that it is not the diffuse hypothalamic nuclei but the medial forebrain bundle traversing that area which release the phenomenon. Further, lesions to the ventral and medial areas of the globus pallidus in rats bring about the same syndrome as lesions of the lateral hypothalamus: such animals cease feeding and die within a few days. Both areas touch essentially

the dorsal and ventral projections, respectively, of the mesodiencephalic activating system outlined here. On the basis of our data the disturbances of feeding developed after the lesion may, however, be interpreted at a more general level. In such animals neither internal nor external environmental stimuli cause activation; as a result, the organization of the fundamental reaction of self-preservation suffers. After lesioning the same areas the avoidance conditioned reflex activity also ceases, showing what fundamental role is played by the structures mentioned in the organization of the self-preservation processes. The ample connexions with the limbic structures are the proof that the brain stem, diencephalon and rhinencephalon are interacting in the vital and differentially organized processes of self-preservation (MACLEAN, 1954).

Neuropathological observations in humans have also called attention to the role of the basal forebrain structures in the maintenance of conscious functions. JEFFERSON (1957), surveying the disturbances of consciousness following lesions to the basal septum and the strio-pallidal system, concluded that, in addition to the "posterior critical point", a point located in the basal septum and the strio-pallidal system also takes part in the maintenance of conscious state: lesioning this anterior area often produces more severe changes than that of the posterior area in the brain stem. According to SCOVILLE (1957), a similar loss of conscious state results if during frontal leucotomy the basal septum and the pallidal areas have suffered an accidental lesion.

According to our present results, meso-diencephalic activation plays a role of fundamental importance in the function of the non-specific diffuse activating system described by MORUZZI and MAGOUN (1949). Its excitation evokes and maintains the attention reaction, and with its complicated somato-motor connexions furnishes the basis for the activation of various complex behavioural reactions. Its role is to be sought in a decoding of the informative content of environmental stimuli, and on other hand the "non-specific" activating system of this circuit represents the essence of the driving force of motivation.

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Elemér ENDRŐCZI, Lajos KORÁNYI, Kálmán LISSÁK, Géza HARTMAN, Orvostudományi Egyetem Élettani Intézete, Pécs.

EFFECT OF ALDOSTERONE ON THE INTESTINAL TRANSPORT OF SODIUM AND POTASSIUM IN RATS

By

A. Spät, Margit Saliga, J. Sturcz, and J. Sólyom

DEPARTMENT OF PHYSIOLOGY, MEDICAL UNIVERSITY, BUDAPEST

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Aldosterone was found to increase the rate of absorption and excretion of sodium, and to diminish the rate of absorption and excretion of potassium in the small intestine of rats. The net result of these two effects is zero. Consequently, in the rat aldosterone seems to exert no influence on sodium and potassium balance by its regulating the transport of cations across the intestinal wall. The effect of aldosterone on the rate of sodium and potassium transport appears to be more expressed in the proximal portions of the ileum.

Aldosterone regulates the concentration of sodium in the intravascular fluid and the volume of body fluids not solely by means of controlling the reabsorption of sodium in the renal tubules; its effect is more general. Thus, for instance, aldosterone is known to influence the distribution of sodium and potassium between the extracellular and intracellular spaces, the concentration of electrolytes in various glandular secretory products, etc. These extrarenal effects of aldosterone are of variable importance; they may, however, considerably influence sodium and potassium concentration in the extracellular fluid.

The homeostasis of electrolytes is greatly affected by the movement of sodium and potassium across the intestinal wall. Therefore, a study was made to examine the effects of aldosterone on the intestinal transport of sodium and potassium.

Methods

Male albino rats of the same strain, weighing from 150 to 200 g, were used in the experiments. The animals had been kept on a standard diet and were deprived of food 24 hours prior to the experiment. Anaesthesia was induced by urethan, 1 g per kg body wt. administered into the peritoneal cavity. Both adrenals were removed through an abdominal midline incision. Two sections of the small intestine, each 12 cm long, were isolated proximal to the site of the ileocoecal valve, by the method of Kertai and Ludány [6]. A cannula was inserted into the proximal end of each segment and the intestinal lumen was washed with approximately 20 ml of distilled water of 38° C. The remaining fluid was removed by air insufflation. Following this procedure, the aboral termination of the segment was ligated and 0.5 ml of a solution tempered at 38° C was introduced into the lumen. In the first group of animals the solution contained 3 or 5 μC of ^{24}Na or ^{42}K, respectively. The total amounts of sodium and potassium were 100 mEq and 15 mEq per liter, respectively. In the other group of animals the solution contained no isotope, but was of the same concentration with respect to sodium and potassium. After the solutions had been introduced, the abdominal incision was temporarily closed with

a forceps. After a 15-minute absorption period the intestinal segments were carefully removed, an incision was made near the aboral termination and the lumen was rinsed with 15 to 20 ml of distilled water. The sodium and potassium contents of the washing fluid were determined by flame photometry, and the activity was measured by a GM counter. In the experimental group, 15 minutes prior to the absorption period, 2.5 μg per 100 g of body weight of d-aldosterone, dissolved in physiological saline containing alcohol, was administered intravenously. The control group received identical amounts of physiological saline containing alcohol, without aldosterone.

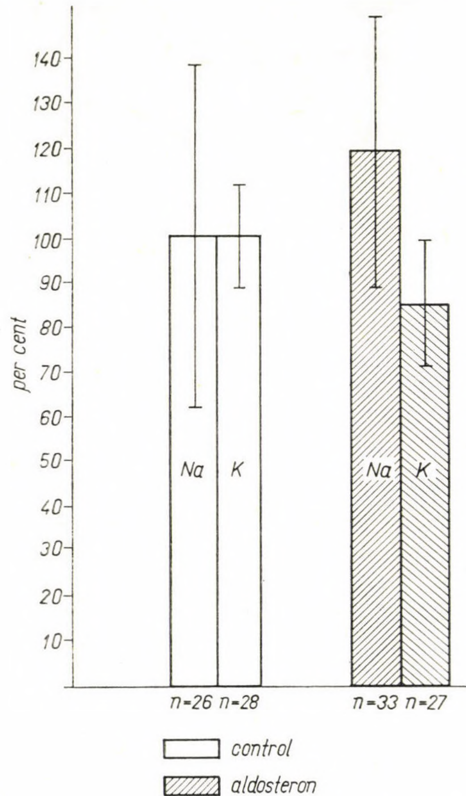

Fig. 1. Effect of aldosterone on ^{24}Na and ^{42}K absorption in the small intestine. Values expressed in per cents of the control values

Results

A. Effect of aldosterone on the absorption of ^{24}Na. — **Aldosterone given 15 minutes prior to the experiment caused a 19 per cent increase in the absorption of ^{24}Na (Fig. 1). Although the values showed great individual variations, the difference between the control and experimental groups was significant statistically ($P < 0.05$). Careful analysis of the results showed that absorption of ^{24}Na was enhanced by aldosterone particularly in the proximal segments of the ileum. As demonstrated in Fig. 2, the rate of absorption was**

increased by 30 per cent in the proximal segments; the difference was signifcant. At the same time, in the distal portion of the ileum the increase in ^{24}Na absorption was only 7 per cent, not significant statistically.

B. Effect of aldosterone on the absorption of ^{42}K. — The absorption of ^{2}K from the ileum was decreased by 15 per cent (Fig. 1). The difference between the experimental and control groups was significant ($P < 0.01$). This effect of aldosterone was again more pronounced in the proximal segment, the difference being 21 per cent ($P < 0.01$) in the proximal, and only 9 per cent in the distal segment; the latter difference was not significant (Fig. 3).

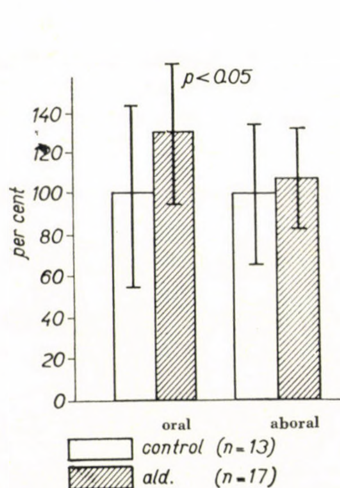

Fig. 2. Effect of aldosterone on ^{24}Na absorption in proximal and distal segments of the ileum. Values expressed in per cents of the control values

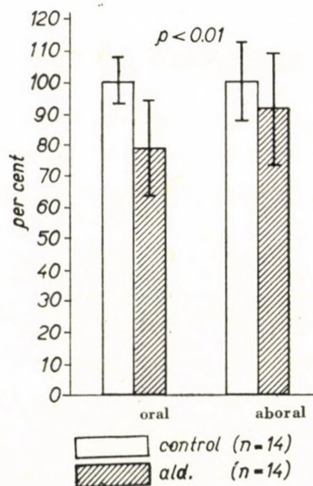

Fig. 3. Effect of aldosterone on ^{42}K absorption in proximal and distal segments of the ileum. Values expressed in per cents of the control values

C. Effect of aldosterone on the absorption of total sodium and potassium. — As demonstrated in Table I, aldosterone failed to produce any change in the total amount of sodium and potassium in the solution introduced into the intestinal segments.

Table I

	Number of cases	Non-isotope remaining in the intestine	
		Na %	K %
Control	24	100±31	100±16
Aldosterone	48	102±23	103±11

Discussion

It was reported by BERGER and STEELE [1] that in cases of secondary hyperaldosteronism, *e.g.* congestive heart failure or incompensated cirrhosis of the liver, the faecal Na/K ratio was diminished. Similar results were obtained by MILNE, MUEHRCKE and AIRD [7], and EALES and LINDER [3], in patients with Conn's syndrome. DAVIS, BALL, BAHN and GOODKIND [2] stimulated the secretion of aldosterone by thoracic caval constriction and observed a decrease of the Na/K ratio in the faeces. After adrenalectomy the Na/K ratio returned to its original value.

The quoted authors examined the electrolyte constituents of the faeces only. This test does not, however, provide reliable information concerning intestinal absorption and excretion, since the electrolyte content of the faeces depends also on other factors, *e.g.* the rate of excretion by the liver and pancreas.

FÖLDVÁRI, CZEIZEL, KERESZTES and PALKOVITS [4], and FÖLDVÁRI, CZEIZEL, PALKOVITS and KERTAI [5] showed that extracts of the subcommissural organ enhanced the absorption of ^{24}Na from the small intestine. The extract was without effect when the adrenals had been removed. On the basis of these data and the changes of nuclear volume in the glomerular zone, they concluded that aldosterone was responsible for the increased absorption of ^{24}Na. However, SIMON, SZÜCS, FÖLDVÁRI and CZEIZEL [9] found that DOC and hydrocortisone were also augmenting the absorption rate of ^{24}Na. This means that it is only from the application of exogenic aldosterone that definite conclusions can be expected.

In the first part of the present experiments the absorption rate of ^{24}Na in isolated segments of small intestine was found significantly to increase following aldosterone administration. Since ^{24}Na has the same physiological characteristics as non-radioactive sodium, increased absorption of the isotope points to increased absorption of sodium. However, the amount of non-isotope sodium in the intestinal lumen remained unaltered. On the basis of this finding it may be supposed that aldosterone, in addition to its stimulating effect on sodium absorption, increases the rate of sodium transport across the intestinal wall in the opposite direction. Therefore, the amount of sodium in the intestinal fluid remains unaffected.

The absorption of ^{42}K was decreased by aldosterone. At the same time, the amount of non-isotope potassium in the intestinal fluid did not change throughout the 15-minute period. Thus, the movement of potassium into the intestinal lumen is probably also diminished by aldosterone.

The effect of aldosterone was more marked in the proximal than in the distal intestinal segments. Similar observations have not been reported and the explanation for the phenomenon is lacking.

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András Spät, Margit Saliga, József Sturcz, János Sólyom, Budapesti Orvostudományi Egyetem Élettani Intézete, Budapest

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РЕЗЮМЕ

ПОПЕРЕЧНАЯ ПОЛОСАТОСТЬ ЛЕТАТЕЛЬНОЙ МЫШЦЫ НАСЕКОМЫХ ПРИ РАЗЛИЧНОЙ ДЛИНЕ САРКОМЕРОВ

Н. ГАРАМВЕЛЬДИ, Й. КЕРНЕР и М. ЧЕР-ШУЛЬЦ

Описаны опыты, проведенные при помощи интерференционного микроскопа на фибриллах летательной мышцы насекомых, обладающих различной поперечной полосатостью. Отдельные изменения поперечной полосатости нельзя объяснить на основании модели «sliding», в частности в области крайнего удлинения. Авторы подкрепляют свои выводы также фотометрическими измерениями электронномикроскопических снимков.

ОБМЕН ПИРОВИНОГРАДНОЙ КИСЛОТЫ В МИТОХОНДРИЯХ ПЕЧЕНИ

Т. КЁНИГ, И. МАРОШВАРИ и А. ЛИПЧЕИ

Авторы исследовали обмен пировиноградной кислоты в митохондриях печени при различных экспериментальных условиях. В аэробных условиях печеночные митохондрии весьма быстро утилизируют пировиноградную кислоту. Расход кислорода при этом, по сравнению с утилизацией пировиноградной кислоты, отстает, но он еще значителен и после того, как в системе больше нельзя выявить пировиноградной кислоты. Часть пировиноградной кислоты превращается в ацетоуксусную кислоту, а остальная, более значительная часть окисляется в двуокись углерода и в воду. При добавлении дикарбоновой кислоты снижается образование ацетоуксусной кислоты и повышается вступление ацетил-CoA в цикл посредством лимонной кислоты. Пока в реакционной смеси имеется значительное количество пировиноградной кислоты, расход кислорода и образование двуокиси углерода, по сравнению с утилизацией пировиноградной кислоты, низкие. Накопляющиеся за это время ацетоуксусную кислоту, лимонную кислоту и α-кетоглутаровую кислоту только отчасти можно считать ответственными за значительно повышенную утилизацию пировиноградной кислоты. В ходе утилизации молочная и фосфоэнолпировиноградная кислоты не накопляются. Наблюдаемой при анаэробных условиях утилизацией пировиноградной кислоты можно пренебречь. На основании измерения расхода кислорода можно сделать вывод, что пировиноградная кислота и в присутствии 0,008 М малоната не превращается полностью в ацетоуксусную кислоту, а некоторая часть и при таких условиях вступает в цитратный цикл.

Сравнение полученных результатов позволяет сделать вывод, что в митохондриях печени значительная часть пировиноградной кислоты сперва превращается посредством связывания CO_2 в дикарбоновую кислоту, и в таком виде вступает в цитратный цикл. Обсуждаются результаты, а также предположительный путь и значение происходящего в печеночных митохондриях синтеза дикарбоновой кислоты из пировиноградной кислоты $+ CO_2$.

СРАВНИТЕЛЬНЫЙ ГЕЛЬДИФФУЗИОННЫЙ АНАЛИЗ НАТИВНОГО И МЕЧЕНОГО ХРОМОМ ОВАЛЬБУМИНА

М. КАВАИ и Г. БАТОРИ

Авторы сообщают результат иммунодиффузионного и иммуноэлектрофорезного анализа полученного описанным ими способом и меченого хромом овальбумина. Иммунохимическими методами было установлено, что хроморование в щелочной среде достоверно не влияло на величины, антигенное свойство и электрофоретическую подвижность белковых молекул.

ИССЛЕДОВАНИЕ АНТИГЕННЫХ СВОЙСТВ И ХИМИЧЕСКОЙ СТРУКТУРЫ ОБЛУЧЕННОГО БЕЛКА

Ш. ДАМЬЯНОВИЧ, М. КАВАИ и Л. КЕСТЬЮШ

Исследовались антигенные свойства и группы **SH** растворов овальбумина различной концентрации до и после облучения рентгеновыми лучами. При применяемой авторами дозе облучения качественных изменений антигенных свойств не наблюдалось. Полученные количественные разницы авторы объясняют — в соответствии с определением числа **SH**-групп — изменением соотношения детерминантной **SH**-группы. Из полученных результатов авторы делают вывод, что при повреждении белков рентгеновыми лучами как в биологическом, так и в радиохимическом отношениях самыми чувствительными оказались **SH**-группы.

ИЗОЛИРОВАНИЕ СВЕРТЫВАЕМОГО БЕЛКА ИЗ СЕКРЕТА СЕМЕННОГО ПУЗЫРЬКА КРЫС

Ш. МАНЬЯИ

При помощи электрофореза на бумаге исследовался секрет семенного пузырька грызунов; в присутствии мочевины удалось отделить различные белковые компоненты секрета. Наличие в секрете сильной основной белковой фракции было характерным и для крыс, и для мышей, и для морских свинок. После энзиматического свертывания секрета везикулязой — секретом коагулирующих желез — в электрофоретической картине белка наблюдаются характерные изменения: сильно основная белковая фракция выпала в осадок у всех трех видов животных. И путем хроматографии на целлюлоидной колонке ДЕАЕ, и осаждением глицерином удалось изолировать тот же свертывающийся белок (свертываемый белок) из секрета семенного пузырька крыс. Изолированный основной белок оказался гомогенным по данным электрофореза на бумаге при различных значениях pH.

РЕГУЛЯЦИЯ ПЕЧЕНОЧНОГО КРОВООБРАЩЕНИЯ

А. ФИШЕР и Л. ТАКАЧ

Авторы исследовали на собаках регуляцию кровообращения в печеночной артерии и в воротной вене. Кровообращение в печеночной артерии измерялось ротаметром, а полный круговорот крови в печени (**EHBF**) определялся при помощи бромсульфалеина.

1. После лигирования печеночной артерии кровообращение в воротной вене не изменилось.
2. В случае отведения крови воротной вены в бедренную вену, циркуляция в печеночной артерии значительно ускорилась. По мнению авторов расширение печеночной артерии является важнейшим фактором регуляции печеночного кровообращения.
3. Введенные в печеночную артерию адреналин и ацетилхолин расширяют, а норадреналин суживает систему печеночной артерии.
4. Наблюдаемое после отведения крови воротной вены расширение печеночной артерии не удалось предотвратить ни дачей атропина, ни дибенамина.

РОЛЬ МЕЗО-ДИЭНЦЕФАЛИЧЕСКОЙ АКТИВИРУЮЩЕЙ СИСТЕМЫ В «РЕАКЦИИ ПРОБУЖДЕНИЯ» ЭЭГ И В УСЛОВНО-РЕФЛЕКТОРНОЙ ДЕЯТЕЛЬНОСТИ

Э. ЭНДРЕЦИ, Л. КОРАНЬИ, К. ЛИШШАК и
Г. ХАРТМАН

В опытах, проведенных на кошках и крысах, в хронических условиях определилось, что после разрушения базальной перегородки, медиального прозэнцефалического пучка, вентральной и медиальной областей **globus pallidus** (разрушается нижняя ножка зрительного бугра), нарушаются как реакция пробуждения в ЭЭГ, так и ранее образованный оборонительный условний рефлекс. Раздражение базальной перегородки ускоряет габитуацию ЭЭГ пробуждения как на раздражения из окружающей среды, так и на раздражение мезэнцефалической ретикулярной формации. Авторы обсуждают некоторые особенности габитуации реакции пробуждения ЭЭГ в частности дегабитуирующее свойство новых дифференцировочных раздражителей.

На основе нейро-анатомических связей авторы предполагают, что мезо-диэнцефалическая активирующая система имеет характер кругового процесса; с помощью своих связей она участвует в поддержании активности ринэнцефалона и коры головного мозга, а также в вызывании реакции внимания.

Нарушение мезо-диэнцефалической активации приводит к изменению декодирования информаций внешних раздражителей и через него к нарушению организации функций самоподдержания, что проявляется в исчезновении восприятия пищи и условной оборонительно-рефлекторной деятельности.

ДЕЙСТВИЕ АЛЬДОСТЕРОНА НА КИШЕННЫЙ ТРАНСПОРТ НАТРИЯ И КАЛИЯ

А. ШПЕТ, М. ШАЛИГА, Й. ШТУРЦ и Й. ШОЙОМ

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