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PREPARATION AND CHARACTERIZATION OF MITOCHONDRIAL MYOSINS OF RAT AND HUMAN LIVER

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This paper confirmed the reality of the mitochondrial myosin (mt-myosin [26]) in human and rat liver. Simultaneously, cytoplasmic myosin (cp-myosin) was prepared from the large particle-free supernatant. The yield of purified mt- and cp-myosin from 1 kg fresh liver was altogether 5—600 mg (= 1—1.2 μmol). Half of the myosin originated from the mitochondrial fraction (composed of about 60 g of mitochondrial protein), while the remaining portion (cp-myosin) was derived from a translucent, but voluminous supernatant (containing about two-third of liver proteins). Comparing the molecular mass of mt- and cp-myosin to the skeletal muscle myosin (about 480 kDa) — on the basis of gel filtration profiles — they proved to have similar profiles. The characteristic properties of both preparations were similar to other myosins developing filamentous aggregations and showing ATP-induced superprecipitations, but they had lower ATPase activities thus being more similar to smooth muscle and cell myosins than to skeletal muscle myosin.

The mitochondria and both myosins contained abundant covalently bound P and their endogeneous Si content was low, 2—3 μmol/g in fresh mitochondria and 5—7 mol Si per mol in the mt-myosin. The Si content was resolved into 2—3, while P into 5—6 fractions as revealed by ion exchange chromatographic technique.

The mt-myosin could be saturated to a higher P level by autophosphorylation than the cytoplasmic myosin. The interaction of actin with myosin induced a release of significant amounts of P, depending on the ATP concentration. The Cu^{2+} treatment of mt-myosin caused also P release, and a limited amount of Cu remained bound in the preparations.

Keywords: mitochondrial and cytoplasmic myosins, endogeneous P content, distribution of P content, autophosphorylation.

It was shown more than twenty years ago [9] and since then it has been comprehensively demonstrated [1] that mitochondria as well as other organelles [20, 34, 49] contain contractile proteins which participate in the motion of the particula.

Lin et al. [37] described that cytochalasin-B inhibited the contraction of the mitochondria following the administration of ATP and Mg^{2+}. The drug also inhibited the synthesis of ATP. The maximum effect developed in about

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the 20th min. The inhibition of contraction, i.e. the effect of cytochalasin-B, has led to the conclusion that contractile proteins are present among the structural elements of the mitochondria. Since then, the above conclusion has been corroborated by the preparation of mitochondrial actin-like protein from mitochondria of corn germ origin [38].

The two main organelles (mitochondrium and chloroplast) have not only their own DNA [2, 5, 11, 52] and RNA but also a protein synthesizing ability [3, 10, 32]. Furthermore, they exhibit not only a special kind of division (division by partition) [17] and at least four forms of motion, but also micropulsation [7, 34, 37, 49]. The above-mentioned observations corroborate the presence of contractile proteins; thus, the conclusion might be drawn that contractile proteins do participate also in the motion of mitochondria.

Until present, two theories have been evolved as to the origin of mitochondria and plasts (endogeneous vs. symbiotic). Both of them are based on peculiar experimental findings and data in the literature; however, the struggle seems to come to an end. Hungarian authors [21], after having reviewed and critically evaluated the relevant evolutionary and genetic data in the literature, have come to the conclusion that the data are in the favour of the symbiotic origin of the organelles. This idea is also supported by the works of other authors [14, 16]. Nevertheless, the epysome (plasmide) theory also seems to be logical [15, 52].

Taking into account the above-mentioned aspects as well as the observations that myofibre preparations of muscle origin contain a mixture of isomyosins [55] while liver preparations consist of smooth muscle myosin-like protein [57], human and rat liver mitochondrial preparations were chosen and used in our investigations. It has been assumed that one source of isomyosins in the muscle is in fact the myofibre, however, there is another one, the mitochondrion. So, the usual mixed myosin preparations contain not only myosins of fibrillar origin but also other ones of mitochondrial (mt-myosin) and cytoplasmic (cp-myosin) origin.

Thorough investigations performed during the past years have elucidated the characteristics of the mitochondrial membranes almost down to molecular level. However, membranes represent only a small portion, 15—25 per cent [51], of the total protein content of the mitochondria. Detailed investigations on matrix proteins representing the larger portion, some 67 per cent on average [20], fell somehow in the background when compared to the investigations on membranes. Nevertheless, the localization of mitochondrial contractile proteins among the structural elements of the matrix is of utmost importance [1, 44].

Encouraged by previous findings [26], we have extended our investigations to mitochondrial myosin prepared rat brain, domesticated rabbit muscle

and human muscle as well as, in some cases, to myosin fractions of cytoplasmic and chloroplastic origin. Also these investigations will be referred to whenever necessary.

Materials and methods

The mass of the liver cleaned of connective tissues and main blood vessels was determined by weighing. Thereafter the liver was immediately placed into ice-cold solution A (containing 250 mM saccharose, 1 mM EGTA and 25 mM Tris-HCl at pH 7.25). The chilled tissue was sliced and repeatedly washed in solution A in order to remove any blood contamination.

Mitochondria were prepared from rat liver according to the method of Schneider and Hogeboom [45] while from human liver according to the method of Benga et al. [6]. Preparative work was done at 2 to 4 °C. Tris-HCl buffer was used instead of the phosphate-containing one in order to prevent unnecessary phosphate uptake by the specimen. To the preparation fourfold volume of solution A was added, and homogenated in a motor-driven Potter-Elvehjen tissue grinder (50 ml in volume) with a tight fitting teflon pestle (1500 rpm for 4×10 s). The lipid layer formed during centrifugation was carefully removed from the surface of the supernatant. Thereafter the sediment was rehomogenated and centrifuged once again as described above. The supernatants obtained were pooled and the raw mitochondrial fraction was separated from them by centrifugation (7000 g for 10 min). The mitochondrial sediment was suspended in a sufficient volume of solution A and centrifuged. The washing process was repeated once again and the mitochondria pooled by centrifugation (10 000 g for 10 min).

For the preparation of large amounts of mitochondria a six-fold volume of solution A was added to the liver then it was placed into a pre-chilled waring-blendor and homogenated (3000 rpm for 3×15 s). The homogenate was filtered through three and six layers of gauze and centrifuged in polypropylene tubes (300 ml in volume) at 1000 g for 10 min. The lipid layer formed during centrifugation was carefully removed from the surface of supernatant. The sediment layer was rehomogenized once again, centrifuged and the supernatant were collected. Mitochondria were finally pooled by centrifugation and washed as mentioned above. The method described resulted in a preparation containing smaller amounts of mitochondria and other particular elements (membrane — derived and mitochondrial fragments) than do other processes. Therefore, this method has not been used routinely.

a) Purification of myosin. In some cases, the first mitochondrial matrix (mitoplast) [29] was prepared from half of the washed mitochondrial fraction in order to prove the matrix origin of myosin. However, myosin was extracted mostly from the washed mitochondrial fraction. Two ml of 2-mercaptoethanol were added to the pellet and then, under continuous stirring, a five-fold volume of 0.6 M KCl solution was added; the pH of the suspension was adjusted to 7.6 by the addition of 1 M $KHCO_3$. The suspension was then centrifuged at about one-hour intervals, at 6000 g for 20 min, and the extraction was repeated with two-fold volume of solution B (containing 0.6 M KCl, 10 mM $KHCO_3$ and 0.1 mM DTT). When the second extract was still markedly opalescent the residue was extracted for a third time and the supernatant were pooled.

Extracts of mt-myosin contained great amounts of lipid; thus, they were markedly opalescent. In order to clear them the extracts were mixed with 2 ml of filamentous DEAE-cellulose (altogether 20 mg) suspension and centrifuged 20 000 g for 30 min. When the volume of the DEAE-cellulose pellet was large it still contained a considerable amount of myosin. Thus, after reextraction with a two-fold volume of solution B and centrifugation, considerable amounts of myosin could be regained. This was then combined with the main fraction.

Myosin (with other concomitant proteins) was obtained in the form of flocullated flakes from the combined supernatant by dialysis. Dialysis were performed first against distilled water changed three times and then against a solution containing 20 mM KCl and 2 mM Tris-HCl (pH 7.2) until complete flocculation. Myosin was collected by centrifugation (10 000 g for 30 min). Since the volume of the supernatant was large and its myosin concentration low, myosin was not wholly precipitated. In order to regain the total dissolved myosin the supernatant was mixed with 5 ml filamentous DEAE-cellulose (weight, 0.5 g) suspension; myosin was thereby bound to the ion exchange cellulose. One hour later the specimens were pooled by centrifugation (5000 g for 10 min) and the sediment was combined with the flocculated main fraction.

Myosin was extracted from the three-fold and then with two-fold volume of solution B and centrifuged (5000 g for 10 min). Since, the extract was highly opalescent its KCl concentra-

tion was decreased to 0.38 M. Then the purification with DEAE-cellulose (pearl form, DE_{32}) was repeated. Altogether 10 mg were mixed with the supernatant obtained from 100 g liver and 10 min later, centrifuged (20 000 g for 20 min) for further purification. Myosin was regained by dialysis and reextraction was undertaken as described above. However, in this case tried to obtain the myosin in a small volume (approximately 10 ml, if possible). Ten mg $DEAE_{32}$-cellulose, plus 1 mg Dowex 1×8 ion exchange resin were added to the myosin solution; the solution was then purified by ultracentrifugation (105 000 g for 20 min; uc-myosin).

b) Purification of cp-myosin. The nucleus- and mitochondrium-free supernatant contains still considerable amount of myosin (cp-myosin). If this is to be regained it must be released from the supernatant. Thus, to the supernatant first 5 ml 2-mercaptoethanol were added then solid KCl up to a concentration of 0.6 M. After stirring for one hour or two the supernatan was put into a dialysing bag and dialyzed first against distilled water and then against 10 mM Tris-HCl buffer (pH 7.2) until the total flocculation of proteins. The flocculated proteins were pooled by centrifugation (5000 g for 20 min). Since myosin was flocculated but partially in the relatively large volume, the portion remaining in the supernatant was collected by DEAE-cellulose adsorption (in a may similar to the corresponding step in the preparation of mt-myosin) and combined with the main fraction obtained by the flocculation procedure.

Myosin was extracted from the combined sediment fraction first with five-fold, then with two-fold, volume of solution B. The insoluble residue was removed by centrifugation (5000 g for 10 min). The myosin-containing supernatants were pooled. The opalescent supernatant was mixed with DEAE-cellulose (0.5 g DEAE-cellulose suspended in 5 ml 10 mM Tris-HCl buffer) and as a further purification step, centrifuged at 20 000 g for 30 min. When the volume of $DEAE_{-32}$-sediment was abundant, it certainly contained considerable amounts of myosin; thus, the sediment was extracted with a three-fold volume of solution B, centrifuged and combined with the myosin fraction.

Since the volume of the myosin solution was still large and its purification grade low, the cp-myosin was again subjected to flocculation (now for the second time) by dialysis and then centrifuged. We attempted to regain the myosin that had remained in the supernatant by the procedure already described that attempts were made to obtain the smallest possible volume during the extraction and purification of the combined pellet fractions.

The last phase of purification was similar to that of mt-myosin and completed by ultracentrifugation (105 000 g for 120 min), in order to remove the contaminations with this denaturated proteins, actomyosin, phospholipids and RNA (uc-myosin).

Mt- and cp-myosins could be obtained by the method described with the smallest loss and best efficacy. When the use of ion exchange column was omitted, the recovery of myosin was variable since flocculation highly depended on the myosin concentration of the solution and on the phosphate and lipid contents of the myosin molecule itself.

The water-clear, transparent solution of uc-myosins was gel filtered on Sepharose 4B column (1.95×86 cm). The column was equilibrated with 0.5 M KCl—8 mM $KHCO_3$ solution. A maximum of 6 ml of the specimen was transferred to the column and the elution carried out with the same solution. The protein content was determined by measuring the UV-absorption (at 280 nm wavelength) in tubes collected by means of a fraction collector. The first fractions were collected as myosin.

Determination of the protein content of the pooled myosin fractions, determination of the total phosphate, phospholipid, lipid and RNA contents of myosin as well as the separation and identification of phosphate-containing fractions of the alkaline hydrolysate were all carried out as described for brain myosin [25]. The P content of the reaction mixture was determined in Si background by the administration of perchloric acid ($HClO_4$) using the micro-scale modification of the reaction described elsewhere [24, 28, 39]. In addition, the samples were checked by another method already described [43]. When the endogeneous Si content of the specimen investigated, the samples hydrolyzed into teflon tubes [24] was to be determined, King's method [33] modified to micro-volumes was used. Further discussion of the phosphorylation and P determination is provided in the legends of the Figures.

Gel filtered myosin preparations did not exhibit phosphatase activity when checked by the method of Ehlers [18].

Homogeneity of the gel filtered preparates was checked by the polyacrylamide-pyrophosphate gel electrophoretic method of Hoh et al. [31]. The electrophoresis resulted but in a single line.

Composition of myosin subunits was investigated on slab gel according to the sodium dodecyl sulphate (SDS)-polyacrylamide gel electrophoretic method of Laemli [35]. The separated subunits were detected by coomassie blue staining according to Burgress [13].

Results

Gel filtration of mt-myosin (A) and that of cp-myosin are shown in Fig. 1. Both myosins were eluted in the very same volume from the same gel column. Even more, the elution profiles of striated muscle myosins were identical with those of the above-mentioned myosins. On this basis the molecular weight of mt- and cp-myosin could be assessed as being within the range of 480—500 kDa.

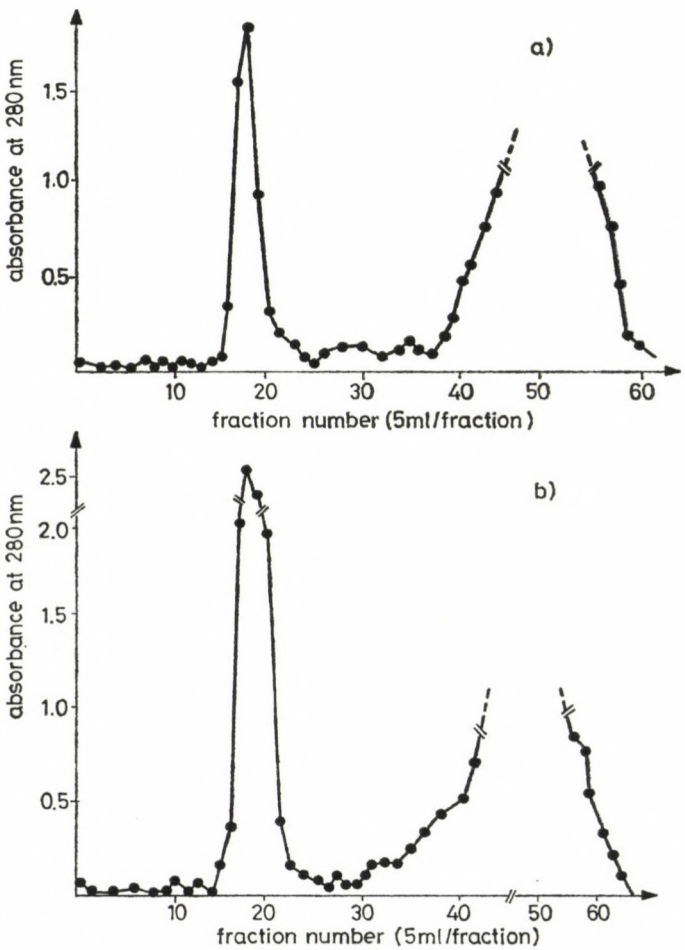

Fig. 1. Gel filtration and elution profile of ultracentrifuged mitochondrial (a) and cytoskeletal (b) myosins on Sepharose 4B column (1.95 × 89 cm). The column was equilibrated and eluted with 0.5 M KCl containing 8 mM $KHCO_3$. The concentration of KCl was increased to 3 M and pH 9 in the myosin samples, and left in refrigerator for 12—24 hours before separation. 78 mg mitochondrial and 120 mg cytoskeletal uc-myosins were applied onto the column. Flow rate, 18—20 ml/h. The protein content was detected by UV absorbance at 280 nm. The tubes under the first peaks were pooled as myosins

Both myosins proved to be homogeneous proteins when investigated by the pyrophosphate gel electrophoretic method of Hoh et al. [31] (Fig. 2). By determining the protein content and comparing the myosin contents gel filtered mt-myosin was found to amount to 25—40 per cent of the uc-myosin transferred to the column. On the average, some 50—85 per cent of the cell-myosins and 80—90 per cent of the striated muscle uc-preparations were

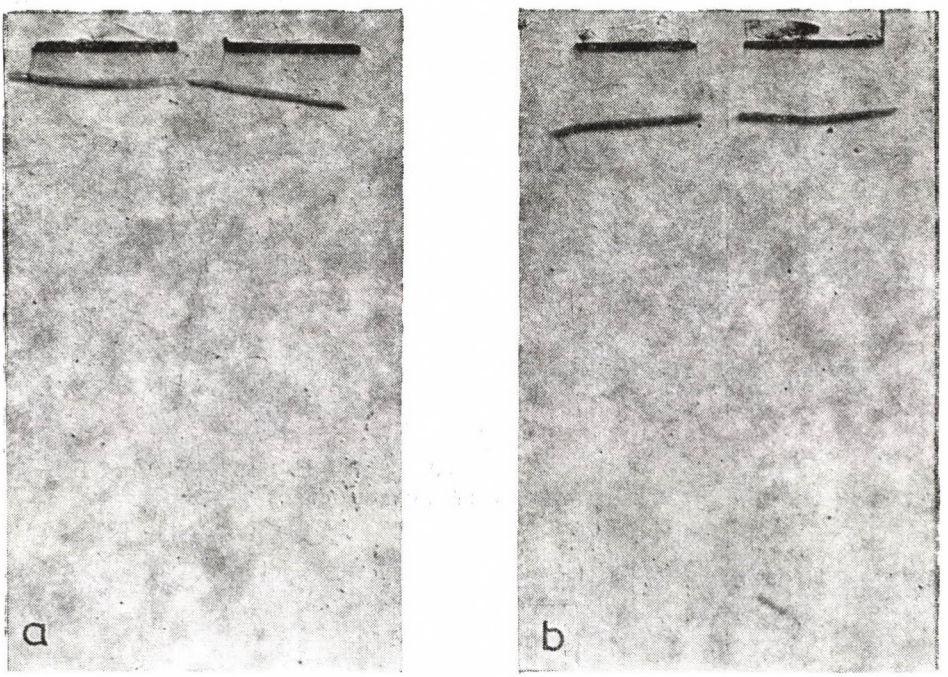

Fig. 2. Separation of mt-myosin (a) and cp-myosin (b) from rat liver in pyrophosphate containing buffer by the polyacrylamide electrophoresis method [31]

reganed as gel filtered myosin. After ultracentrifugation mt- and cp-myosin preparations of liver origin contained far more concomitant proteins than did uc-preparations of striated muscle myosin. Successful and proper preparation (sharp elution peak) could be obtained if the KCl concentration of uc-myosin was increased to 3 M before gel filtration and the specimen stored for at least one might.

For sake of comparison, some mt-myosin preparations are shown in Table I. As seen, when only one DEAF-cellulose treatment was performed, just like in the case of purification of brain myosin preparations [25], the yield was very low. The highest yield could be obtained only in the case of repeated and systematic administration of DEAE-cellulose as has been described in detail in the previous section. Not even the data of the most successful prepara-

tions shown in the Table indicate reasonably the true mitochondrium and myosin contents of the liver. On one hand, the amount of mitochondrial fraction is rather low due to the washing processes and, on the other, the total amount of myosin cannot be recovered even by the manipulations newly introduced into the processes of purification, since a considerable part of myosin will be removed in the form of actomyosin.

Table I

Yield and P content of mitochondrial myosin from rat and human liver

Species	Serial N°	Liver (wet weight) g	Yield of total protein content in final mitochondrial pellet		Yield of gelfiltered myosin		Total P content in myosin	P content of lipid-free myosin	Lipid P in myosin	Ribose content in myosin
			g+	%*	mg+	%*	mol P/mol			
Rat	1	40	0.165	0.41	5.25	0.013**	90.2	59.1	25.1	3.5
	2	110	0.520	0.47	3.31	0.003**	74.5	48.8	20.1	4.3
	3	123	0.53	0.43	32.98	0.027	114.4	72.4	32.0	4.5
	supernatant		cp-myosin		17.0	0.014	72.1	45.95	22.0	tr
	4	113	0.83	0.74	11.1	0.015	137.7	80.4	40.0	11.5
	supernatant		cp-myosin		48.45	0.048	103.3	60.0	35.0	3.0
	5	111	1.05	0.95	38.0	0.034	94.2	54.1	33.0	2.7
	supernatant		cp-myosin		33.5	0.030	73.6	40.0	26.1	2.8
Human	6	290	0.261	0.09	48.3	0.014**	136.5	78.2	53.0	tr
	supernatant		cp-myosin		86.4	0.029	95.0	58.5	40.0	tr
	7	98	3.80	3.98	121.0	0.124	126.6	80.1	44.5	tr
	supernatant		cp-myosin		88.2	0.09	88.5	53.0	33.2	1.5

+ determined by biuret micro method
* calculated for liver wet weight
** prepared by the method of [25] applied DEAE-cellulose treatment before ultracentrifugation only and homogenized in Waring blendor

One gram of wet mitochondrial pellet washed and pooled by centrifugation contained 120–150 mg of protein. Mitochondrial protein, 15—49 mg can be obtained from one gram liver. (Of course, the low values are due to the mitochondrial fraction obtained from either the homogenates prepared by the Waring-blendor or samples washed too many times.) It is easy to calculate from the above data that 1.5 per cent, or maximally 4.8 per cent, of the fresh liver weight was obtained as mitochondria which amounts to 7.5 or 24 per cent of the total protein content of the liver. (For the calculations of liver protein content was considered to be 20 per cent.) The data in the literature on the mitochondrium content of rat liver are highly variable. The 23 per cent yield found by Schneider—Hogeboom [45] and cited in the Section Methods represents a gross average value while, on the basis of volume distribution measured in two different samples, the 7 and 19 per cent [42] values of the total volume of rat liver cells seems to be rather low when compared to the data of other

authors [15, 20, 49, 51, 53]. It is also possible that mitochondria are denses than the average cell.

More than one-third or, sometimes, even more than the half of the myosin in our preparations originated from mitochondria while the other two-third or other half remained in the mitochondrium-free supernatant. Fragments of mitochondria and, partly, the so-called "small mitochondria" mostly remained in the "large particles-free" supernatant of the Waring-blendor homogenate. Thus, preparations of cytoplasmatic origin will contain various amounts of mt-myosin.

The protein content of the liver is 200 g/1000 g on the average. From 1000 g of liver a total of 5—600 mg (= 1.0—1.2 μmol) myosin can be obtained which corresponds to 0.25—0.30 per cent of the total protein content. Half of the myosin (250—300 mg) originates from the approximately 60 g protein of the mitochondrial fraction; thus mt-myosin represents 0.42—0.50 per cent of the mitochondrial fraction. The other half of the myosin fraction (cp-myosin) can be obtained from the voluminous supernatant which contains 60 per cent of total liver proteins.

Mt- and cp-myosins exhibit low Ca-ATPase activity (4—8 nkat $\times$ mg^{-1}, i.e. 0.25—0.50 μmol Pi $\times$ min^{-1} $\times$ mg^{-1}), similarly to cell myosins. ATPase activity can be increased to 2-to 6-fold of the basal value by administration of actin of rabbit striated muscle origin.

Lipid- and P-lipid contents of myosin preparations are higher than those of muscle and cell-myosin preparations. The content of human liver mitochondrium preparations is even higher than that of preparations of rat origin. Most probably, this is due to the higher inherent lipid content of human mitochondria [6].

P content of mt and cp-myosins are the highest in fresh preparations of lipid-free samples. Slow release of the P content can be observed during storage (Table II) and, parallel with this, an increase can be seen in the in-

Table II

Decrease of P content of rat and human liver mitochondrial myosin during storage

Species	Duration of storage	P content of lipid-free mitochondrion mol P/mol	myosin from cytoplasma mol P/mol
Rat			
	at gel filtration	72.7	49.0
	after day 8	48.8	31.0
	after day 15	15.3	18.0
Human			
	at gel filtration	78.2	58.5
	after day 8	50.1	37.0
	after day 15	30.1	27.0
	after day 21	6.1	8.07

organic P content. About one-fourth of the active P content will be released during storage for 4—5 days. Intramolecular motion and release of phosphate can be stopped by precipitation with acetone. A comparison of previous and present investigations indicated the extent of P release to be an inherent characteristic depending on the origin of the sample investigated. In accord with the above features, this mobile P content had previously been called "acid labile, preparative" and then "preformed or active" phosphate [22, 23, 24, 25].

We described in detail that a maximum of nine molibdate-positive fractions could be separated from the alkaline hydrolyzates of lipid-free myosin samples [25]. Mt-myosin hydrolyzates show the smallest molibdate-positive fractions on the chromatograms, namely, the 3rd (Pt-Arg), 4th (Pi), 7th (N^{π}-P-His), and 8th (N^{τ}-P-His) fractions. In addition, there are two further fractions (N° 1 and N° 2) which have not yet been identified (Fig. 3).

The small but easily separable molibdate-positive fractions of mt-myosin enabled us to continue our study on the method of P determination, in order to obtain calibration or the individual fractions. As a matter of fact, the endogeneous Si content of myosin was kept in mind which could not be separated and identified due to the Si background originating from the walls of the glass tubes used for hydrolysis. Since considerable amount of Si had been detected in the mitochondria [54] its presence or absence were still to be proved in our preparates. The completion (or rather repetition) of the investigations was thought to be reasonable and urgent since investigations on muscle myosin samples hydrolyzed in the formerly used "teflon" tubes had failed to yield any reliable results.

Due to the high P content of mt-myosin far less protein hydrolyzate (5—10 mg) suffice for the separation of the P-containing peaks and for the identification of the still unknown fractions N° 1 and N° 2. Only polyethylene or polypropylene vessels, equipments and centrifuge tubes were used for the preparation of mitochondria and myosin. The chemicals used were all checked as to their Si content and stored exclusively in plastic boxes. Special attention was paid to the purity of NaOH, KOH, KCl, and $KHCO_3$.

A column was prepared from freshly washed and activated Dowex 1×8 resin for the separation of samples hydrolyzed in "teflon" tubes. The hydrolyzate was first diluted to 50 ml and the P and Si contents were determined in its aliquot. Then the dilution was continued until a KOH concentration of 0.005 M, in order to allow the medium to be transferred to the column as well as its separation.

The phosphate content was determined in a 1.75 ml aliquot of each tube and the Si content in 2.0 ml. Previously the influence on colour development of the reduction mixture and that of ascorbic acid, both used by King [33] for Si determination, had been investigated. For certain advantages not to be

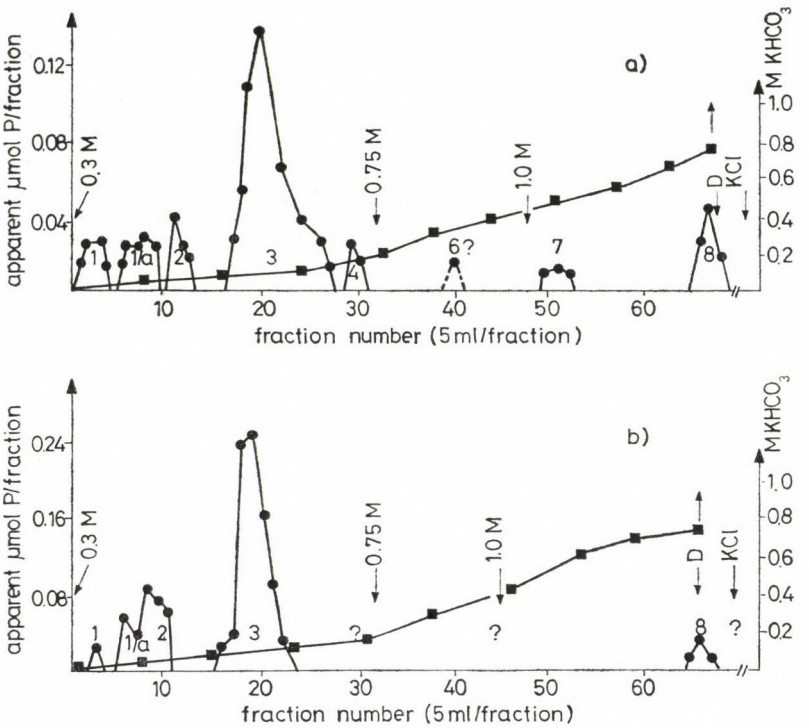

Fig. 3. Elution profile of amino acid phosphates from hydrolysate of mitochondrial (a) and cytoskeletal (b) myosins. Ten mg of protein were precipited by acetone and after removal of lipid the samples hydrolysed (in 3 M KOH, at 105°, 10 h) in a sealed "Pyrex glass" ampulle. The hydrolysates were diluted to a volume of 50 ml and in their aliquot the P was determined before application to the column. There after the hydrolysates containing 1.56 and 1.16 μmol P were diluted up to 0.005 M KOH concentration and percolated through Dowex 1×8 columns (0.9×6 cm). The separation was performed with linear step gradient chromatographic technique using a mixing chamber with a capacity of 160 cm^3 containing 0.02 M $KHCO_3$ and 120 ml of 0.30 M $KHCO_3$ in the reservoir, equipped with an electromagnetic stirrer. The $KHCO_3$ concentration was increased and changed as indicated by the arrows. 1 M $KHCO_3$ was applied onto the column directly (D) without mixing, and 2 M KCl for the regeneration of column. The P content was determined in 1.75 ml aliquot of all tubes in presence of $HClO_4$. The P-containing peaks are represented by the P content of tubes in μmol per fraction

discussed here in detail ascorbic acid was used in the present experiments (0.05 ml 1% ascorbic acid added to 2.5 ml final volume). The results obtained are shown in Fig. 4 and the Legend to the Figure discussed.

Si-positive tubes were found in the region of peaks 1 and 2 as well as in peaks containing P-Arg and inorganic P. By thorough investigation it could be demonstrated that mt-myosin in fact contained endogeneous Si which can be separated from the protein samples hydrolyzed in teflon tubes into 2 to 4 fractions.

In one experiment 2.69 μmol P was detected in 15 mg lipid-free protein hydrolyzate before separation, a value corresponding to 87 mol P/mol myosin.

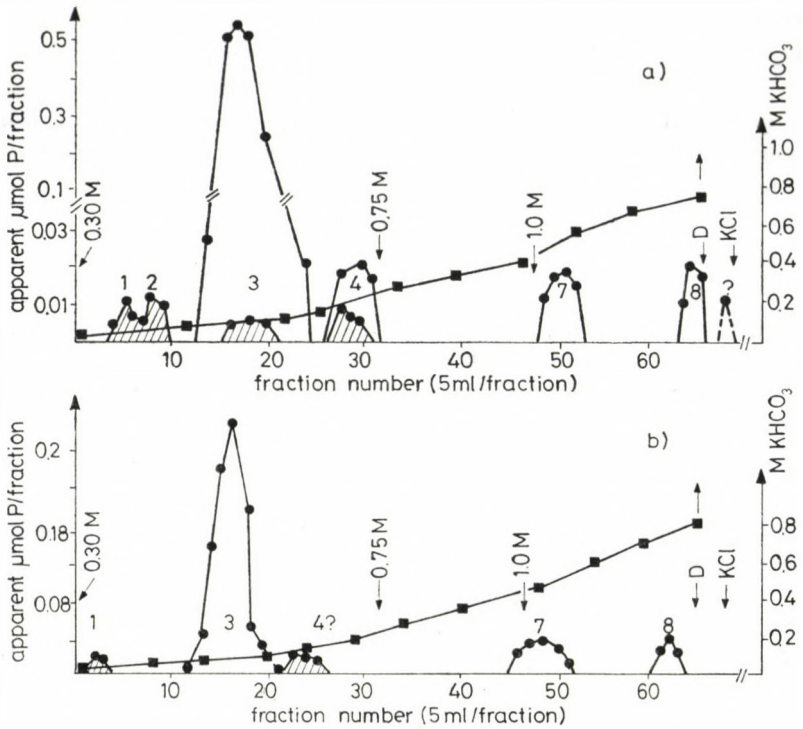

Fig. 4. Elution profile of amino acid phosphates and endogenous silicate content in "teflon ampulle" hydrolysed mitochondrial (a) and cytoskeletal (b) myosin samples. 15 an 10 mg lipid-free mitochondrial and cytoskeletal myosins were hydrolysed in thick-walled (6 mm) teflon ampulles with a capacity of 10 ml, furnished with screw-caps under similar circumstances as in Fig. 2. The hydrolysates were diluted to a volume of 50 ml and in an aliquot of then the P and Si contents were measured before separation. The hydrolysates were diluted to 0.005 M KOH concentration; percolation, chromatographic separation were similar to the methods shown in Fig. 3. From every effluent tube an aliquot, 1.75 ml and 2 ml were used parallel for P and Si determination, respectively. Si elution profile signed by dashed line

The same sample contained 0.20 µmol Si, corresponding to a value of 6.4 Si/mol myosin. The contents of other mt-myosin samples varied also between 5.0—6.6 mol Si/mol myosin. As it can be seen on chromatograms, the ratio of P to Si corresponds to the pre-separation composition. In smaller amounts of protein hydrolyzate P can hardly be detected. This holds true also for Si content. Muscle tissues and muscle myosins contain less Si than do mitochondria and mt-myosin [54]. In muscle myosin preparations levels of only 1—2 mol Si/mol myosin were found.

Since myosin contains Si, perchloric acid has to be given to each sample before determination of the P content in order to exclude the endogeneous Si from the molybdate reaction [24]. Molybdate-positive reaction occurs — even in the presence of perchloric acid — in tubes preceding the P-Arg peak in

hydrolyzates of either large amounts of striated and smooth muscle myosins or cell myosins of high P content. The tubes obtained prior to the P-Arg peak can be separated into 2 to 4 peaks depending on the origin of the samples. At least one of the peaks is NH_3-positive and gives Nessler's reaction [48]. The process of alkaline protein hydrolysis has not yet been properly elucidated making the detection of the origin of NH_3 difficult. However, it is known that arginine is hydrolyzed in several steps during the process of alkaline hydrolysis, and neither arginine nor the by-products of hydrolysis bind to Dowex 1 column, except if they are already phosphorylated.

Since the N-P binding is highly alkali stable phosphate in the medium will protect against the hydrolytic splitting of P-Arg. Only phosphorylated arginine and its hydrolytic, similarly phosphorylated derivates remain on the ion exchange column.

Since of the phosphorylated amino acids of myosin P-Arg is present in the highest concentration the molybdate-positive peaks before P-Arg consist of phosphorylated hydrolytic derivates of P-Arg. The P- and NH_3-containing peak is the reaction product of the slow dissolution and must to be considered as the phosphoramidate originating from the final step of the hydrolysis of P-Arg.

P-amidates originate exclusively from the alkaline hydrolysis of P-Arg; thus they are unequivocally to be regarded as belonging to the endogeneous P-arginine of myosin. All these considerations could be proven by the separation of P-containing by-products appearing during the hydrolysis of synthetic P-Arg as well as by other specific reactions. Identification of the by-products is discussed in detail elsewhere.

Since the alkaline-resistant N-P bond provides a high degree of protection for P-Arg, the difference caused by hydrolytic N-P by-products is not more than 5 per cent. However, when the tubes preceding the P-Arg peak are taken into account the difference exceeds 10 per cent. Differences of 10 per cent or even more can be observed in samples of muscle origin and of low P content. Namely, not only P-Arg but also its mono- and probably also its dimethyl- and the lysin methylated hydrolytic derivates might be present in phosphorylated form in the muscle myosin samples.

Since NH_3 and its various by-products (NH_4HCO_3, $(NH_4)_2CO_3$, NH_4-formate, NH_4-acetate), when present in a concentration over 0.1 mol, interfere with the phosphomolybdate reaction, the detection of the phosphate-containing fractions of the hydrolyzate is rather vague whenever the gradient chromatographic method using NH_4-containing volatile buffer is employed. In view of the above data henceforward we used the chromatographic separation method employing $KHCO_3$-containing gradient.

When the KOH concentration of the hydrolyzates is diluted only to 0.03 mol before the transfer to the ion exchange column, the peaks of the

molybdate-positive substances of the tubes preceding the P-Arg peak cannot be observed any more, for these substances will not bind to the Dowex 1 column. (Furthermore, if due care is not taken even P-Arg will not bind of the column.) However, the hydrolytic P derivates of P-Arg may still interfere with the results even if the above considerations are taken into account. Therefore deceptive technical manipulations should not be used since they may falsify the quantitative evaluation of the endogeneous P content of myosin. And this evaluation was exactly our aim in all cases.

Effect of phosphorylation on mt-myosin

Freshly gel filtered preparates are the most capable of taking up the largest amounts of phosphate. With the duration of storage, myosin takes up gradually smaller amounts of phosphate. The dependence on the ATP concentration of phosphate saturation of mt-myosin is demonstrated in Fig. 5(a). Mt-myosin can take up phosphate in higher amounts than does cp-myosin (for comparison see Part b of the Figure).

The same phenomenon was observed when the time dependence of phosphate saturation was investigated (Fig. 6). Fresh mt- and cp-myosin isolated

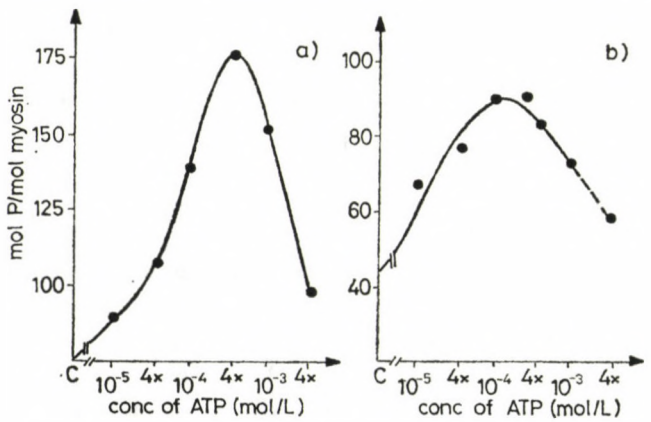

Fig. 5. The phosphorylation of mitochondrial (a) and cytoskeletal (b) myosins in the presence of increasing concentration of ATP. In a final volume of 2.5 ml we incubated 0.485 and 0.46 mg gel filtered myosin samples. Composition of the medium: 140 mM KCl, 15 mM Tris-HCl buffer, pH 7.28, 10 mM $MgCl_2$, $5 \cdot 10^{-5}$ M $CaCl_2$, 60 mM NaCl. The concentration of ATP was varied. The phosphorylation was started by the addition of ATP, incubated at 37° and terminated at 3 min incubation time by the addition of five volumes of ice-cooled acetone. The samples were stored in refrigerator overnight to precipitate the protein. The protein was sedimented by centrifugation and excess nucleotide removed by a "washing solution" (20 mM KCl, 10 mM NaCl, 2 mM $MgCl_2$, 0.1 mM $CaCl_2$, 5 mM $NaHCO_3$, 0.1 mM EGTA) applied five times, then washed once more with 15 mM Tris-HCl buffer, followed by the removal of lipids by Chl-MeOH mixture (2 : 1 v/v). The lipid-free samples were combusted in the presence of cc HNO_3, and the inorganic P content was determined. The P (including the incorporated P) of samples are represented in mol P/mol myosin versus ATP concentration. C, control sample incubated without ATP

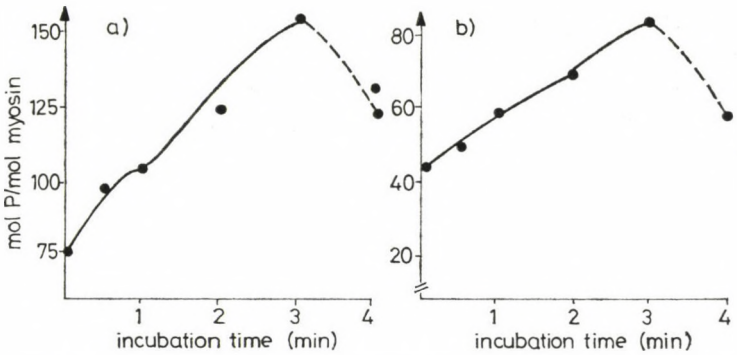

Fig. 6. Effect of incubation time on the phosphate saturation of gel filtered mitochondrial (a) and cytoskeletal (b) myosins. The incubation medium was the same as in Fig. 4 except that it contained 0.75 mM ATP. The reaction was terminated at proper time by ice-cooled acetone. The incorporated P is shown in mol P/mol myosin versus incubation time

simultaneously from the same liver sample can presumably be maximally saturated during incubation for 3 min. Prolonged incubation results in increased phosphate release; thus, in this case, less phosphate incorporation into the samples can be observed than during a shorter period of incubation.

On the basis of its behaviour mt-myosin seems to be an independent protein characteristic of the mitochondrium, different in many features from other particle preparates. All these statements can most strikingly be supported by the P content of mt- and mt-myosin* isolated simultaneously from trained muscle. The mitochondrium content of trained muscles is considerably higher; thus, mitochondria can be prepared from them in an amount sufficient for investigating the characteristics of myosin. The phosphate content of mt- and cp-myosin of the gastrocnemius muscle of two sportsmen are compared in Table III.

The active P content of mt-myosin can be decreased by Cu^{2+} treatment in a way similar to that used for previously investigated myosins. The treatment denaturates and then precipitates myosin, leading to a complete loss of biological activity (including ATPase activity). After removal of the unnecessary Cu^{2+}, lipid-P and released P, the administration of 0.42 mM Cu^{2+} resulted in a well-characterized, low grade and stable P content (Table IV).

Any further decrease in the P content occurring in parallel with subsequent washing processes is only virtual since a low decrease ("wearing") of the proteins of the sample can also be observed. Thus, after precipitation with acetone and washing twice with a solution containing EGTA, further washing processes are indicated only in the case of phosphorylated samples. Prolonged storage (at 2 to 4 °C for 20 days) results in a mt-myosin P content even lower than that

* Myofibrillar myosin.

Table III

P content of the mitochondrial and myofibrillar myosin lipid-free preparation from exercised human skeletal muscle (m. gastrocnemius medialis)

Preparation	Experiment	mol P/mol myosin
Mitochondrion		
	1	51.92
	2	96.9
Myofibril		
	1	13.34
	2	16.62
Prepared from Entire muscle*		
	1	40.1
	2	36.6
	3	28.5

* From unparticulated muscle containing the mixture of isomyosins.

Table IV

Decrease of P content of liver mitochondrial myosin following Cu^{2+} treatment

	P content of lipid-free myosin	
	gel filtered mol P/mol	Cu-treated mol P/mol
Rat	56.26	18.76
	48.78	24.3
	56.97	19.03
	72.7	21.6
	45.6	15.6
	50.6	19.0
Human	74.0	13.5
	Average	18.82 ± 5.32

obtained by the Cu^{2+} treatment. For instance, P content of the human mt-myosin preparate decreased to a value of 6.1 mol P/mol myosin. In our opinion, phosphate will not be released to the same extent by Cu^{2+} treatment from different kinds of binding of mobile phosphates.

In contrast to the lowest P content, a saturation degree even higher than the maximum P content demonstrated in the Figures can be observed in mt-myosin when the samples are incubated in the presence of $KHCO_3$ (pH 9.3). In an extremely alkaline incubation medium as much as 380 mol P/mol myosin can accumulate in the preparations within three minutes. Thus, in extremely alkaline medium the behaviour of mt-myosin is similar to that of the muscle myosin preparations of striated muscle where the extreme P

saturation was first observed. It was assumed that further P binding sites released by the alkaline medium would also be saturated. In accord with the above-mentioned considerations, P saturation when depicted as a function of pH, shows an unusual profile when compared to the extreme P values, since not more than half of the phosphorylated sites are saturated in the medium at pH 7.2 to 7.6.

Effect of actin on the P contents of myosin

As already described actin decreases the P content of myosin [22]. However, we succeeded in demonstrating the effect of actin in a truly clear-cut and acceptable way in present experiments only. Incubation of actin containing a low but well-known amount of P with P-containing mt-myosin (which can be produced in practically unlimited amounts) results in a well-detectable and evaluable phosphate release. The effect of actin on the P content of myosin is shown in Table V.

Table V

Effect of actin on P content of mitochondrial myosin

Exp.	Protein content in incubated sample (mg)	Quantity of sample μmol	Determined P content μmol P** in sample	Calculated P content mol P	Expected total (mol P)
Myosin	(0.48)	1	0.056	56.0	
Actin*	(0.42)	10	0.045	45.0	
Myosin, actin	(0.48 + 0.61 = 1.09)	14.5	0.050	50.0	(56 + 65.2 = 121.2)
Myosin, actin	(0.48 + 1.082 = 1.562)	25.8	0.036	36.0	(56 + 116.1 = 172.1)

* The monomer of skeletal muscle actin was calculated for 42 kDa, and that of myosin for 480 kDa

** 1 μmol P equal to 3.12 E at 720 nm in 2.5 ml reaction volume; 1 μmol actin sample contains 4.5 μmol P

The obtained results indicate that during incubation for three min (interaction between actin and myosin) the decrease of the P content of myosin depends on the concentration of actin. When the incubation is carried out with an higher than optimal amount of actin, there is no proportionally increasing P release, while the minimum P content of the myosin can be reached within a shorted incubation period. The phenomenon seems to be connected with muscle contraction. Namely, if ATP is present but in low concentration (exhausted muscle) the P content of myosin will decrease as a result of repeated stimulation at short intervals, since there is no possibility of rephosphorylation.

Discussion

Active motion of mitochondria were demonstrated by kinematographic methods quite early [36]. Such investigations on mitochondria were performed in the seventies when different forms of motion could be demonstrated, viz. peristaltic, saltatoric, alternate extension-contraction (pulsation) and individual motion of compartmental parts (cristae) [4, 7, 8, 41]. The opinion is generally upheld that mitochondrial motion be the result of the presence of contractile proteins. This view has been given unequivocal support by mt-myosin preparates obtained so far and further confirmed by the production of cp-myosin. It has already been demonstrated by the investigations of Schnaitman and Greenwelt [44] that contractile proteins should be searched for in the matrix of the mitochondrium since the contractile cycle of the outer, membrane-free preparates (mitoplast) will remain unchanged after digitonin treatment.

It has been a striking result of our investigations that nearly half of the myosin content of the liver originates from mitochondrial proteins representing 16 per cent [20, 42, 47], while of the total protein content other authors found 22 per cent [53]. This finding indicates that the total myosin content of the mitochondrium is at least four or five times higher than that of the cytoplasm.

The characteristics of mitochondrial myosin differ from that of preparations of myofibrillar and cyto- (and sarco-)plasmatic origin. Lipid content of gel filtered mitochondrial preparations is higher than that of cp-myosin, while samples of human origin represent the highest values so far found.

The endogeneous Si content of mt-myosin is higher than that of the cytoskeletal preparations. This feature might be an inherent characteristic since the mitochondrium itself has a higher Si content than does the whole liver or cytoplasmatic fraction (also including muscle mitochondria) [54].

On the basis of phosphorylation experiments and other phenomena (dependence of phosphorylation on ATP concentration and on the time duration of incubation, investigations on ATPase activity and on interactions) mt-myosin can be considered to be a muscle myosin with special features of the mitochondria. Mt-myosin can take up at least twice as much P than does cp-myosin. The P saturation of cp-myosin failed to reach that of mt-myosin in any preparations so far studied. Myosin samples incubated in extremely alkaline medium (pH 9.3) are capable of taking up approximately twice as much P, i.e. twice as much as the value detected in the pH range of 7.2 to 7.6.

The effect of an extremely alkaline pH is rather universal. Myosin preparations of striated muscle reach their highest phosphate saturation also at extremely alkaline pH. Myosins of fast twitch muscles take up twice as much phosphate in alkaline than in normal medium.

Several characteristics of mt-myosin are similar to those of other myosin preparations. On the basis of the profiles observed during gel filtration the molecular weight of mt-myosin is identical with that of preparations of cytoskeletal and muscle origin and can be estimated to be 480—500 kDa.

Mt-myosin shows one band when investigated by the pyrophosphate polyacrylamide gel electrophoretic method of Hoh et al. [31] and so does also cp-myosin.

Both mt- and cp-myosin exhibit low ATPase activity and can be activated by actin just as can striated muscle- and cell-myosins.

During storage the P content of mt-myosin decreases to a greater extent than that of cp-myosin preparations. As a result of storage the lowest value detected (6 mol P/mol) was measured in mt-myosin preparate while the value found in cp-myosin was 8 mol P/mol.

The performed P contents that can be separated into molybdate-positive fractions from the alkaline hydrolyzate of both myosins are less than that derived from the hydrolyzate of other myosins. The number of P-containing peaks is a total of five when the peaks are separated from protein samples hydrolyzed in teflon tubes and measured in the presence of $HClO_4$. Parallel investigations on the Si content of the samples indicated that not only P but also endogeneous Si was eluated in the region of Peak 1 and Peak 2, despite the presence of $HClO_4$. When one to four smaller peaks were separated from the hydrolyzate of mt-myosin, P-containing and P-free molybdtae-positive peaks also appeared. The latter one was a N-containing derivate of the hydrolysis and it also originated from P-Arg. It is worth to mention that numerous drawbacks of alkaline hydrolysis have been known for a long time and one of them interferes with the guanidino group of free arginine also involved in our investigations. Hydrolysis of the guanidino group of phosphorylated arginine is much less due to the high alkaline stability of the N-P bonds. (The production of alkaline stable phosphoramidates and their hydrolytic derivates both present in the hydrolyzate, as well as their identification and the concomitant amino acids of the P-containing fractions have all been dealt with elsewhere.)

The P content of mt-myosin is decreased by Cu^{2+} treatment to a nearly constant level (19 mol P on the average). Also this characteristic seems to be universal since Cu^{2+} treatment decreases, even though to different degrees, the active P content of all the myosins so far investigated. Long-lasting storage (20 days) is more effective as far as the P content of mt-myosin is concerned since it diminishes the P content to a value even lower than that observed after Cu^{2+} treatment, this value most probably corresponds to the level of the ester-type phosphates.

The mobile P content of mt-myosin is decreased by actin. During interaction (when mt-myosin is incubated with actin of striated muscle origin for

three min) the extent of the diminution is proportional to the concentration of actin. When the P content of individual samples is evaluated in term of the mol to mol ratio of actin and myosin concentrations it will turn out that it can be nothing else just the 1.0 to 1.3 mol content consisting of actin and the residual endogeneous ester-type phosphate content of myosin. When interpreted as the function of the muscle the investigations give rise to the idea that during contraction the mobile phosphates of myosin are transiently placed onto the actin from where the inorganic phosphate is released. Moreover, actin accelerates and increases P release both from myosin and ATP. The latter phenomenon is also known as "actin-activated myosin ATPase activity".

It has been demonstrated in experiments on actin phosphorylation that actin takes up P from the ATP-containing incubation medium within a short period of time (actually within one min) and will be saturated to a value of 9 mol P/mol. During longer incubation periods smaller amounts but more firmly bound P remains in actin [27].

Summing up the above discussed observations and comparing them with the findings of others we can make a rough evaluation not only as to the P content, characteristics and function of mt-myosin but also as to its origin in the mitochondria. It appears especially on the basis of the independent fusion and division movements of mitochondrial crystae, that myosins should be present in the matrix [4, 10, 20]. This assumption was to be proved by experiments. Though myosin seems to be an independent isoenzyme its synthesis has never been observed in the matrix. Now we know not only the sequence of mtDNA of different origin, among them that of high-rank organs such as is the rat liver, but also the proteins coded and synthesized by the sequence. In spite of this, the above assumption has not yet been supported by direct observations, for no sequences coding myosin HC and LC have so far been isolated [2, 3, 11, 20, 49]. It has been experimentally demonstrated that mt-myosin, like most of the matrix proteins, is also coded in the genom of the muscles. Mt-myosin is synthesized extramitochondrially and reached the matrix from the cytoplasm either in a precursor form via the well-known continuous substance flow or in peptide form [30, 40, 47].

Wydro et al. [55] demonstrated in striated muscle that, "Detailed analysis of one embryonic and one adult myosin heavy chain gene shows that the coding sequences are interrupted by numerous intervening sequences whose number, size and distribution do not appear to be conserved in the same organism or between species". The existance of 11 to 13 MHC genes has been proved. So, at least 11 to 13 peptide chains are involved in the synthesis of the heavy chain assuring a wide range of possibilities for the development of isoforms of MHC (isomyosin). Ordinary and sarcoplasmatic MHC genes and clones could not be separated from sarcomeric MHC genes, a fact rendering probable

that non-sarcomeric MHC genes are coded in the nucleus in an independent gene category. Thus, it may be suggested that there exists an independent line, ancient in origin, of DNA sequences of particular and sarcoplasmatic myosins [12, 35, 55, 56].

The experiments are in favour of the symbiotic theory but not in the simple old way. It has to be taken into account that the ancient particular MHC genom is integrated into the nucleus [49, 56] and mt-myosin prepared by us is a result of the mitochondrial genom which underwent peculiar modification after cessation of the independent endosymbiotic existance.

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SHORT-TERM STIMULATION OF LYMPHOCYTE PROLIFERATION BY INDOMETHACIN IN VITRO AND IN VIVO

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The effect of short-term (up to 24 h) in vitro and in vivo treatment with indomethacin was studied on the blastogenesis of mouse spleen cells. Indomethacin in itself induced a strong proliferation of the lymphocytes starting after 6 h treatment both in vitro and in vivo. Besides, significantly enhanced the blastogenesis of splenocytes in response to various doses of PHA and Con A. The stimulation of lectin-induced lymphocyte proliferation occurred after indomethacin treatment both in vitro and in vivo. Indomethacin had no major effect on the distribution of Lyt-1+ and Lyt-2+ subsets within the spleen cell population. An important role of the prostaglandins in the early phase of lymphocyte activation is suggested.

Keywords: indomethacin, lymphocyte proliferation

The possible role of prostaglandins (PGs) in the regulation of lectin-induced blastogenesis has lately prompted extensive investigations [1, 3, 7, 8]. Lectin-induced blastogenesis was stimulated in vitro in the presence of indomethacin (INDO), a specific inhibitor of PG synthesis [1, 3]. In contrast to data in vitro, administration of INDO in vitro for 4 days [7] or 8 days [8] inhibited the lymphocyte proliferation in response to lectins. Following an 8 day treatment, the augmentation of Lyt-1—2+, while the reduction of Lyt-1+2— splenic lymphocyte subsets were associated with a decreasing blastogenic response (8). Early events after in vivo INDO administration have not been investigated, although it may have an important role in lymphocyte activation, and may explain the conflicting results in lymphocyte proliferation after in vitro and in vivo INDO treatment.

Materials and methods

Animals

Male CBA mice, 4—6 weeks old, bred and maintained in our institute, were used throughout the experiments.

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Lymphocyte proliferation assay

Spleen cells were obtained by squeezing the spleen. After filtering through cotton screen, the lymphocytes were isolated by centrifugation on Ficoll-Uromiro gradient [2] at 2000 rpm for 10 min, and resuspended in RPMI 1640 tissue culture medium supplemented with 10% heat-inactivated fetal calf serum (Phylaxia, Hungary), 25 mM HEPES buffer (SERVA, GFR), 2 mM L-glutamine (GIBCO, USA), 100 IU/ml penicillin, 100 μg/ml gentamycin and 7.5 μg/ml amphotericin B. Four hundred thousand spleen cells in 200 μl medium were placed in each flat-bottomed microplate well (Greiner, GFR) using four parallel samples. Phytohaemagglutinin (PHA, Leucoagglutinin, Pharmacia, Sweden) in doses of 2, and 10 μg/ml, or concanavalin A (Con A, Pharmacia, Sweden) in doses of 1, 5, and 10 μg/ml were added to the cells. Control cultures without the lectins were included in each experiment. The plates were incubated at 37 °C in a humidified atmosphere with 5% CO_2 for 72 h. The cultures were pulsed with 0.4 μCi ^{3}H-thymidine (^{3}H-TdR, UVVR, Czechoslovakia) 24 h before termination. The cells were harvested onto filter paper disks by an automated sample harvester (Dynatech, GFR). Isotope determinations were made in a liquid scintillation counter (Nuclear Chicago Isocap 300, USA). The results were expressed in cpm using the arithmetic mean of four replicate values [6].

Treatment with indomethacin in vitro and in vivo

Indomethacin (INDO, SIGMA, USA) was dissolved in ethanol, and diluted to 1 mg/ml in phosphate buffered saline (PBS), containing 10% ethanol, before use. To evaluate the in vitro effect of INDO on lymphocyte proliferation, mouse spleen cells were pre-treated with 10 μg/ml INDO (4) for 2, 6 and 24 h, then washed twice with culture medium. To determine the in vivo effect of INDO, mice were injected intraperitoneally (ip) with 0.7, 4 and 8 mg/kg INDO. These procedures were reported to block prostaglandin synthesis [5]. Control mice were injected ip with PBS that also contained 10% ethanol. Ethanol in itself in the doses applied had no major influence on blastogenesis either in vitro or in vivo.

Analysis of Lyt phenotypes of spleen cell populations

Mouse splenocytes were suspended at 1×10^7 cells/ml in anti-Lyt monoclonal antibody (Cedarlane Laboratories, USA) at a dilution of 1/20 and incubated for 45 min at 4 °C. After being washed, the cells were resuspended in fluoresceinconjugated and 1/10 diluted antimonoclonal antibody. The distribution of Lyt-1+ and Lyt-2+ phenotypes in the mouse splenocyte population was studied by fluorescence microscopy Leitz-Wetzlar, GFR).

Statistics

Data are given as mean +/— S.E. of n experiments. Statistical analyses were performed with the Student's *t*-test.

Results

Effect of INDO on blastogenesis in vitro

The proliferation of mouse spleen cells was studied in response to the lectins, PHA and Con A. Prior to the proliferation assay, the lymphocytes were treated with 10 μg/ml INDO, then washed twice. This concentration of INDO was found to be the most effective in stimulating blastogenesis in vitro (data not shown). In control cultures, without mitogens, a short-term pretreatment with INDO induced a strong proliferation of mouse splenocytes. After 6 h treatment, INDO significantly stimulated the blastogenesis of lymphocytes and this was further enhanced after 24 h treatment.

On the other hand, after 6 h treatment INDO further stimulated lymphocyte proliferation in response to 5 and 10 μg/ml Con A, while not to PHA.

Table I

Effect of INDO on blastogenesis of mouse spleen cells in vitro

	Hours		p	6 hr	p	24 hr	p
	0	2					
Spontaneous	490 ± 174	783 ± 333	ns	2 138 ± 189	<0.02	22 825 ± 6 248	<0.05
PHA 2 μg/ml	2 333 ± 84	18 576 ± 9 846	ns	20 259 ± 15 240	ns	90 889 ± 3 225	<0.01
PHA 10 μg/ml	1 492 ± 331	8 480 ± 4 192	ns	21 613 ± 6 079	ns	28 741 ± 10 410	ns
Con A 1 μg/ml	1 268 ± 496	43 320 ± 38 611	ns	11 882 ± 4 180	ns	65 312 ± 20 619	ns
Con A 5 μg/ml	-11 955 ± 3 931	63 814 ± 35 471	ns	27 996 ± 3 754	0.01	73 462 ± 6 352	0.01
Con A 10 μg/ml	8 723 ± 2 802	57 038 ± 33 446	ns	87 702 ± 11 301	0.02	63 245 ± 22 854	ns

Data represent ^{3}H-TdR-incorporation in cpm, mean ± S.E, in four separate experiments. Mouse spleen cells were pretreated in vitro with 10 μg/ml INDO, then washed twice prior to the lymphocyte proliferation assay. P values indicate the effect of in vitro INDO treatment in comparison to corresponding data using the paired *t*-test.

Table II

Dose-dependent effect of in vivo INDO treatment on the blastogenesis of mouse spleen cells

INDO treatment	Control	0.7 mg/kg	p	4 mg/kg	p	8 mg/kg	p
Spontaneous	245 ± 41	573 ± 93	ns	2 238 ± 407	<0.02	3 505 ± 826	<0.05
PHA 2 μg/ml	3 812 ± 795	7 589 ± 1 000	<0.05	7 085 ± 584	<0.02	7 195 ± 1 220	ns
PHA 10 μg/ml	5 042 ± 2 239	5 715 ± 540	ns	6 352 ± 1 536	ns	5 440 ± 626	ns

Data represent ^{3}H-TdR-incorporation in cpm, mean ± S.E., in five experiments. Mice were treated intraperitoneally with INDO 2 h before the blastogenesis assay in doses of 0.7, 4 and 8 mg INDO/kg body weight. P values refer to the effect of in vivo INDO treatment on ^{3}H-TdR-incorporation by lymphocytes as compared to the data of control mice, using Student's *t*-test.

Table III

Short-term in vivo effect of INDO on the blastogenesis of mouse spleen cells

	Hours		p	6	p	24 hr	p
	0	2					
Spontaneous	508 ± 67	824 ± 169	ns	1 749 ± 351	<0.05	15 945 ± 863	<0.001
PHA 2 μg/ml	4 562 ± 547	8 719 ± 1 067	<0.05	13 952 ± 2 808	<0.05	46 205 ± 2 958	<0.001
PHA 10 μg/ml	3 344 ± 529	4 704 ± 116	ns	11 356 ± 3 022	<0.05	16 256 ± 1 552	<0.01
Con A 1 μg/ml	1 679 ± 626	5 369 ± 2 615	ns	20 167 ± 6 889	<0.05	89 942 ± 8 516	<0.001
Con A 5 μg/ml	14 214 ± 4 209	25 685 ± 8 617	ns	161 449 ± 17 323	<0.01	94 730 ± 9 639	<0.02
Con A 10 μg/ml	32 010 ± 13 833	27 703 ± 7 791	ns	170 625 ± 13 907	<0.01	33 391 ± 3 730	ns

Data represent ^{3}H-TdR-incorporation in cpm, mean ± S.E. in five experiments. Mice were treated intraperitoneally with 4 mg/kg body weight INDO, 2, 6 and 24 h prior to the blastogenesis assay. P values show the effect of in vivo INDO treatment on ^{3}H-TdR-incorporation by lymphocytes as compared to the data of control mice, using Student's *t*-test.

Lymphocyte proliferation after 5 and 10 μg/ml ConA+INDO was significantly greater than to Con A or INDO alone. Similar results were obtained after 24 h treatment with INDO at 2 μg/ml PHA and 5 μg/ml Con A doses, respectively

Effect of INDO on blastogenesis in vivo

After 2 h in vivo treatment, INDO in itself already stimulated the proliferation of mouse splenocytes in a dose-dependent manner (Table II). This effect increased in parallel with the time of treatment (Table III).

Blastogenesis of lymphocytes in response to 2 μg/ml PHA was enhanced after 2 h in vivo treatment with INDO, while after 6 h and 24 h treatments blastogenesis was increased at all PHA and all but one (10 μg/ml) Con A doses. The proliferative responses to lectins + INDO were greater than those to lectins or INDO alone.

Analysis of Lyt phenotypes of spleen cell populations after in vivo treatment with INDO

After 2, 6 and 24 hrs in vivo treatment, INDO had no major effect on the distribution of Lyt-1+ and Lyt-2+ phenotypes in the mouse splenocyte population (Table IV).

Table IV

Effect of INDO on the distribution of mouse spleen cell subsets (per cent ± S.E., n = 4)

Hour	Lyt 1+	p	Lyt 2+	p
0	67.0 ± 3	—	22.0 ± 2	—
2	67.0 ± 3	n.s.	23.0 ± 3	n.s.
6	70.0 ± 4	n.s.	23.0 ± 3	n.s.
24	68.0 ± 3	n.s.	22.0 ± 4	n.s.

n.s.: not significant.

Discussion

The present report suggests that short-term treatment with INDO, both in vitro and in vivo, stimulates the lectin-induced blastogenesis of mouse spleen cells. In addition, INDO alone exerted a strong proliferative effect on lymphocytes. The lymphocyte proliferation in response to both a lectin and INDO was considerably greater than to a lectin or INDO alone. This indicates that the proliferative effect of lectins and INDO is based on mechanisms not interfering with each other.

Concerning the stimulation of both spontaneous and mitogen-induced blastogenesis, the similar results obtained during in vitro and in vivo studies may refer to a common mechanism of INDO in these systems. Since short term INDO treatment did not affect the Lyt phenotypic distribution of splenocytes, the enhancement of lymphocyte proliferation by INDO may not be related to the reduced number of T cells with suppressor phenotype [8]. These observations give evidence for the putative in vivo role of short-lived suppressor cells [3] regulating lymphocyte blastogenesis by PG production.

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LIVER BLOOD FLOW DURING HAEMORRHAGIC SHOCK IN THE DOG TREATED WITH PHENOXYBENZAMINE

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The liver blood flow has been extensively studied in hemorrhagic shock, but considerable disagreement exists as to the nature of hemodynamic changes and their controlling mechanism. The present investigation was undertaken in order to determine the effects of hemorrhage and phenoxybenzamine (PBZ) on the participation of hepatic artery (HAF) and portal vein flow (PVF) in total liver blood flow (LBF) changes. The dynamics of LBF (H_2 washout method), HAF and PVF (electromagnetic flowmeter) during 3-hours posthemorrhagic hypotension (90 min. = 50—60 mmHg; 90 min. = 25—30 mmHg) and one-hour postretransfusion period were investigated on 20 mongrel dogs under chloralose anesthesia. All animals were divided into 2 groups (control and PBZ-treated — 5 mg/kg b.w. 30 minutes following first bleeding). Half an hour following bleeding there occurred a significant decrease of LBF ($P < 0.001$) in dogs of both experimental groups. This degree of decrease was due to equal decrease in the PVF and HAF. The infusion of PBZ caused a slight tendency towards increase of LBF, while the subsequent decrease in blood flow values during second hypotensive period in the treated dogs was not so pronounced as in the untreated dogs. Although retransfusion led to an increase of LBF, HAF and PVF in both groups, the restauration was significantly better in PBZ-treated animals. The degree of metabolic acidosis was more pronounced in the untreated dogs than in PBZ-treated.

Keywords: posthemorrhagic hypotension, phenoxybenzamine, liver blood flow, hepatic arterial blood flow, portal venous blood flow, acid-base balance

The blood supply to the liver is unique, since the organ is supplied by systemic arterial as well as portal venous blood draining the gastrointestinal tract. Liver circulation has been extensively studied in hemorrhagic shock, but considerable disagreement exists as to the nature of the haemodynamic changes and their controlling mechanism. It is generally accepted that total liver blood flow [3, 4], portal venous [5] and mesenteric blood flow [2] decrease after hemorrhage. Hepatic arterial blood flow has been reported to decrease [4, 5], increase, or to remain unaffected [6].

The aim of the present investigation was to analyze liver circulatory dynamics during haemorrhagic shock. The following questions were studied.

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1. How does the liver blood flow in the dog react to experimental haemorrhagic shock?

2. How does the hepatic artery and portal vein blood flow participate in total liver flow changes during posthaemorrhagic hypotension and retransfusion?

3. Whether phenoxybenzamine had a beneficial effect on liver circulation and on evolution of irreversible changes in the treatment of haemorrhagic shock?

Materials and methods

A total of 20 mongrel dogs of both sexes aged between one and two years weighing 13 to 20 kg were used in the experiments. Anaesthesia was induced with 1% chloralose (0.1 g · kg^{-1} i.v.). After preparation the animals were allowed to stabilize for 30 minutes before control values were taken. The dogs were then bled to a constant arterial pressure of 50—60 mm Hg and kept at this level for 90 minutes (I hypotensive period). At the end of this period the animals were bled to 25—30 mm Hg and kept at this level for 90 minutes (II hypotensive period) and after retransfusion passed through a postretransfusion period for 60 minutes. The animals which survived this experimental procedure were observed for 24 hours, than they were killed by exsanguination and the liver was weighed.

All animals were divided into two groups: control (untreated) and PBZ-treated (phenoxybenzamine 5 mg · kg^{-1} in a total volume of 10 ml, slow infusion from 30 to 40 minutes during the I hypotensive period).

Blood pressure, heart rate and respiratory rate were recorded using adequate transducers. Noncannulating electromagnetic blood flow probe (Nycotron Blood Fowmeter 376) was placed around the hepatic artery to estimate hepatic arterial blood flow (HABF). Parameters were displayed on a recording multi-channel Polygraph Grass 7D. Total liver blood flow (TLBF) was measured with the hydrogen washout method [1]. As the TLBF was determined in $ml \cdot min^{-1} \cdot 100\,g^{-1}$ liver tissue, HABF was transformed into the same units by dividing the total HABF by liver weight, to obtain the value of portal vein blood flow (PVBF) the HABF was subtracted from TLBF [4].

In the arterial and venous blood samples the complete acid-base parameters were determined using an ABL-1 (Radiometer) divice.

Results

The acid-base parameters showed all characteristics of metabolic acidosis. The symptoms of metabolic acidosis were more pronounced in the untreated dogs, particularly in the second hypotensive period and during the postretransfusion period. At these stages differences between the groups in the value of pH, base excess and plasma and standard bicarbonate were statistically highly significant. Representative parameter — pH — is presented in Fig. 1.

The data of liver blood flow changes during posthemorrhagic hypotension and after retransfusion are given in Fig. 2.

The data concerning survival of the animals were as follows.

Untreated group: three dogs died during the second hypotensive period, five animals died within 12 hours after the end of the experiment, one within the next 12 hours, while only one untreated dog survived 24 hours after the end of the experiment.

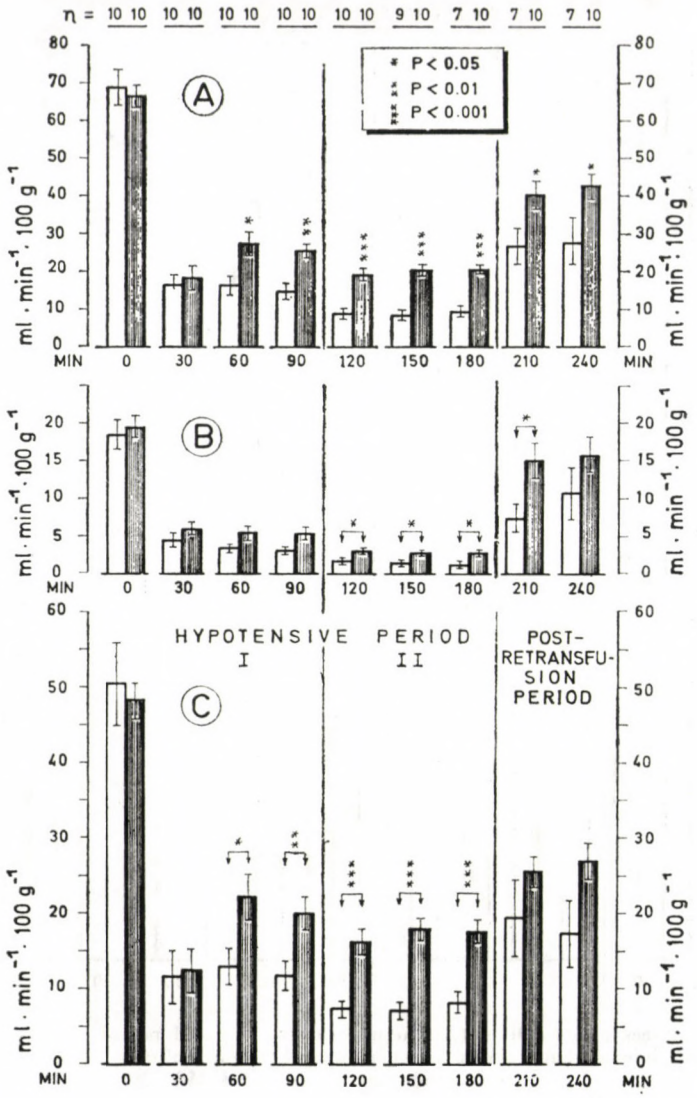

Fig. 1. Differences of arterial and venous blood pH changes during haemorrhagic shock between control (hollow columns) and PBZ-treated (filled columns) dogs (mean ± S.E.M.)

In the PBZ-treated group all animals survived the experimental procedure, two dogs died within 12 hours and one within the next 12 hours after the experiment, the remaining seven survived 24 hours after the end of the experiment.

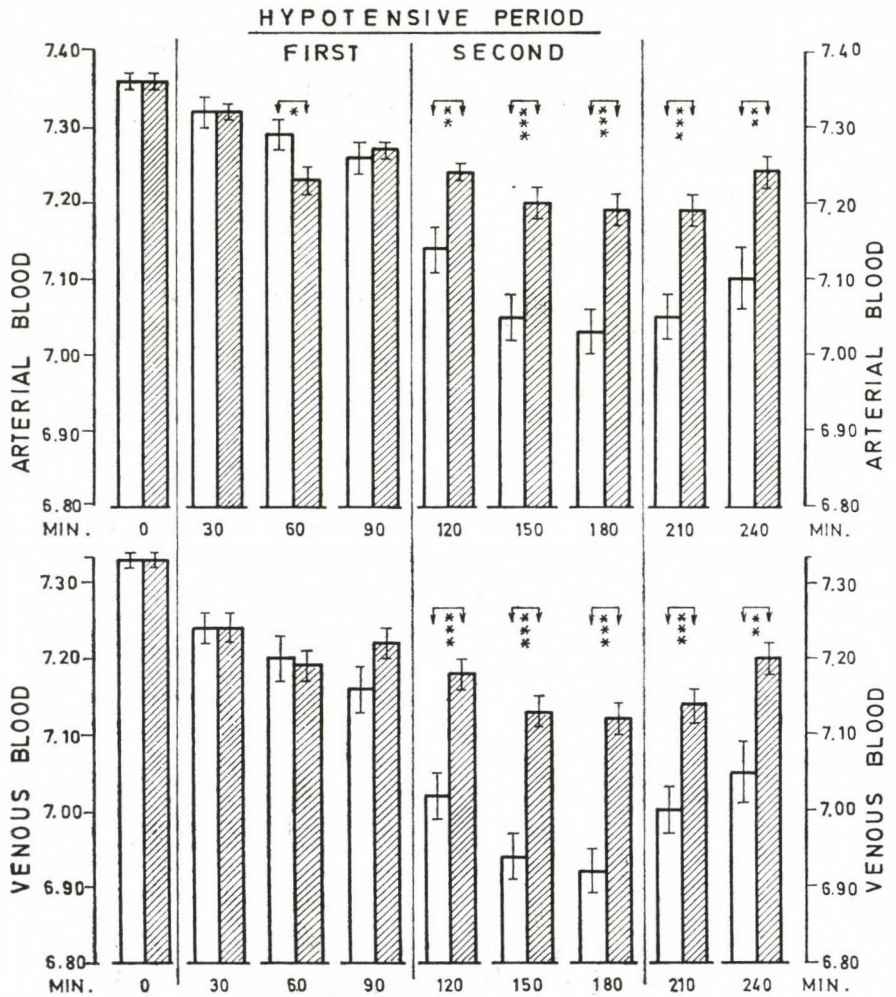

Fig. 2. Differences of total liver (A), hepatic artery (B) and portal venous (C) blood flow changes during haemorrhagic shock between control (hollow columns) and PBZ-treated (filled columns) dogs (mean ± S.E.M.)

Conclusions

1. It is apparent from the results that the haemorrhagic shock procedure caused a significant decrease in TLBF. The degree of decrease was equal in PVBF and HABF.

2. PBZ-treatment caused a tendency towards an increase of TLBF, while the subsequent decrease during the II hypotensive period in the treated dogs was not so pronounced as in the controls, All TLBF values after PBZ ad-

ministration in the treated dogs were significantly higher than in the untreated dogs. It was associated with a linearly related increase in PVBF.

3. The degree of metabolic acidosis was more pronounced in the untreated than in the PBZ-treated dogs.

4. All experimental results suggested that PBZ-treatment had a protective effects on the evolution of irreversible changes during the haemorrhagic shock procedure. All animals of the treated group survived the experimental procedure and lived considerably longer after the end of the experiment, while three dogs of the untreated group died during the experiment and the rest showed a shorter survival after the end of the experiment.

5. Regarding the mechanism of PBZ, our opinion is in agreement with data in the literature in that the drug abolishes the vasoconstrictive effect of alpha adrenergic hyperactivity. On basis of the PVBF data, it seems that the effect is markedly expressed in the portal drainage system.

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EFFECT OF PHENYLEPHRINE AND ISOPROTERENOL ON MESENTERIC BLOOD FLOW IN EXPERIMENTAL POSTHAEMORRHAGIC HYPOTENSION

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In previous reports it was found that in mesenteric blood vessels exist profound alpha-adrenergic, and less profound beta-adrenergic receptors. Our experiments were done on 20 mongrel dogs, both sexes, weighing 6.5—22.5 kg, age from 1—2 years, which were divided into 2 groups: experimental (14 dogs) and control (6 dogs). Experimental group of animals passed through the posthemorrhagic hypotension (180 min. at 5.3 kPa). Aim of investigation was to clarify mesenteric vascular reactivity changes during post-hemorrhagic hypotension. In order to reach this goal, phenylephrine and isoproterenol were injected alternatively in one hour intervals through cannulated superior mesenteric artery first proximal branch. Our results indicate that mesenteric blood flow responses to those drugs changed quantitatively, but never ceased. It is apparent from our experiments that those quantitative changes are due to $beta_2$ adrenoceptors changes during the posthemorrhagic hypotension.

Keywords: posthemorrhagic hypotension, mesenteric vascular reactivity, phenylephrine, isoproterenol, $beta_2$-adrenoceptors

The mesenteric vascular bed is considered to be a major contributor to circulatory homeostasis. In mesenteric blood vessels alpha-adrenergic receptors are abundant, while beta receptors are less numerous [3]. It also seems that beta receptors are of great importance in the control of transcapillary fluid shift to blood during hypovolaemia [4]. It was therefore of interest to investigate mesenteric vascular reactivity during posthaemorrhagic hypotension. Previous reports [1] have shown for instance that reactivity of the blood vessels to epinephrine may completely disappear in the irreversible stage of post-haemorrhagic shock.

Materials and methods

Experiments were done on 20 mongrel dogs of both sexes aged 1—2 years weighing 6.5—22.5 kg. They were divided into 2 groups experimental (14 dogs) and control (6 dogs).

The dogs were anaesthetized with 1% chloralose (0.1 g/kg i.v.). Blood pressure was measured via a blood pressure transducer (Statham P23D). Noncannulating electromagnetic

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blood flow probe (Nycotron Blood Flow Meter 376) was placed around the superior mesenteric artery for mesenteric blood flow measurements. Parameters were displayed on a recording multi-channel polygraph Grass 7D. The experimental group was subjected to posthaemorrhagic hypotension for 180 minutes (at 5.3 kPa) and then retransfused. Control dogs were not treated and remained normotensive during the experiment. Drugs (phenylephrine and isoproterenol 1 μg/kg) were given alternatively in 1 hour intervals during the whole experiment through a cannula placed into the first proximal branch of the superior mesenteric artery [2].

All values are presented as means ±S.E.

Results and discussion

From the experiments it was apparent that the reactivity of mesenteric blood vessels to alpha and beta agonists never completely disappeared during posthaemorrhagic hypotension. Reactivity changes were only quantitative. During normotension phenyolephrine decreased and isoproterenol increased mesenteric blood flow in all experiments in both groups of animals. In the experimental group, phenylephrine generally decreased mesenteric blood flow during both hypotension and postretransfusion (Table I). The same happened

Table I

Response of superior mesenteric artery flow to L-phenylephrine (1 μg/kg)

Experimental time	Application time (min)	Blood flow change, per cent				Blood flow before application (ml/min/kg) ± SE
		decrease	increase	biphasic	no change	
CP	—30	38.3 ± 6.2 (9)	—	—	—	12.55 ± 2.7 [9]
	30	20.8 ± 2.9 (8)	—	—	0 (1)	3.2 ± 1.3 [9]
HP	90	15.9 ± 2.5 (6)	—	+6.7 ± 1.4 —8.6 ± 2.1 (3)	—	2.8 ± 1.1 [9]
	150	19.7 ± 4.3 (5)	20.51 (1)	+10 —30 (1)	0 (1)	2.46 ± 0.9 [8]
	210	42.8 ± 10.3 (6)	—	+33.6 ± 6.4 —42.3 ± 12.2 (2)	—	42.8 ± 10.3 [8]
RP	270	3.4 ± 5.6 (5)	—	+24 —24 (1)	0 (1)	3.4 ± 5.6 [7]

Experimental group

Blood flow changes are expressed as mean precent of increase (+) or decrease (—) of values measured before L-phenilephrine application ±SE. Parentheses indicate number of cases in which the change had occurred, brackets indicate number of animals.

CP — control period; HP — hypotensive period; RP — period after retransfusion

without exception in the control group. On the other hand, the effect of isoproterenol during hypotension and retransfusion differed from that in normotension (Table II). During hypotensive and retransfusion periods, isoproterenol either increased or decreased mesenteric blood flow or had a biphasic effect. In the control group there were no changes in the effect of isoproterenol during the experiment.

Table II

Response of superior mesenteric artery flow to isoproterenol (1 μg/kg)

Experimental time	Application time (min)	Blood flow change, per cent				Blood flow before application (ml/min/kg) ± SE
		decrease	increase	biphasic	no change	
CP	—30	—	61.38 ± 11.29 (14)	—	—	10.54 ± 5.79 [14]
	30	9.38 ± 3.32	32.04 ± 9.98	—23.07 +7.69	0	3.03 ± 0.95
		(4)	(6)	(1)	(1)	[12]
HP	90	8.39 ± 0.8	39.01 ± 8.50	+11.36 ± 3.35 —11.46 ± 1.6	0	2.85 ± 1.07
		(3)	(3)	(3)	(2)	[11]
	150	9.77 ± 4.51	17.85 ± 6.39	+18.53 ± 6.47 —10.92 ± 5.74	0	2.41 ± 0.85
		(2)	(5)	(2)	(1)	[10]
RP	210	48.78 ± 26.7 (3)	32.19 ± 7.54 (6)	—	—	3.83 ± 1.26 [9]
	270	8.33 (1)	21.47 ± 6.23 (5)	—	0 (1)	2.90 ± 1.01 [7]

Experimental group

Blood flow changes are expressed as mean percent of increase (+) or decrease (—) of values measured before isoproterenol application ±SE. Parentheses indicate number of cases in which the change had occurred brackets indicate number of animals.

CP — control period; HP — hypotensive period; RP — period after retransfusion.

Mesenteric blood flow changes are believed to be the result of alpha (decrease) or beta (increase) adrenergic receptor stimulation. Our results indicated that beta-adrenergic receptors during posthaemorrhagic hypotension underwent changes while the reactivity of alpha-adrenergic receptors remained at the control level. This fact may be of importance when considering the role of beta receptors in the control of transcapillary fluid shifts to the blood.

The experiments did not point to any direct connection between mesenteric vascular reactivity and the onset of the "irreversible" stage of haemorrhagic shock.

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CORRELATIONS BETWEEN POSITIVE AND NEGATIVE AEROIONS, TISSUE REDOX-STATE POTENTIAL AND HEART FREQUENCY IN RATS

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It was observed in rats that following positive aeroionization the redox-state potential (E_0') in skeletal muscles and liver was decreased, and the heart frequency increased. After negative ionization these interrelationships took place inversely. It was also established that upon adding an oxidant (menadione) i.v., the E_0' was decreased (compensatorily) in the organs mentioned above, parallel with the increment of heart frequency. Following injection of reductants (cysteine, thiamine) a reverse image was observed. Applying simultaneously positive ionization and reducing agents, the E_0' change and the heart frequency alteration failed to appear. The phenomenon was the same after simultaneous application of negative ionization and oxidant (menadione) injection. Because the heart effect of positive and negative ionization could readily be prevented by a respective redox agent, it seemed that actions of aeroions are exerted through shifts in tissue E_0'. The most probable site of action of E_0', is the pacemaker mechanism, but an action on serotonin liberation may also be assumed.

Keywords: positive aeroions, negative aeroions, redox-state potential, skeletal muscle, liver, heart frequency

Although the significance of aeroions in physiological and pathophysiological processes is widely accepted, the questions of the mechanism(s) and site of action(s) are not clear.

According to Krueger et al. [6] the tracheal changes attributed to positive air ions can be increased by an intravenous injection of serotonin. These effects can be reversed by negative air ions. It seems therefore to postulate that positive air ions are serotonin releasers and that a local accumulation of serotonin in the trachea is the immediate cause of the effect of positive ion effects. It can further be postulated that negative air ions reverse the positive ion effect by speeding up the rate at which serotonin is oxidized. Like other oxydase systems, monoamine oxydase is thought to consist of a dehydrogenase linked to a respiratory chain, which may include cytochromes or flavins. Krueger's experiments demonstrating that negative ions have a direct action

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on cytochrome oxydase and accelerate the cytochrome-linked conversion of succinate to fumarate would suggest that this same action may produce a cytochrome-linked oxidation of serotonin. Tal et al. [20] confirmed that negative ionization reduced the serotonin release significantly. It should, however, be emphasized that serotonin may not be the sole factor, as no studies have been made on the possible involvement of other biogenic amines such as acetylcholine or of immunologic factors like histamine [7].

Taking into account the effects of aeroions, one would conclude that the character of their modifying, regulatory actions is chiefly that of a regulator (modulator?) of the whole internal milieu. Since positive or negative ions readily influence redox potential values in vitro, it was assumed that such an effect may occur also in organisms. This accumption was corroborated by the observation that both positive ions and redosis (the relative decrease of E_0') increase the symphatetic dominance, while negative ions and oxidosis (the relative increase of E_0') increase parasymphatetic dominance in the organism. We have therefore studied the redox-state potential in skeletal muscles and liver of rats together with their heart frequency following positive or negative ionization.

Materials and methods

The experiments were carried out on CFY rats of both sexes, weighing 300—350 g. They were anaesthetized with Ketanest (14 mg/100 g body weight). The redox-state potential (E_0') was measured by the potentiometric method of Cater and Phillips [2]. An electronically sharpened platinum wire was inserted (tip diameter 200 μm) into the muscles (vastus medialis) and the liver. The electrodes were enamel insulated to 0.5 mm of their tips. The reference electrode (Ag/AgCl) was placed into the intraperitoneal cavity. After compensation of the electrode potentials, the potential difference between the platinum-Ag/AgCl electrodes gave the E_0' value in mV. As a measuring instrument, a digital potentiometer of high input resistance (Radelkisz OP-208) was used, the amplifier of which was regulated automatically by a thermosensor, also placed in the intraperitoneal cavity at 22 °C, ambient temperature and normal body temperature being maintained automatically by means of infrared lamps.

Heart frequency was measured by ECG with limb (two electrodes on the fore and one on the hind limbs) leads.

The animals were placed into a slightly fenestrated ionization chamber (V = 8L) and ionized with a BION 80 type ionisator (Medicor) of 10^9 ion/cm^3 capacity. Upon the effect of the 3000 V potential, at the tip of the apparatus a coronadischarge comes about, which ionizes the air particles. From the ionized particle pairs, in dependence on the polarity of the plugged voltage either the positive or negative ions will be neutralized, while those of the opposed sign will be ejected. The electrical configuration of these ions is under debate. According to Mohnen (10) the following suggestions exist: $H^+(H_2O)_n$, $(H_3O)^+(H_2O)_n$, $(H_3O)^-(H_2O)_n$, $O_2^-(H_2O)_n$, $OH^-(H_2O)_n$, $O^+(H_2O)_n$, $NO_2^-(H_2O)$, $O_2^-NO(H_2O)$, $NO_2^-(SO_2)$. Application of redox agents was carried out through a cannula fixed into the jugular vein. As oxidant, 5 mg/100 g body weight menadione, and as reductant 5 mg/100 g body weight cysteine + thiamine was injected. Because after injection of these agents in some minutes significant changes were observed in the parameters measured in the muscles, the liver or the hearts, it was certain that they have reached the target organs.

Although menadione, cysteine and thiamine penetrate cell membranes, this had no significance in our case, because according to Clark et al. [3] and Sun et al. [18] there are transplazmamembrane redox enzyme systems which are capable to mediate the redox-state between the extra and/or intracellular compartments.

The number of experiments was 9 in every series. The p values given below relate to the data between the 0 and 16 minutes.

Results

As it is seen in Fig. 1, following positive ionization the E_0' decreased significantly both in the muscles ($p < 0.002$) and the liver ($p < 0.001$), while the heart frequency increased ($p < 0.001$). On the other hand, after ionization with negative ions (Fig. 2) a mirrorlike image was observed; E_0' increased in both muscle ($p < 0.001$) and liver ($p < 0.001$) and the heart frequency decreased ($p < 0.001$).

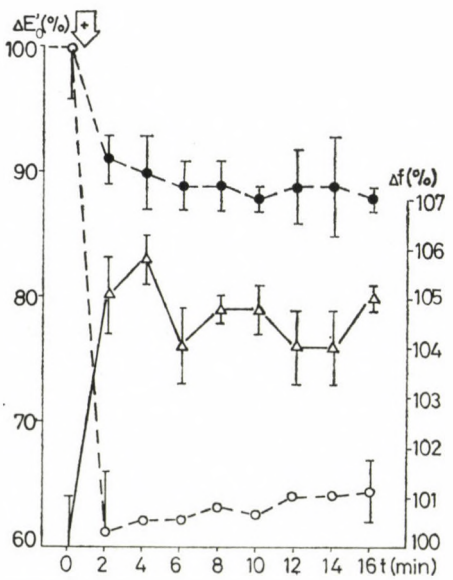

Fig. 1. Effect of positive aeroions on the redox-state potential (E_0') of the vastus medialis muscle (●) of the liver (○), and on heart frequency (△), in per cent. f — heart frequency. Following the start of ionization, (arrow) data were registered every 2 min. Vertical bars: ±S.E.M.

Figure 3 demonstrates that after and i.v. injection of oxidant the E_0' decreased and parallel with this the heart frequency increased ($p < 0.001$). Application of the reductant caused an inverse picture (Fig. 4) in that E_0' showed an elevation ($p < 0.002$) while heart frequency declined ($p < 0.001$). From this observation a general rule could be deduced: Independently of the origin of the exciting factor, a negative correlation exist between the E_0' level of the organism and heart frequency.

In order analyze as to whether this interrelation was a simple epiphenomenon, or there was a causal relationship between them, we simultaneously applied positive ionization and reducing drugs, or negative ionization and an oxidant (menadione). It is shown in Fig. 5. that under the effect of positive ionization and reducing drugs the decrease of E_0' caused by positive ions was

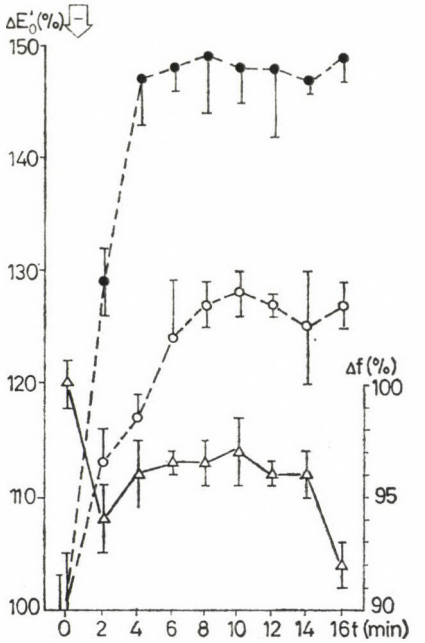

Fig. 2. Effect of negative aeroions on the redox-state potential (E_0') of the vastus medialis muscle (●) of the liver (○) and on heart frequency (△) in per cent. f — heart frequency. Following the start of ionization, (arrow) data were registered every 2 min. Vertical bars: ±S.E.M.

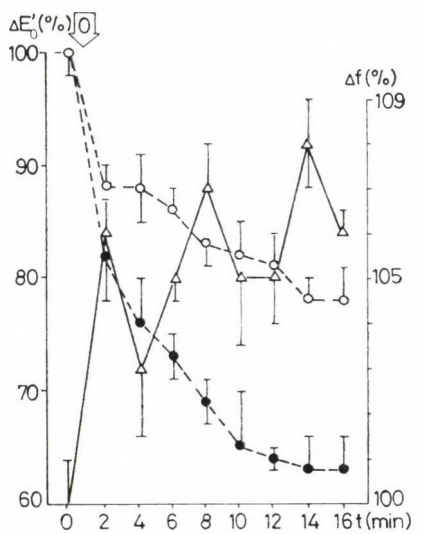

Fig. 3. Effect of oxidant (menadione 5 mg/100 g body weight) on the redox-state potential (E_0') of muscle (●) and liver (○) and on heart frequency (△) in per cent. f — heart frequency. Following the injection (arrow) data were registered every 2 min. Vertical bars: ±S.E.M.

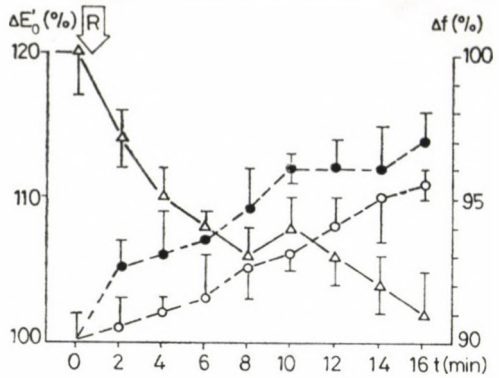

Fig. 4. Effect of reducing drugs (2.5 mg cysteine + 2.5 mg thiamine) (100 g body weight on the redox-state potential (E_0') of muscle (●) of liver (○) and on heart frequency (△). Following the injection (arrow) data were registered every 2 min. Vertical bars: ±S.E.M.

substantially reduced and heart frequency showed only minor oscillations around the mean of controls. A significant increase was registered only in the second minute of application ($p < 0.05$). On the other hand, after simultaneous application of negative ions + menadione, there was a weak decrease of muscle E_0' only for 4 minutes, and heart frequency did not change (Fig. 6). Since the effect of negative or positive ions on heart frequency could be prevented or at least decreased with redox agents, the main site of action of positive or negative aeroions was probably to decrease or increase, respectively, the tissue redox-state potential.

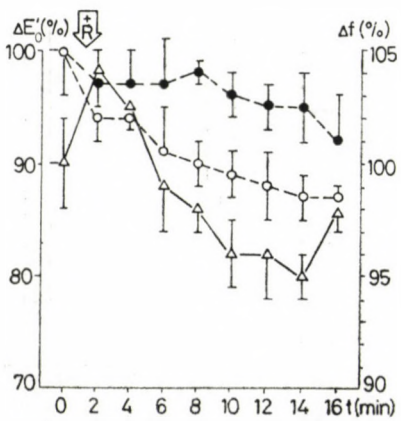

Fig. 5. Effect of simultaneous application of menadione (5 mg/100 g body weight) and positive ionization on redox-state potential (E_0') of muscle (●), of liver (○), and on heart frequency (△). Following the start of operations (arrow) data were registered every 2 min. Vertical bars: ±S.E.M.

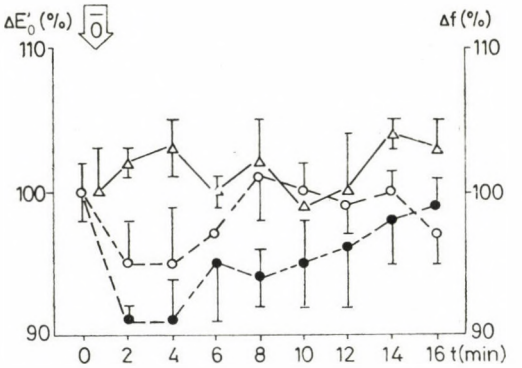

Fig. 6. Effect of simultaneous application of reducing drugs (2.5 mg cysteine + 2.5 mg thiamine) (100 g body weight) and negative ionization on redox-state potential (E'_0) of muscle (●), and liver (○), and on heart frequency (△). Following the start of operations (arrow) data were registered every 2 min. Vertical bars: ±S.E.M.

The E'_0 changes were more intensive in the liver following positive, and more expressed in the muscle after negative ionization. Following oxidant or reductant application the phenomenon did not take place.

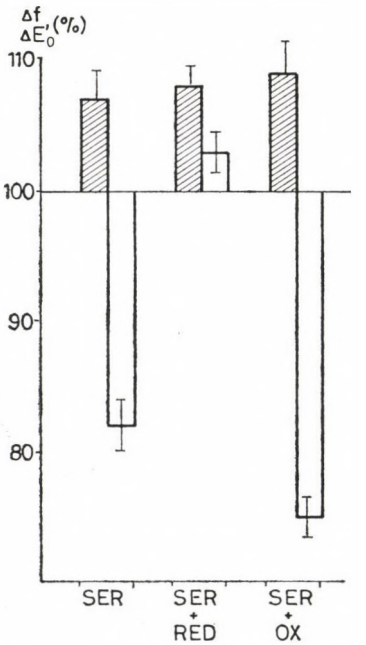

Fig. 7. Effect of serotonin (20 μg/100 g body weight), serotonin + oxidant (menadione, 5 mg/100 g body weight) and serotonin + reducing drugs (2.5 mg cysteine + 2.5 mg thiamine/100 g body weight) on redox-state potential of liver (E'_0, □) and on heart frequency (f, ▨), 2 min after application. Vertical bars: ±S.E.M.

Because many authors attached importance to serotonin in the mechanism of aeroions, we examined the question whether the serotonin action was influenced by changes in E_0'. In these experiments (Fig. 7), 20 μg/100 g body weight serotonin was injected i.v. It caused a 7% increase in heart frequency and a 18% E_0' decrease in liver. Following the application of serotonin + oxidants or reductants, the E_0' decreased or increased, respectively, but the positive chronotropic action of serotonin did not change significantly. Thus the direct heart effect of serotonin was not influenced by the decrement or increment of E_0'.

Discussion

It is evident from the present results that positive aeroions evoke a relative decrease in E_0' while negative ions elicit a relative increase in E_0'. It is known, that a change in tissue E_0' is an important factor in the regulation of numerous physiological and pathophysiological functions [4, 15, 16]. Our recent results seemed again to corroborate with this notion.

It has been shown that there is a negative correlation between the increase of E_0' and heart frequency, and a positive correlation between the decrease of E_0' and heart frequency independently of the fact that the redox change was evoked by aeroions or by application of redox agents [recent data and 8, 12, 13, 14, 19, 21].

The changes caused in E_0' by negative or positive ions are evidently a biochemical compensation, because in simple solutions positive ions increase and negative ions decrease the E_0' [4, 15, 16].

As to the mechanism of the effect of positive and negative ions, with Krueger [6] we suppose, that the increase in serotonin level following positive, and its decrease after negative ionization might be an important site of action in the inverse effects of positive or negative aeroions. The ions exert this effect indirectly, through changes in the tissue E_0'. This mechanism seemed to be supported by the present findings showing that the effect of positive ions on heart frequency may be prevented by exogenous reductants, and the action of negative ions may be counteracted by exogenous oxidants. Redox control of the liberation of bioactive agents occur also with acetylcholine [1], glutamine [22] and adrenaline [8]. Not only the control of serotonin liberation might play a role in the redox influence, but also the rate of its oxidation. A decrease in E_0' will inhibit while its augmentation speed up the oxidative formation of 5-hydroxyindole acetic acid (5-HIAA).

The supposition that the change of E_0' influences the direct effect of serotonin on the heart should be excluded on the basis of our recent results (Fig. 7).

It is less probable that an increase or decrease of E_0' would evokes a reverse effect on the liberation of acetylcholine or adrenaline, because an increase will stimulate the liberation of both acetylcholine [1] and adrenaline [8]. Moreover, it reverses the type of acetylcholine effect [14], so if the increase in E_0' produced by negative ions had increased the liberation of acetylcholine, a positive chronotropic action should be expected. Hence, in all probability the liberation of acetylcholine or adrenaline is not a decisive mechanism in this phenomenon.

On the other hand, the increase in E_0' decreases and a fall in E_0' increases the activity parameters of the autorhythmic heart beat [9, 11, 13, 14, 5, 23]. Because the redosis after positive and oxidosis following negative ionization elicited an increment or decrement of heart frequency, this mechanism seems to be the most probable site of action of the redox changes evoked by aeroions.

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INDUCTION OF HEPATIC MITOCHONDRIAL ALPHA-GLYCEROPHOSPHATE DEHYDROGENASE BY L-TRIIODOTHYRONINE IN SINGI FISH (*HETEROPNEUSTES FOSSILIS* BLOCH)

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A single injection of L-triiodothyronine (T_3) in different doses (0.25, 0.5, 5, 20 and 50 μg/g) increased the hepatic mitochondrial cytochrome-linked α-glycerophosphate dehydrogenase (α-GPD) activity and mitochondrial protein content of Singi fish, as observed on the 3rd day. A non-linear dose-response relationship with respect to enzyme activity was observed with different doses of T_3. A low dose of 0.1 μg of T_3 per g failed to cause any change in α-GPD activity and mitochondrial protein content of the liver. The enhancement of α-GPD activity over the control level with a low and a high dose of T_3, viz., 0.5 and 5 μg/g, was followed from the 1st to the 7th day, when it was found that enzyme activity reached the maximum level on the 3rd day and then gradually declined to the control value on the 7th day. The percentage increase in enzyme activity with 5 μg/g was higher than that with 0.5 μg/g from the 2nd to 5th day. Compared to the control, these two doses of T_3 caused an increase in α-GPD activity from the 1st to the 6th day. Cycloheximide inhibited the T_3-induced increase in α-GPD activity, mitochondrial and total protein content of liver. Immersion of Singi fishes in thiourea-containing (1 mg/ml) medium for 30 days showed a fall in hepatic α-GPD activity in comparison to the euthyroid control. A single injection of T_3 (0.5 μg/g) to the hypothyroid fish recovered α-GPD activity to more than the euthyroid control level. An increase in mitochondrial protein content in the T_3-injected hypothyroid fish has been observed. DNA content of the fish liver remained unchanged in every experimental condition. The results thus showed the significant responsiveness of the fish liver to thyroid hormone.

Keywords: L-triiodothyronine, thyroid hormone, α-GPD, fish liver, hypothyroid fish

Mitochondrial cytochrome linked α-glycerophosphate dehydrogenase (α-GPD), which is involved in hydrogen transport, can dramatically be induced by thyroid hormones in various organs in mammals except in human and guinea-pig liver [9, 16, 22, 23, 24, 29]. The activity of this enzyme in the liver varies with different thyroidal states. In hyperthyroid condition it is increased markedly to 10 to 20-fold more than the euthyroid control and in hypo-

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thyroid condition this enzyme activity is decreased significantly [15, 17, 23, 24, 31]. Naturally, mitochondrial α-GPD is one of the good biochemical indexes to show the thyroid hormone response [9]. There are some evidence of the thyroid hormone-induced *de novo* synthesis of this enzyme protein [9]. A relationship between the saturation of the putative thyroid hormone receptors in the rat liver nuclei and the degree of simultaneous induction of α-GPD has also been observed [23, 24]. It has, therefore, been suggested that this mitochondrial effect is mediated through the nuclear activation by thyroid hormone since this enzyme is synthesized in the cytosol and then incorporated into mitochondria [9]. The synthesis of α-GPD has also been reported to be inhibited by α-amanitin [6, 23].

In fish, thyroid hormones affect protein and nucleic acid synthesis, nitrogen excretion, growth, development, etc. [14, 27, 28, 19, 7, 8]. Recently it has been reported from our laboratory that thyroid hormones have significant influences also on protein, nucleic acid, lipid and carbohydrate metabolism and on the activity of α-GPD in fish brain [11, 12, 13, 20]. The presence of putative nuclear thyroid hormone binding sites in the liver of the rainbow trout was demonstrated by Van der Kraak and Eales [32]. Darling et al. [3] have compared the binding characteristics of fish liver nuclei with those of rat liver nuclei. Still, we found no report on the effect of thyroid hormone on α-GPD in the liver of tropical fresh water fish, and this has prompted us to study the effect of triiodothyronine (T_3) on the activity of that enzyme in the liver of euthyroid and hypothyroid Singi fish. Cycloheximide was used to show the specificity of induced enzyme activity in fish liver.

Materials and methods

Animals and Injections

Singi fish (*Heteropneustes fossilis* Bloch) of 6—9 g body weight were collected from the local supplier and acclimated to laboratory conditions at 25 ± 1 °C for about 2 weeks. The fishes were fed with *Tubifex tubifex ad libitum*. The experimental fish were divided at random in different groups and kept in tap water (1 litre per fish) in polythene trays. The water was changed every second day. L-T_3 (Sigma Chemical Company, USA) was dissolved in a minimum volume of 0.1 M NaOH and the required volume was made up by 0.65% saline. Various doses of T_3 (0.1, 0.25, 0.5, 5, 20 and 50 μg/gram body weight) were injected intraperitoneally in the fish, the injected volume did not exceed 40 μl. Control fish were injected with an equivalent amount of 0.65% saline, made alkaline with 0.1 M NaOH. Some of the T_3-injected (0.5 μg/g) fish were kept simultaneously in a cycloheximide (Sigma Chemical Company, USA) — containing medium (0.5 mg/litre). The fishes were kept at 25° ± 1 °C and starved during the experimental period. Some fish were immersed in thiourea-containing medium (1 mg/ml) for 30 days to make them hypothyroid. They were fed during this period.

Separation of the mitochondrial fraction

The animals were sacrificed on the third day of the experiment or daily up to the seventh day as required. The liver was quickly dissected out, weighed and homogenized in cold 0.25 M sucrose with a glass-teflon homogenizer. The nuclei with cell debris were separated by centrifug-

ing the homogenate in cold at 700 g for 10 minutes. The supernatant was then centrifuged in cold at 16 000 g for 15 minutes to collect the mitochondria. The mitochondrial pellet was washed in cold 0.25 M sucrose, then suspended in cold 0.25 M sucrose for assaying α-GPD activity.

Assay Method

The mitochondrial α-GPD activity was measured by the method of Lee and Lardy [16] with the synthetic electron acceptors phenazonium methosulphate (PMS) and 2-p-iodophenyl-3-phenyltetrazolium-nitrophenyl-5-chloride (INT), obtained from Sisco Research Laboratories Private Ltd., India. In the reaction mixture used for measuring the hepatic mitochondrial α-GPD activity, formazan production followed zero order kinetics and linearity was observed with a renge of 50—135 μg of hepatic mitochondrial protein. Generally 90—120 μg of mitochondrial protein was used in each assay and terminated after 15 minutes. The livers from 6 or 7 animals were pooled in each set and the mean specific activity of α-GPD from seven such sets was expressed as Mean ± SE of change in optical density at 500 nm per min per mg of protein (mitochondrial) and DNA.

Protein content was measured by the method of Lowry et al. [18]. The DNA content of the liver homogenate was estimated by the method of Burton [1] as modified by Croft and Lubran [2]. All data were statistically analysed by *t*-tests. Values of $P \leq 0.05$ were taken as significant.

Results

Mitochondrial protein and total DNA content of fish liver (Tables I and II)

A single injection of various doses of T_3 (0.25, 0.5, 5, 20, and 50 μg/g) caused a significant increase in the mitochondrial protein content on the 3rd day in comparison to the control. but T_3 dose of 0.1 μg/g did not alter the mitochondrial protein content. Almost the same level of increase in protein content was observed with the T_3 doses of 0.5, 5, 20 and 50 μg/g, while 0.25 μg/g had a weaker effect. In hypothyroid (thiourea treated) fish the mitochondrial protein content remained unchanged. A single injection of T_3 (0.5 μg/g) to hypothyroid fish also increased the mitochondrial protein content above the euthyroid control level. The DNA content of the total liver homogenate remained unaffected on the 3rd day in the euthyroid fish injected with T_3 of various doses (0.1—50 μg/g) and in thiourea treated hypothyroid fish injected with 0.5 μg/g of T_3.

Hepatic mitochondrial α-GPD activity (Tables I and II, Fig. 1)

Since the hepatic mitochondrial protein content was increased after T_3 injection, α-GPD activity was measured as ΔOD/min/mg of protein and also DNA. Compared to the control, T_3 in doses of 0.25, 0.5, 5, 20 and 50 μg/g increased the α-GPD activity as expressed per mg of mitochondrial protein. T_3 in a dose of 0.1 μg/g was ineffective in this respect. α-GPD activity was higher with higher doses of T_3 (5, 20 and 50 μg/g), the activity level was about the same (no statistical difference) with these doses. There was no significant

Table I

Effect of a single injection of T_3 in different doses (0.1, 0.25, 0.5, 5, 20 and 50 μg/g) on mitochondrial α-GPD activity, mitochondrial protein and total DNA content of liver homogenate of singi fish (Heteropneustes fossilis Bloch)

Dose of T_3 (μg/g)	Mitochondrial α-GPD activity, ΔOD/min per mg protein	Mitochondrial protein mg/100 mg tissue	Mitochondrial α-GPD activity ΔOD/min per mg DNA	Total DNA μg/100 mg tissue
	Mean ± SE			
Control (A)	0.079 ± 0.004	1.08 ± 0.03	0.36 ± 0.01	237.8 ± 10.9
0.1 (B)	0.089 ± 0.004	1.04 ± 0.05	0.38 ± 0.01	236.0 ± 12.8
0.25 (C)	0.098 ± 0.006^{a}	1.20 ± 0.03^{aA}	0.44 ± 0.01^{bB}	232.7 ± 9.4
0.5 (D)	0.106 ± 0.005^{bA}	1.30 ± 0.02$^{bB\alpha}$	0.51 ± 0.03$^{bB\alpha}$	234.0 ± 17.8
5 (E)	0.126 ± 0.007$^{bB\beta*}$	1.32 ± 0.04$^{bB\alpha}$	0.64 ± 0.03$^{bB\beta**}$	244.7 ± 29.2
20 (F)	0.135 ± 0.008$^{bB\beta**}$	1.29 ± 0.02$^{bB\alpha}$	0.69 ± 0.04$^{bB\beta**}$	242.2 ± 10.9
50 (G)	0.140 ± 0.008$^{bB\beta**}$	1.32 ± 0.03$^{bB\alpha}$	0.75 ± 0.04$^{bB\beta**\S}$	252.7 ± 12.8

The fish were kept at 25 ± 1 °C in fasting condition and sacrificed on third day after injection. The livers from 6 or 7 animals were pooled in each set and the data of seven such pooled sets were expressed as Mean ± SE.

SE = Standard error. *t*-test probability differences: 'p' value: a, A, α, *, § = $P < 0.05$, b, B, β, ** = $P < 0.01$, where lower case letters denote comparison between the control and different treated groups, capital letters between 0.1 and 0.25, 0.5, 5, 20 or 50 μg/g; Greek letters between 0.25 and 0.5, 5, 20 or 50 μg/g; star between 0.5 and 5, 20 or 50 μg/g, and § between 5 and 50 μg/g.

difference in the increase in enzyme activity caused by 0.25 and 0.5 μg of T_3/g (Table 1), and all doses of T_3 except 0.1 μg/g enhanced α-GPD activity in comparison to the control. Generally, the higher the dose of T_3 the more was the enzyme activity, except between the doses of 5 and 20 μg/g and 20 and 50 μg/g. A significant difference was observed between the doses of 5 and 50 μg/g.

In another set of experiment, percentage increase in α-GPD activity (ΔOD/min/mg of mitochondrial protein) over the control level was observed on different days with a low and high dose of T_3, viz., 0.5 and 5 μg/g (Fig. 1). The enzyme activity of both the treated groups reached the maximum on the 3rd day and then gradually declined to almost the control value on the 7th day. Significant differences in the percentage increase in enzyme activity between the two injected groups (0.5 and 5 μg/g) were observed on days 2, 3, 4 and 5 ($P < 0.05$ or 0.01). In comparison to the control an increase in α-GPD activity after these two doses of T_3 was found from the lst to 6th day ($P < 0.05$ or 0.01), the average control value being 0.081 ± 0.004 ΔOD/min/mg protein.

In thiourea-treated hypothyroid fish the liver α-GPD activity decreased (Table II) significantly (about 30% below the euthyroid control value). A single injection of T_3 (0.5 μg/g) to these hypothyroid fishes increased α-GPD activity to about 46% above the control value on the 3rd day.

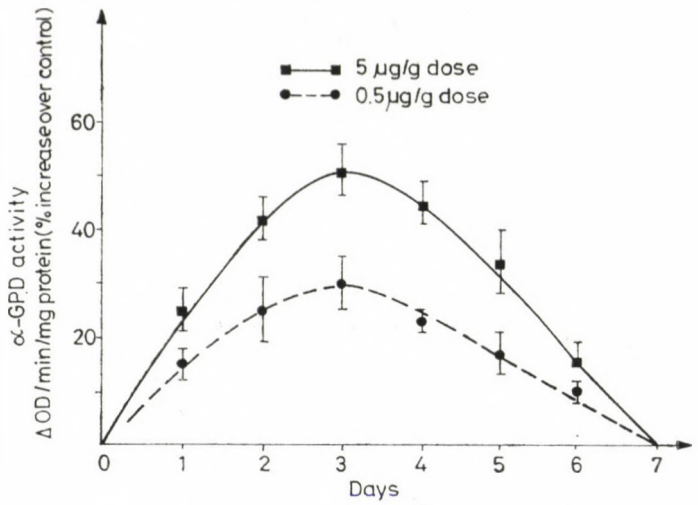

Fig. 1. Percentage increase in hepatic α-GPD activity of Singi fish over the control level at different days after a single injection at day 0 of a lower and a high dose of T_3 (0.5 and 5 µg/g). Each point represents the mean percentage increase in enzyme activity from 7 pooled sets, each set containing livers from 6 or 7 animals. The vertical bars in the figure indicate the standard error of the mean. The control value of α-GPD activity was 0.081 ± 0.004 ΔOD/min/mg protein. A significant increase in enzyme activity was found from 1st to 6th day with these two doses of T_3 in comparison to the control ($P < 0.05$ or 0.01). The higher dose of T_3 (5 µg/g) caused a higher increase in enzyme activity than did the low dose (0.5 µg/g) from 2nd to 5th day ($P < 0.05$ or 0.01)

Table II

Effects of thiourea treatment (hypothyroid) by immersion (1 mg/ml) for30 days and a single injection of T_3 (0.5 µg/g) to hypothyroid animals, on α-GPD activity, mitochondrial protein content and total DNA content of liver of Singi fish

Treatments	Mitochondrial α-GPD activity, ΔOD/min per mg protein	Mitochondrial protein, mg/100 mg tissue	Mitochondrial α-GPD activity, ΔOD/min per mg DNA	Total DNA, µg/100 mg tissue
	Mean ± SE			
Control	0.081 ±0.004	1.08 ±0.03	0.41 ±0.02	235.96 ±11.90
Hypothyroid	0.058^{a} ±0.002	1.10^{d} ±0.04	0.28^{g} ±0.05	238.0^{d} ±7.5
Hypothyroid + T_3	0.119^{bc} ±0.009	1.30^{ef} ±0.05	0.65^{hi} ±0.07	236.4^{j} ±5.4

SE = Standard error. *t*-test probability differences: 'p' value: a, c, e, f, g, i = $P < 0.001$; b, h = $P < 0.01$; d, j = Not significant.

a or d or g — denotes comparison between control and hypothyroid group,
b or e or h or j — denotes comparison between control and hypothyroid + T_3 group,
c or f or i — denotes comparison between hypothyroid and hypothyroid + T_3 group.

Dose-response relationship at 72 h (Fig. 2)

The increase in T_3-induced hepatic α-GPD activity was also determined as the enzyme activity per min per mg of protein and DNA above the control level and the mean percentage of the maximum response with each dose was calculated. The dose-response relationship was expressed by the application of equation $E = \frac{D}{D + D1/2}$, where E is the induced enzyme activity, D is the

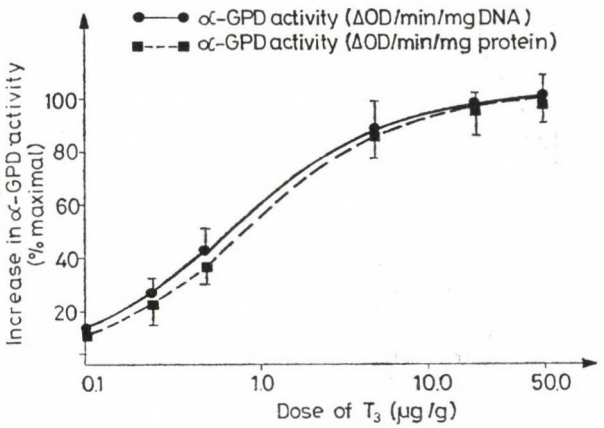

Fig. 2. Mean percentage increase in hepatic α-GPD activity of Singi fish over the control after a single injection of various doses of T_3 (0.1, 0.25, 0.5, 5, 20 and 50 μg/g). The ordinate represents the mean percentage increase in mitochondrial α-GPD activity above the control value, expressed as percent of maximum response. The abscissa represents the injected different doses of T_3 on a logarithmic scale. The livers from 6 or 7 animals were pooled in each set and the data of 7 such pooled sets were expressed as Mean ± SE. The equation used for the expression of the dose response relationship was $E = \frac{D}{D + D1/2,}$, where E is the induced enzyme activity and D the dose of T_3 (μg/g). The D1/2 for hepatic α-GPD was 0.8 μg of T_3 per g body weight, by calculating the enzyme activity per mg of protein per min and 0.66 μg of T_3 per g by calculatin, the enzyme activity per minute per mg of DNA on the 3rd day

dose of T_3 (μg/g) injected and D1/2 is the dose required to obtain half of the maximum response. In liver, the D1/2 for α-GPD was found to be 0.8 μg of T_3/g of body weight when calculated per mg of protein per min, and 0.66 μg of T_3/g of body weight when calculated per mg of DNA per min.

Effect of cycloheximide on tne T_3-induced changes in the liver (Fig. 3)

The T_3 (0.5 μg/g)-induced increase in α-GPD activity and mitochondrial and total protein content of the liver was inhibited by cycloheximide (0.5 mg/litre) on the 3rd day. In hormone injected fish, cycloheximide treatment maintained the level of these cellular constituents at almost the same height level as in the control.

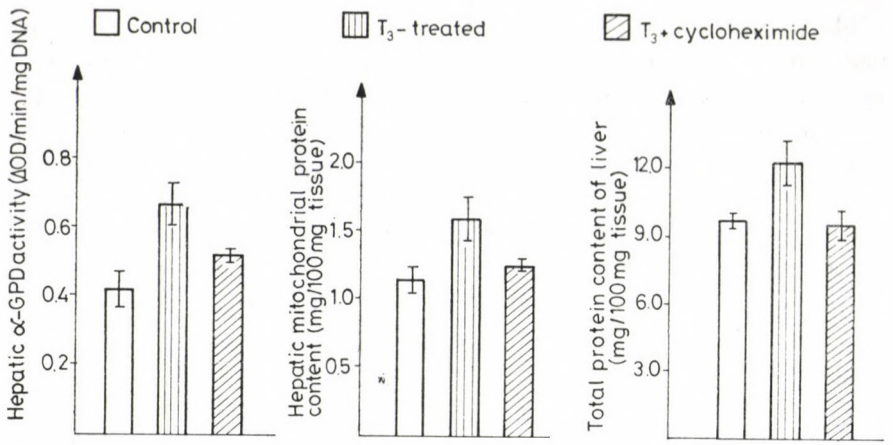

Fig. 3. **Effect of cycloheximide on T_3-induced changes in hepatic mitochondrial α-GPD activity, mitochondrial and total protein content of Singi fish. A single injection of T_3 (0.5 μg/g) was given at day 0, enzyme activity and protein contents were measured on day 3. Vertical bars indicate the standard error of mean value for 7 pooled sets, each set containing 6 or 7 animals**

Discussion

In continuation of our attempts to show the thyroid hormone actions in freshwater fish, the present experiments have been undertaken by using hepatic mitochondrial α-GPD activity as a biochemical index, to demonstrate the thyroid hormone responsiveness of Singi fish. It is evident from the present data that T_3 is effective in inducing the hepatic α-GPD enzyme depending upon the dose of the hormone, but mostly in a non-linear fashion. The result were consistent with those of the previous studies on rat [23, 24, 9]. The increase in hepatic mitochondrial α-GPD activity by T_3 in injection may be due to induction of *de novo* synthesis of this enzyme protein as observed in various other organs of the rat [30, 23, 24, 9] and also in fish brain [12, 20]. This assumption is supported by our present observations on the inhibition of T_3-induced increase in hepatic α-GPD activity as well as mitochondrial protein content by cycloheximide. Moreover, total protein content of liver of Singi fish increased by a single injection of T_3 (0.5 μg/g) which was counteracted by cycloheximide. The inhibitidon of T_3-induced α-GPD activity in fish liver by cycloheximide reflects a possible extramitochondrial synthesis of α-GPD enzyme protein, probably through nuclear action under the influence of thyroid hormone. Apart from the specific α-GPD protein, other proteins might also be synthesized by T_3 in the cytoplasm. A fall in α-GPD activity in thiourea treated hypothyroid fish may be due to the lack of endogenous level of T_3. The subsequent recovery of the enzyme activity to more than the euthyroid control level by a single injection of T_3 to the hypothyroid fish supports the thyroid hormone

inducible nature of α-GPD in fish liver. Increase in mitochondrial protein content and a concomitant rise in α-GPD activity in the hypothyroid plus T_3-injected fish indicats that the rise in α-GPD activity may be due to T_3-induced synthesis of α-GPD enzyme protein.

A dose-response relationship at 72 h was evident from the present observations showeing the maximum increase in α-GPD activity with higher doses of T_3 (20 and 50 μg/g) which might have been the result of a full saturation of the hepatic nuclear T_3-ginding sites. The T_3 dose required to obtain half the maximum response of the hepatic enzyme (DY2) was found to be 0.8 μg/g, and 0.5 μg/g in Singi fish brain, when the activity was expressed per mg of protein per min, and 0.66 μg of T_3/g per mg of DNA per min against 0.35 μg/g in Singi fish brain [12]. Since there was a change in hepatic mitochondrial protein content after T_3 treatment, expression of the results of α-GPD activity appeared to be more convincing with unit weight of DNA. This was also evident in the case of fish brain [12]. The higher dose of T_3 required to obtain half the maximum response of liver α-GPD in comparison to brain of Singi fish of almost the same body weight reflects the presence of a greater number of nuclear T_3-binding sites in the liver than in the brain. The number of nuclear T_3-binding sites in Singi fish liver and brain has, however, not been shown. The maximum increased activity of hepatic α-GPD on the 3rd day with 0.5 of 5 μg or T_3/g indicates the time required for the hormone for total expression of physiologic response in Singi fish liver besides other hormonal actions at the molecular level. A gradual decline in the response after the 5th day and the subsequent reduction to the control level on the 7th day might have been due to a gradual fall in the T_3 effect resulting from the fractional metabolism to hormonally inactive compounds.

The T_3-induced changes in the liver of Singi fish in the present experiments may be aphysiological resulting probably from the high plasma T_3 level after injection. During the experimental period no toxic side effect of T_3 was observed and there was no change in body weight of Singi fish kept at 25 °C after a single injection of different doses of T_3 (0.1—50 μg/g). It has been shown that the fish kept at 25 °C require higher doses of thyroid hormone for the biphasic action with respect to nitrogen excretion and protein and RNA metabolism [27, 19]. Moreover, thyroxine doses of 0.01 and 0.1 μg/g injected for 5 consecutive days failed to cause any change in the liver of Lata fish, *Ophicephalus punctatus*, at 25° [25]. Also, a single injection of 0.1 μg/g T_3 failed to affect the mitochondrial protein content and α-GPD activity. Naturally, the effects of thyroid hormone in fish cannot be compared to those in mammals whose body temperature is much higher. Moreover, T_3 uptake by some fish liver nuclei is slower than that by rat liver [21, 4, 32]. Although the endogenous T_3 level of Singi fish is not known, it has been reported that T_4 concentrations in some other teleosts were in the range of 8.4—17.0 ng/ml or more, depending

on the season of the year [5] or a T_3/T_4 level of about 500 ng dl^{-1} or more [8]. It has also been reported that due to binding to plasma proteins about 5% of the total endogenous T_4 or T_3 in fish is free [10]. Whatever the transient rise of thyroid hormone in the body fluids after a single injection, there is no reason to believe that all free hormone will reach the liver. There should be a preferential distribution of free thyroid hormones in different organs of the fish, depending on the affinity and number of receptors. In our experiments a dose-response relationship existed: an increase in the dose of T_3 was associated with a significant rise in α-GPD activity, and high doses of T_3 (20 and 50 μg/g) caused the maximum response. It may, therefore, be assumed that the present results with respect to increase in α-GPD activity, mitochondrial and total protein content of Singi fish liver are physiological effects of thyroid hormone, and thus the Singi fish liver may be considered to represent an important thyroid hormone target organ.

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TISSUE OXYGENATION IN SEPTIC RESPIRATORY FAILURE

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The relationships between tissue oxygenation and the different haemodynamic and respiratory parameters were studied in 20 patients with ARDS of septic origin. Good regressions were found between O_2 delivery and cardiac index ($r = 0.8507$), O_2 delivery and systemic vascular resistance ($r = -0.7051$), O_2 extraction ratio and mixed venous O_2 saturation ($r = 0.8978$), O_2 consumption and cardiac index ($r = 0.6593$), O_2 consumption and systemic vascular resistance ($r = -0.6548$), and O_2 consumption and mixed venous O_2 saturation ($r = -0.7068$). The correlation among the parameters of tissue oxygenation was more expressed between O_2 extraction ratio and O_2 consumption ($r = 0.7285$), than between O_2 delivery and O_2 consumption ($r = 0.6095$). A better result was achieved by multiple regression analysis, where the multiple r was 0.9748 between O_2 consumption and O_2 delivery + O_2 extraction ratio, whereas the other variables did not increase the multiple r significantly. These regressions also proved the relationship following from the Fick equation, that is O_2 consumption is the result of O_2 delivery multiplied with the O_2 extraction ratio.

Keywords: tissue oxygenation, oxygen consumption, oxygen delivery, oxygen extraction, Fick equation, haemodynamics, ARDS, sepsis

Septic diseases are a major cause of death in critically ill patients. Despite the careful use of powerful antibiotics, adequate haemodynamic monitoring, respiratory support and surgical interventions, a large number of patients neverthless die a few days after the full manifestation of sepsis, with acute respiratory failure, with ARDS.

In the early phase of sepsis there is no great problem in the haemodynamic and respiratory state, but a severe circulatory failure and an uncorrectable tissue hypoxia develop at the final stage of the illness [4, 12, 13]. The limitation of aerobic metabolisms has an important pathogenetic role in morbidity and mortality [1, 9—11]. For this reason the interest of clinicians has turned toward the changes of tissue oxygenation, since recognition of the oxydative processes in the cells could help in clarifying the exact pathomechanism.

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Several authors [3, 6, 8] have stated that O_2 consumption ($\dot{V}O_2$) depends greatly on O_2 delivery ($\dot{D}O_2$). It has also become clear that sufficient $\dot{D}O_2$ alone is not a sure sign of adequate tissue oxygenation [7, 9, 10] because it is uncertain whether the cells can utilize the delivered O_2. Our purpose was to examine the relationships between the various measured and derived haemodynamic and blood gas parameters as well as the parameters of tissue oxygenation to determine the factors affecting the $\dot{V}O_2$ of the body.

Materials and methods

20 patients (12 men and 8 women, aged 14—71, mean 36 years) with ARDS of septic origin were studied. Sepsis was diagnosed on basis of a positive blood culture or if the same organism was isolated from different body fluids. During the examinations each patient was placed on a volume-cycled ventilator using 5—15 cm H_2O of positive end expiratory pressure and various levels of inspired O_2 fraction. The different pressures and cardiac output were measured with a Swan—Ganz flow directed thermodilution catheter (Edwards Lab.) placed into the pulmonary artery. Blood gas parameters were determined from arterial and mixed venous blood. The haemodynamic and respiratory measurements were taken strictly one after the other. The measurements taken were independent of the different therapies or the course of illness. A patient had a maximum of 4 measurement sequences.

In the analysis we have been using the following parameters: cardiac output (CO), cardiac index (CI), stroke volume, pulmonary arterial and capillary wedge pressure, left and right ventricular stroke work index, pulmonary and systemic vascular resistance (PVR, SVR), arterial and mixed venous pH, pO_2, O_2 saturation (SaO_2, $S\bar{v}O_2$), O_2 content (CaO_2, $C\bar{v}O_2$), alveolo-arterial O_2 difference, shunt fraction (Qs/Qt), haemoglobin concentration, $\dot{D}O_2$, O_2 extraction ratio (O_2ER) and $\dot{V}O_2$. Tissue oxygenation was calculated from the formulae,

$$\dot{D}O_2 = \frac{CO \times CaO_2 \times 10}{\text{body weight}} \quad \text{(ml/min/kg)}$$

$$O_2ER = \frac{CaO_2 - C\bar{v}O_2}{CaO_2}$$

$$\dot{V}O_2 = \frac{CO \times (CaO_2 - C\bar{v}O_2) \times 10}{\text{body weight}} \quad \text{(ml/min/kg).}$$

Further parameters were calculated by using conventional equations.

The associations were analysed with the SPSS statistical software package.

Results

Table I shows the statistical characteristics of those parameters which indicated significant and also clinically important relationships.

A good correlation was found between $\dot{D}O_2$ and CI and a similar one between $\dot{V}O_2$ and CI (Fig. 1). The relationship between SVR and $\dot{D}O_2$ as well as $\dot{V}O_2$ (Fig. 2) was also similar. Using partial correlation test between $\dot{D}O_2$—SVR — CI ($r = -0.6285$) and $\dot{V}O_2$ — SVR — CI ($r = -0.5616$) it was found that the correlation coefficient became significantly lower ($r = 0.0718$ and $r = 0.0605$) when the effect of CI had been eliminated. These relationships

Table I

Mean values ± S.D. of variables having a significant role during examination

CI(l/m²)	3.2 ± 0.9	n = 65
SVR (dyn · sec · cm^{-5})	1419 ± 445	n = 65
$S\bar{v}O_2$	0.809 ± 0.079	n = 50
Qs/Qt (per cent)	28.8 ± 12.2	n = 50
$\dot{D}O_2$ (ml/min/kg)	12.5 ± 4.5	n = 65
O_2ER	0.206 ± 0.069	n = 50
$\dot{V}O_2$ (ml/min/kg)	2.5 ± 1.0	n = 50

support that the real connections are between $\dot{D}O_2$ — CI and $\dot{V}O_2$ — CI, and not between $\dot{D}O_2$ — SVR and $\dot{V}O_2$ — SVR. The $S\bar{v}O_2$ showed a close negative association both with O_2ER and $\dot{V}O_2$ (Fig. 3). The $\dot{V}O_2$ of the whole body correlated significantly with $\dot{D}O_2$, but the connection was more expressed between $\dot{V}O_2$ and O_2ER (Fig. 4).

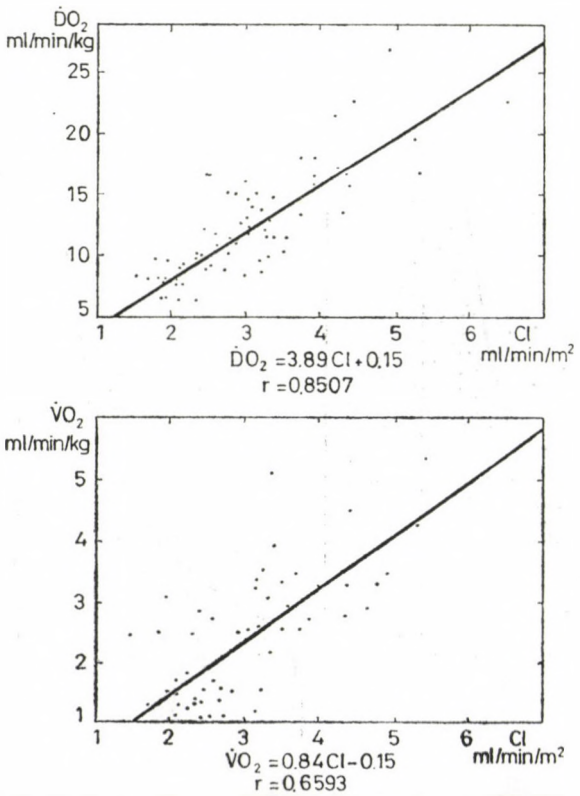

Fig. 1. Relationship between CI—$\dot{D}O_2$ (upper part) and CI—$\dot{V}O_2$ (lower part)

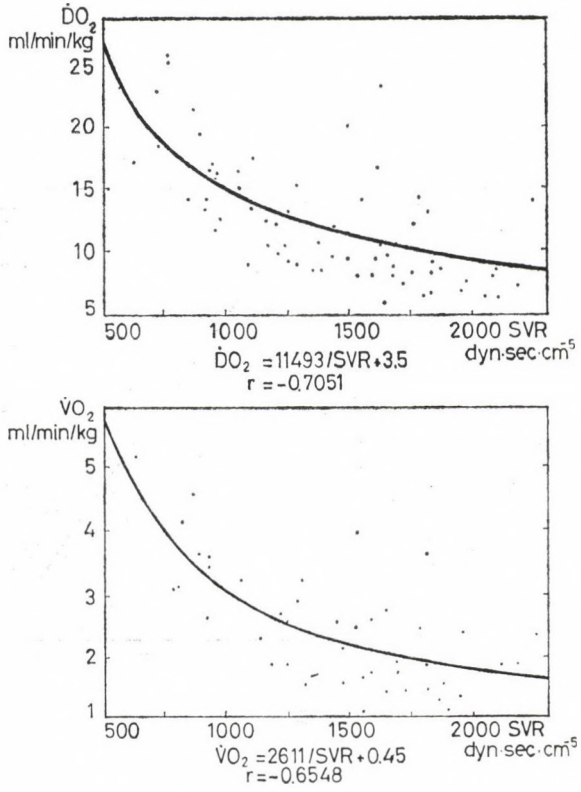

Fig. 2. Relationship between SVR—$\dot{D}O_2$ (upper part) and SVR—$\dot{V}O_2$ (lower part)

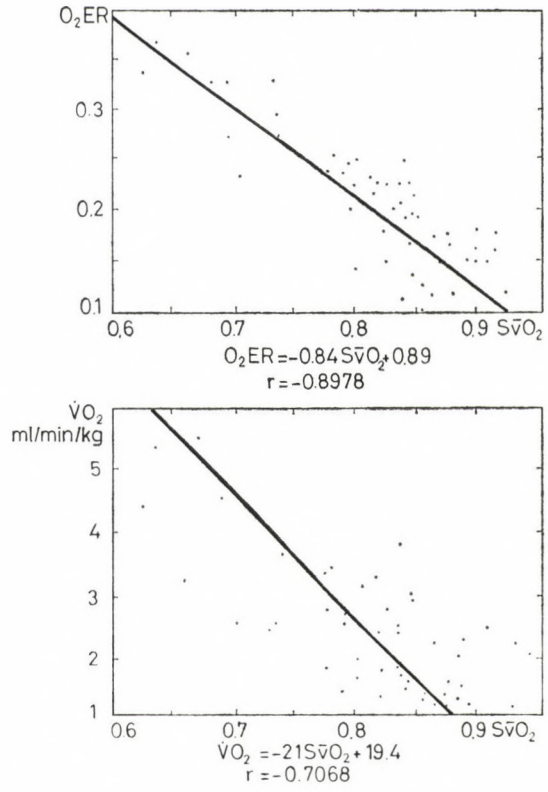

Fig. 3. Relationship between $S\bar{v}O_2$—O_2ER (upper part) and $S\bar{v}O_2$—$\dot{V}O_2$ (lower part)

Table II shows the result of multiple regression analysis performed to determine the factors affecting $\dot{V}O_2$. The first two variables, O_2ER and $\dot{D}O_2$ alone determined $\dot{V}O_2$ precisely (the multiple r was 0.9748), whereas the other variables did not increase the multiple r significantly.

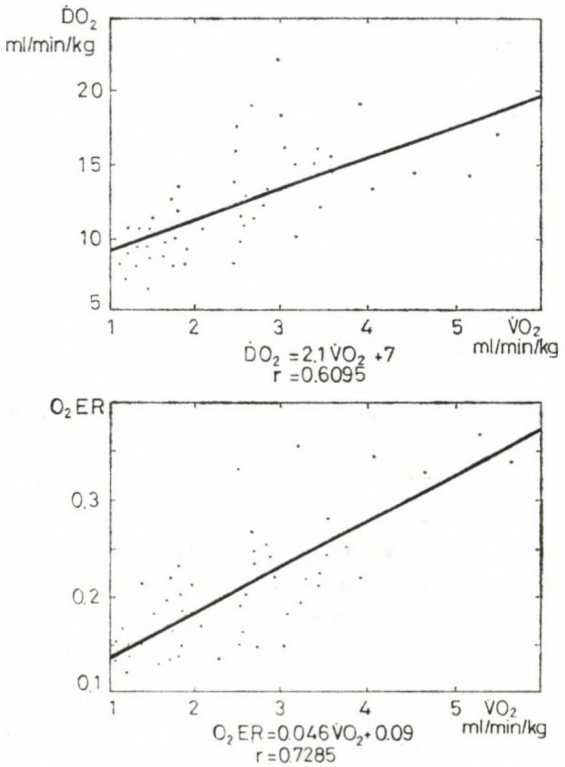

Fig. 4. Relationship between $\dot{V}O_2$—$\dot{D}O_2$ (upper part) and $\dot{V}O_2$—O_2ER (lower part)

Table II

Multiple regression analysis of the factors influencing $\dot{V}O_2$

Dependent variable:	$\dot{V}O_2$
Variables:	Multiple r:
O_2ER	0.7285
$\dot{D}O_2$	0.9748
CI	0.9781
SVR	0.9834
Qs/Qt	0.9855
$S\bar{v}O_2$	0.9858

Discussion

Examining the influencing parameters of tissue oxygenation using a multiple correlation test we found a close relationship between $\dot{V}O_2$ — $\dot{D}O_2$ — O_2ER. This direct connection can be expressed on basis of the Fick equation as

$$\dot{V}O_2 = CO \times (CaO_2 - C\bar{v}O_2) \quad \text{(equation 1)}$$

Substituting $CO \times CaO_2 = \dot{D}O_2$ and $\frac{CaO_2 - C\bar{v}O_2}{CaO_2} = O_2ER$ into equation 1, after simplification we receive the following simple relationship:

$$\dot{V}O_2 = \dot{D}O_2 \times O_2ER \quad \text{(equation 2)}$$

In other words, the oxygen consumption at a given time is exactly determined by multiplying the value of oxygen delivery with the oxygen extraction ratio.

This aspect of the Fick equation has clinical importance. It is obvious from the formula that a reliable picture of $\dot{V}O_2$ is received when $\dot{D}O_2$ and O_2ER are examined together. To conclude from $\dot{D}O_2$ alone the changes of $\dot{V}O_2$, would cause severe mistakes.

Among the tissue oxygenation parameters, $\dot{D}O_2$ is a dynamically changing parameter since cardiac output, one of its main factors, is regulated by numerous physiological mechanisms according to the demands of the body. This is the only parameter of tissue oxygenation that can be controlled well in clinical practice at present.

Nevertheless, in many cases it was observed that an increase of $\dot{D}O_2$ would not increase $\dot{V}O_2$ in all cases, because due to certain mechanisms the arterial O_2 content reappeared on the venous side without being utilised [1, 5, 7, 9, 14]. This process is well reflected in O_2ER. The increase in O_2ER can be considered a possibility for compensation. An increased O_2ER with a given $\dot{V}O_2$ always represents a decreased $\dot{D}O_2$. This is the case when by an increase of $\dot{D}O_2$ we can significantly increase $\dot{V}O_2$ in clinical practice. A decreased O_2ER, which is characteristic for septic respiratory failure [2, 7, 13], means an oversupply in oxygen, when there is no use of increasing $\dot{D}O_2$, as $\dot{V}O_2$ will not change substantially. The real therapeutic method would be in this case to restore the oxydative metabolism of the tissues. But for the time being, our present therapeutical possibilities are limited.

Acknowledgements

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INFLUENCE OF HORMONAL IMPRINTING ON HORMONE LEVEL. PERMANENT RECEPTOR DAMAGING EFFECT OF PITUITARY HORMONES ADMINISTERED TO NEWLY-HATCHED COCKERELS

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The overlapping effect of TSH and FSH on the gonad and on the thyroid gland can be demonstrated in cockerels even at the age of five weeks. These hormones influence the secretion of testosterone in a similar way and to a similar extent, while on the thyroxine level the influence of the specific hormone is more pronounced. Neonatal FSH and TSH treatment considerably decreased the basal testosterone level measured at the age of five weeks. Neonatal FSH treatment increased the basal T_4 level while TSH treatment decreased it. The effect of TSH treatment administered at the age of five weeks in increasing the testosterone level was weakened after neonatal pretreatment with any iodine hormone. The effect of TSH treatment could only be inhibited by neonatal FSH pretreatment. Neonatal pretreatment with any of the trophormones caused a diminution of the T_4 level augmenting of FSH and TSH administered at the age of five weeks.

Keywords: hormonal imprinting, pituitary hormones, receptor damage, neonatal influences, TSH, FSH

Membrane receptors capable of binding hormones in a specific way develop during ontogenesis of animals. The specificity — selectivity — of hormone receptors which is characteristic of adulthood, develops during the maturation process in the perinatal period [2]. The maturing receptor is characterized by a selectivity (specificity) lower than that in adulthood and by a certain plasticity. Due to their low specificity, receptors during the maturation period are able to bind not only the hormone which will be later the adequate one but also other hormones similar in structure and hormone analogues as well [3]. Due to the increased plasticity this period is the critical phase of receptor maturation [the phase of hormonal imprinting, 4, 5, 6], since various effects of the environment on the receptor such as non-physiological hormone

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concentrations, binding of non-adequate hormones or hormone analogues, alter maturation of the receptor. Consequently, in adulthood sensitivity to the specific hormone of the receptor and that of the cell itself will be different, increased or decreased, from the physiological one.

Numerous experiments have been performed with two glycoprotein-type hormones similar in structure [12, 14] during the maturation period of FSH and TSH receptors. The structure of the testicle and thyroid gland was investigated by quantitative histological analysis. These investigations, together with studies on hormone secretion have revealed that, due to the lower specificity — plasticity — of the receptors, the above-mentioned two hormones exert an overlapping effect on each other's target cells in the perinatal period. The phenomenon of hormonal imprinting was also demonstrated by morphological investigations on cockerels: when FSH or TSH had been administered immediately after hatching and the determinations carried out at the age of five weeks, the sensitivity to FSH and TSH of the testicle and thyroid gland proved to be altered [7, 8, 10, 11, 13].

It seemed, however, necessary to prove the existence of hormonal imprinting also by direct functional evidence in an experimental arrangement similar to that used in our previous investigations. Thus, in the present experiments, newly-hatched cockerels were treated with FSH or TSH and it was studied how the serum thyroxine and testosterone levels would change after FSH and TSH treatment applied at the age of five weeks.

Materials and methods

Newly-hatched Hubbard broyler cockerels were treated subcutaneously with 200 μg FSH (National Biochemical Co., Cleveland, USA) or 200 μg TSH (Ambinon, Organon, Oss, The Netherlands) or 0.1 ml physiological saline solution on the day following hatching. At the age of 33 days each group was divided into three subgroups and the animals in the subgroups were given either physiological saline solution or 200 μg FSH or 200 μg TSH subcutaneously. The animals were decapitated 90 minutes after the hormone administration. Blood samples were collected and the serum thyroxine (T_4) and testosterone levels determined. For this purpose we used the T_4 RIA-kit (Isotope Institute of the Hungarian Academy of Sciences, Budapest, Hungary) as well as the RIA method according to Bodrogi and Fehér [1].

Ten animals were investigated in each group.

Results and discussion

Results are shown in Tables I and II.

Hormonal overlapping at the age of five weeks

The animals having received physiological saline at hatching and FSH or TSH at the age of five weeks were compared with the absolute control ones. This experimental arrangement allowed to investigate whether FSH or TSH

Table I

Thyroxine (T_4) levels (nmol/l) of treated and untreated cockerels at hatching and at five weeks of age (Mean ± S.D.)

	At hatching		
At age of 5 weeks	Physiol. saline	FSH	TSH
Physiological saline	4.6 ±2.179	6.17 ±1.499	2.59 ±0.787
FSH	5.74 ±1.397 ↑	3.91 ±1.050 ↑	2.58 ±0.993 ←
TSH	11.31 ±5.610 ↑	8.34 ±0.982 ↑	7.39 ±0.461 ←

Ten animals were evaluated in each group.
Significance: $p < 0.05$ between the group marked by arrow and the group pointed at by arrow.

Table II

Testosterone level (nmol/l) of treated or untreated cockerels at hatching and at the age of five weeks (Mean ± S.D.)

	At hatching		
At age of 5 weeks	Physiol. saline	FSH	TSH
Physiological saline	0.91 ±0.473	0.59 ±0.445	0.37 ±0.102 ←
FSH	2.00 ±0.917 ↑	1.07 ±0.280 ↑ ←	1.93 ±0.90 ↑
TSH	1.90 ±0.438 ↑	1.47 ±0.624 ↑	1.22 ±0.372 ↑ ←

Ten animals were evaluated in each group. Significance: $p < 0.05$ between group marked by arrow and the group pointed at by arrow.

administration at the age of five weeks would result in an overlapping effect on the testicle and thyroid gland.

Even a single, acute FSH or TSH treatment administered at the age of five weeks increased significantly the serum thyroxine and testosterone levels. In the case of testosterone, practically no difference was observed as far as the extent of the effect of FSH and TSH was concerned. This finding clearly demonstrated that in species phylogenetically different from mammals the overlapping effect of FSH and TSH on the gonads may persist also in adulthood, as it has been demonstrated in our previous investigations on adult frogs [9].

FSH exerted a considerably weaker effect on the thyroid gland than did TSH, the specific hormone. This finding was in good accordance with our previous morphological investigations [10, 13], where the overlapping effect of FSH and TSH was considerably weaker on the thyroid gland than on the testicle both in the neonatal period and at the age of five weeks.

Hormonal imprinting

From the experiments the conclusion might be drawn that neonatal treatment with either FSH or TSH significantly decreased the basal level of testosterone by the fifth week. This basal level will be altered upon the effect of trophormone treatment in adulthood. The level observed in animals treated neonatally with physiological saline will however only be reached after neonatal TSH treatment followed by FSH administration at the age of five weeks. Even after FSH treatment, the testosterone level of animals treated with FSH neonatally was only half the control value. Interestingly enough, TSH treatment administered at the age of five weeks exerted a more pronounced effect on the testosterone level of animals treated with FSH neonatally than on that of animals treated with TSH. In the control animals FSH and TSH had increased the testosterone level practically to the same extent. In contrast, the animals pretreated with TSH in the neonatal period, TSH administered at the age of five weeks failed to produce that serum level which had been observed in the control animals. Thus, the experiments demonstrated that neonatal trophormone treatment (with either FSH or TSH) exerted a more negative than positive effect on the response to trophormone administered in adulthood. On the other hand, morphological evaluation of our previous experiments [7, 8, 10, 13] revealed that the diameter of the gonads and that of the seminiferous cords had increased, i.e. a response was obtained which could be regarded as positive from the morphological point of view. Thus, the similarity of the morphological pictures does not necessarily mean a similarity in the functional response. Nevertheless, both the morphological picture and the functional response observed in the present investigations clearly demonstrated that one single neonatal hormone treatment would result in fundamental alterations of the whole system.

As far as T_4 is concerned, neonatal FSH treatment significantly increased while neonatal TSH treatment decreased the basal T_4 level. In our previous experiments on rats FSH treatment also decreased the basal T_4 level measured in adulthood; so, differences could be found between the response of the rat and that of the cockerel. These differences might probably be explained by an unlike degree of maturation.

Both neonatal FSH and TSH treatment decreased, though not to the same extent, the effect on the thyroxine level of trophormone (FSH or TSH)

treatment administered in adulthood, in accordance with the results of our previous investigations on cockerels and rats. The effect of TSH treatment to decrease the effectivity of trophormone treatment administered in adulthood was more pronounced than that of neonatal FSH treatment. In experiments on rats exactly the opposite tendency was seen.

Summing up, the conclusion might be drawn the the effect of hormonal imprinting in cockerels is well marked not only from the morphological but also from the functional point of view. Still, the morphological picture does not necessarily correspond to the functional response. In the case of both parameters, i.e. testosterone and T_4 level, it is of especial interest that the inhibitory, hormone-level decreasing, response to neonatal treatment with the adequate hormone, FSH in the case of testosterone and TSH in the case of T_4, proved to be more pronounced than that of a treatment with the related hormone. This observation calls attention to the phenomenon that though imprinting takes place in every species, its extent and direction may be different. So, a given species might react more intensely to its own hormone while the other one to an alien hormone.

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A SIMPLE MODEL DESCRIBING THE ELASTIC PROPERTIES OF HUMAN UMBILICAL ARTERIAL SMOOTH MUSCLE

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Elastic properties of cylindrical segments of 71 normal double and 4 single human umbilical arteries were studied. This specimen is rich in vascular smooth muscle in comparison with other great arteries. Outer diameter versus intraluminal pressure characteristic curves were taken with decreasing pressures *in vitro* in different contraction states. Tangential force and circumferential incremental elastic modulus were computed. A sum of 222 curves was analysed. The investigations showed that if we take tangential force and outer radius values measured at the same pressure levels but in different contraction states, then a similar proportional change in tangential force will induce a similar proportional passive change in the outer radius, to some extent independently of the degree of active tangential shortening of the segment. For example to induce a 10% passive decrease of the outer radius from values measured at 100 mm Hg intraluminal pressure, tangential force had to be decreased by 76.7 ± 9.9% in single relaxed arteries, and by 79.6 ± 0.8% in normal double relaxed segments. These values corresponded to intraluminal pressure levels of 26.4 ± 4.9 mm Hg and 23.1 ± 0.9 mm Hg, respectively. In 30% active spontaneous shortening to reach the same 10% passive decrease in outer radius from the value measured at 100 mm Hg, tangential force had to be decreased by 75.2 ± 1.9% which corresponded to 29.6 ± 20 mm Hg. The same values in 5-HT induced contraction, 30% active shortening were 76.7 ± 2.0% and 28.4 ± 2.6 mm Hg, respectively. In addition to the similarity of relative changes in tangential force, the pressure levels were to some extent also similar. These data suggest that elastic elements in the human umbilical arterial smooth muscle may be organized in such a way as to ensure similar prestretch of similar elastic elements at similar pressures independently of the degree of active shortening of the circumference.

Keywords: artery, smooth muscle, mechanics, elasticity

There is general agreement in the literature concerning the circumferential elastic properties of vessels from different species, with different calibres and from different vascular territories as follows:

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1. Stiffness increases with increasing intraluminal pressure.

2. Active contraction decreases stiffness if it is expressed as a function of intraluminal pressure, and increases it if it is expressed as an absolute value of the radius.

There seems to be no exception from the above two laws. The elastic moduli actually measured may, however, vary widely among vessels from different sources, also with the absolute value of intraluminal pressure applied, etc. [1, 2, 6, 7, 12, 14, 17, 19, 21, 32, 43]. Several attempts have been made to find connections between the collagen, elastic and smooth muscle content and arrangement, and the measured stiffness [1, 2, 6, 11, 13, 15, 17, 22, 24, 25, 37, 38, 42, 46, 47]. Several models, some of them constructed in our laboratory were intended to explain the effect of smooth muscle shortening on the elastic properties [2, 5, 6, 7, 8, 10, 13, 14, 16, 17, 19, 20, 21, 22, 23, 26, 31, 32, 33, 34, 41].

Most vessels are complex structures where experimental or theoretical separation of individual elastic elements can be made only with difficulty. To get more data on the elastic properties of the vascular smooth muscle component itself with as little influence from the part of collagen and elastin as possible, the human umbilical artery seemed to be the proper model [38].

The human umbilical artery has no adventitia, has no continuous elastic lamellae and it is characterized by a scarcity of thick collagen fibres [29, 36, 38,4 0, 44, 45]. Using this specimen we hoped to characterize the elastic properties of vascular smooth muscle with intact structure.

Materials and methods

71 arterial segments from human umbilical cords were prepared from healthy mature newborns. 4 segments with the morphological malformation called single umbilical artery were also added. The segments were between 10—25 mm in length and after cannulation at both ends they were axially extended by 10% of their original length. The measurements were made in normal Krebs-Ringer solution. The composition of the Krebs-Ringer solution was: Na^+: 144.9 mM, K^+: 4.5 mM, Ca^{2+}: 2.5 mM, Mg^{2+}: 1.2 mM, $H_2PO_4^-$: + HPO_4^{2-}: 1.2 mM, SO_4^{2-}: 3.6 mM, Cl^-: 125.9 mM, HCO_3^-: 22.5 mM, glucose: 5.56 mM. The bath was bubbled with 95% O_2 and 5% CO_2 that kept pH at 7.4. The temperature was maintained at 37 °C. The drugs used were 5-hydroxytriptamine (Reanal), EDTA (Reanal), EGTA (Reanal).

Intraluminal pressure was increased and decreased at constant rates from 0 or 4 mm Hg up to 100 or 150 mm Hg and down again. For this task a reservoir vessel and a continuous flow pump (Harvard Apparatus Model 907) were used. The rate of pressure change varied in these experiments between 0.33—7.7 mm Hg/sec but according to our experience it had no significant effect on the parameters discussed here. A Statham P23DC pressure head continuously measured the intraluminal pressure. The outer diameter of the segments was continuously measured with a strain gauge displacement measuring device. Intraluminal pressure-outer diameter plots were recorded on a Riken-Denshi X-Y plotter.

Diameters and intraluminal pressures were read from calibrated sheets. Cross sections of the segments were computed in such a way, that diameters of maximally contracted closed segments were measured. To compute the parameters discussed in this paper the following equations were used:

$$F_{tang} = r_i \cdot p$$

where F_{tang}: tangential force, r_i: inner radius. This latter was computed from the outer radius using cross section of the segments, and isovolumy of the wall material was supposed. p: actual value of intraluminal pressure.

$$E_{inc} = \frac{2r_i^2 r_0}{(r_0^2 - r^2)} \cdot \frac{\Delta p}{\Delta r_0}$$

where r_i is the inner radius, r_0 is the outer radius, Δp is the change in intraluminal pressure between two points where the elastic modulus is computed, and Δr_0 is the change in outer radius in answer to the pressure change.

More detailed description of the measuring methods see in [12, 32].

Results

The course of a characteristic experiment is seen in Fig. 1. At the beginning of measurements, the segments are in maximally contracted, closed state. This spontaneous contraction in normal KR solution bubbled with O_2 but without any constricting agent added, is a characteristic feature of the umbilical artery. In a large part of the experiments vasodilator agent $NaNO_2$ was added to help relaxation. In some experiment to induce relaxation, EDTA or EGTA was added in Ca^{2+} free media in concentrations of 1 or 5 mM. In the experiment shown in Fig. 1, 5 mM $NaNO_2$ was in the bath during the dilatation period. Intraluminal pressure was raised from 0 up to 150 mm Hg (20.0 kPa) and down again at rates of 4.8 mm Hg/sec (0.64 kPa/sec). Several cycles were applied until the relaxed state was reached. On Fig. 1 curve No. 1 marks the curve taken during the first dilatation cycle, curves No. 2 and 3 were

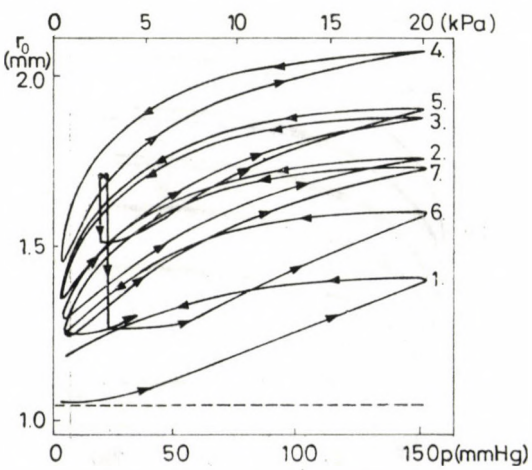

Fig. 1. Intraluminal pressure (p), outer radius (r_0) of hysteresis loops in a characteristic individual experiment. No. 1: Spontaneous contraction, 1st cycle, 5mM $NaNO_2$; No. 2: Spontaneous contraction, 21st cycle, 5 mM $NaNO_2$; No. 3: Spontaneous contraction, 39th cycle, 5 mM $NaNO_2$; No. 4: Relaxed, nKR; No. 5: Spontaneous recontraction, 1st cycle, nKR; No. 6: 2.5×10^{-6} M 5-HT, 1st cycle; No. 7: 2.5×10^{-6} M 5-HT, 14th cycle

taken during the 21st and 39th cycle, respectively, with 5 mM $NaNO_2$ still in the bath. Curve No. 4 is the relaxed curve. We call it relaxed, despite the fact that spontaneous contraction can still be observed. Further dilatation with repeated cycles caused a hardly observable creep of the characteristic curve in the course of 10 cycles and mostly made the segments less sensitive to the contracting agent due to smooth muscle exhaustion. Curve No. 5 shows the spontaneous recontraction phenomenon. It was taken in such a way that the segment was kept at a relatively low pressure of 21 mm Hg (2.8 kPa) in normal oxygenized KR media, without any vasoconstrictor agent added, for 10 minutes. Then the pressure cycles were resumed and the 1st cycle was curve No. 5. After reaching a relaxed state again due to repeated cycles, intraluminal pressure was set at 22 mm Hg (2.93 kPa) (for easy comparison) and 2.5×10^{-6} M 5-HT was added. After 10 minutes, with the contracting agent still in the bath, the pressure cycles were resumed. The 1st and 14th cycles are marked No. 6 and 7, respectively.

It was observed that the upward and downward routes followed different lines, the cycles were hysteretic. In the case of curves Nos. 1, 5 and 6 it was seen that the upward routes are less stable, their courses seem to be dependent on the previous mechanical events. On the contrary, the decreasing routes were more stable. A simple dilatation cycle seemed to give regular character to the pressure-radius characteristic curves. One may realize the remarkable fact that the decreasing routes of curves Nos 2 and 7 as well as 3 and 5 were

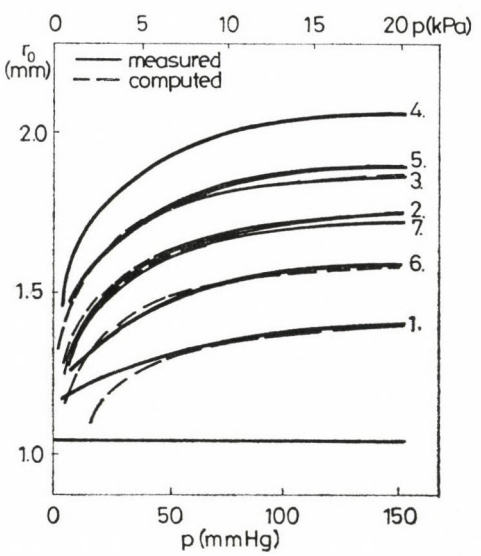

Fig. 2. Comparison of measured and computed pressure-radius characteristic curves in an individual experiment identical with the experiment shown on Fig. 1. Continous lines: measured curves. Dotted lines: computed curves

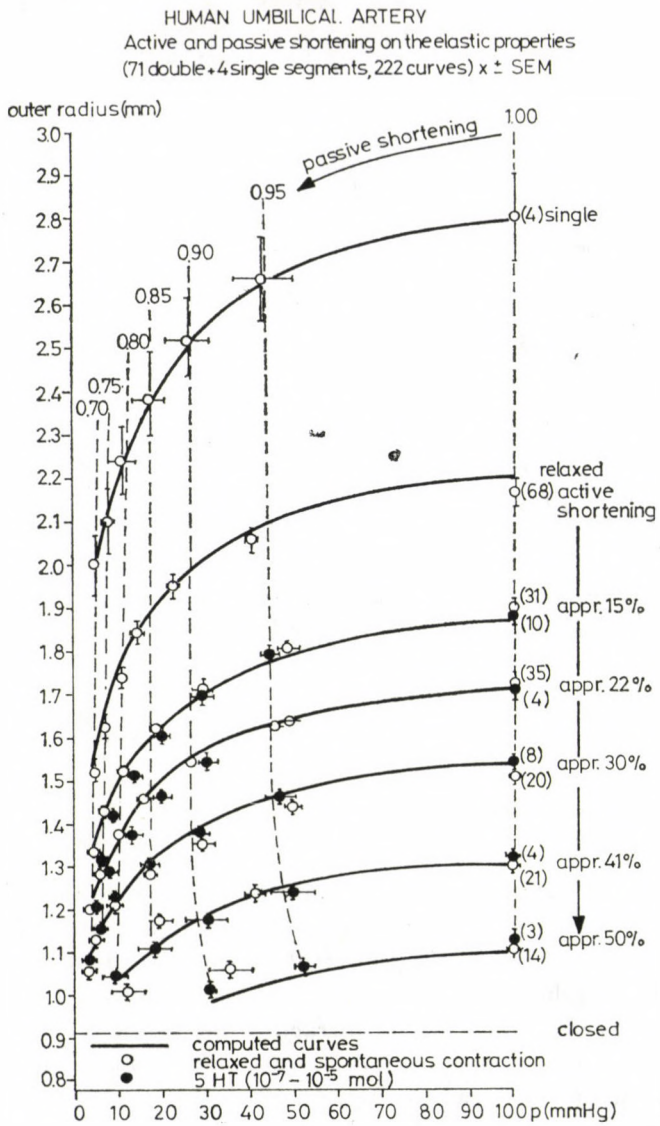

Fig. 3. **Pressure-radius characteristic curves measured at different degrees of active tangential shortening. Computed curves are drawn with continuous lines**

almost identical despite the different conditions they had been taken. For this reason we decided to analyse the decreasing routes of the pressure-radius hysteresis loops. Those of the experiment of Fig. 1 are shown on Fig. 2.

To characterize the elastic properties of the human umbilical artery as a function of active contraction the sum of 222 pressure-radius curves was

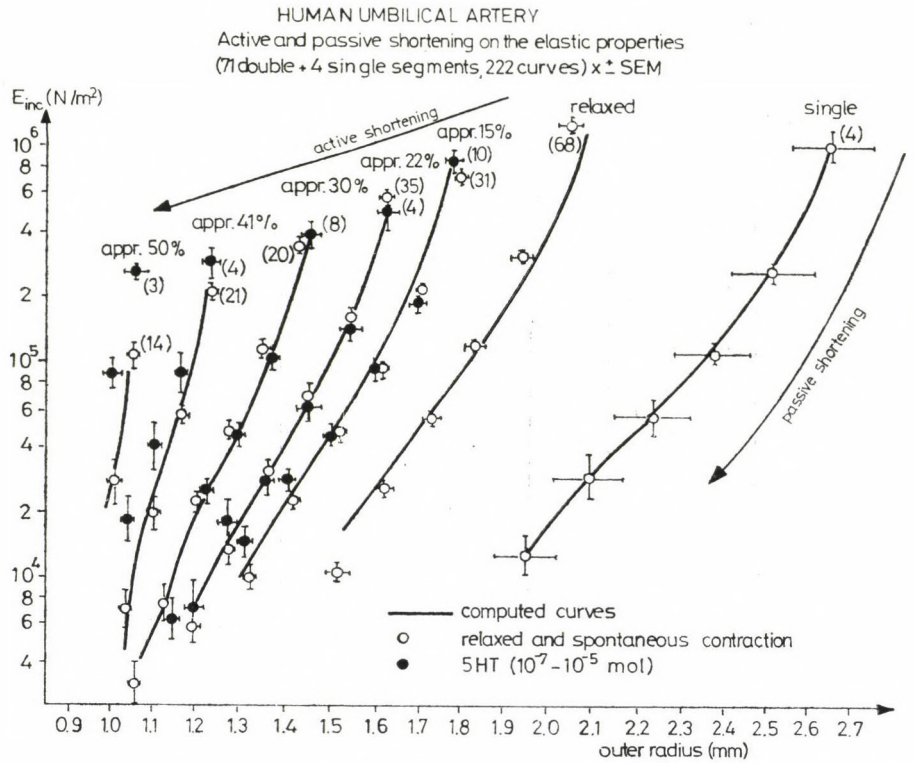

Fig. 4. Interconnection between elastic modulus (E_{inc}) and outer radius at different levels of active and passive shortening. Computed curves are drawn with continuous lines

plotted and analysed (Figs 3 and 4). Spontaneous contraction and spotaneous recontraction were grouped together. 5-HT contractions were induced by concentrations in the ranges of 10^{-7}—10^{-5} M. It is seen that an increase in passive tension, i.e. in intraluminal pressure, augmented tangential stiffness in all contraction states (Fig. 3). Active contraction increased tangential stiffness, i.e. the tangential elastic modulus, if it was expressed as a function of the absolute value of the outer radius (Fig. 4).

In Figures 3 and 4 it is indirectly included that stiffness decreases with active contraction, if it is expressed as a function of intraluminal pressure.

Figures 1, 2, 3 and 4 in addition, show some regularity, if we compare the decreasing routes taken with different degrees of active circumferential shortening.

Discussion

The fact that stiffness increases with increasing pressure, and that active contraction increases stiffness as a function of radius and decreases it as a

function of intraluminal pressure, is in accordance with the general rules of vessel elasticity as described in the Introduction [1, 2, 6, 7, 12, 14, 17, 19, 21, 32, 43].

The regularity of pressure-radius and radius-elasticity curve (Figs 1, 2, 3 and 4) presented an intriguing question. After several attempts, partly following the explanation given by Hardung [21], we succeded in establishing a simple rule that seems to govern the tangential elasticity of the human umbilical arterial smooth muscle.

If we take the outer radii measured at a pressure e.g. of 100 mm Hg as units and we plot the tangential forces that are needed to induce passive shortenings to 0.95, 0.90 . . . 0.75 value of the outer radius, we find that similar proportional changes in tangential force are needed to induce the same proportional change in outer radius at different contraction stages (Fig. 5). E.g. to induce a 10% passive change in outer radius from values measured at 100 mm Hg, the tangential force had to be decreased by 76.7 ± 9.9% (26.4 ± ± 4.9 mm Hg) in malformed, single relaxed arteries, by 79.6 ± 0.8% (23.1 ± ± 0.9 mm Hg) in normal, double relaxed segments, by 74.3 ± 1.4% (29.7 ± ± 1.7 mm Hg) in 15% active spontaneous contraction, by 75.0 ± 1.7% (29.3 ± 2.3 mm Hg) in 15% active 5-HT contraction, by 77.2 ± 1.5% (27.0 ± 1.9 mm Hg) in 22% active spontaneous contraction, by 73.9 ± 1.7% (30.6 ± 1.4 mm Hg) in 22% active 5-HT contraction, by 75.2 ± 1.9% (29.6 ± 2.0 mm Hg) in 30% active spontaneous contraction, and by 76.7 ± ± 2.0% (28.4 ± 2.6 mm Hg) in 30% active 5-HT contraction. At very low radii the interconnection is less explicit. This was explained by the fact that at low levels of radius the internal circumference may become irregular, as folding of the internal smooth muscle layers occurs. Not only the relative changes in tangential force, but also the intraluminal pressure levels corresponding to the same passive shortening levels are relative stable at different degrees of active shortening (Fig. 3).

On Fig. 2 dotted lines show the computed pressure radius curves. The computation was made supposing that similar proportional changes in tangential force are needed to induce similar proportional changes in outer radius. These computed curves are in good agreement with the measured contracted curves. Figure 5 demonstrates the regression lines computed for each passive shortening level. These lines will not cross the ordinate at 0 point, but at a little distance from it, and give a somewhat better approximation even at low pressures and at high degrees of active shortening. On Figs 3 and 4 computed curves using the regression equations are also shown, and a good fit between computed and measured values is seen. Based on the data presented, it seems probable that force bearing elements of the human umbilical arterial smooth muscle are organized in the following way. Similar force bearing elements are similarly prestretched at physiological pressures at different degrees of active

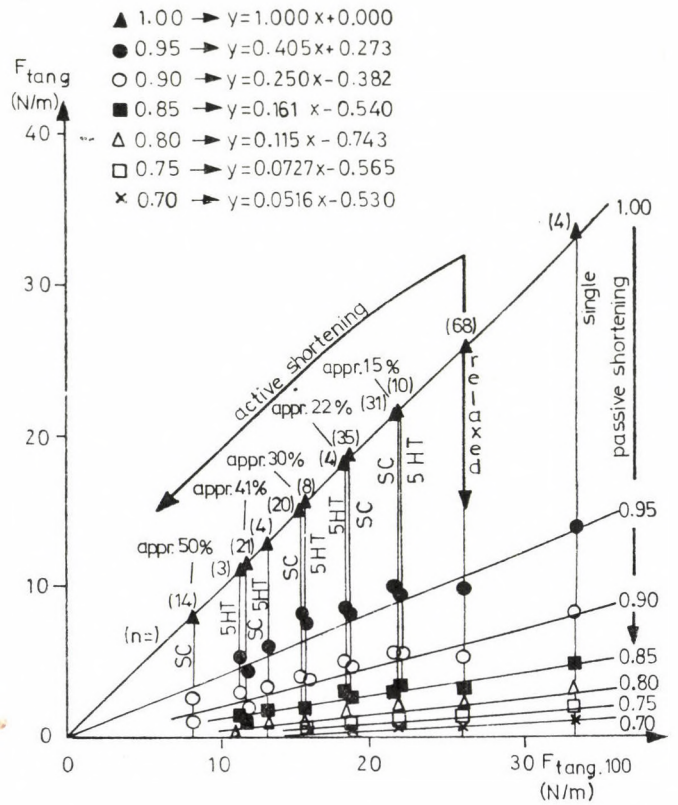

Fig. 5. Interconnection between proportional changes in tangential force and passive proportional changes in outer radius at different degrees of active tangential shortening. $F_{tang\ 100}$: Tangential force measured at 100 mm Hg intraluminal pressure which was taken as unit. F_{tang}: measured value of tangential force corresponding to different changes of passive and active contraction. Regression lines for each passive shortening level are drawn and marked on the top of the Figure

circumferential shortening. The number of parallely and serially connected elements should change with active shortening in such a way as to ensure similar stress at the individual elements at physiological pressures. This way the elastic elements could work around a similar working point at different contraction states at physiological pressures. This mechanism could also give the characteristics of a pressure sensor to the vessel wall as this might result from the fact that deviations from physiological pressures would cause a similar increase or decrease of the stress on each elastic element, independently of the degree of active shortening.

Our values concerning the relaxed state must, however, be taken with some criticism. The characteristic active spontaneous contraction, a typical phenomenon of this specimen may contribute to the fact, that relaxed state

elasticity is not essentially different from active state elasticity if elasticity is expressed as it was showed above. If we speculate what the elastic elements involved should be, from the morphological point of view the following structures can be taken into consideration: collagen fibres connecting the smooth muscle cells, basement membranes surrounding the individual smooth muscle cells, fine elastic fibres surrounding small branches of smooth muscle cells, acto-myosin cross bridges, actin filaments, intermediate filaments. Either of them or a characteristic group of them may be at the basis of the elastic properties found in these studies [1, 5, 6, 7, 8, 9, 10, 11, 13, 15, 16, 17, 18, 19, 20, 22, 24, 25, 26, 31, 34, 36, 37, 38, 40, 41, 42, 43, 44, 45, 46]. These observations give further support to the view that haemodynamics play a crucial role in the development of geometrical and viscoelastic properties of the vessel wall [3, 4, 27, 28, 30, 35, 39, 46, 47, 48]. While the umbilical artery is a unique specimen, we still believe that the rule found by us reflects to some degree some general properties of the vascular smooth muscle. Similar properties may play a role in the function of other vessels with more complicated structure.

Acknowledgement

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A SINGLE NEONATAL TREATMENT WITH METHYLCHOLANTHRENE OR BENZO(A)PYRENE ALTERS MICROSOMAL ENZYME ACTIVITY FOR LIFE

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A single neonatal exposure to methylcholanthrene or benzo(a)-pyrene altered the hepatic microsomal enzyme activity of rats for a long time. Methylcholanthrene depressed, whereas benzo(a)pyrene enhanced the activity of the microsomal enzyme system. The former had a greater influence on males than on females, whereas the latter acted practically uniformly on both sexes.

Keywords: Methylcholantrene, benzo(a)pyrene, microsomal enzyme system, neonatal imprinting

Although the cell receptors are genetically encoded structures, they require a functional adaptation during ontogenetic development [7]. The critical period of receptor adaptation is the perinatal period. The perinatal presence of the adequate ligand — e.g. of the hormone — at the adequate time and concentration accounts for lifelong functioning of the receptor [5, 8]. If, however, molecules structurally different from the ligand, yet able to bind to its receptor, appear in the critical perinatal period, they may alter the cellular response to the adequate bioactive molecule for life [5, 7, 8]. Hypothalamic (steroid) receptors [2, 13] and the receptors of perinatal target cells (of amino acid, polypeptide and steroid hormones) [7, 8, 11] may equally changes their behaviour under the influence of primary interaction with "foreign" molecules. Not only the hormone receptors, but also the receptors of other materials such as drugs [9] or carcinogenic hydrocarbons [10], are able to undergo this perinatal adaptation (imprinting) for perfect (or imperfect) function later in life.

In this paper we report on the impact of neonatal exposure to carcinogenic agents on the adult hepatic microsomal enzyme activity of rats.

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Materials and methods

Newborn CFY rats were treated within 24 h after birth with a 1 and 3 mg dose of benzo(a)pyrene (Fluka, Buchs, Switzerland) or methylcholanthrene (Fuka, Buchs, Switzerland) by the subcutaneous route. At 30 days of age the animals were separated from their mother and kept under constant conditions in metal cages and fed a standard chow (LATI, Gödöllő). Still, as benzo(a)pyrene and methylcholantrene treated groups had been examined in two different periods of the year, only intra-Table values were comparable.

Livers were homogenized with 4 vol of 0.15 M KCl—0.1 M Tris-HCl buffer pH 7.4 in a Potter-Elvehjem glass homogenizer equipped with a motor-driven Teflon pestle. The homogenates were centrifuged at $9000 \times g$ for 20 min and the supernatant (postmitochondrial fraction) was used for subsequent enzyme assays. The microsomal fraction was obtained by the calcium aggregation method [4], resuspended in 0.15 M KCl—0.1 M Tris-HCl buffer pH 7.4 containing 25% glycerol for the determination of cytochrome P-450. Total amounts of cytochrome P_{450} were assayed by the carbon monoxide difference spectrum of dithionite reduced microsomes, using an extinction coefficient of 91 mM^{-1} for $A_{450-490}$ [19]. Phenobarbital inducible cytochrome P_{450} was determined by metyrapone induced difference spectra of reduced microsomes as in [17]. An extinction coefficient of 52 $mM^{-1} \cdot cm^{-1}$ for $A_{446-490}$ was used [16]. Microsomal hydroxilase and demethylase activities were measured using the postmitochondrial supernatant; p-hydroxilation of aniline was estimated from the formation of p-aminophenol [3], N-demethylation was quantified by measuring the formaldehyde released from the substrate aminopyrine [15]. Enzyme activities were measured under conditions which were within the linear range of the assay. Significance was evaluated by using analysis of variance.

Results and discussion

The impact of perinatal treatments on adult microsomal enzyme activity has been studied by several investigators. For example, neonatal exposure to phenobarbital increased the cytochrome P_{450} activity of both male and female rats [1]. Phenobarbital treatment also enhanced the binding of digoxin and methylcholanthrene to the Ah receptor [18]. The progeny of female mice exposed to benzo(a)pyrene between days 13 and 18 of pregnancy showed an altered microsomal enzyme activity and an increased susceptibility to malignant transformation [20]. Since the receptors are prenatally to a certain degree protected against external influences, and are unchangeable in the late postnatal period, the most critical period of receptor adaptation (plasticity) is the first day of life [in the rat-11, 13]. In view of this we examined the impact of neonatal exposure to carcinogenic hydrocarbons on the adult microsomal enzyme activity of rats, with special regard to the sex differences referred to in the literature [1, 20].

As can be seen from Tables I and II, both polycyclic hydrocarbons altered the adult PSMO enzyme activity, but by different trends of action. Methylcholanthrene depressed the activity of all enzymes, although not always significantly, and to a greater degree in males than in females, whereas benzo(a)-pyrene generally enhanced microsomal enzyme activity to a similar degree in both sexes.

The considerable, partly significant, changes had been induced by a single neonatal treatment. Taking into consideration that, in adulthood, the

Table I

Influence of neonatal methylcholantrene (M) treatment of rats on the polysubstrate monooxygenase enzyme system in adult age

Group	Cytochrome P_{450} Co-complex	Cytochrome P_{450} Metyrapone Complex	Cytochrome b_5	Aniline p-hydroxylase	Aminopyrine N-demethylase
	(nmol/g liver)			(nmol/g liver /min)	
♀ —/—	17.9 ± 1.38	7.3 ± 0.28	8.4 ± 1.25	16.1 ± 0.44	202.4 ± 6.65
♀ M/—	16.3 ± 1.16	4.1 ± 0.65**	7.7 ± 0.97	15.7 ± 0.15	182.4 ± 7.51
♂ —/—	20.7 ± 1.05	6.6 ± 0.17	8.9 ± 0.47	26.9 ± 1.81	274.2 ± 13.96
♂ M/—	15.4 ± 2.76	5.6 ± 0.20*	7.1 ± 2.07**	14.7 ± 2.07**	199.1 ± 4.76**

* $p < 0.05$
** $p < 0.01$

Table II

Influence of neonatal benzo(a)pyrene (B) treatment of rats on the polysubstrate monooxygenase enzyme system in adult age

Group	Cytochrome P_{450} CO-complex	Cytochrome P_{450} Metyrapone Complex	Cytochrome b_5	Aniline p-hydroxylase	Aminopyrine N-demethylase
	(nmol/g liver)			(nmole/g liver/min)	(nmol/g liver/min)
♀ —/—	14.6 ± 1.04	3.8 ± 0.44	7.0 ± 0.63	10.2 ± 0.62	182.9 ± 3.64
♀ B/—	18.0 ± 0.93*	7.1 ± 1.17*	7.1 ± 0.56	13.4 ± 1.18*	192.7 ± 9.81
♂ —/—	15.6 ± 2.30	4.2 ± 1.32	6.0 ± 0.28	13.1 ± 1.43	208.4 ± 7.66
♂ B/—	24.7 ± 2.42*	7.8 ± 1.25	8.9 ± 0.40**	14.0 ± 1.69	238.8 ± 9.14*

* $p < 0.05$
** $p < 0.01$

enzyme inducing action of polycyclic hydrocarbons lasts only about 72 h, there is reason to postulate that the single neonatal treatment had induced radical changes which persisted for several months. The effect of polycyclic hydrocarbons on the Ah (cytosolic) receptor may have been essentially similar to hormonal imprinting [5, 7, 8]. Since, however, the hydrocarbon molecule binds (as a ligand) not only to the receptor, but (as a substrate) also to the microsomal enzymes, imprinting could have occurred also by the latter interaction [12]. Receptors and enzymes being equally subject to degradation and resynthesis, a lasting interrelationship between them necessarily presupposes a transmission of the "memory" of the primary interaction (imprinting) from one cell generation to the other, in other words, fixing of the neonatal impact (imprinting) at the gene level. This seems to be feasible in the case of the cytosolic (Ah) receptor, which is being transposed from the cytoplasm to the nucleus [14], where it actually acts at the gene level. This action if it develops in the critical stage of ontogenesis, will influence the later receptor level events for life. The presumed molecular changes induced in the enzymes by inter-

action with the ligand (substrate) cannot, however, be reconciled with the current concepts on DNA transcription.

The interaction of the maturing hormone receptor with a "foreign" molecule (which it is able to bind) accounts for a decrease (less often an increase) in the maximum receptor number in adulthood. One might postulate that the carcinogenic hydrocarbons gave rise, by a similar mechanism, to a decrease in the number of microsomal enzyme molecules. This is, of course, only a hypothetical implication, but the fact remains that a single neonatal treatment with methylcholanthrene or benzo(a)-pyrene did considerably alter the adult microsomal enzyme activity of rats, and the trends of influence differed between the two tested types of aromatic hydrocarbons.

Since nowadays exposure to aromatic hydrocarbons may frequently occur in the critical period of ontogenesis, especially under urban conditions, the adverse influence of such effects should be taken into account as an additional environmental damage.

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POSSIBILITY OF BRAIN RECOVERY AFTER ELECTRICALLY INDUCED CARDIAC ARREST AND REANIMATION IN DOGS

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The changes of aortic blood pressure (BP), carotid artery flow (CAF), power spectrum of analysed EEG, neurologic deficit and survival rate were determinated in dogs after experimental cardiac arrest of different duration. Following artificially induced ventricular fibrillation of 1, 4, 10, 12 and 15 min duration successful cardiopulmonary resuscitation was performed in 30 experimental animals. Alterations of power spectrum during and after reanimation procedures, severity of the neurologic state and the survival rate deteriorated in parallel with the increasing duration of circulatory stop. Advantageous effect of direct heart massage could be demonstrated by the measuring circulatory parameters. Following a 15 min fibrillation, all animals were lost in a few hours despite the successful restoration of circulation and ventilation. Considering the various experimental and clinical conditions experimental cardiac arrest lasting for 12 min seems to be useful in extrapolating the results to human cases. The suggested model allows to study the brain function recovery after circulatory stop and resuscitation.

Keywords: resuscitation, EEG recovery, power spectrum, neurologic deficit

On the basis of comprehensive statistical evaluation of resuscitated patients after circulatory arrest several ascertainments have been made. Successful restoration of the circulation could be expected in the one third of the cases. However, only 8 to 20 percent of the patients were discharged from hospitals in satisfactory condition [5, 14, 20, 25]. Recently some improvement is apparent [2, 3, 4, 12, 23, 24]. Secondary ischaemic brain damage is believed to be responsible for the loss of most of the primarily resuscitated patients. During the brain reperfusion that follows cardiac arrest, a wide range of ultrastructural cell lesions may occur. The mechanisms underlying these pathologic events have not been clarified yet, anyhow, cell and mitochondrial membrane damages seem to be verified [6]. Due to this membrane lesion, the abnormal electrolyte and water transport may result in swelling of the brain tissues causing an increase in vascular resistance and impeding reperfusion. Thus

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hypoxia of the edematous brain may be prolonged which further deteriorates the ischaemic lesions.

The study of secondary hypoxic brain damage requires a proper model which provides severe but not fatal cerebral ischaemia. Previously we investigated the acute effects of cardio-pulmonary resuscitation carried out by different methods. The brain surface oxygen level, the reappearance and quality of analysed EEG were studied as prognostic signs of the success. Owing to the short duration of observation period the survival and neurologic damages could not satisfactorily be assesssed [21]. It was therefore necessary to find a proper model and to record the survival rate, the circulatory parameters the changes of EEG and the neurologic state of the animals in a long term investigation.

Materials and methods

Thirty mongrel dogs weighing 12 to 30 kg were used. Under sterile conditions two electrodes were fixed into drilled holes of the left parietal region of the skull for bipolar leads.

Several weeks later, after Droperidol® and Fentanyl® premedication and a light barbiturate anaesthesia the narcosis was maintained with ventilation of $N_2O + O_2$ (3 : 1) gas mixture. Also under sterile conditions a catheter was introduced through the femoral artery into the thoracic aorta and connected to a Medicor EM 41 electromanometer. A cannula was inserted into the other femoral artery to obtain blood samples for determination of pPO_2, pCO and pH. In the neck, the left common carotid artery was exposed and a Nycotron N 376 electromagnetic flow meter probe was fitted to the vessel. The previously fixed electrodes of the skull were connected to a Medicor 4760 type 8 channel polygraph. ECG (standard leads) was taken simultaneously. Blood pressure (BP), carotid artery flow (CAF), EEG and ECG were continuously recorded.

Using a PCM converter the EEG was stored on tape with an AKAI 4000 DS recorder. Under replay and visual control to exclude artifacts, 6 s epochs were selected from each period of the experiments. After digitalization the EEG signals were analyzed by Fast Fourier Transformation with an R 22 computer (sampling frequency of 100 Hz) using a special program [1]. The obtained power spectrum yielded a relative numerical value of the intensity of frequency components of EEG waves. The sum of these individual values made up the total power in arbitrary units. The power spectrum was evaluated between 1 and 30 Hz with a resolution of 1 Hz. Results represent EEG power in delta (1—3 Hz), theta (4—7 Hz), alpha (8—13 Hz), and beta (14—30 Hz) bands. The method has been described earlier (22).

After the surgical preparations, control parameters were recorded and following relaxation of the dogs ventricular fibrillation was induced electrically. Depending on the duration of the circulatory arrest the animals were divided in five groups, each containing six dogs. In the first (I) and second (II) groups the fibrillation was maintained for 1 and 4 min, respectively. Thereafter external thoracic massage was carried out, simultaneously with intraarterial norepinephrine injection, oxygen ventilation and correction of pH alterations. Lastly electric defibrillation was attempted. In the remaining three groups (III, IV and V) fibrillation was sustained for 10, 12 and 15 minutes, respectively. The relatively long period allowed a quick and sterile thoracotomy (in the left fourth intercostal space) and pericardiotomy. Simultaneously with the beginning of direct heart massage and oxygen ventilation, the vasomotor tone and pH changes were corrected with norepinephrine injection and $NaHCO_3$ solution, respectively, via the intraaortic cannula then intrathoracic defibrillation was performed. After stabilization of the circulation the chest was closed; following an observation period of two hours and sufficient restoration of paO_2 (10 to 15 kPa) and $paCO_2$ (4 to 5 kPa), the dogs were carried to their cages.

Results

Group I

The average control BP (12.2 ± 2.1 kPa) decreased to 1.7 ± 0.2 kPa, the CAF changed from 68 ± 12 ml/min to 0 during fibrillation (Fig. 1). At the end of the massage period (1.4 min on the average) the mean BP and CAF

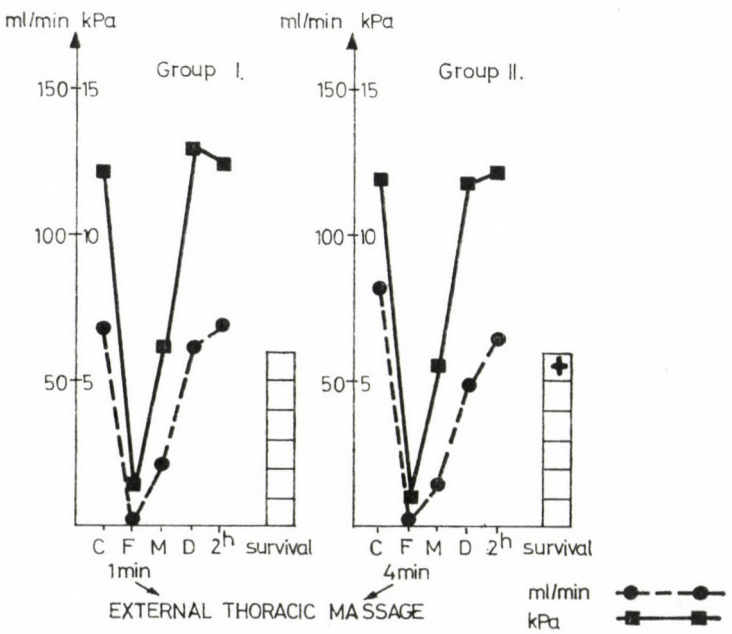

Fig. 1. Changes of aortic blood pressure (BP) and left common carotid artery flow (CAF) of the external massaged dogs. The solid line denotes BP, the dashed one represents the CAF values. Values: C, control; F, at the end of fibrillation; M, during the course of massage; D, immediately after defibrillation; 2^h, at the end of observation period. Open squares of the columns at the right side of the diagrams indicate the number of survivors, those marked with a cross the animals which perished

were 6.1 ± 0.8 and 21 ± 9 ml/min, respectively. After defibrillation, BP (12.8 ± 2.1 kPa) exceeded the control, while the CAF almost achieved it (63 ± 14 ml/min). At the end of the observation time the values returned to the original levels (12.4 ± 2.0 kPa and 69 ± 10 ml/min).

Group II

The tendency of the changes in the circulatory parameters was similar to that obtained in the first group. The BP fell to a lower level (1.1 ± 0.4 kPa) at the end of the longer fibrillation period (4 min). The massage (3.1 min

duration) was less effective, i.e. at 5.5 ± 0.7 kPa of BP the CAF rose only to 16 ± 7 ml/min. Immediately after the restoration of circulation the BP achieved the initial pressure, while the CAF was still significantly lower (48 ± 12 ml/min) than the control value. At the end of the observation period the circulatory parameters approached the normal values (12.2 ± 2.5 kPa and 65 ± 10 ml/min).

The animals of Groups I and II showed awakening similarly to other dogs undergoing thoracic surgery. All the animals survived the procedure without abnormal neurologic symptoms, except one in Group II which died on the first postoperative day.

Group III

The longer duration of fibrillation (10 min) allowed thoracotomy and pericardiotomy to be carried out for direct heart massage. The control values for the circulatory parameters decreased to zero in the course of cardiac arrest. Using the method of direct massage a BP of 7.6 ± 1.1 kPa and a CAF of 31 ± 11 ml/min could be achieved at the end of the massage period lasting for 4 min on the average. Following defibrillation the pressure and flow values

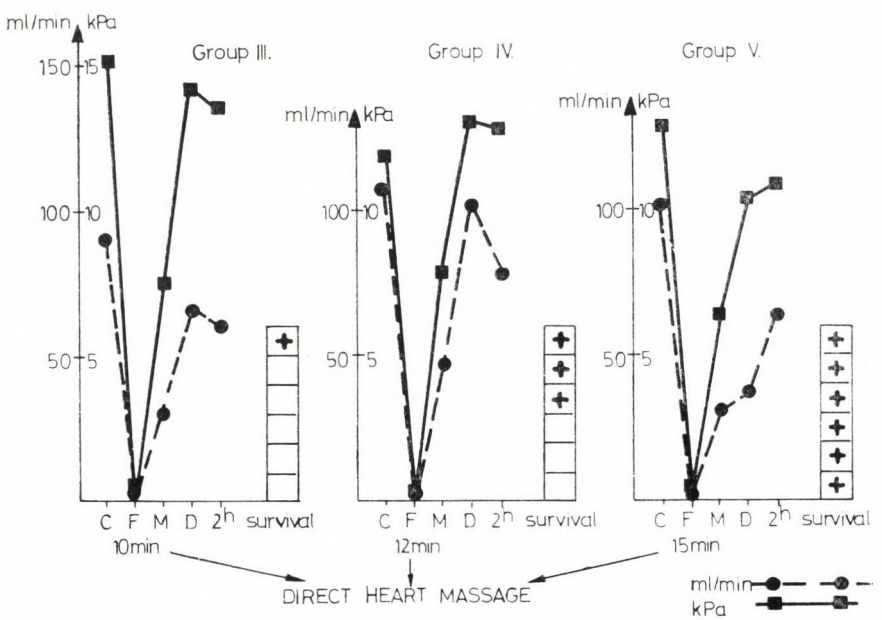

Fig. 2. Changes of haemodynamic parameters and of mortality in directly massaged animals. Legends are the same as in Fig. 1

approached the control data (14.2 ± 3.0 kPa and 66 ± 12 ml/min). Two hours later both of them became stabilized on somewhat lower levels (13.4 ± 2.9 and 60 ± 12 ml/min; Fig. 2).

Group IV

The measured circulatory parameters showed the same tendency of changes as those in Group III, nevertheless, the highest mean BP and CAF values could be found in this group (7.8 ± 2.2 kPa and 47 ± 12 ml/min, resp.). After the resuscitation procedure (3.9 min) the circulatory parameters almost returned to normal (12 ± 2.7 kPa, 77 ± 10 ml/min, resp.).

The awakening of the dogs of Groups III and IV was extremely protracted. For hours after surgery the drowsy animals could be awakened only by strong stimuli, their consciousness seemed to be clouded. On the first postoperative day the reflexes of cranial nerves were present, but the ability of erection was absent. All the animals refused food and water and most of them showed increased extensor tone in the limbs.

One animal of Group III and three of Group IV were lost between 6 and 18 hours after successful cardiopulmonary resuscitation without regaining their consciousness (Fig. 2). The autopsies did not reveal any cause of death macroscopically.

Group V

After fibrillation for 15 min the required massage and defibrillation averaged 7.1 min. The efficiency of massage was the smallest (6.4 ± 1.7 kPa and 31 ± 6 ml/min, resp.) in this group and the CAF did not change significantly even after defibrillation (36 ± 6 ml/min). Despite the satis factory improvement in the circulatory parameters at the end of the experiments (10.8 ± 2.2 kPa, 64 ± 10 ml/min, resp.), all the six dogs died between the 6 to 18 postresuscitation hours without demonstrable reason of death.

Figure 3 shows the distribution of the components of the analyzed EEG spectrum in dogs resuscitated with external thoracic massage. In the control phase the proportions of the four bands were near equal with a small prevalence of the lower frequency waves. During fibrillation, owing to the electric silence, no appreciable spectrogram was obtained. At the end of the short massage period high total powers were recorded with extreme dominance of the delta and theta frequencies. Following the restoration of circulation in Group I a rapid, while in Group II a delayed EEG normalization was seen with decreasing low frequency components and increasing prevalence of high frequency waves. On the first postoperative day and subsequently the EEGs in both groups were practically normal.

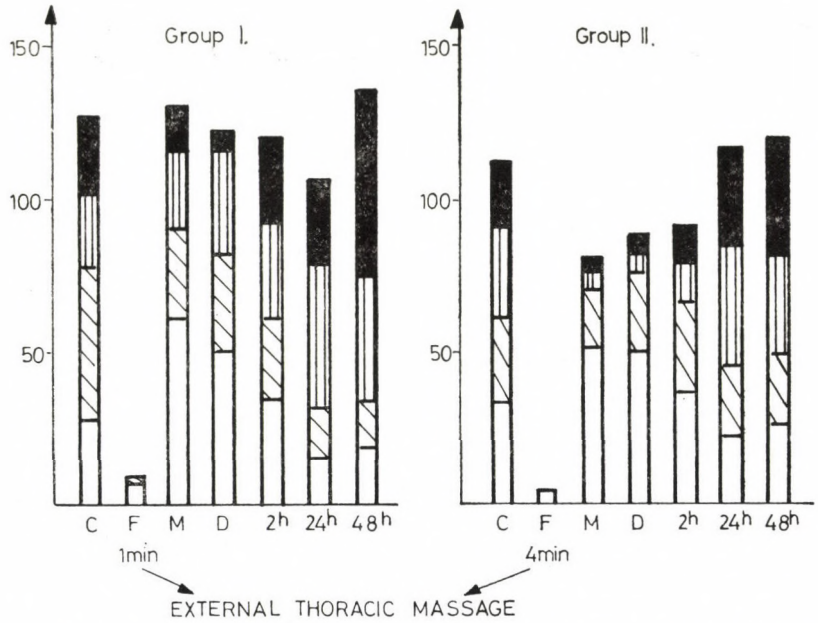

Fig. 3. Distribution of delta □, theta ▨, alpha ▥ and beta ■ frequencies of the computer analyzed EEG, recorded during the short observation period and on the first and second postoperative days. The figures express the values of total power in arbitrary units

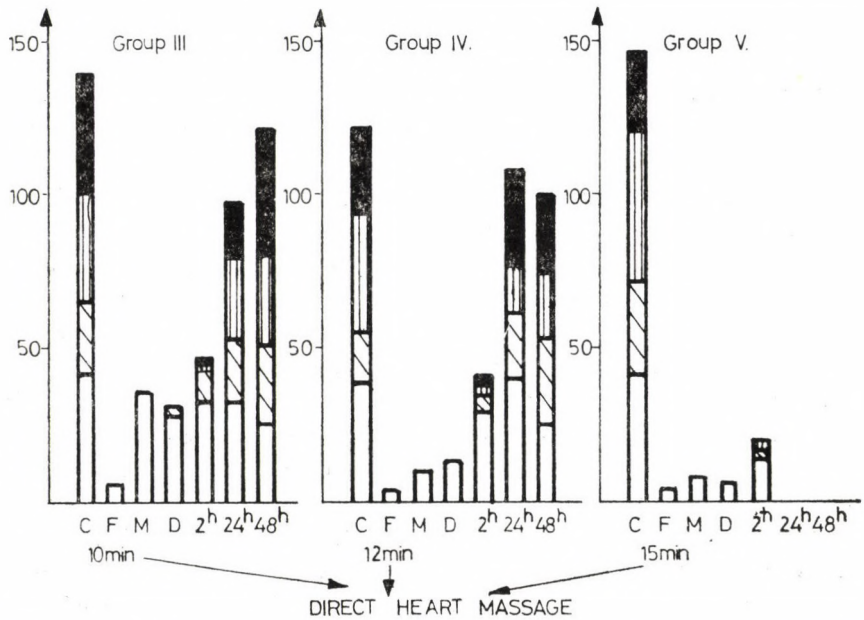

Fig. 4. Changes of the computer analyzed EEG spectrogram in the directly massaged groups. Legends are the same as in Fig. 3

Considerable differences could be observed in the analyzed EEG spectra after fibrillation for longer periodes (Fig. 4). The changes during fibrillation were similar to those in the first two groups, however, during massage and after defibrillation very low frequency waves with short amplitudes were obtained. At the end of the observation period somewhat improving EEG signs could only be recorded in Groups III and IV. The survivors of these animals showed near normal spectrogram 24 hours later, the EEG becoming normal on the second postoperative day. The recorded cerebral electric signs of the dogs of Group V appeared as delta activity with low amplitudes, even at the end of the experiments.

The survivors of Groups III and IV showed a gradual improvement of their neurologic state. The increased extensor tone of the limbs released. The ability of erection returned. The capability of standing and walking reappeared usually within a week and only some incoordination of gait remained.

Discussion

According to experimental [8, 17] and clinical [3] studies following circulatory arrest the most favourable prognosis is to be expected if quick restoration of cardiorespiratoric functions is achieved. To increase blood flow to the brain for the prevention of neurologic deterioration and at the same time to improve coronary perfusion by maneuvres or drugs are the most important preconditions of success [17].

In the present experimental series the effect of ventricular fibrillation of different duration was investigated. The cardiac arrest lasting for 1 min did not substantially affect the vitality of the experimental animals. Although the circulation stopped immediately, the vasomotor tone kept the aortic pressure near to 2 kPa even at the end of fibrillation. The extrathoracic massage led to the achievement of sufficient BP and CAF values prior to defibrillation. Thereafter the circulatory parameters were rapidly normalized. The wakening and later the behavior of the dogs did not differ from other animals subjected to thoracic surgery.

According to Ljunggren et al. [9] 70 per cent of the available energy sources of the brain cells are utilized within 1 min of total ischaemia; despite this observation the ability of quick normalization was apparent in the EEG spectrum. After short complete ischaemia during massage a mean incomplete ischaemia of 1.4 min, the EEG reappeared in only a moderately altered pattern. Overall power level did not change the control theta dominance, however, was replaced by a delta band combined with decreased beta activity. After restoration of heart function a tendency for the reduction of delta dominance was found and within few minutes the EEG frequencies already showed the initial distribution.

The analysis of the data revealed more marked abnormalities in the dogs of Group II. At the end of fibrillation arterial tone was less (mean BP = 1.1 ± ± 0.4 kPa) than in Group I, therefore even the longer lasting revival manoeuvres were unable to produce higher pressure and flow values. Although following defibrillation the circulatory parameters became nearly normal and awakening and behaviour were acceptable, one of the six dogs was lost in the second postoperative day without any demonstrable cause of the death. Certain disorders of the brain was revealed in the EEG following fibrillation. The total power being decreased, the higher frequency waves almost disappearing, delta dominance prevailing until the end of the observation period. EEG analysis of the animal lost in this group showed no differences in the mean distribution of frequencies. The deterioration of the EEG spectrum following a 4 min long circulatory arrest and further 3.1 min incomplete brain ischaemia is known on the basis of some experimental observations. It was reported that the depletion of the energy reserves of brain cells occurred between 3 to 5 min after complete cerebral ischaemia [9, 18]. Similar exhaustion of the energy reserves was shown in our earlier experiments by demonstrating the characteristic elevation of brain surface extracellular potassium level [7].

The data of Group III provided sufficient evidence for the advantages of direct heart massage. In spite of the absence of vasomotor tone (mean BP = 0 kPa) resulting from the considerably longer fibrillation period (10 min) during the hardly extended heart massage time (4 min), higher pressure and flow values could be recorded. The mortality in this Group was equal to that of Group II, despite the increased duration of clinical death and the additional thoracotomy. The changes of the EEG spectrum verified the severe condition of the brain. During cardiac massage and after defibrillation electric silence could be observed. Even two hours later delta dominance and decreased total power were seen. In the surviving animals the normalization of EEG occurred only on the second day.

The awaking of the animals was protracted and the neurologic symptoms disappeared only after a week. There were no significant differences between either the circulatory parameters or the EEG spectrum of Groups III and IV, however, in the latter one a slower restoration of the severe condition could be observed and 3 of the six dogs died on the second postoperative day. The survival rate also supported the benefit provided by the direct massage, as compared to the indirect one. Safar et al. [17] lost all six dogs weith external massage after 12 min fibrillation.

Resuscitation was most difficult in the last group. Restoration of the circulation and ventilation occurred slowly. Despite the fact that at the end of the experiments acceptable parameters were recorded, the EEG spectrum revealed severe lesion of the brain and none of the animals survived the procedure.

The EEG as an indicator of the brain cell action potentials depends on the actual ATP content of the cell which maintains the ion transport across the cell membranes [18, 19]. Cerebral ischaemia leads to rapid cell energy depletion, causing the pumping action to stop within seconds. Simultaneously with this energy decrease marked reduction in the EEG frequencies occurs and electric silence ensues within 20 to 30 seconds. After restoration of the circulation the reappearance of the EEG is not the same process with an inverse direction. Following a brief brain ischaemia (not longer than one min) the first low frequency EEG waves can immediately be recorded after recirculation and the normalization of EEG also takes place quickly.

As the duration of ischaemia increases the period of EEG restoration is prolonged exponentially [7]. There is reason to believe that circulatory arrest longer than 5 min causes the free cell energy reserves to be exhausted with disintegration of the essential cell and subcellular structures. During reperfusion the restoration of these destroyed structures obviously precedes the reinstitution of ion pump function. This view seems to be supported by the finding that the phosphocreatine and ATP contents of brain cells were found to be restored after 30 min of ischaemia and 90 min of reperfusion [10, 11], while ischaemia of this duration cannot be expected to lead to normalization of the EEG.

The restoration process of the brain cells is rather difficult during incomplete ischaemia of the reanimation period. After a circulatory arrest of 10 to 15 min duration an approximately 50 per cent lower BP sustains 30 to 40 per cent of the initial CAF as seen in the present experiments. The high pressure oxygen ventilation increases the paO_2 to more than twice the control value [21]. Consequently during massage a slight incomplete ischaemia develops with sufficient oxygen supply. Nordström et al. found that incomplete ischaemia results in less favourable outcome than the complete one of the same duration [10, 11]. Recent investigations have provided explantation to this paradoxical phenomenon through the increased lipid peroxidation of cell membranes caused by the aggressive free oxygen radicals [15, 19]. Nevertheless, further studies are needed to prove this concept.

According to clinical data circulatory arrest is caused by different pathologic lesions. The existing alterations and atherosclerosis of the vessels decrease the chances for the success of resuscitation. On the other hand, in young healthy experimental animals with intact vessels a well trained team will obviously solve the problems involved in artificially induced ventricular fibrillation not combined with any other pathologic lesions. Utilizing the favourable effects of norepinephrine injection [13, 23] and pH correction as well as of the direct massage and oxygen ventilation, the defibrillation may be carried out relatively easily. Under such circumstances resuscitation may be successful even after 12 min cardiac arrest, while in the clinical practice the situation is quite dif-

ferent. Starting of the reanimation procedures 5 min after the onset of circulatory stop will incur an extremely poor probability of success.

On the basis of available experimental and clinical data it appears that the characteristic brain lesions modelling human pathology develop only after 12 min fibrillation in canine experiments.

In absence of the continuous control and treatment of the animals — at least on the first and second postoperative days — it can not be formed a judgement of the real cause of the dogs' death. It is probable that more vigorous therapy would have increased the survival rate or, eventually, would have kept alive some of the animals in the last group.

We therefore have decided to extend our neurologic investigations on resuscitated dogs subjected to prolonged ventricular fibrillation in further studies.

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PRESENCE (HPL, PROSTAGLANDIN) AND ABSENCE (TRIIODOTHYRONINE, THYROXINE) OF HORMONES IN TETRAHYMENA: EXPERIMENTAL FACTS AND OPEN QUESTIONS

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The RIA technique detected prostaglandin (PGF_2) and human placetal lactogen (hPL) in Tetrahymena cultures grown in bacto tryptone + yeast extract medium which, however, itself contained these hormones. About one to two per cent of the total hormone content of the medium was demonstratəd intracellularly. Treatment with diiodotyrosine (T_2), which is known to stimulate the growth of Tetrahymena, was followed by a decrease in the intracellular prostaglandin level. Triiodothyronine and thyroxine were not detected in Tetrahymena or in the medium, and did not appear in it on induction with TSH either. In the light of these observations it might well be doubted that prostaglandin was native in Tetrahymena: the use of synthetic media, and/or a reliable demonstration of the hormone content of the growth medium is recommended for evidence of hormone biosynthesis by unicellular organisms.

Keywords: Hormone phylogeny, Tetrahymena, hPL, prostaglandin, thyroxine

Although the unicellular organisms represent the lowest phylogenetic level among eukaryotes, certain species are highly differentiated. For example the unicellular Tetrahymena, being an aquatic organism, is able to respond to a great variety of environmental chemical stimuli [6, 7]. If the steric structure of the environmental molecules is complementary to the structures presented by the protozoon, they induce the formation of receptors in the membrane (amino acid and polypeptide hormones, etc.) or probably, in the cytosol (steroid hormones) of Tetrahymena. These receptor structures persist over many subsequent generations and account for a prompt and increased response to the hormone on reexposure(s) [3, 4, 5]. Tetrahymena however, possesses not only hormone receptors but also hormones, some of which are characteristically involved in the endocrine regulation of higher (vertebrate, mammalian) organisms [2, 3, 14.] It is an experimental fact that Tetrahymena contains polypeptides of an immunobiological action similar to insulin [15], somatostatin [1], beta endorphine, ACTH [16], relaxin [17, 18], dihydroepiandro-

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sterone [6, 9] and to a lesser degree testosterone and estradiol-like steroids. The steroid-like non-hormone digoxine [10] and the fatty-acid like hormone prostaglandin [12] have also been demonstrated in Tetrahymena. In view of this we have ventured the following hypothetical conclusions.

1) Only those hormones are lacking in Tetrahymena, for which it is not scrutinized.

2) The hormones represent, at the unicellular level, mere by-products of biosynthesis, for they cannot ab ovo play a role in the regulating mechanisms of the unicellular.

3) The hormones regarded as native to Tetrahymena represent components taken up from the nutrient medium rather than biosynthetic products.

To obtain more information on the problem, we examined a previously not tested polypeptide hormone and two amino acid type hormones, as well as prostaglandin which had been tested earlier [12], for influence on the Tatrahymena with special regard to a possible uptake from the medium. We have also studied whether the intracellular level of the tested hormones was influenced by hormone binding to Tetrahymena at the receptor level.

Materials and methods

Tetrahymena pyriformis GL cells, cultured in 0.1% yeast extract containing 1% Bactotrypton medium (Difco, Michigan, USA) for 48 h at 28 °C, were used in the logarithmic phase of growth. The cells were washed twice in Losina solution before the hormone assay. The cell counts were always determined before treatment.

Determination of human placental lactogen (hPL)

The washed cultures were sonicated twice for 60 min, and then centrifuged for 20 min at 1800 g. A ^{125}I-hPL RIA kit was used for hormone assay.

Determination of prostaglandin F_2

The washed cultures were sonicated twice for 60 min, precipitated with ethanol, and centrifuged for 20 min at 1800 g. The supernatant was dialysed, and the dialysate was assayed for prostaglandin content with a ^{125}I-prostaglandin F_2-alpha-thyrosine methyl-ester kit.

In some experimental series the Tetrahymena cells had been treated with diiodotyrosine (T_2, Fluka, Buchs, Switzerland) for 48 h before hormone assay. In other series 48 h exposure to T_2 was followed by return to plain medium for 3 days, and by a second exposure to T_2 for one h. Only after the second T_2-exposure were the cells washed, sonicated, dialysed, etc. for hormone assay. In a third series the cells were transferred to a physiological (Losina) medium for 24 h before prostaglandin assay by the above technique.

Determination of triiodothyronine (T_3 and thyroxine (T_4)

The washed cells were homogenized by ultrasonication, centrifuged, and assayed for hormone content with ^{125}I-T_3 and ^{125}I-T_4 kits, respectively.

In further experimental series the cells were pretreated before hormone assay as follows.

1. Exposure to thyrotropic hormone (TSH, Ambinon, Organon, Oss, Holland) for 48 h.
2. Exposure to TSH for 48 h, followed by return to plain medium for 24 h.

3. Exposure to TSH for 48 h, followed by return to plain medium for 3 days, and re-exposure to TSH (10^{-7} M) for one h.

All kits used for the hormone assays were obtained from the Radioisotope Institute of the Hungarian Academy of Sciences.

All experiments were performed in 5 replicates, and were repeated on three occasions.

Results and discussion

The applied techniques detected hPL and prostaglandin, but failed to detect T_3 and T_4 in Tetrahymena (Table I). It should, however, be taken into consideration that while hPL and prostaglandin were present in the maintenance medium, T_3 and T_4 were not present in it. In this light it appears that the hormone content of Tetrahymena originated from the maintenance medium, since hormones lacking in the latter did not appear in the body of the unicellular. This was certainly the case in the present experiment, but Roth and coworkers [1, 15, 16, 18], who had used a synthetic maintenance medium devoid of macromolecules (hormones), also had detected hormones in Tetrahymena. Thus, these hormones seemed to be as native to the Tetrahymena as the steroids and digoxine, which had been lacking in the non-synthetic medium used by us. On the other hand, Hokama and others [12], had used a protease-peptone-liver extract medium, which may well have contained prostaglandin seem to have confused the storage of prostaglandin with its biosynthesis.

The hPL- and prostaglandin F_2 α-levels detected in the entire Tetrahymena population represented 1—2% of the total hormone content of the

Table I

Detection of prostaglandin (PG) and human placental lactogen (hPL) in Tetrahymena cells and growth medium

Group	Cell count	PG (pg/100 μl)			PG	
		in the medium	in all Tetrahymena cells (100 μl)	in 10^6 Tetrahymena cells	in total medium (μg)	in all Tetrahymena cells (pg)
—	—	35.8			35.8	
Control	1×10^6	18.0	19.0	19.0	18.0	950
T_2 for 48 h	2.02×10^6	14.0	16.0	7.92	14.0	800
T_2 for 48 h + 1 h	4.04×10^6	22.0	17.5	4.33	22.0	875
Control in Losina solution for 24 h	1×10^6		10.2	10.2		
		hPL (μg/ml)			hPL	
—	—	2.3			230 μg	
Control	1.4×10^6	1.95	2.05	1.46	195 μg	2.05 μg

medium. The intracellular hPL-concentration seemed to be high, considering that 1 ml blood of a woman pregnant for 15 weeks [11] contained as much hPL as 10^6 Tetrahymena cells did, while their volume was much less than 1 ml. Since Tetrahymena takes up its food through its pharynx by endocytosis, the hormone concentration in question may well have been taken up from the nutrient medium, and may have, with regard to the enormous difference between the intra- and extracellular prostaglandin concentrations, accounted for the maintenance of a constant intracellular hormone level. Since, however, the materials incorporated by endocytosis break down intracellularly, a concentration decrease should necessarily take place in the medium with progressing time. This is portrayed by the difference between the hormone content of the original medium and of the Tetrahymena cells. At the same time, the relative proportion of the hormone contents of 100 ml Tetrahymena extract and of an equal volume of medium was not conclusive of the actual conditions, for the centrifugal separation of the cells accounted for enrichment of their hormone content.

In the case of prostaglandin, the cell counts changed under the influence of T_2-treatment. Determination of the prostaglandin content of the total cell population thus disclosed little, if any, concentration increase with the population increase. It is known that one treatment with T_2 increases the population twofold, and two T_2 treatments increase it fourfold. Still the total prostaglandin content of the cells changed hardly with the population increase. If, however, the intracellular prostaglandin level is related to 10^6 Tetrahymena cells, a gross difference, practically a halving of the prostaglandin content parallel to the number of divisions is found. This may be explained by supposing that the T_2-enhanced high mitotic rate [5, 6, 9] prevented endocytosis, whence the prostaglandin level present during the normal cell cycle decreased to one half with progressing division. An other explanation is that prostaglandin, whose presence is required for the division of *Tetrahymena* [12], is being increasingly used up during the enhanced division. As a third alternative it may be taken into consideration that T_2, which acts at the unicellular level more intensively than the hormone proper (T_3 and T_4) [8] stimulates not only the growth, but also the metabolism of Tetrahymena, which is portrayed by the intracellular concentration decrease of prostaglandin.

It is known that Tetrahymena cells do not grow in physiological (Losina) solution, but survive in it for at least 24 h. The prostaglandin content of the cells decreased by about 50% during incubation in the physiological solution. It appears that the intracellular prostaglandin was used up (released) also without division, and its intracellular presence was due to storage rather than to biosynthesis.

As to hPL, the present observations do not exclude that it was native to Tetrahymena. Since, however, hPL was present in the applied growth

medium, evidence is lacking whether it had been taken up from the latter or had in fact been biosynthesized.

It is an experimental fact that while certain hormones (epinephrine, ACTH, somatostatin, relaxin, insulin, endorphine, steroids, etc.) are native to Tetrahymena, T_3 and T_4 are not. Thus, Tetrahymena cannot synthesize all hormones, only certain types of amino acid, polypeptide and steroid hormones. Considering that non-hormone steroids, among others tetrahymanol, also are native to Tetrahymena, there is reason to postulate that the hormone-like steroids and polypeptide-like materials observed in it represent by-products of biosynthesis or, in other words, evolutionary attempts of a highly differentiated unicellular organism.

The hormones not native to Tetrahymena (T_3 and T_4) cannot be induced by exogenous tropic hormone (TSH) treatment, whereas those native to it or stored by it (e.g. prostaglandin) show an intracellular concentration decrease in response to hormone (T_2) exposure.

It follows that extreme caution should be exercised in stating that certain hormone are being *synthesized* by Tetrahymena, and evidence of their biosynthesis should be based on cultures grown either in synthetic medium, or in a medium carefully analysed for hormone content.

Futher studies are required to clarify why prostaglandin and hPL are present in the applied tryptone + yeast extract medium.

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ONE-TRIAL INSTRUMENTAL CONDITIONING IN RATS

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A new method for a single-trial operant conditioning task with food reward in rats is described. The non-contingent reward of the first random lever press resulted in a higher response rate in both 24 and 48-hour retention tests as compared to contingently rewarded individuals. The latency to press the lever was shorter in non-contingently rewarded subjects than in contingently rewarded rats. The response rate in the first 5-min interval of the 24-hour retention test was higher in non-contingently rewarded subjects.

The neuroleptic haloperidol (500 μg/kg., i.p.) given immediately following the "event to be remembered" significantly depresses the response rate compared to that for saline-treated controls.

The advantages of the new method are discussed with respect to its applicability for memory research in animals and especially in neurophysiology and psychopharmacology.

Keywords: Operant conditioning, one-trial learning, memory, haloperidol.

In an one-trial learning experiment, such as the passive avoidance paradigm for rats (PAP), the time during which learning takes place can precisely be specified, and time-dependent investigations of experimentally induced changes of "memory" processes have been widely used to determine the effect of treatment on memory performance. The PAP is simple because aversion can be acquired in a single session and a large number of animals can be tested in a short time. The latency to re-entrance into the dark compartment of a conditioning box by the rat is the dependent variable and can readily be quantified in seconds. This latency of the PAP, used as a reliable measure of memory performance, has been criticized by different authors with regard to the large number of non-associative components, e.g. fear-induced high arousal and/or emotionality, which is, of course, unrelated to the "event to be remembered" (ETBR) [2, 3, 5, 6, 9, 10, 11, 12, 14].

The present experiments were designed to check whether an instrumental single-trial, positively rewarded reinforcement [15] can be used as a valid

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measure of the "strength of association" between a single contingent lever press and the subsequently given food in an operant conditioning procedure and may serve as an index of associative learning or memory.

Materials and methods

Animals. Naive male Wistar rats weighing 270—320 g were used. They were kept under standard conditions, with 12-hour light 12-hour dark cycle, the period for light starting at 6.00 a.m. The rats were housed in groups (4—5 animals per cage ($43 \times 38 \times 22$ cm) in perspex boxes), handled daily and adapted to the laboratory. They had free access to food and water. All testings took place between 9.00 and 12.00 a.m.

Apparatus. The apparatus was a perspex Skinner box measuring $20 \times 28 \times 36$ cm with a grid floor. At the back wall, the box contained a feeder 2 cm above the floor and a plastic lever 17 cm above the floor. The apparatus was illuminated by a 60 W bulb, 50 cm above the box.

Experimental design. In the first week the body weight of the rats was reduced to 85% by food-deprivation (water ad libitum). In the second week, on days 1—4, rats were individually placed for 15 min per session into the Skinner box, from which the lever had been removed for these four days. In this time the rats were trained to eat in intervals of 20—60 s, the chow being food-pellets of 100 mg each, irregularly delivered by the feeder. On day 5 the animals were randomly assigned to one of two groups (treatment (T) and control (C) subjects) of equal size and the lever was reinserted into the conditioning box. For the C subjects the first random lever press was not reinforced. In this case, independently of their behaviour and lever press activity, the rats received 3 food pellets in an interval of 50 s. By contrast, the first random lever press of the T subjects was immediately reinforced by the delivery of 3 food pellets. After they had consumed the pellets, the rats were removed from the Skinner box and returned to their home cages.

The 24 and 48-hour retention tests were carried out on days 6 and 7. The rats were placed individually into the conditioning box and their lever press activities (responses) were recorded electronically in 15-min sessions as a fixed extinction period and separately counted for 3 intervals of 5 min each. The latency from the placing of the animals into the box up to the first lever press was also measured. Thus, for every individual C and T subjects, four different measurements existed: the 24 and 48-hour lever press activity counts per session of 15 min each, the latency to touching the lever, and the sequence of responses in the 5-min intervals.

Treatment. In a second experiment, the rats were divided into two groups of equal size. The animals in one group were treated on day 5 with the neuroleptic haloperidol (Gedeon Richter, Pharmaceutical Works, Budapest in a dose of 500 μg/kg i.p. in a volume of 0.5 ml per rat and administered immediately following the ETBR (the first contact with the lever on day 5). The rats in the control group received an i.p. injection of saline of equal volume at the same time.

Statistical analysis. Statistical evaluation of the data was performed with the Wilcoxon matched-pairs signed-rank test (13) and with the F-test. A probability level of <0.05 (two-tailed) was accepted as significant.

Results and discussion

Reinforcement of the first random lever press for the T subjects resulted in a significant enhancement of the lever press activity relative to the C subjects, as measured in the 24 and 48-hour retention tests (Fig. 1a). It seemed that the T subjects developed an association between the random lever contact and the subsequent reward by food pellets. This interpretation is supported by the fact that the latency to the first lever contact was significantly shorter in the T subjects (296.9 ± 101.1 s) than in the C subjects (563.7 ± 109.0 s), $T = 1$, $p < 0.01$. The rate of responses in the first 5-min interval of the 24-hour

retention test was significantly higher in the T subjects (4.1 ± 1.44) than in the C subjects (1.1 ± 0.52), $F = 7.66$, $p < 0.01$. The time of information input and forming the association (contact to the lever and subsequent food reward) could easily be determined. Thus, it was possible to evaluate precisely the extinction curve for the response activity in time. On the other hand, the results indicated a goal-directed use of the lever on the basis of previously acquired information. This demonstrated, however, that the rats associated

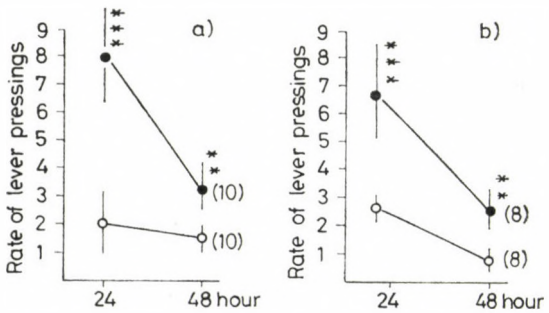

Fig. 1a: Effects of a non-contingent (treatment subjects) and a contingent (control-subjects) food reward on the lever press activity of rats in a single-trial operant conditioning procedure. ●——● treatment subjects, ○——○ control subjects. * $p < 0.02$ ($T = 3$), ** $p < 0.01$ ($T = 0$, $F = 8.48$). (Number of animals in parentheses)

Fig. 1b: Effect of haloperidol on lever press activity of non-contingently rewarded rats in a single-trial operant conditioning procedure. ●——● saline-treatment, ○——○ haloperidol (500 μg/kg, i.p.), post-trial administration. * $p < 0.02$ ($T = 2$), ** $p < 0.01$ ($T = 2$, $F = 31.05$). (Number of animals in parentheses)

the existence and the use of the lever with the food reward. This effect was not obvious in the C subjects.

Post-trial treatment of T subjects with haloperidol in a dose of 500 μg/kg (i.p.) significantly decreased the response rate in the 24 and 48-hour retention tests as compared to saline-treated controls (Fig. 1b). The drug is known to affect memory performance in a negative sense in rats [1, 4, 7]. In our experiments, haloperidol disrupted the development of associations which otherwise could formed be readily in saline-treated or untreated T subjects. Furthermore, there was no difference between the saline-treated and the haloperidol-treated individuals in the latency to the first lever press (saline-treated rats 277.9 ± ± 87.5 s; haloperidol-treated rats 248.8 ± 87.7 s, $T = 12$, $p > 0.10$, n.s.). The data show that the treated rats did not differ significantly in their motor behaviour (general motor patterns, food searching activity, rearing), but they did differ in their goal-directed reaction to the lever.

The response rate in the first 5-min interval of the 24-hour retention test was 2.38 ± 1.46 for the saline-treated controls while it was 1.0 ± 0.31 for the haloperidol-treated rats. ($F = 22.57$, $p < 0.01$).

The data indicate, however, that post-trial treatment with haloperidol in this test paradigm also blocked the development of associations in a single-trial learning task but did not affect the latency to the response to the lever. It seems that the single-trial operant conditioning technique is a useful method for the investigation of memory phenomena in rats, and that it has certain advantages over the PAP. Unlike the PAP, the new test contains no fear components. Fear affects the behaviour of rats as a non-associative element, enhancing species-specific defense responses and the arousal level of the animals and which can cover the response to the ETBR. The conditioning of mammals with a food reward will have in general a higher priority in the ETBR process than punishment conditioning. The variable, strength of association between the lever press activity and the food reward, can easily be quantified in this test as the sum of the responses to the lever at different times according to the information input or the lay-down in the brain of the rat. The approach to the lever indicates that association(s) are established between these events and that traces of these events are formed in the central nervous system [8]. This test seems to be a useful technique in memory research in animals, and is readily applicable to experimental work on psychopharmacology.

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INHIBITION OF PROGESTERONE SECRETION AND DECREASE OF OVARIAN BLOOD FLOW BY ISOXAZOLE IN PREGNANT RATS

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Pregnant rats were treated on day 11 of pregnancy with 0.62, 1.25, 2.5 or 5.0 mg of isoxazole per 100 g of body weight (bw) (Experiment I) or with 2.5 mg of isoxazole per 100 g of bw (Experiment II). In Experiment I animals were anaesthetized with pentobarbital at 48 hr. In experiment II animals were similarly anaesthetized at 0, 12, 24, or 48 hr following administration of isoxazole. Blood was collected separately from the ovary or from the uterus via a thin cannula which was inserted into the utero-ovarian vein. Blood was also collected from the vena cava inferior. The concentrations of progesterone (P), 17β-oestradiol (E_2), and corticosterone were measured in blood samples by RIA. All of the animals exhibited reduced rates of ovarian venous blood flow and rates of P secretion when pregnancy was terminated by isoxazole (2.5 mg/100 g bw or greater). The concentration of P was found to be correspondingly reduced by 90% in ovarian venous blood and by 50% in peripheral blood, relative to the controls. After isoxazole had been administered, the rate of P secretion and also the concentration of P in peripheral blood continued to decrease rapidly and to remain at low levels for at least 48 hr. By contrast, the concentration of corticosterone in peripheral blood decreased during the first 12 hr but then it returned to the control levels by 48 hr. The ovarian secretion rate of E_2 and the concentration of E_2 in peripheral blood remained unchanged during the same 48 hr period. The results suggest that isoxazole causes a decrease in ovarian blood flow in parallel with a decrease in the secretion of P which may promote the irreversible damage of luteal function.

Keywords: isoxazole, inhibition of 3β-hydroxysteroid dehydrogenase, progesterone secretion and blood levels, ovarian blood flow, corticosterone

Isoxazole is a synthetic, heterocyclic steroid which was found to terminate pregnancy in rats on days 8—12 of gestation. Termination of pregnancy could be prevented by administration of progesterone (P) [3, 4]. Several non-steroidal compounds also have been found to interrupt pregnancy during this same period of gestation [5, 8, 11]. Pregnancy termination by isoxazole has been associated with inhibition of the enzyme Δ^5-3β-hydroxysteroid dehydrogenase in the ovaries and adrenal cortex [6, 9]. Rats that were ovariectomized on day 15 of pregnancy required at least 7 ng/ml of P in peripheral blood plasma which was critical for maintaining pregnancy [10]. When isoxazole terminated preg-

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nancy in rats the reduced concentration of P in peripheral blood plasma was, unexpectedly, about four times higher than this "critical" value [4].

The present study was designed to determine in pregnant rats the effects of isoxazole on the secretion rate of P in the ovary and to correlate this with the concentrations of P in uterine venous blood and peripheral blood. The effects of isoxazole on the adrenal cortex were also evaluated by measuring changes in the concentration of corticosterone in the peripheral blood plasma and correlating them with changes in the concentration of P in the same blood samples. The specificity of inhibition by isoxazole of P secretion in the ovary was examined by determining in parallel the rates of secretion in the ovary of 17β-oestradiol (E_2) and the concentration of E_2 in the blood. In luteal ovaries, the secretion of P and rate of blood flow have been shown to undergo parallel changes [15, 18]. Therefore, we considered the possibility that the isoxazole-induced inhibition of the secretion of P may induce a decrease in the rate of ovarian blood flow.

Materials and methods

Isoxazole was synthesized in our laboratory and exhibited the reported physical and spectroscopic characteristics (12). Experiments were conducted on 200 g to 260 g CFY rats maintained on a 13 h light/11 h dark photoperiod. The first day that sperm was noted in vaginal smears was designated as day 1 of pregnancy. For surgery, the animals were anaesthetized with pentobarbital (4 mg per 100 g bw).

Experiment I

On day 11 of pregnancy, the rats (N = 26) were given intramuscular injections of 0.62, 1.25, 2.5, or 5 mg per 100 g bw of isoxazole (in 0.1 ml of sunflower oil). Control rats were injected with 0.1 ml of the vehicle. After 48 hr, the animals were anaesthetized for surgery.

Experiment II

Rats on day 11 of pregnancy (N = 62) were given intramuscular injections of 2.5 mg per 100 g bw of isoxazole in 0.1 ml of sunflower oil or of the vehicle alone. These animals were anaesthetized at 0, 12, 24, or 48 hr after treatment.

Laparotomy was performed on both groups of animals, and a 20 cm polyethylene cannula (1 mm O.D. and 0.6 mm I.D.) was inserted into one of the utero-ovarian veins. The distal end of the cannula was fixed 4 cm below the inferior vena cava. The uterine branch of the utero-ovarian vein was clamped and the volume of ovarian venous outflow was measured as a function of time. Blood samples were collected separately from the ovarian vein (Experiments I and II), from the uterine vein, and from the inferior vena cava (Experiment II). Blood clotting was prevented by heparin. Following the collection of blood, the pregnant uteri were excised, trimmed, weighed, and the numbers of embryos were counted. The viability of each embryo was evaluated visually with a stereomicroscope. The conceptus weight quotient (refered to as the conceptus weight) was taken as the weight of the pregnant uterus divided by the number of embryos. Ovarian venous blood flow and haematocrit were measured and the collected blood samples were assayed by RIA for P [17] and E_2 [1]. Corticosterone was measured in the peripheral blood plasma [7, 13]. From the values of ovarian blood flow, haematocrit, and plasma concentrations of P and E_2 in ovarian venous blood samples, the rates of secretion of P and E_2 were calculated.

Analysis of variance and the Dunnett multiple range test [14] were used for statistical evaluation of the data. The calculations of secretion rates and concentrations of hormones were carried out using logarithmic transformation of data and the geometric means were presented in the tables and figures.

Results

Experiment I

The lowest dose of isoxazole which terminated pregnancy in *all* of the animals was 2.5 mg per 100 g bw. The ovarian venous blood flow and the rate of secretion of P and also the concentration of P in ovarian venous blood plasma

Table I

Dose dependent effects of isoxazole on day 11 of pregnancy in rats

Isoxazole dose mg/100 g bw	Conceptus weight mg	Ovarian blood flow rate μl/min/100 g bw	Ovarian progesterone		Pregnancies terminated/pregnant animals
			conc. μg/dl	secretion rate ng/min/100 g bw	
0	361.6[a] ±15.3 (n = 6)	37.51 ±4.52 (n = 5)	232.6[b] (5.45 ± 0.31)[c] (n = 5)	52.56 (3.96 ± 0.07) (n = 5)	0/6
0.62	351.2 ±6.8 (n = 5)	30.50 ±5.47 (n = 5)	311.5 (5.74 ± 0.21) (n = 5)	49.69 (3.91 ± 0.20) (n = 5)	0/5
1.25	328.2 ±53.7 (n = 5)	24.06 ±6.62 (n = 5)	93.2 (4.54 ± 0.39) (n = 5)	13.26* (2.58 ± 0.56) (n = 5)	2/5
2.50	245.9 ±54.1 (n = 6)	22.40 ±5.37 (n = 5)	105.3 (4.66 ± 0.35) (n = 5)	5.89** (1.77 ± 0.68) (n = 5)	6/6
5.00	161.7** ±4.4 (n = 6)	17.40** ±3.47 (n = 4)	54.9* (4.01 ± 0.53) (n = 4)	4.89** (1.58 ± 0.69) (n = 4)	6/6

a = mean ± S.E.
b = geometric mean.
c = (ln mean ± S.E.)
n = number of experiments; *p < 0.05; ** p < 0.01

decreased in response to increase in the administered dose of isoxazole in the range of 0.62 to 5 mg per 100 g bw (Table I).

Experiment II

After injecting isoxazole (2.5 mg per 100 g bw), the pregnant rats were autopsied at 0, 12, 24, and 48 hrs. Pregnancy was terminated during each of these time periods (except at 0 hr). The number of concepti remained unchanged which showed that resorption of the dead embryos was incomplete (Table II). The rate of ovarian venous blood flow decreased during the first 12 hr following isoxazole administration. After 12 hr, this rate gradually returned to control.

Table II

The number of embryos and weight of concepti following the administration of isoxazole (2.5 mg/100 g bw) on day 11 of pregnancy

	0 hr	12 hr		24 hr		48 hr	
	C	C	T	C	T	C	T
Number of embryos	12.0[a] ±0.9	12.6 ±0.5	11.0 ±0.4	11.7 ±0.6	10.6 ±0.7	11.5 ±0.6	11.9 ±0.3
Weight of conceptus (mg)	157.5 ±9.8 (n = 7)	221.4 ±4.9 (n = 9)	206.8* ±5.1 (n = 12)	217.6 ±5.5 (n = 10)	182.0* ±7.1 (n = 9)	350.4 ±10.2 (n = 6)	187.7** ±15.2 (n = 9)

C = control, T = isoxazole treated
a = mean ± S.E.
n = number of animals in each group
* $p < 0.05$; ** $p < 0.01$

The concentration of P in ovarian venous blood and the rate of secretion of P in the ovaries decreased in a time-dependent manner (Fig. 1). The concentrations of P in both the uterine and peripheral blood continuously decreased for at least 48 hr. The concentration of corticosterone in peripheral blood decreased

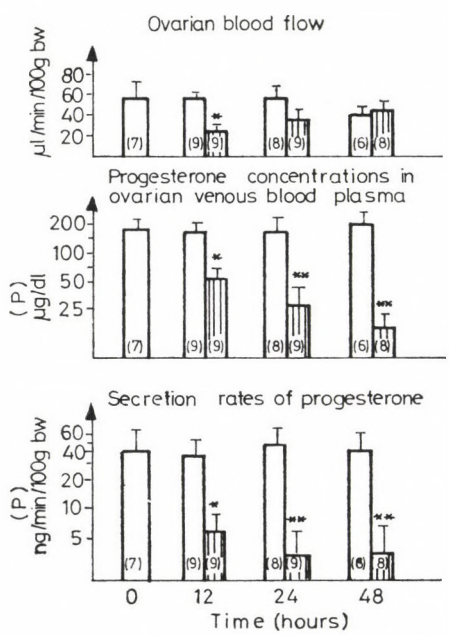

Fig. 1. Effects of isoxazole on ovarian blood flow and progesterone secretion 0, 12, 24 and 48 hrs after the administration of 2.5 mg per 100 g bw of isoxazole (hatched bars) or sunflower oil (open bars). Mean ± SE; the number of experiments are in parentheses; * $p < 0.05$; ** $p < 0.01$

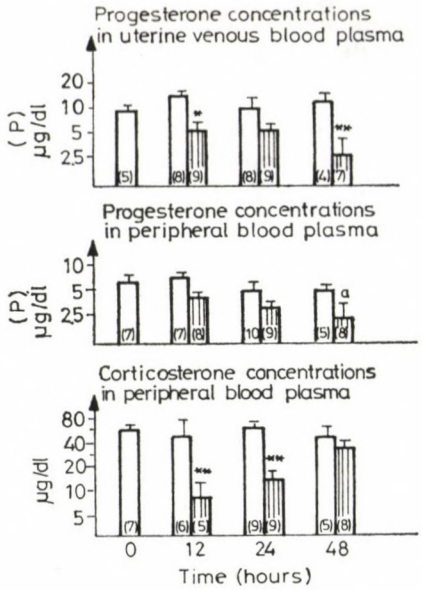

Fig. 2. Effects of isoxazole on the concentrations of progesterone and corticosterone in uterine and peripheral blood plasma 0, 12, 24 and 48 hrs after administration of 2.5 mg per 100 g bw of isoxazole (hatched bars) or sunflower oil (open bars) Mean ± SE; the number of experiments are in parentheses; * $p < 0.05$; ** $p < 0.01$; a = significant difference between the treated and control groups

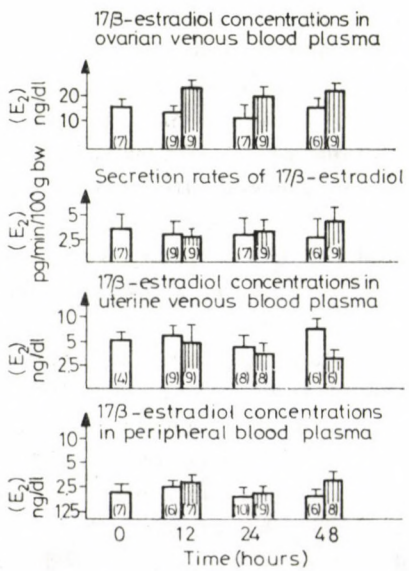

Fig. 3. Effects of isoxazole on the rate of secretion of 17β-oestradiol in the ovary and on its concentration in uterine and peripheral blood 0, 12, 24, and 48 hrs after administration of isoxazole (hatched bars) or sunflower oil (open bars) Mean ± SE; the number of experiments are in parentheses

during the first 12 hr, remained low during the succeeding 12 hr, and then returned to the control level by 48 hr (Fig. 2).

There was a slight elevation in the concentration of E_2 in ovarian venous blood as ovarian blood flow was decreasing. However, the rate of E_2 secretion from the ovary remained practically constant. Similarly, the concentration of E_2 remained unchanged both in the uterine venous blood and also in peripheral blood (Fig. 3).

Following the administration of isoxazole, the rate of secretion and concentration of P in ovarian venous blood decreased by 90% (Fig. 1). The concentration of P in peripheral blood decreased by 50% (Fig. 2). By 12 hr, the concentration of corticosterone in peripheral blood was diminished by 85%. In the control group of animals, the concentration of P in the ovarian venous blood was 15 to 20 times higher than in the uterine blood and 30 to 40 times higher than in the peripheral blood. Isoxazole caused these relationships to undergo dramatic changes. Thus, 48 h after administration of isoxazole, the concentration of P in ovarian venous blood fell to a level which was only about five times greater than the concentration of P in uterine venous blood or peripheral blood.

Discussion

When pregnancy was terminated on day 11, the embryos were found to have lost viability during the first 12 hr after administration of isoxazole. At this time, level of P in peripheral blood plasma was reduced to 30 ng per ml (3 μg/dl, Fig. 2). This appears to be consistent with the concentration of P that was previously reported to be 20 ng per ml in peripheral blood plasma under similar experimental conditions [4]. A similar reduction in the concentration of P in peripheral blood was observed after isoxazole treatment but ovariectomy or aminoglutethimide treatment caused much greater decreases in the P content of peripheral blood [9]. Between day 8 and day 12 of pregnancy we found that there was an increase in the output of P from the ovary [18] and others have shown that there is a greater requirement for P between day 8 and day 12 than before day 8 or after day 12 [8]. This may be associated with placental development. Although the "critical" or minimum concentration of P in peripheral blood for maintaining pregnancy in day 15 pregnant rats was proposed to be 7 ng per ml [10], the concentration of P in peripheral blood was three to four times higher than this values when in the present study pregnancy was terminated by 2.5 mg 100 g bw of isoxazole.

The termination of pregnancy by isoxazole is accompanied by decreased concentrations of P in uterine venous blood and peripheral blood, a decrease in the concentration of corticosterone in peripheral blood, and by the inhibition of Δ^5-3β-hydroxysteroid dehydrogenase in the ovaries and adrenals [6, 9].

In the present study, it was presumed that the synthesis of P was inhibited by isoxazole because a 90% decrease in the rate of secretion of P in the ovaries was observed (Fig. 1). Also, the concentrations of P in the uterine venous blood and peripheral blood decreased by about 50%. The differences between the two rates of decrease suggest an accumulation of P in peripheral blood. This implies that isoxazole interferes with P metabolism and its clearance from peripheral blood.

Possibly, isoxazole terminates pregnancy in rats by interacting with cellular P receptors in analogy with what has been found with other antiprogestagens [20], and with Danazol [2]. Administration of P (4 to 5 mg per rat) was found to prevent isoxazole-induced termination of pregnancy. The exogenous P was presumed to compensate for the reduced output of P from the ovary [3, 4]. The administration of P caused a several-fold increase in the concentration of P in peripheral blood relative to the controls. Most likely the elevated concentrations of P effectively protect the P receptors in the uteroplacental unit against isoxazole by P occupying all of the receptor binding sites.

Isoxazole inhibited the output of P from the ovaries and it also decreased ovarian blood flow. These observations agree with earlier findings that changes in the secretion of P and ovarian blood flow often occur in parallel [15, 18]. However, 48 hr following the administration of isoxazole the rate of ovarian blood flow in the animals of Experiment II returned to that of the controls but the rate of secretion of P continued to decrease (Fig. 1). Evidently, the doses of isoxazole which terminated pregnancy irreversibly reduced the rate of secretion of P in the ovaries by impairing luteal function. However, these are not necessarily the limiting factors in terminating pregnancy because the viability of the embryos was lost during the first 12 hr after isoxazole was administered.

In contrast to the changes in the concentration of P in peripheral blood plasma, the concentration of corticosterone in peripheral blood returned to the control level 48 hr after the administration of isoxazole (Fig. 2). These data suggest that adrenal cortex function was reversibly inhibited by isoxazole. Even though the concentration of corticosterone in peripheral blood decreased during the first 12 to 24 hr after administration of isoxazole this could not have contributed to the termination of pregnancy in the rats because it has been shown that following adrenalectomy pregnant rats deliver normal numbers of pups at term [16].

The inhibition of ovarian P synthesis induced by isoxazole seems to be highly specific. This conclusion is based on the observation that following the administration of isoxazole the rate of secretion of E_2 and the concentrations of E_2 in uterine venous blood and in peripheral blood remained unchanged throughout the experiments (Fig. 3) while the secretion of P steadily decreased. However, the P/E_2 ratios underwent a continous decrease because while the

secretion of P continuosly decreased the secretion of E_2 remained constant. The change in P/E_2 ratios may be another factor which contributed to the termination of pregnancy in the rats by isoxazole.

Measuring the ovarian blood flow and also the output of P from the ovaries was useful in revealing the nature of the inhibitory effects of isoxazole on the ovaries in pregnant rats. These data have shown that marked decrease in ovarian blood flow caused by isoxazole occurs simultaneously with the decrease in the rate of secretion of P. The results point to the regulatory relationship between the process of P production and ovarian blood flow (19). Even though the substrates for hormone synthesis and the necessary stimulating hormones are carried to the ovaries by the blood, the decrease in ovarian blood flow caused by isoxazole probably contributes to the irreversible damage of luteal function.

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SEPTAL DEAFFERENTATION PRODUCES CONTINUOUS RHYTHMIC SLOW ACTIVITY (THETA) IN THE RAT HIPPOCAMPUS

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Hippocampal EEG was recorded in behaving rats in which the brain stem afferents to the septal region were previously damaged. In these animals rhythmic slow activity (RSA or theta) was continuously present, including drinking and immobility. The average frequency of RSA, however, was significantly higher during running (8—9 Hz) than during drinking or awake motionless state (6—8 Hz). In normal rats irregular sharp waves, rather than RSA were present during drinking and immobility. The results suggest that brain stem afferents are necessary to suppress the rhythmic firing of septal "pacemaker" cells.

Keywords: hippocampus, septum, theta, EEG, deafferentation

Circumstantial evidence indicates that the rhythm generator of hippocampal rhythmic slow activity (RSA or theta) is the cholinergic and noncholinergic pacemaker cell assembly of the medial septum and the nucleus of the diagonal band of Broca [3, 9, 15]. Axons of the pacemaker cells reach the hippocampal formation via the fimbria-fornix, the supracallosal stria and a ventral path [5]. Neurones of the septal area innervate also the entorhinal cortex which, in turn, supplies the major input to the hippocampal formation (8, 11). One of the main targets of the hippocampal outputs is the lateral septum [6, 7].

Hippocampal RSA may be due to a rhythmic series of somatic IPSPs in pyramidal neurones and granule cells coupled with rhythmic, but phase-shifted, EPSPs at the level of dendrites. The somatic dipoles are formed by GABAergic interneurones interposed between the septal projection and the principal cells of the hippocampus [2, 4]. The excitatory dipoles, on the other hand, are set up by rhythmic excitation of the principal cells by the entorhinal input [4, 13].

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At the behavioural level, RSA is correlated with preparatory-exploratory behaviour such as walking, rearing, sniffing, head turning. Immobility and consummatory activities, on the other hand, are associated with irregular sharp waves [4, 12].

Regarding the septal control of RSA the question is what regulates the pacemaker versus irregular states of the neurones in the septal area. Is the integrity of the hippocampo-septal connections necessary for the rhythmic firing of the pacemaker cells (6), or is the rhythm generation in septal neurones triggered by subcortical afferents to the septum. The answers to both questions are probably "no" because at least some cells in the deafferented septum and furthermore, the cells of the isolated septal area continue to fire in rhythmic bursts *in vitro* [14].

The present experiments raised the questions whether the deafferented septo-hippocampal system was capable of sustaining hippocampal RSA in the freely moving rat, and whether in such preparations the behavioural control of hippocampal EEG was preserved.

Methods

Seven male hooded rats (250—350 g) were anaesthetized with pentobarbital (40 mg/kg) and placed in a stereotaxic apparatus with bregma and lambda in the horizontal plane. A special knife was used to cut all subcortical afferents to the septum; the tool was introduced through the lateral ventricle 1.5 mm anterior to the bregma, 2.3 mm from the midine and 7.5 mm from the dura. A horizontal knife of 2.0 mm was pushed from the needle and the tool was moved 3.0 mm cranially. The knife was then moved up to the surface of the brain and removed. From two weeks to 4 months after the operation the rats were implanted with hippocampal recording electrodes as described elsewhere [4]. After recovery they were trained to run in a Wahman wheel for water reward [4]. The EEG was recorded on magnetic tape and by a polygraph. After the experiments the rats were perfused with saline, 4% K-ferrocyanide solution (Prussian blue reaction) and 10% formalin. Frozen sections were cut (30 μm) and stained with Nissl method.

Results

Isolation of the septum from subcortical inputs appeared to be complete in 6 animals. The cut isolated the vertical limb of the diagonal band from the horizontal limb. Slight damage of the caudate-putamen and thalamus was present in all rats. The contralateral septum was isolated only incompletely (Fig. 1).

Large amplitude hippocampal RSA of 8—9 Hz was present in all animals during wheel running and walking. The most striking difference from normal rats was the absence or low frequency of sharp waves during drinking and immobility. Instead, continuous and regular RSA was observed during these behaviours (Fig. 2). The frequency of RSA, however, was significantly lower (6—7 Hz; $t = 2.95$, $p < 0.002$) during drinking a than during running (8—9 Hz).

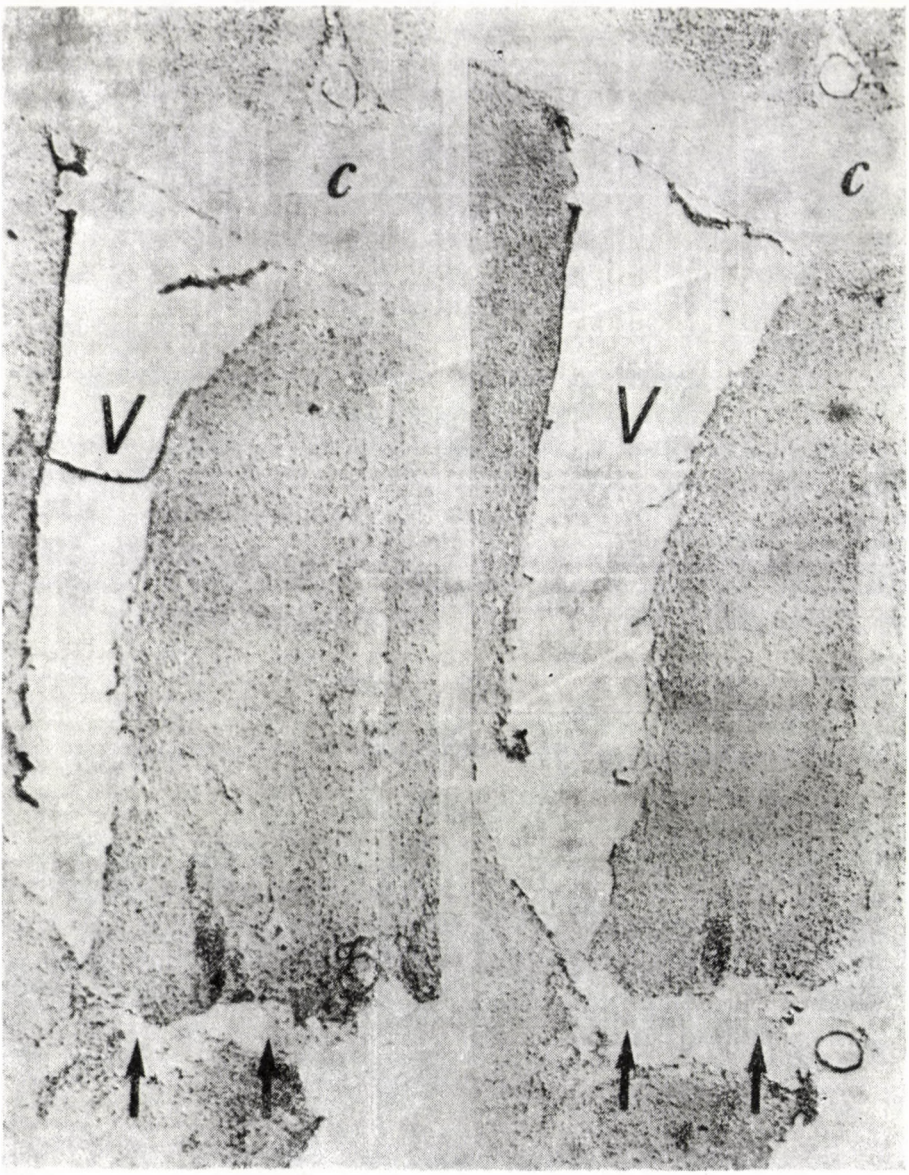

Fig. 1. Frontal sections at two different levels of the septum. Arrows indicate the knife cut. Nissl stain. V: ventricle, c: corpus callosum

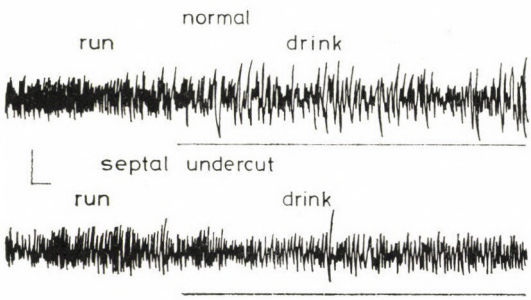

Fig. 2. EEG activity recorded from the molecular layer of the dentate gyrus during running-drinking transitions. Horizontal line: drinking. Above: normal rat, below: animal with deafferented septum. Note persistence of rhythmic slow activity during drinking in the deafferented rat

Discussion

The present experiments showed that hippocampal RSA is preserved after undercutting to the medial septal area. Moreover, RSA dominated the hippocampal record also during immobility and drinking, behaviours which are normally associated with irregular sharp waves.

The results thus supported the view that neurones of the septal area are capable of sustaining their rhythmic or "pacemaker" properties even in the absence of brain stem afferents [14]. Furthermore, the findings allowed the conclusion that it is the irregular EEG state of the hippocampus which requires an intact brain stem control, probably via suppressing the rhythmic activity of the septal pacemaker cells. One such afferent path may be the dopaminergic system. Dopamine containing neurones in the mesencephalic A 10 cell group exert a tonic inhibitory control on the cholinergic septohippocampal pathway [10]. Dopamine-depleting 6-hydroxydopamine injections in the septum resulted in an increase in choline uptake in the hippocampus. In the present experiments the continuous presence of RSA may have reflected an increased choline uptake.

The importance of the surviving hippocampo-septal connections is stressed by the finding that the frequency of RSA was significantly higher during running than during drinking. This finding suggests that the remaining afferents to the septo-hippocampal system exert an important modulatory role on the frequency of RSA in intact animals as well. Such a candidate may be the entorhinal input-hippocampus-septum path [7]. It may be assumed that the intact brain stem-entorhinal connections are still capable of exerting context-specific influence on the neurones of the entorhinal cortex projecting to the hippocampus.

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AUTOPHOSPHORYLATION OF UNTRAINED AND WELL-TRAINED SKELETAL MUSCLE MYOSIN

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Earlier the autophosphorylation of myosin and the labile phosphate (P) content of rabbit skeletal muscle was reported [6, 7, 9]. The present paper describes that the endogeneous preformed P level in fresh preparation of exercised muscle is higher than that of untrained control one.

It was revealed that the presence of a significant amount of mitochondrial myosin (with much higher P content) in the well-trained human muscle preparations falsified the appreciation of myofibrillar myosin. Therefore, a reliable myofibrillar preparation with correct P content from exercised subjects was obtained only after the separation of mitochondrial fraction. The P content of fresh preparations can be increased by phosphorylation even in the exercised muscle myosins up to the most higher level in human samples.

The phosphoryl group incorporation from [γ-^{32}P]ATP into the rabbit and hare myosins was checked by radioactive tracer technique, and confirmed by total P content determination performed parallel with molybdate test.

It was stated that under present circumstances the labelled ^{32}P incorporation was lower even at an optimal substrate concentration than that of P value obtained directly with molybdate method; because the total P content of preparations had not exchanged during 2 min incubation.

So it has been concluded from [γ-^{32}P] phosphoryl group assayments that much higher amount of P was incorporated into P-Arg, N^{π}-P-His and fraction 2 as compared with unappreciated labelled P level of the inorganic P (P-Ser, P-Thr), P-Lys, N^{τ}-P-His and minor fractions. From these observations it has been considered that the P-Arg, N^{π}-P-His and fraction 2 take part in the contraction mechanism and in the course of physical training.

Keywords: exercised muscle myosin, phosphate content, effect of exercise, phosphorylation capacity, active P distribution

It was reported the myosin of rabbit dorsal muscle contains average 12 mol P/mol myosin [5]. The P contents of fresh myosins from the muscles of well-exercised persons were also described because they had always higher values than the preparations of an average conditioned human or mammalian samples [8]. Moreover, a phosphate (P) release was observed during storage from the preformed (endogeneous) P contents of myosin preparations of rabbit

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skeletal muscle. Later on that was revealed the P content of myosins can be increased by incubation in a suitable phosphorylating medium; namely 3—5 mol P incorporation was found into myosin from the ATP-containing medium [6, 7]. Then, the investigations of phosphorylating capacity revealed the number of binding sites for phosphoryl groups which is higher in myosin of exercised muscles than is in control preparation [12].

Further experiments have been confirmed of the higher amounts of "preparative phosphate" of exercised muscle myosins, revealed that, the surplus of P content consist of the acid labile, N—P binding types of amino acid phosphates. In addition to a hare was observed having bred for one half a year in a cage, moreover some sportsmen, who stopped their activity, in consequence of this it was experienced that the preformed P content of myosin returns to the P level of rabbit or human level with average activity [7, 8].

In previous study is described the basic amino acid phosphates of myosin samples are provided by P-Arg, P-His and a small P-Lys [7], those can be separated from the alkaline hydrolysates of myosin [9]. The amino acid phosphates give about the 70% of P contents in gel filtered myosin hydrolysates. As above-mentioned there are found 3—5 minor P containing peaks, too. So, that was altogether 9 peaks observed in hydrolysates [10].

The P contents of the chromatographic fractions do not decrease by the similar scale during the storage or phosphorylation. This has directed our attention for to study the different mobility of the P containing peaks. We find necessary to clear up that which peak's phosphoryl group is exchanged or is labelled during the short incubation time in the medium containing [γ-^{32}P]ATP and which molybdate-positive peak's P contents does not vary during the incubation.

The competence of the experiment is considred because of the mobile amino acid phosphates take part in the contractile processes of the muscle. Moreover in the study was only recognized by the "in vitro" investigations that the "active" P contents of myosin is reduced by the actin directly depending on its concentration.

Materials and methods

The experiments were carried out with myosin preparations purified from long dorsal muscles of 9 weeks old domesticated rabbits, and adult rabbits (half years old), wild rabbits, and hares (1.5 years old), and the myosin preparations from medial gastrocnemius muscles of sportsmen.

For the phosphorylating studies are used labelled [γ-^{32}P]ATP (specific activity 0.4—0.7 GBq, 10—19 mCi/mmol purchased from institute of isotopes of Hung. Acad. Sci., Budapest, Hungary) applied in 90—100 000 cpm per 1 mmol ATP in all normal incubation medium, however, was higher when the incorporated P distribution were investigated to be perceived the minimal P exchange in small fractions, too.*

* The used chemicals were: Tris-(hydroxymethyl)-amino-methan, KCl, NaCl, $KHCO_3$, $NaHCO_3$ (Reanal, Budapest); HNO_3, H_2SO_4, ammonium molybdate, 2-mercaptoethanol

The freshly excised muscle was cooled among ice cubes to 0 °C and cleaned from the connective tissue was minced in a pre-cooled mincer. 100 g mince was mixed with 5 vol of 100 mmol KCl solution containing 15 mmol Tris-HCl buffer, pH 7.2, and centrifuged at 4000 g, 20 min in order to get rid of the soluble proteins. Then the washed mince was suspended in 3 vol solution of 0.6 M KCl, 15 mmol Tris-HCl, pH 7.2, 2 mmol 2-mercaptoethanol and stirred it slow mixing to extract the myosin. After 10 minutes the insoluble residue was removed by centrifugation (15 000 g, 20 min). The supernatant was diluted with icy distilled water to 11 times its original volume in order to the myosin to be precipitated. When the forming of additional myosin flakes were not observed, the precipitation was sedimented by centrifugation (15 000 g, 20 min). Then the pellets was suspended in a minimal volume of 2 M KCl, and diluted it with a solution of 0.6 M KCl, 8 mmol $KHCO_3$, 1 mmol DTT under a stirrer, so that the protein concentration be reduced to about 10—15 mg/ml. The gained myosin solution was turbid it was centrifuged (20 000 g, 30 min) to clear up and the sediment throwed away. From the translucent supernatant the myosin was flocullated by dialysis at first against distilled water followed with 25 mmol KCl solution. The myosin flakes were collected by centrifugation again and dissolved as earlier.

The RNA and lipid remnants from transparent myosin solution were removed with DEAE-cellulose treatment [10]. The denaturated proteins and the actomyosin traces were removed by ultracentrifugation (105 000 g, 120 min). The purification were finished by gel filtration using Sepharose 4B (2.3 × 84 cm) column.

The protein phosphatase activity of preparations were checked with Ehler's method [4]. The phosphatase activity was not perceived in gel filtered myosin, it disappeared with the first DEAE-cellulose treatment from the preparation.

The protein contents of the preparations were estimated by biuret micro method [14] and uv absorbtion measured at 280 nm and checked by the dry material determination of the lipid- and salt-free myosin proteins gained by gravimetric measurement (at 105 °C after 5 hours).

The P contents were determined by the method of Fiske and Subbarow [13], except the final reduction reached with 1% ascorbic acid by Lowry and Lopez's method [21]. The total P contents were measured in the inorganic residues of the gel filtered, lipid-free, non-phosphorylated, phosphorylated and control samples. The traces of RNA and nucleotide in the gel filtered and phosphotylated preparations were determined by Schneider's pentose analysis method [28], and the results were taken into correction at the calculation of real P content.

The phosphorylation of myosin in case of rabbit skeletal muscle was performed 5 mg protein containing samples. However, in exercised muscle samples — as has higher preformed P content — was used far less myosin for phosphorylation. The incubation and removal of excess nucleotide and adsorbed phosphate was carried out in the earlier described way [10].

The radioactive phosphorylating mixture was containing 1.4 mg myosin from skeletal muscle, 25 mmol Tris-HCl, pH 7.6, 12 mmol $MgCl_2$, 1 mmol ATP or 1 mmol labelled [γ-^{32}P]ATP (90—100 000 cpm, except in two marked investigation, per 1 mmol ATP), the phosphorylation was started by the adding Mg-ATP mixture and exactly at 2 min incubation mixed with 60 mmol NaCl and immediately stopped with icy acetone. The further manipulations (as excess nucleotide and P removal, and lipid removal) was performed in the already mentioned way. The P incorporation was determined after solubilised samples in alkali (0.5 M NaOH) in lipid-free protein by measuring the radioactivity, and parallel the P content determination in the inorganic residues of labelled ATP samples with molybdate test.

When the dependence of myosin P saturation on ATP concentration was examined, the samples incubated a labelled and a non-labelled ATP containing medium with rising concentration, and stopped the phosphorylation at the time of maximum saturation by the addition of ice-cooled acetone.

On examining the phosphate saturation depending on the incubation time, the myosin is incubated in 1 mmol ATP containing medium so, that the phosphorylation terminated at a gradually increasing incubation time by adding acetone.

Finally, the incorporation of labelled P in the myosin samples of exercised muscles were investigated, as described above. However, the excess nucleotide, phosphate and lipide are removed from the phosphorylated samples and hydrolyzed they in 3 M KOH (105 °C,

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(Merck, Darmstadt); ATP, DEAE-cellulose, Dextran blue, Serotonin sulphate, Dithiothreitol (Serva, Switzerland); Sepharose 4B(CL 4B) (Pharmacia); EGTA (Fishmans, England); DTNB = 5,5'-Dithio-bis-/2-Nitro-Benzoe-saure (Fluka, Switzerland).

10—16 hours) and separated each hydrolyzate by ion-exchange chromatography method to obtain P containing fractions [9, 10].

The radioactivity of different samples were measured by fluid scintigraphy method by determination the Cherenkov radiation (Beckman Ls). Simultaneously, the P contents are measured in the same samples directly with the molybdate test.

Results

In the lipid-free myosin samples preparated from hare's muscles the P contents is higher than in the preparations of homologous muscles of wild rabbit and domesticated rabbit with less movement.

The gel filtration profile of hare's myosin preparation is shown in the Fig. 1.

Although the gel filtration profile shows one peak, and numerous investigations proved that the gel filtered preparations contain the mixture of at least 3 muscle myosins [20, 22, 23, 26, 27, 31]. It can be supposed that the source of isomyosins are not only the myofibrils, but have the mitochondrion and besides — by other experiments — the sarcoplasm, too. Already earlier has been observed, that the number and the size of mitochondria from exercised muscles is higher than in control ones [15, 16, 18]. That is way we found reasonable that the mince of exercised muscles to be separated in some cases to mitochondrial and to myofibrillar fractions. In these cases the myosins are prepared separately as myofibrillar and mitochondrial ones. On Figures 2 and 3 are shown the gel filtration profile of mt-myosin (mitochondrial myosin) and mf-myosin (myofibrillar myosin) from muscles gastrocnemius medialis of two exercised sportsmen.

On comparing the figures it could be seen that when the two myosins are gel filtered together, the mt-myosin present in a lower concentration is

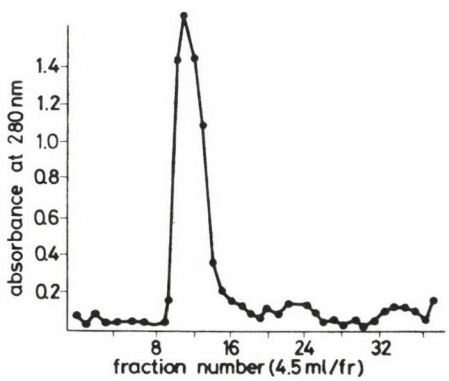

Fig. 1. Gel filtration profile of the skeletal muscle myosin on Sepharose 4B column (1.9 × 84 cm). Myosin was prepared from m. rectus femoris of hind leg of bare (Lepus europeus). 84 mg ultracentrifuged myosin in 4.5 ml volume was applied on column and were the tubes of first peak collected as myosin

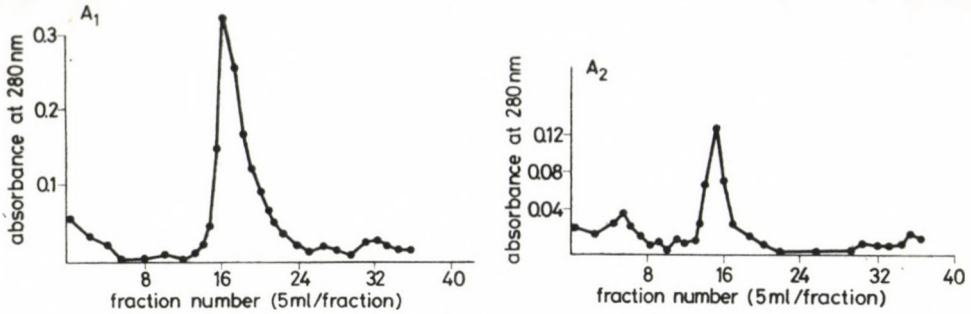

Fig. 2. Gel filtration profile of exercised human skeletal muscle myosins (fencing champion) on Sepharose 4B column (1.9 × 84 cm) prepared separately from myofibrillar (A_1) and mitochondrial (A_2) fractions of m. gastrocnemius medialis. A_1 = Gel filtration profile of myofibrillar preparation yielded 15 mg myosin (0.30%) from 5 g muscle mince. A_2 = Profile of mitochondrial preparation yielded 2 mg myosin (0.040%) from the same quantity of muscle mince

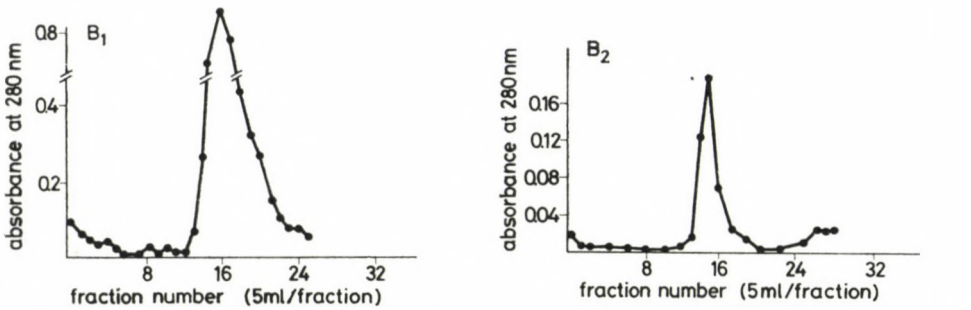

Fig. 3. Gel filtration of exercised human skeletal muscle myosins (of a football player) on the same column those were prepared from myofibrillar (B_1) and mitochondrial (B_2) fractions of m. gastrocnemius medialis. B_1 = profile of myofibrillar preparation yielded 27.66 mg myosin (0.346%) from 8 g muscle mince. B_2 = profile of mitochondrial preparation yielded 1.85 mg myosin (0.023%) from the same quantity of mince

concealed and a component in the common peak; similarly it is present in the myosin preparations without particle fractioning from the whole muscle. When the two myosins were prepared from distinct fractions the molecular weight — on basis of gel filtration profile — appears to be higher in mitochondrial preparation. Taking into consideration the higher lipid [1] and total P content of mt-myosin the apparent higher molecular mass seems to be reasonable.

The data of preparations purified and investigated under similar circumstances are collected in Table I for comparison.

The Table I shows that the lipid-free myosin samples from homologous muscles of domesticated rabbit and wild rabbit have the lowest P content, while the hare long. dorsi and human gastrocnemius medialis muscle preparations have the highest.

Table I

Yield and P content of myosin from the exercised rabbit hare, and human skeletal muscle

Sample	Mince, g	Yield of gel filtered myosin		$E_{280/260}$	P content of lipid-free myosin	Lipid P	Ribose content	P content in phosphorylated lipid-free sample[a]	
								control*	$KHCO_3$**
		mg	%		mol/mol				
M. long. dorsi									
rabbit	95	3390	3.56	1.50	8.85		0.18		10.8
rabbit	90	2697	2.99	1.52	4.59	1.14	3.0	1.19	44.8
wild rabbit	150	2670	1.19	1.60	7.0		0.18	2.95	16.6
wild rabbit	140	3400	2.4	1.61	6.3		0.46	4.0	20.0
hare	300	530	0.176	1.53	33.6	4.29	1.06	3.7	59.6
hare[b]	140	300	0.21	1.44	10.96	6.0	4.3	1.06	49.6
M. gastr. med.									
human^{+}	6	43.8	0.73	0.97	110	22.3	4.25	14.5	85.7
human^{+++}	4.3	45.2	1.05	0.98	30.8	12.0	9.0	12.85	66.5
human^{++}	5	mf. 15	0.30	1.18	13.62	4.4	2.2		
		mt. 2	0.04	0.84	156.1	25.1	0.9		
human^{++++}	8	mf. 27.6	0.346	1.20	12.34	6.34	1.0		
		mt. 1.85	0.023	0.85	52.0	51.8	6.0		

[a] Effect of incubation in ATP-free medium, control*, causing P release in sample compared with lipid-free sample

** The high pH of medium (9.3) maintained and adjusted by $KHCO_3$ produces the highest P incorporation in myosin

[b] This hare was breeding in cage for five months so he is one year-old at the time of myosin preparation

[c] Human muscle samples origin from tendom-break sportmen obtained at the time of local restore operation after the accident on the day 2^{+}, 3^{++}, 10^{+++}, 11^{++++}

When the hare — after capturing — for a long period as 5 months is bred under similar conditions as the domesticated rabbit the P content of myosin decreases to 1/3rd values compared to the muscle myosin P of the captured hare.

In the last 2 columns of Table are displayed the behaviour of samples under different phosphorylating circumstances. It can be seen that in the control samples (incubated without ATP) the quantity of P falls further compared to the fresh lipid-free preparation, however, the samples are incubated a medium with extremely high pH (9.3) they saturated maximally, i.e. higher than in the medium with pH 7.25.

When the myosin from mitochondria of exercised muscle is prepared separately it is revealed that the P content of mt-myosin being the highest, and the P quantity of mf-myosin is lower than that of the non-particulated myosin preparations. It is not economical to isolate the mitochondria and to make myosin preparation from homologous (white) muscles of domesticated

rabbits. Only a great quantity of muscle gives a sufficient mass of mitochondria enough for isolation of myosin. However, can be prepare mt-myosin from the sportsmen because the mitochondrial protein gives the 7.24 and 6.6 per cent of total muscle protein of them, respectively.

It was observed in the case of human preparations, that the resting-time before the operation has effect on the amount of phosphate in myosin, because the P content of preparations from exercised muscles decreases parallel with the number of days of restcure, and this could be perceived in the mitochondrial preparations, too.

Here must be call attention, that in column 3 of the table only the mt-myosin is indicated the approaching quantity of myosin that estimated in muscle. Myosin preparations from whole muscle, and myofibrils can not indicate the real values, because for that to be produced actin-free myosin was only applied 10 minutes of extraction time, and the a precipitation method with dilution technique by which could be get the obtainable myosin proportion as are evaluated and presented in per cent of muscle mass.

In spite of the higher P contents of hare myosin has incorporated a considerable quantity of phosphoryl group during phosphorylation. The phosphoryl group intake of myosin depend on the Mg^{2+} (Fig. 4) and ATP concentration (Fig. 5) in incubation medium.

High Mg^{2+} and also ATP concentration (above 3—4 mmol), respectively, prevents the incorporation of phosphoryl group.

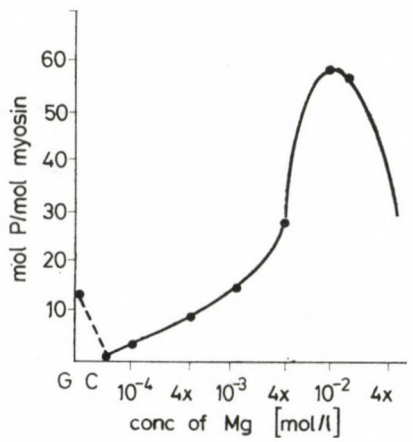

Fig. 4. Effect of Mg^{2+} concentration on the phosphate incorporation of samples of gel filtered hare myosin. The phosphorylation were assayed in 2.5 ml of a solution containing 1.89 mg myosin and increasing amounts of $MgCl_2$ added before preincubation at 30 °C in the same medium described in method. The phosphorylation was started with 1 mmol labelled ATP in final volume (spec. act. ≃100 000 cpm/mmol [γ-^{32}P]ATP added to each incubation medium), and terminated with the addition of ice-cold acetone at maximum phosphate saturation (2 min). The lipid- and nucleotid-free samples were dissolved in 2 M NaOH and an aliquot of sample was applied for radioactivity measurement. C = control, incubated in ATP-free medium

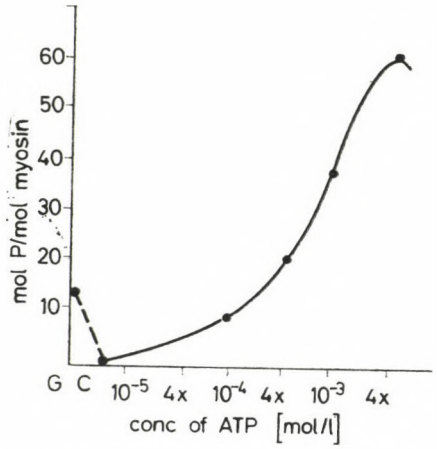

Fig. 5. Effect of ATP concentration on phosphorylation of gel filtered hare myosin. The samples containing 1.89 mg myosin in the same incubation medium that described earlier and increasing amounts of labelled ATP (spec. act. ≃100 000 cpm/mmol [γ-^{32}P]ATP) were applied in proportional rate to proper final ATP concentration. The phosphorylation was started with the addition of myosin (0.5 ml) in each incubation medium, and terminated at 2 min of incubation time. The others were performed by legend of Figure 4

The skeletal myosin preparation of the young (2 months old) domesticated rabbits incorporated more phosphoryl groups (up to 10—15 mol P/mol), than that of the older animals. However, has never reached — not even approached — the phosphate saturation of preparations of hares (Fig. 6).

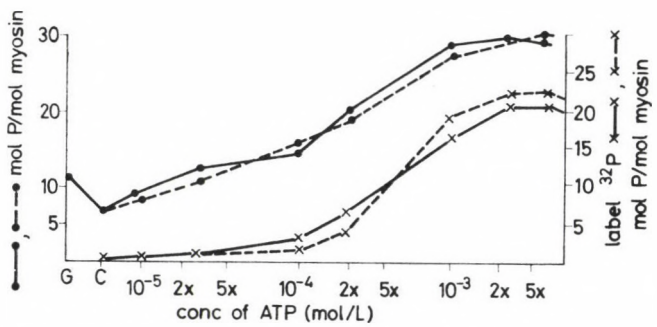

Fig. 6. Increase of bound P content of a young rabbit skeletal muscle myosin in course of ATP-dependent phosphorylation. 7.1 mg myosin was incubated in each incubation medium in the presence of increasing ATP or labelled ATP. Both ATP concentration were used at a proportional rate to final amounts; specific activity ≃100 000 cpm/mmol [γ-^{32}P]ATP in the same medium as described. The phosphorylation was started with the addition of Mg-ATP mixture containing ATP in an appropriate proportion shown at abscisse, and terminated at 2 min incubation by ice-cold acetone. G = lipid-free gel filtered myosin, C = control incubated in ATP-free medium, ●——● and ● — — — ● total P content calculated on the basis of direct P determination (molybdate method); radioactivity ×——× and × — — — × by liquid scintillation was determined in an aliquot of NaOH dissolved lipid- and nucleotide-free myosin samples. (Liquid scintillation in Beckmann LS 35, efficiency: 44%)

During the 2 min incubation the phosphoryl groups of myosin preparations at a low concentration of ATP in medium do not exchange in an observable quantity. That is why for the total P quantity in the parallel series were always gained with higher values by the direct molybdate test determination than the data obtained from the labelled phosphoryl group incorporation studies, calculated for mol P/mol myosin.

The effect of some chemicals and hormones on the P saturation was also investigated. The results can be seen on Table II. It could be known by data, that the DTNB and Cu^{2+} given before the incubation prevent the transfer of the phosphoryl groups, in addition decreases the P content, while the cAMP, serotonin and triiodtyrosin — in the examined concentration — inhibit only slightly the P incorporation.

Table II

Phosphate incorporation into rabbit's skeletal muscle KCl-myosin (The P content are calculated to mol P/mol myosin)

Experiment	Incubated in 1 mmol unlabelled ATP-containing medium determined by directly with molybdate method	Incubated in presence 1 mmol [γ-^{32}P]ATP-containing+ medium	
		determined by directly with molybdate method	measured by scintillation method parallelly
Gel filtered and lipid-free sample	5.5		
Control*		6.7	1.8
1 mmol ATP	22.0	27.5	22.2
1 mmol ATP, 0.31 mmol cAMP	23.0	21.0	18.5
1 mmol ATP, 1 mmol Ser	24.0	26.0	13.8
1 mmol ATP, 1 mmol triiodtyrosin	14.2	11.2	8.8
1 mmol ATP, 2 mmol DTNB	4.15	3.85	2.1
1 mmol ATP, 0.42 mmol Cu^{2+}	4.1	4.8	0.20
1 mmol ATP, sodium carbonate buffer**	47.0	43.0	28.0

+ All incubation medium contain 1 mmol labelled ATP (spec. act. 394 000 cpm/mmol [γ-^{32}P]ATP)

* The labelled ATP added to medium after acetone precipitation

** Incubated in 100 mmol $Na_2CO_3/NaHCO_3$ buffer, pH 9.3

As the estertype phosphates (P-Ser, P-Thr) hydrolysed in alkali, in consequence of hydrolysis producing inorganic phosphate which could be easily determined.

For this verification was investigated the labelling of P containing fraction separated from alkaline hydrolysates of hare myosin preparations. A larger quantity of freshly gel filtered myosin (9.2 mg) was phosphorylated parallel

from the same hare preparation that is presented in Figs 4 and 5. The phosphorylated nucleotid- and lipid-free myosin was hydrolysed in a teflon ampoulle and after determined the total P content and the radioactivity from an aliquot of the hydrolysates the P containing fraction were separated by chromatographic technique. Prior to separation the KOH concentration of the hydrolysates was diluted to 0.005 mol and percolated it through a Dowex I X8 column, then the P containing fractions were separated by the method of [9]. In every effluent tube or in its aliquot the P contents was determined with molybdate method and by the measurement of the radioactivity. The results are represented in the Fig. 7.

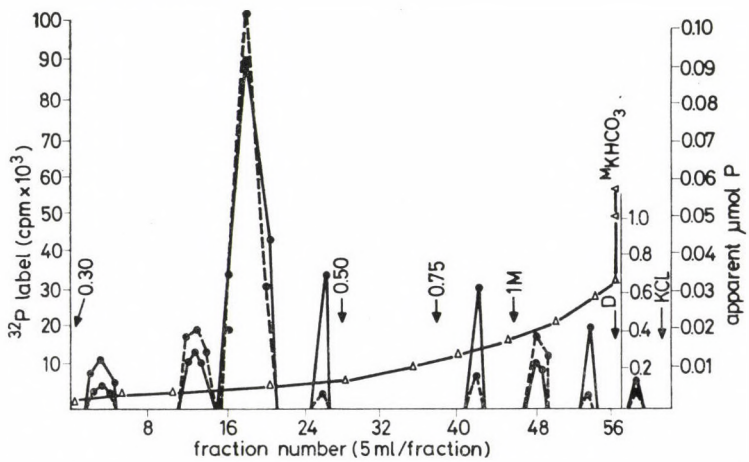

Fig. 7. Incorporation of phosphoryl groups from 1 mmol [γ-^{32}P]ATP (specific activity $\simeq 5 \times 10^6$ cpm/mmol) (into hare's muscle rectus femoris) myosin. Incubation mixture contains 9.2 mg myosin in 5 ml of medium that described in method. After 5 min preincubation the phosphorylation was started by the addition of Mg-ATP mixture and stopped at 2 min incubation with 4 volume ice-cooled acetone. After the usual procedure the lipid- and nucleotide-free phosphorylated myosin was hydrolysed in teflon ampoulle in 3 mls of 3 M KOH (10 h, 105 °C) and diluted to 0.005 M KOH concentration to be percolated through Dowex 1 X8 (0.9 × 6 cm) column. The separation was performed with a linear step gradient chromatographic technique as detailed in [9, 10]. The radioactivity was determined in each effluent tube by liquid scintillation technique, followed by P content determination directly on the basis of specific molybdate test. Every P containing peak is represented as µmole P and total scintillation in each tube (5 ml), applied the same numbering for peaks as the Table III

It can be seen on the figure that the radioactivity of the fractions 4, 8, 9 do not show an appreciable activity, the fractions 1 and 6 are labelled moderately. Maximum phosphoryl group incorporation occurred into fractions 3 (P-Arg), 7 (N^{π}-P-His) and the non identified fraction 2. On the basis of results from 7 P containing fractions have only 3 fraction very mobile, exchangeable P content in myosin.

The approaching quantity and the distribution of P containing fractions are summarized in Table III.

Table III

Distribution of amino acid phosphates in P-containing fractions of hydrolysates of phosphorylated hare's skeletal muscle myosin (determined by molybdate test)

Fraction	Apparent μmol P/fraction	%
1. ?	0.023	6.92
2. ?	0.031	9.33
3. P-Arg	0.168	50.60
4. Pi	0.033	9.83
6. P-Lys	0.031	9.33
7. N^{π}-P-His	0.021	6.30
8. N^{π}-P-His	0.023	6.92
9. ?	0.012	3.40
Total	0.342 μmol P	

? = Unidentified P containing fractions

From the chromatographically recovered fractions the total phosphate contents is calculated 17.84 mol P/mol myosin. That is lower than the quantity that was applied on the column, because prior to separation the P content is calculated to 55.2 mol P/mol myosin. That is, was recovered the 32.3 per cent of the applied P quantity on column. When the quantity of hydrolysates used for determination of P content is taken into consideration then into correction, the sum of the recovery is 45%. This value is correlated with the mean value of recovery that could be regained from chromatographic columns by the separation of the samples.

Discussion

The results of our previous experiments [5, 6, 7, 8, 10] suggesting a higher quantity of endogenous phosphate in the myosin of exercised muscles of well-trained animals and sportsmen related to the specimens of the homologous muscles of controls. This difference is extremely prominent when compared the values of fresh specimens. Namely, it was observed a slow but continuous phosphate release from the myosin preparations examined up to now during storage. That is why that only RNA- and lipid-free samples of freshly preparated myosins garantee and stand requirements of comparison

Up until now a lot of basic statements had been made iregarding the changes occurring in the muscle after exercise, for example concerning the protein synthesis and decomposition, adaptation of fibrils and mitochondria thinking to changes in the morphometry and structure of these [2, 3, 11, 12,

15, 16, 17], but not enough to attempt the analysis of muscle myosins by the order of ideas arisen up here. The nowadays revealed multigeneous origin concerning the heavy chains of myosins [20] makes this a difficult task.

In these investigations concerning the heterogeneity has been come to the point that revealed the existence of the mitochondrial myosin and it is interpretted as an original myosin. The results also revealed the change of P contents in myosin in connection with the requisition, and performance and endurance training of muscles. In the structure of the myosin molecule (wonderfully constructed) the possibility is given for its P contents to change, to remain on a higher level in the muscles of a well-trained organism and to be able to fill the higher requirements. As earlier experiments documented [7, 12] the P level of the myosin with physical training can not only be kept on a higher level but it can be phosphorylated, i.e. the concentration of the mobile phosphate content can be increased. The myosin of muscles with lower load and movements is able to take up less but the well-trained muscles are capable to incorporate more phosphoryl groups. In the real life this can be one component why the athletes on the verge of their maximum capacity with further long-term physical training can reach some more performance increase.

Lastly, where is the maximum capacity of P content in myosin? On theoretical basis this can be equal to the number of the basic amino acids. For example on the basis of amino acid analysis data of Huszar and Elzinga [19] in the myosin of the white muscle of domesticated rabbit this would be identical with the sum of the basic amino acids (Lys 465, His 75, Arg 225 altogether 765). In reality in phosphorylated form so far was only found a given per cent of the residues of basic amino acids in peptide chains of the fresh myosin, namely in domesticated rabbit 1—1.2, in hare 2—2.5, and in sportsmen 3—10%. In vitro phosphorylation these can be enhanced corresponding to the above list 2—2.5, 7—8, 8—15 per cent of the basic amino acids, respectively.

From the shape, structure of myosin and structure of the subunit of myosin molecule has been come to the conclusion, that not all sites of the basic amino acids but only on a certain localized domains of the molecule get phosphorylated.

By the effect of physical training in all cases has been increased the number of phosphoryl group binding sites, by which there is given a possibility for greater performance. An anomalous, but good testimony of this conception is given by the control hare, of which's specimen the P contents level returns to a lower concentration during a half year reared in cage, become similar to that of the domesticated rabbit.

The healthy human muscle samples — that can be comprehended as a control — show a wide P content range. Among these far lower and approaching P values can be found that similar to samples of the exercised muscle.

This is all right this way, because that is being discovered at the investigation of human specimen there is difference (bias) in the individual way of life of them (sedentary or mobile) — although they were thought to be normal. The observation made on hare is confirmed by the fact, that P contents of human specimen also decreased and correlated with the restcure time to the operation [8].

During training lots of other biochemical, physiologcial and morphological change occur parallel. That is why it became an important fact of the myosin purification demanding more care in the investigations concerning to myofibrillar myosin — mainly in case of exercised muscles — to make the mitochondrion-free fraction, so that they as mentioned above to be referred to only myosin derived from myofibrils. A similar consideration seems to be reasonable for the mitochondrial fraction [18] and mt-myosin of exercised muscle.

Whether is, the observed maximum phosphoryl group incorporation in the myosin of fast-twitch (white) muscle at extreme basic pH means in reality the number of all the phosphorylating sites — is to be confirmed. With the confirmation of this could be gained a suitable method for the determination of the sum of the total potential phosphoryl group binding sites, so that, through this could be got a control for the effect of training from the side of performance. It is thought the observed phenomenon is not only analogous with increased ATPase activity on effect of basic pH — observed by Padykula and Herman [25] —, but in somehow is in connection with those directly.

A new period of our experiments has begun a few years ago with the identification of binding site types, distribution and enhancing of P contents by phosphorylation. These precedents made possible to the observed the increase of " preparative phosphate" surplus by the binding types. As it is indicated by the experiments that the phosphorylation increased the P content and also the training similarly, firstly the amount of P-Arg [7, 10, 11] that can reach the 50—70 per cent of all P content — depending on the source of myosin. This means that from the total arginin (225) of muscle myosin about 50—75 (= 20—32% Arg) or more can be in phosphorylated form.

Of all the basic amino acid phosphates is precisely known that the free P-Arg is being the most instable, as well as the non-identified P containing fraction 2 (that is a hydrolysed product of P-Arg which — by our newer studies — likely can be identified with phosphoramidate). Luckily, it is experienced that the release of bound P from myosin occurred by a rather slower scale at least with two orders of magnitude than it was being from free P-Arg. The observations nowadays give an possibility that the interpretation of the usage of P binding types will be realised. The ester phosphate binding is directly interpreted for the P-Ser (P-Thr), which's increase during the short time (2 min) phosphorylation could not be detected even with ^{32}P labelling, neither during 5 min of incubation. The lowest detected level of this was 3—4 mol P/mol in

specimen of white muscle myosin of domesticated rabbit. As mobile P contents are considered the all other P in peptide chains of myosin are "active P" because during 2 min incubation all fractions of these show ^{32}P labelling, only with different rapid incorporation. (For example the P-Lys, N^{τ}-P-His, fraction 1 in relation to the P-Arg, N^{π}-P-His and fraction 2.) From this observation is considered the three of all fractions as quickly labelling amino acid phosphates and called "active" or N—P binding type, acid-labil phosphates for these participated with prominent role in contraction (de- and rephosphorylation) in training and in satisfying higher demands.

It is arouse some incertitude from methodical incompletion (the nonsufficient knowledge of alkaline hydrolysis) and from origin of myosin specimen, that could be observed in case of hydrolysis of higher quantity of myosin still 2—3 minor P containing derivates reached some per cents of the all fractions that resolved to 9 peaks chromatographically. Because of some bias all that has been said the calculated mol P/mol myosin values could not be interpreted so absolute value only just have to be considered as data for comparison.

Acknowledgements

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Book reviews

R. H. ADRIAN, H. zur HAUSEN, E. HELMREICH, H. HOLZER, R. JUNG, R. J. LINDEN, P. A. MIESCHER, J. PIIPER, H. RASMUSSEN, U. TRENDELENBURG, K. ULLRICH, W. VOGT, A. WEBER (Eds)

Review of Physiology, Biochemistry and Pharmacology

Volume 101. Springer-Verlag Berlin, Heidelberg, New York, Tokyo 1984. 247 Pages, 22 Figures and 2 Tables, price DM 98,— ISBN 3-540-13679-7

The new volume of the series contains three studies.

The first paper is The Mammalian Muscle Spindle and Its Central Control by M. Hulliger. This article focusses on the properties of spindle afferents and efferents and on their role during natural movement. The paper is divided in four parts:

1. Structure and Innervation of the Mammalian Spindle
2. Functional Properties of Spindle Afferent and Fusimotor Neurones
3. Central and Reflex Actions on γ-Motoneurones
4. Spindle Afferent and Fusimotor Activity During Natural Movement

The second study deals with the Interaction of Viruses with Tumor Promoters, by N. Yamamoto. It summarizes studies on the interaction between the persisting oncogenic herpes EBV (Ebstein-Barr virus) and tumour promoters, with special reference to virus induction and the transformation of normal lymphocytes by EBV.

In the last paper D. W. Busija and D. D. Heistad disscuss the Factors Involved in the Physiological Regulation of the Cerebral Circulation. It deals with the recent studies concerning the role played by metabolic stimuli, perfusion pressure, chemical stimuli and autonomic nerves in the regulation of cerebral blood flow and cerebrovascular resistance.

In the concluding remarks it is mentioned that several new concepts have been emerged recently, namely large arteries are important resistance vessels in cerebral circulation, metabolic activity matched closely with blood flow, sympathetic nerves participitate in the growth and development of cerebral arteries and play an important role in the regulation of CBF (cerebral blood flow) during hypertension, hypercapnia, hypoxia and hypotension and finally, that central nervous system pathways may be involved in the regulation of CBF, independently of changes in cerebral metabolism.

E. VARGA

J. BURES, Olga BURESOVA and J. P. HUSTON (Eds)

Techniques and basic experiments for the study of brain and behavior

Second, revised and enlarged edition Elsevier Science Publishers B. V. Amsterdam 1983. 326 pages with 179 figures and 9 tables. Price: paperback: 90 Dfl, hardbound: 188 Dfl. ISBN 0-444-80448-X, ISBN 0444-80535-4

During the last three decades a large number of handbooks, reviews and theoretical works have been published on the basic principles of brain sciences. The number of publications dealing with basic techniques of giving an overview of experimentation for the study of brain

and behaviour is, however, rather low. This book is an excellent presentation of the practical and conceptual issues associated with behavioural tests. It provides a clear description of different behavioural, electrophysiological and histological techniques. At the end of every section the authors give a summary of procedures, the suggested experiments and interpretation of the results. Description of experiments follows the convention of journals that publish paper in physiological psychology. Using this method the book becomes an excellent teaching manual, a guide-book for both students and scientists. It provides a large amount of information that is always useful and often difficult to obtain elsewhere.

The book was first published in 1976. Since that time interest in brain research has grown at a rapid rate. This second revised and enlarged edition consists of general updating of the text and includes new methods and experiments illustrating developments in the field.

The first chapter of the book (Physiological Psychology Research) starts with a short review of basic concepts in physiological psychology, and contains suggestions of how meaningful experiments should be planned. Different procedures of animal care, handling, sacrificiing, perfusion and removal of the brain are discussed in detail. Simple and more sophisticated histological techniques are described (cresyl violet, neutral red and the Klüver-Barrera stain, reduced silver stain for normal axons and silver impregnation of degenerating fibres). In this second edition new sections are included on histofluorescence of monoaminergic neurones, neuroanatomical tracing with horseradish peroxidase and on double labelling fluorescence methods. The use of different filter combinations of the fluorescence microscope and the application of different fluorescence tracers are discussed. The second part of the chapter deals with basic electronics, measuring and recording techniques, the properties of electrical stimulation and with the description of electronic components and circuits. The reader can easily learn the construction of integrating and differentiating circuits, the use of different types of amplifiers and the programming with TTL logic.

The second and third chapters (Innate and Motivated Behaviour, and Learning and Memory) contain the description of neurological and behavioural tests in rats. Experiments for measuring general activity and drive intensity, open field activity, pain or isolation induced aggression, submissive behaviour, schedule induced polydipsia and sexual behaviour are presented. The third chapter details the procedures of classical conditioning, the different forms of avoidance responses (including conditioned taste aversion and swim-escape), the operant conditioning, discrimination learning and experiments on memory. Examples are given for construction of experimental chambers and apparatus and the text is illustrated with clear, well understandable figures. The section of "Suggested experiments" contains the presentation of behavioural tests combined with stimulation, lesion or recording techniques.

In chapter 4 (Ablation and Stimulation of the Brain) different types of brain damage (electrolytic, aspiration and neurochemical lesions) and procedures for brain stimulation are described. The theoretical basis of application of neurotoxins is given and the use of 6-hydroxydopamine, kainic acid and ibotenic acid is described. This chapter includes a section on spreading depression and another one on chemically elicited consummatory behaviour. Experiments for stimulation induced consummatory behaviour and technical questions of electrical self-stimulation are also presented.

Special experimental tools in neurophysiology are shown in chapter 5 (Electrophysiological Correlates of Behavior). Methods for recording spontaneous EEG activity, unit activity and evoked responses, and procedures to elicit focal epileptic seizures are demonstrated. Technical details of production of microelectrodes, head-mounted micromanipulators and of recording equipments are discussed. In the last chapter (Pathological States) examples are shown for measurement of turning behaviour, the application of electroconvulsive shock and for the observation of reflex immobility (animal hypnosis).

The collection of methods described in this book covers all aspects of experimentation in physiological psychology. The style is clear and consice and the text is well-illustrated. The book is sightly recommended for physiologists, psychologists and pharmacologists.

L. LÉNÁRD

D. J. CHIVERS, B. A. WOOD and A. BILSBOROUGH (Eds)

Food acquisition and processing in primates

Plenum Press, New York, London 1984. 576 pages with 142 figures and 29 tables. Price US $85.00. ISBN 0-306-41701-4

Paleoanthropological results based on the discovery of different fossil hominoids and hominids have basically modified our views on human evolution. The new theoretical framework is highly supported by ethological observations of living primates. These discoveries have further stimulated extensive research using comparative anatomical and physiological methods on monkeys and primates. This book is a summary of results obtained with such a comparative approach.

The book contains the lectures and discussions of a two-day symposium and a three-day workshop held in Cambridge, Great Britain, in 1982 and sponsored by the Primate Society and the Anatomical Society of Great Britain and Ireland.

The aim of the meeting was to review and synthetize recent research on food acquisition and processing in primates. The main topics were a) primate habitats and niche structure, b) feeding and positional behaviour in evolutionary and ecological contexts, c) energy balance, metabolic requirements, digestive problems and body size, d) social and positional behaviour in relation to different feeding strategies, e) craniel, jaw, tooth and gut morphology in relation to diet and body size, and f) variation in trends in tooth and jaw morphology in fossil hominoids, including hominids.

The workshop was concerned with coordinating ecological and evolutionary models including information on energy requirements, diets, feeding strategies and morphology of primates. Such models may help researchers when extrapolating from living primates to fossil species.

The first section of the book contains 8 papers on "Ecological diversity and food acquisition". These examine habitats, plant chemistry, diet and feeding strategies of monkeys and include case studies of chimpanzees and grey langurs. One of the most interesting papers of this section deals with allometric comparisons (Body size, brain size and feeding strategies, by R. D. Martin). It contains the definition of the empirical allometriv formula, the criticism of allometric analysis and the problem of biological interpretations of allometric data. The author concludes that species with high-energy strategies are typically found to have significantly larger brains than their ecological relatives with low-energy strategies. The brain size largely reflects maternal metabolic turnover during gestation and the correlation between dietary habits and relative brain size is secondary rather than indicative of a direct causal relationship. According to Martin it is possible that natural selection operates primarily on maternal metabolic turnover in relation to dietary habits, and that differential brain size is to a large extent a passive consequence of maternal energy strategy.

The second section of the book contains 11 papers on "Food processing in living primates". These papers give a comparative anatomical analysis of the gastrointestinal tract, gape, jaw and teeth. Morphological results are analysed together with physiological mechanisms, mathematical formulations are also presented. Comparisons are made between measures of the gastrointestinal tract and of diet for mammalian species of different body size and adaptive strategy. With these methods it becomes possible to delimit the ecophysiological tendency of each species in relation to others (Diet and gut morphology, by D. J. Chivers and C. M. Hladik). Examples are given of how cumulative patterns of molar wear are related to diet and thus, how such wear patterns might be used to predict diet in extant species where the ecology is not known, or to reconstruct the possible diet of a fossil species (Prediction of primate diets from molar wear patterns, by C. M. Janis).

The third section (Evolutionary perspectives on feeding) begins with an evolutionary model (P. Andrews and L. Aiello: An evolutionary model for feeding and positional behaviour). This model examines habitats with reference to their use by primates and this information is combined with information on dietary preferences, dental morphology, locomotor capacity and post-cranial morphology of living primates. It concludes that the cercopithecoids evolved in tropical to subtropical savanna while the higher primates and hominoids had their origins in tropical forest environments. This section of the book includes a review of the anatomy and behaviour of extinct primates (R. F. Kay) and of the evolution of bipedalism in relation to food acquisition (M. D. Rose). The last two papers deal with the morphology of African Australopithecines (F. E. Grine and B. A. Wood).

The concluding discussion is a collaborative attempt to elaborate an integrated scheme relating different aspects of habitat, species characteristics (body size, group size, etc.), positional behaviour, food type and their mechanical, chemical and digestive properties.

The appendix contains mathematical formulations, statistical procedures and estimates related to allometric coefficients.

The book is recommended to physiologists, anatomists, dentists, anthropologists and psychologists.

L. LÉNÁRD

Cell fusion

Ciba Foundation Symposium 103. Pitman, London 1984. Published by Pitman Books, London; Price £ 25.00, approx US $35.00; ISBN 0-272-79750-2

Cells are individual units. Their integrity is assumed by the plasma membrane. If these membrane coated units are very closely paoked, the plasma membrane is fused by deformation of the sole and rearrangement of the common lipid bilayer. This membrane fusion is very rare under physiological conditions, but giving certain substances (PEG, Sendai virus) to the culturing media could be endhanced and regular. The artificial and planned fusion of cells is important not only from the theoretical point of view (to know what is the interrelation between different types of cell, how occurs the degradation of foreign substances, e.g. proteins, to study immunological reactions between the two foreign, e.g. animal and plant cells) but is has practica importance, too, e.g. in hybridoma technology and agriculture. The mechanism of cell fusion is not clear and we do not know how the so-called fusogens affect the cell membrane. To clear these problems in the summer of 1983 a Ciba Symposium was held and published in this book. It contains papers of the best researchers of the field and in addition good and keen discussions equal in length with the lectures.

The opinions of the participants diverged so much that one of them, Pethica, the chairman of the Symposium doubted even the bilayer structure of cell membrane. Nevertheless the volume is useful, containing new, more physiological methods for fusion (e.g. electro-fusion), model fusions and fusions of specialized cells (e.g. myoblasts). The book includes the sperm-egg fusion, which has an outstanding role in the process of fertilization, and methods for plasma membrane protein transfer between cells, by microinjection of protein and ribonucleic acid into cells and determination of their fate.

Cell fusion is not only a biological phenomenon, but a very important biological method, which helps to answer many questions and raising new ones. Most of them had not been solved by the contents of this book, but the reader with understand the problems better and may be inspired to new thoughts.

G. CSABA

Ferenc HAJÓS—Eduard BASCO

The surface contact glia

Advances in anatomy, embryology and cell biology Vol. 84.

Springer-Verlag Berlin, Heidelberg, New York, Tokyo 1984. 81 pages with 25 figures, price DM 52,—; approx. US $19.40; ISBN 3-540-13243-0

A significant amount of information has accumulated in the last decades about the functional significance of the neuroglia and a number of clinical aspects have been raised by recent advances in glia research. The investigations summarized in this volume are focussed on the "radial glia" a cell population of the immature brain present from the earliest stages of neural tube formation and characterized by long straight processes spanning the wall of the neural tube in a direction nearly perpendicular to the outer surface. The authors point out that there appears to be one feature fundamentally common to the radial glia derived cells: all of them contact a brain surface, either pial or ventricular. Therefore, they term all radial glia-

derived populations as the surface contact glia. Based on evidence provided by light microscopy, light and electron microscopic autoradiography, silver impregnation, immunocytochemistry and horseradishperoxidase uptake, they propose a new comprehensive classification of the astroglia. The astroglia comprises two main groups of cell derived from the embryonic radial glia: 1) Mature astrocytes of the deep neuropil having the functions ascrobed generally to the astroglia, 2) The surface contact glia, consisting of two subpopulations: (a) transforming type, (b) permanent types (cerebellar Bergmann glia, Müller cells of the retina, diencephalic tanycytes, etc.). The surface contact glia may have the functions of establishing communication and material transport between brain fluid spaces, guiding neuronal migration at certain sites and developmental stages, and transferring information from the brain surface to places of cell proliferation. Its embryonic form may give rise to astrocytes.

The book is well written, the illustrations are outstanding. The list of references contains more than 200 publications. The layout of the book is of excellent quality.

This volume of Advances in Anatomy, Embryology and Cell Biology is warmly recommended to all those interested in developmental neurobiology in the first place.

B. Halász

Harris, P., Pool-Wilson, Ph. A. (Eds)

Advances in Myocardiology

Plenum Medical Book Company, New York and London 1985, Price US $55.00
ISBN 0-306-41756-1

The fifth volume of the series "Advances in Myocardiology" contains the full texts of the invited lectures held at the 11th World Congress of the International Society for Hearth Research. This Congress was held 1983 in London, and provided an opportunity to review some of the growing points in the knowledge of the structure and function of the myocardium such as the investigation of the heart with nuclear magnetic resonance, the technique and molecular basis of electrophysiology, calmodulin research, the synthesis and degradation of myocardial proteins, the role of oxygen-free radicals in the reperfused heart muscle, the structural components of the myocyte, the sarcolemmal sodium exchange in the cardiac muscle and the influence of lipids on myocardial membranes. From the 26 lectures presented in the book the presidential address excels in his originality and curiosity. Peter Harris takes, namely, the liberty of doubting the widespread view that congestive heart failure is due to an inability of the heart to supply the body with necessary oxygen and demonstrated that the syndrome might be explained by an inappropriate and prolonged stimulation of the neurohumoral defence reaction that has developed during the evolution to support exercise and preserve life. Most of the remaining lectures deal with ion exchange, particularly with that of calcium in the heart tissues, and with the extracellular myocardial structures investigated by ultrarapid freezing techniques. The book gives a good review about the state of research in 1983 in these fields.

G. Pogátsa

Masao Ito (Ed.)

The cerebellum and neural control

Raven Press, New York 1984. 580 pages, with 216 figures. ISBN 0-89004-106-7

The author of this monograph is an outstanding export on cerebellar physiology, Masao Ito is one of the authors of the excellent book entitled: The cerebellum as a Neuronal Machine (J. Eccles, M. Ito and J. Szentágothai, 1967). Since the time of publication of the latter book, new techniques have been introduced to study cerebellar functions. Due to these technical advances a remarkable progress in cerebellar physiology has been achieved. In this monograph the author reviews the data obtained during the past fifteen years and incorporates them into concepts of how the cerebellar neuronal machine operates.

The monograph consists of five parts. Part I. (Cerebellar Cortical Network) deals with normal and abnormal morphology of the cerebellar cortex, the development of Purkinje cells and other cell types, influences of malnutrition on dendritic formations and the development of climbing and mossy fibres. Recent results on acetylcholine-related enzymes and receptors and the role of monoaminergic afferents on cerebellar cortical networks are also discussed. Chapter 10 of Part I (Neuronal Network Model) contains the author's remarkable experiments on the floccular lobe which is specially related to the vestibuloocular system. The "flucculus hypothesis" of the vestibuloocular reflex control is described and verified in delicate experiments. The flocculus that projects Purkinje cell axons to relay cells of vestibuloocular reflex receives ventibular signals as a mossy fibre input and visual signals as a climbing fibre input. Conjunctive stimulation of mossy fibres and climbing fibres results in long-term more than one hour depression, of the parallel fibre synapses. Thus, the conjunctive stimulation effectively depresses the vestibular mossy fibre responsiveness of flocculus Purkinje cells. A similar depression can be induced if glutamate activation substitutes for the parallel fibre activation of the Purkinje cell dendrites. It is well-known from Ito's earlier work that Purkinje cells have an inhibitory action on nuclear cells of the cerebellum. The author supposes that the long-term depression of Purkinje cells will be convertedc to long-term potentiation of the nuclear cells and so in the output lines from the cerebellum. Long-term depression and long-term potentiation may be similar in that both are due to Ca^{++} action on glutamate receptors though in opposite directions. The significance of this plastic ability of the cerebellar neuronal network would be its participation in memory processes and learning related to motor processes and the acquisition of skills.

Part II of the monograph (Corticonuclear Complex) deals with cerebellar and vestibular nuclei. The sagittal zonal organization of the cerebellum is discussed. Cortical microzones, corticonuclear microcomplexes and their functional significance are analysed in detail (Chapter 15: Microzone and Microcomplex Model).

The Cerebellar Control System is the title of Part III. This includes anatomical and functional descriptions of input—output systems. Chapter 24 (Control System Model of the Cerebellum) contains theoretical considerations on feedforward and feedback modes, adaptive and predictive control, multivariable control, and describes the sidepath model, different loop models and cascade connections.

Part IV (Cerebellar Functions) reviews currently accumulated data with deference to the control system structures of the cerebellum. Chapter 25 (Vestibuloocular reflex) contains the description of experiments in which the flocculus hypothesis has further been tested and supported by recording of Purkinje cell responses and by examining effects of lesions of the flocculus. In Chapter 30 (Voluntary Limb Movement and Behaviour) the role of cerebellar network in reformulation of the premotor command and updating is discussed. The voluntary control of fine finger movements and its relation to cerebellar mechanisms are detailed. A possible involvement of the cerebellum in complex neural functions such as learning in general and motivated behaviours is discussed. The bibliography (Part V) includes large amount of references.

This excellent book provides a comprehensive review, of recent results on the cerebellum but it is not only a reference handbook. It presents important theoretical considerations, conceptual cerebellar models and the "flocculus hypothesis" of the author verified in exciting experiments. The book is highly recommended to physiologists, anatomists and neurologists.

L. LÉNÁRD

B. MESS, Csilla RUZSÁS, L. TIMA, P. PEVET (Eds)

The pineal gland, current state of pineal research

Proceedings of the Third Colloquium of the European

Pineal Study Group, Pécs, Hungary, August 13—17, 1984. Symposia Biologica Hungarica 28. Akadémiai Kiadó, Budapest 1986. 372 pages

This book contains 24 invited lectures presented at the Third Colloquium of the European Pineal Study Group. The papers cover almost all interesting aspects of current pineal research. In the introductory session, the history of the European Pineal Study Group, comparative aspects and new trends in research of the pineal gland are summarized. Under the

heading morphological and cytological aspects, the structure of the pineal gland of mammals, ultrastructural study of the pineal-hypothalamo-hypophyseo-gonadal axis in mammals, central innervation of the mammalian pineal organ, CSF-contacting neurones and pinealocytes are discussed. One chapter deals with pineal hormones and regulatory mechanisms of pineal secretion. In this chapter the cellular biology of the pineal organ, pineal peptides, neural control of pineal indole metabolism, control of the circadian rhythm in pineal melatonin secretion, factors influencing melatonin secretion are described. Three papers are concerned with the interactions between the pineal and the environment, with special reference to seasonal reproduction, and two papers summarize recent findings on the interactions between serotoninergic and non-serotoninergic neurotransmitters and melatonin upon the reproductive activity in the rat. Two papers are devoted to extrapineal melatonin and the action of melatonin on the brain. The last chapter deals with various aspects of the pineal gland of primates including men. The authors of the papers are internationally known experts, besides presenting their recent findings they give excellent reviews of their subjects.

This well-produced book is a fairly comprehensive survey of the pineal gland. All those morphologists, physiologists, biochemists, endocrinologists, comparative biologists as well as clinicians interested in the pineal gland with find useful information in it.

B. Halász

J. Rivolier, P. Cerretelli, J. Foray, P. Segantini (Eds)

High altitude deterioration

International Congress for Mountain Medicine, Chamonix, France 1984 Vol. 19 Medicine and Sport Science

S. Karger, Basel—München—Paris—London—New York—Tokyo—Sydney
1985. XVI + 282 pages with 65 figures and 32 tables. Price SFr. 145.—; DM 174.—; US $ 61.75.
ISBN 3-8055-3972-X

This volume is devoted to the papers read at the Congress organised by the Medical Commission of the Union Internationale des Associations d'Alpinisme (UIAA) in France. In addition to its contextual and domain importance, it is a milestone on the way of progressing medical specialization and, at the same time, a fine evidence of the need for, and the untiring efforts made in, the re-integration of medicine. Beyond any a doubt, the ground on which this new science is taking shape and in which research strives to dig deeper is practical experience itself. Congress organizers, authors and editors share the view that high altitude deterioration phenomena are basically well-known, nevertheless, they may arise from such a multitude of causes or sources that they essentially need an attitude of complex approach.

The same is reflected by the structure of the volume, extending from basic physiology ("Aerobic and Anaerobic Metabolism During Exercise at Altitude" by Cerretelli and di Prampero) through special aspects of physiology and biochemistry (the chapter on blood flow, heart, respiration) to problems of pathology. Further chapters bear the titles "High Altitude Edema", "Phychobiology", and "Cold Injuries". Practical considerations overtly dominate in the chapter under the heading "Miscellaneous", and the four studies contained in it are worth mentioning by title: Research in Altitude Illness, Medical Statement Analysis of 3200 High Altitude Climbers, Indications and Contraindications for Cardiac Patients in Altitudes, and Cold Tolerance.

The studies of Rivollier (A Neuropsychological Approach of Mountain Deterioration) and of Defayolle (Deterioration of Mental Performance) which constitute the chapter on psychobiology are particularly interesting and of a very high standard. They are a Great help in understanding the possible routes along which body and mind communicate as well as the interconnexions between altitude and performance deterioration.

The closing chapter contains conclusions of the sessions on physiology and biochemistry (written by Cerretelli) and of those dealing with pathology (by Banssillon), offering a very useful, concise, and most interesting reading even for the not dedicated physician, if for no other reason, then merely by serving a unification of concepts in, and attitude to, this field. It is quite certain that the essence here is the interdisciplinary paradigm, similarly to a number of other domains of medicine. This approach has given an equal, if not greaster, emphasis to point of

altitude deterioration rather than acclimatization which had previously ruled our mental associations. This is what may be argued about, yet this is what reality dictates.

Present-day alpinism though standing much less in the limelight than the top ranks of other sports, yet to an extent very similar to those, meets extremely demanding conditions. Consequently, medicine here must answer the challenging question, where are the limits of the physical and mental strain that are still allowable, and within what bounds can efforts such as these be accepted at all. The well-selected material of this volume offers the correct answers. Altitudes of certain height and strains above certain limits will impair the functions of all organs and will interfere with many or all mechanisms of the body. High altitudes can, however, be defeated when the physical strain is kept within the individual's capacity to adapt himself or herself. Moreover, even the risks run by a heart patient with affected can be held within reasonable bounds, provided that the mentioned condition is met. On the other hand, exertion at the same altitude is explicitly forbidden to all who suffer from chronic respiratory disease.

In spite of all the risks, high-mountain tourism is gaining ground in very society and its spread apparently cannot be stopped. This state of affairs creates an exceptionally fruitful opportunity for medical research, in addition to the devotion of medicine to preventive and therapeutic work. The study of the effects of extreme environmental conditions and associated physical strain promises new vistas for basic research. The editors make an emphatic point of promoting the mutual understanding between alpinists and physicians. Medicine has paid much of its dabt by this volume. What remains to be answered in the future is to what extent practice is willing to accept the results provided by research.

R. FRENKL

H. SCHNEIDER, J. DANCIS

In vitro perfusion of human placental tissue

× + 190 pages with 36 figures and 23 tables, hard cover, 1985. Price SFr. 130.—; DM 156.—; US $55.50. ISBN 3-8055-3986-X

Recently a newfound enthusiasm could be seen in the field of placental physiology which is best demonstrated by the emergence of the European International Placenta Group.

Studies during human pregnancy are limited by concerns for the safety of the mother and the fetus. Perfusion of the postpartum placenta is without the burdening ethical and medico-legal problems of clinical testing in vivo. It provides an easily manageable, controllable and interpretable experimental model, in which placental transport and metabolism could be adequately studied.

The perfused placenta retains many complex and special functions that could be of interest to biochemists and obstetricians. According to recent knowledge, amino acids are transported across the in vitro perfused placenta by a stereospecific transport mechanism, with a net result of a gradient towards the fetus. For instance, D-glucose transport is increased threefold above the diffusion rate. Some of the placental peptides such as HPL, are released preferentially into the maternal circuit.

This book is a collection of papers presented at an international workshop, which dealt with the methodology of the placental perfusion techniques, with special emphasis on transport problems.

In the morphology section the reader with find valuable details of the maternal and fetal circuits, analysed by presently available standard morphology techniques. The influence of ischaemia on artificial perfusion is also outlined, based on ultrastructure and morphometric studies. The fetal vasculature is studied by combination of corrosion techniques and SEM.

Especially interesting is the dual perfusion of an isolated lobe for which the technique is fully described.

In the physiology section the dual perfusion technique seems especially useful for toxicology studies which might require long perfusion times such as 12 hours.

Another contribution deals with the simple model of equivalent pores, but this cannot provide a consistent quantitative description of the passive properties of the placenta. The placenta displays the characteristics of a heteroporous membrane and its multilayered structure should not be overlooked.

New conclusions may be found concerning the diffusional permeability of the human placenta. For rapidly diffusing molecules, there seems to be an element of flow limitation in transplacental passage: namely, the rate of transfer could be controlled by supply to, and removal from, the exchange sites rather than the rate of diffusion across these pores. Therefore, flow is playing a determining role in controlling the exchange of small molecules.

Interesting data on the magnitude of overlapping between the two circulations and on the extent of shunting on both sides are presented.

A recurring criticism is whetter an in vitro system may be used as a model of in vivo events. The basic argument is that the mother and fetus which continuously modify the perfusates in vivo, are not exerting their influences in the in vitro system. Therefore placental perfusion should be considered a simplified approach to placental physiology, but it still has the capacity of supplying useful data, since the level of organization of the perfused placenta suggests that the results are likely to be close to the in vivo performance.

The text concludes with 15 short communications on transplacental transport in vitro.

The book may be recommended as a useful reference material to those engaged in placental physiology, pharmacokinetic studies and biochemistry.

J. HERCZEG

Anees A. SHEIKH, Katharina S. SHEIKH (Eds)

Imagery in Education

Imagery in the Educational Process

Baywood Publishing Company, Inc., Farmingdale, Ny 1985. 247 pages with 8 figures, price US $16.50

The second volume of the Series Imagery and Human Development contains studies on creative thinking in education, visual education, imagery. In the volume there are ten papers an follows.

1. The Ebb and Flow of Mental Imagery in Education, by Gisela E. Speidel and Mark E. Troy
2. Imaginative Play: Its Implication for the Process of Education, by Diana Shmukler
3. Educational Applications of Mnemonic Pictures: Possibilities Beyond Your Wildest Imagination, by Joel R. Levin
4. The 4Rs: Readin', 'riting, 'rithmetic, and the Right Hemisphere. A Review of the Application of the Brain Laterality Model to Education, by Robert G. Ley and Melvin Kaushansky
5. Mental Imagery and Problem Solving: Implications for the Educational Process, by Geir Kaufmann and Tore Helstrup
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7. Guided Imagery in Education, by Beverly-Colleene Galyean
8. Imagery Research with Children: Implications for Education, by Nancy S. Suzuki
9. Imagery Research with Adults: Implications for Education, by Kathryn Lutz Alexandrini
10. The enhancement of Imaging Ability by Anees A. Sheikh, Katharina S. Sheikh, and L. Martin Moleski.

A detailed subject index can be found at the end of the book. As the book deals with the most outstanding recent studies on the relationship of mental imagery to education, it is recommended to those who are interested in the modern problems of education.

E. VARGA

Mary TAUB (Ed.)

Tissue culture of epithelial cells

Plenum Press, New York and London 1985. 288 pages, Price US $45.00; ISBN 0-306-417405

Though they possess a diverse number of functional properties, epithelial cells share some common characteristics. In addition to their morphological polarity, all epithelial cells, bound together by occluding junctions, possess the capacity for vectorial transport. Tissue cultures of kidney tubular epithelial cells are especially suited for the study of fundamental properties of such cells, like dome formation, electrical phenomena and the dependence of transport on metabolism. This book is a valuable contribution to the topic of investigation in vitro of intrinsic cellular functions.

The first section of the volume is dedicated to the problem of dome formation in cultured epithelial monolayers. Such domes are results of a lifting of the epithelial cells owing to active transport of ions and water across the cell monolayer in an apical to basolateral direction, trapping a bubble of fluid between the cell layer and the culture dish. Julia E. Lever discusses the role of inducers in dome formation. Section II deals with the electrophysiology and ion transport of MDCK (Madin-Darby canine kidney) cells in essays written by Gonzáles-Mariscal et al., Fernández-Castillo et al. and by Milton H. Saier Jr., respectively. In the third section sugar transport, amino acid transport and the bioenergetics of these transport phenomena in LLC-PK_1 cells are described by James M. Mullin and Arnost Kleinzeller, by Francisco V., Sepulveda and Jeremy D. Pearson and by Dayton J. Misfeldt and Martin J. Sanders. Section IV. entitled Membrane Proteins and Hormone Receptors contains three essays: on the use of viruses in the study of the polarity of epithelial membranes (by Mary Taub): on hormone receptors (by Kathryn E. Meier and Paul A. Insel) and on monoclonal antibodies (by J. Craig Venter et al.). The last part of the book (Section V) is devoted to the interactions between epithelial cell cultures and the hormonally defined medium: Joachin G. Liehr and David A. Sirbasku describe oestrogendependent kidney tumours, David Barnes discusses the use of hormonally defined, serum-free media in epithelial tissue cultures, and finally, Mary Taub analyses the importance of such media in general for in vitro studies concerning epithelial transport. Due to the fact that, in serum-free media, fibroblast overgrowth does not disturb the whole picture and that under such conditions cells retain differentiated functions, serum-free cultures offer quantitatively new perspectives for the study of epithelial tissues in general. On the other hand, as shown by Taub, such systems allow to study the effect of various hormones which is of outstanding impotance in understanding the mechanisms of transepithelial transport.

In summary, this book is a valuable tool not only for members of the tissue culture club but for all those interested in basic functions of epithelial cells. A good subject index helps orientation in the vast factual material contained in this volume. Addition of a general Author Index would have been even more helpful.

B. CSILLIK

D. G. WEISS and A. GORIO

Axoplasmic Transport in Physiology and Pathology

Springer-Verlag, Berlin, Heidelberg, New York 1982. 190 pages, 76 figures, price DM 78,— approx US $31.20, ISBN 3-540-11663-X

The volume contains the contributions presented at the Workshop on Axoplasmic Transport held at Schloss Elmau in Bavaria, 1981. It summarizes the physiological, pathological and experimental aspects of axoplasmic flow based on a wide variety of morphological, physiological, biochemical methods and pathological and clinical observations. Thus it offers a truely interdisciplinary presentation of the topic. The general physiologist or cell biologist, however, who estimates his knowledge to be rather weak in this field, after having read the book can console himself that the lack of proper information is not mainly his or her fault. The present volume gives a series of interesting details but the synthesis of observations and explanation of events will be the topic of future workshops.

After a short introduction written by D. G. Weiss on the general properties of axoplasmic transport, the papers are focussed on five main topics. In the first section the physiological role of axosplasmic transport is detailed. A. Dahlstrom deals with the role of axoplasmic transport in sympathetic nerves and examines the appearance of noradrenaline-containing granules at different distances from the cell body after pharmacological transmitter-depletion by high doses of reserpine. Three papers investigate the fate of nucleosides and proteins, i.e. their transport-properties in the axons and decomposition and secretion from different parts of the neurone. As the amount (but not the velocity) of transported and secreted adenosine is increased by an increase of neural activity, speculations on the possible physiological role of adenosine are also discussed. The last paper of this chapter concerns the biosynthesis and processing of neuropeptides and neurophysinss in the hypothalamo-hypophyseal system.

The second chapter focusses on the role of exoplasmic flow in growing and regenerating nerves. The paper of K. H. Pfenninger investigates with morphological techniques the insertion of new membrane fragments into the growing axon. He identifies the large, clear subplasmalemmal vesicles (SPV) as the vehicles of new membrane pieces to be inserted into the plasmamembrane at the tip of growing neurites whereas integral proteins arrive at the distal end by lateral diffusion in the membrane. In the next two papers biochemical methods are used to comspare the composition of the material transported by axoplasmic flow in normal and regenerating nerves. The team of A. Gorio followed the time-course of appearance of the enzyme acetylcholinesterase in the regenerating axon and the innervated muscle tissue after transection of the sciatic nerve. These data are correlated to physiological parameters as resting membrane potential and transmitter-release and to morphological observations on the distribution of end plates on muscle fibres.

The next chapter deals with experimental neuropathies induced by 2,5-hexanedione, acrylamide, iminodi ropionitrile or disulfiram. The morphological changes detectable with light or electron microscopy are detailed as well as changes of the composition of transported proteins revealed by SDS-PAGE. The fourth chapter consists of a collection of observations on the participation of alterations of axoplasmic flow in the pathogenesis of different diseases such as Alzheimer disease, diabetic neuropathy, compression of peripheral nerves, retinal ischaemia and consequences of high intraocular pressure, and the spread of virus infections in the nervous system.

The last chapter gives a short account on axoplasmic transport as a tool in neuroanatomy and neurophysiology. Application of the retrograde transport of isotopically labelled transmitterrelated molecules, of horseradish peroxidase and fluorescent compounds are detailed.

Erzsébet LIGETI

G. C. WHITTOW and H. RAHN

Seabird Energetics

Plenum Press, New York 1984. I—XII + 317 pages ISBN 0-306-41- 819-3. Price US $55.00

The book consists of the proceedings of a symposium sponsored by the Comparative Physiology Section of the American Physiological Society. The Symposium was held on August 23—24, 1983, in Honolulu.

The foreword by J. B. Nelson and a preface by the editors is followed by 14 chapters, written by 20 contributors. The book is completed by an index.

The subjects discussed comprise problems of egg formation, energetic budget of the egg, incubation and egg-parent interaction (Chapters 2—6). Other chapters (7—13) detail the metabolic response to temperature, energetics of breeding, free ranging of seabirds and penguins. Thermoregulation of the adults is also covered. It is interesting how energy requirements of the seabird-population can be modelled. The 1st and 14th Chapter give a very good general information on the problems associated with seabird energetics, with special reference to the competition for fish between seabirds and man. The negative largescale oceanographic changes of the last three decades (El Nino) calls attention to the importance of knowledge energetics of the approximately 285 seabird species, who are a minor fraction of the 8600 known species of birds.

The book on Seabird Energetics is interesting mainly for bird physiologists, biologists and zoologists, but natural scientists who are interested in comparative aspects will also appreciate the volume.

G. PETHES

R. YAGIL

The Desert Camel

Comparative Physiological Adaptation XII + 164 pages with 21 figures and 11 tables. Karger Edition Basel 1985, price SFr. 128,— DM 153,—, US $54.50. ISBN 3-8055-4065-5

This book is the fifth in the series Comparative Animal Nutrition by the same editor.

The Arabian Camel, which under most temperate areas can be seen merely in zoos, seems a most interesting object for the comparative physiologists. Being adapted both to a hot, daily severely changing environment, and to scarcely available fond, gives a unique example for manyfold adaptation of the regulatory mechanisms of the organism.

The book consists of 11 chapters. Following an introduction (general informations), thermoregulation and metabolism, water metabolism and renal function, urea metabolism, alimentary canal, digestion, nutrition, respiration and acid-base balance, cardiovascular system and plasma, blood cells, endocrine system, lactation and reproduction are dealt with. A 12th chapter, entitled "epilogue", argues why camel husbandry will be of growing importance for men living in arid areas of the world. The book is completed by a list of references and a subject index.

The specialized water metabolism, heat regulation and metabolism linked closely together, provide the possibility of quick adaptation to environmental temperature changes, with a decreased rate of heat production and water loss. The role of urea metabolism in maintaining renal reabsorption of water as well as that of body water equilibrium are of special interest. Description of the specialized cooling and therefore water conserving mechanism provided by the nasal vein and head artery circulatory (countercurrent) system originally suggested by Schmidt-Nielsen et al., is also a very interesting part of the volume.

A "physiological hypothyroidism" seems to participate in water retention in the dehydrated camel and also results in hyperglycaemia. Aldosterone governs water and urea reabsorption both in the kidney, sweat glands and aldmentary tract in accordance with gastrin, which enhances water and urea recycling into the rumen.

The above-mentioned facts are a few examples only for the unique organization of camel's organism. Need for a better understanding of the camel's physiology is emphasized by the author when he calls attention to the fact, that in drought-stricken areas of the world only the camels survive and produce milk and calves, while other ruminants are everely decimated.

Considering the fact that famine in this area is forecasted for a long period, mass rearing of camels seems an important task. This book is therefore more than a simple description of a very unique species, the camel. Anybody who has affinity to comparative (mammalian) physiology will read this comprehensive book with great interest and much pleasure.

G. PETHES

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RECENT ADVANCES CONCERNING THE COUGH REFLEX

(CHAIRMAN'S INTRODUCTION)

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First of all, I would like to express my thanks to the representants of the Hungarian Physiological Society who kindly provided the possibility to hold on the occasion of the 50th Congress a symposium on respirology, dealing, among others, with the problems of cough.

Cough belongs to the most important defense reflexes of the human organism. Cough may be of physiological character if it fulfils its role, or of pathological character if it is abundant or not effective. It may be a symptom of some disease, or represent the cause of some disorders, being a negative reflection of significant changes in thoracic pressures acting on the organism during coughing [6].

A single cough consists of a rapid deep inspiration followed by forced expiration against a closed glottis, and then by a sudden opening of the glottis, under continuous expiratory effort [25]. This basic pattern may show variants both in man and in experimental animals. It may be repeated to produce a paroxysm or it may be fragmented, for example by repeated closing and opening of the glottis, during a single expiratory effort. The relative contributions of inspiration and expiration may vary.

In animals it can be shown that different patterns of cough are produced by stimulation of different sites, or when different types of stimuli are used. Stimuli of chemical irritants to the lower respiratory tract are due to strong inspiratory components on coughing, rather than mechanical stimuli.

Because of the given patterns of cough, the cough reflex has attracted attention of both researchers and clinicians. Cough occurs in almost all diseases of the respiratory organs and is not only one of the main signs, but often the only sign of the disease. In chronic bronchitis, for instance, it is one of the main diagnosic criteria. It is thus not a banal sign, although in practice it is often not fully appreciated either by the physician or the patient. It may

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happen that even reasonable people tend to pass over such a "little thing as" a cough. Underestimation and incorrect evaluation of the significance of cough may cause irreparable damage.

The underestimation of cough passed into the consciousness of the people in our country so far that it is used as a term expressing lack of interest, saying "I cough on the matter", or "I do not care a cough for", "cough it up", etc.

From the clinician's point of view, cough represents a contradicting phenomenon as an apparently banal cough which is ignored but may mask a serious disease, while a persistent, stubborn cough, the cause of which is benign or extrarespiratory, may give rise to numerous investigations in the search for a chest disease, an affection of the pleura or of the upper airways [11].

From the said before it comes out that there must be a required interest about the theory and clinics of cough. Essentially it is about categories of the common and special, which are dialectically tied together, and therefore it is impossible to apply successfully the approach of therapy without complete understanding of the mechanisms of cough, for instance.

Coughing can easily be provoked by mechanical or chemical stimulation of the mucosa of the larynx, trachea and large bronchi, the sites where cough receptors are found.

Nervous receptors in the respiratory tract may be grouped into four general categories [26], first, the slowly adapting end-organs stretch receptors responding to stretching of the airway wall, responsible for the Breuer—Hering reflex, second, the rapidly adapting end-organs, irritant receptors, responsible for defence reflexes such as cough, sneeze, and the expiration reflex, and also for reflex actions to inhaled irritants and to some respiratory diseases, third, the receptors, with non-myelinated nerve fibres, J-receptors which, being polymodal, are stimulated by damage of tissues, by oedema, and by mediators released in these conditions, fourth, the specialized receptors such as those for taste and swallowing, and those around joints and in skeletal muscles.

The situation, however is not so simple. According to Paintal [14] there is no evidence that the slowly and rapidly adapting receptors would constitute a functionally homogeneous group, Paintal and Ravi [13] compared their findings to the results of Sant' Ambrogio [16] and found that the properties and responses of the slowly and rapidly adapting receptors to fourteen different stimuli out of 18, were similar. In spite of these results, numerous observations were described by Sant'Ambrogio [16, 17, 18], Coleridge and Coleridge [2], Widdicombe et al. [24, 26], Richardson and Peatfield [15] and others. The participitation of stretch receptors at a forced expiratory effort is undoubtful as it was confirmed by Hanáček et al. [4] and later by Sant' Ambrogio [16] and Sant' Ambrogio et al. [18]. This is in connection with [1], the significance of the initial inspirium to the expiratory effort to cough.

The fundamental cause of all the obscure matters is the fact that all the given receptors were identified indirectly only, on basis of their discharges and reflex actions but without simultaneous histological identification. Further, it has been stressed that many mechanical and chemical stimuli do not activate only one group of endings, and that there are similarities in the reflex responses of two groups of endings. It is therefore probable that under many pathological conditions in the lungs with the release of mediators, more groups of receptors and their reflexes are involved. Widdicombe [26] concluded that the precise pattern of responses will depend on the balance of the stimuli affecting the receptors, and on the interplay of the reflex motor changes. The complexity of those mechanisms is not surprising since other visceral and somatic tissues have similarly complicated innervations.

One of the aspects of receptor physiology, about which we know so little, is the effect of mucus. It is well known as a powerful stimulus to coughing. What is less certain is, whether it has a protective function, i.e. a particular role in limiting the access of irritant stimuli to the receptors. It is interesting that if the airway epithelium has few or no mucus secreting cells, no epithelial nerve endings could be found there, e.g. in the tracheal epithelium of the ferret and the mouse [7].

In addition, almost nothing is known of the central nervous organization of the cough reflex, although a medullary cough centre has been postulated. Our experiments performed in collaboration with Kulik and her collaborators in this direction were less successful than those performed by Jakuš et al. [5] who will present their findings. Our group consisting of my collaborators Sadloňová, Salát, Salátová and Palaček was aimed to study of objectively registered cough sounds is not less interesting if one wants to consider the disease, its treatment and also the screening of respiratory tract diseases.

It is clear that in the case of the given disagreement, in accordance to various points on cough, its therapy should be very pretentious. The idea to treat cough causally, by using antibiotics, has often failed and we have to review the use of some effective substances. It is necessary to use antitussive drugs of non-narcotic character, able to depress cough or its undesirable components, e.g. bronchospasm, initial inspiration etc. To synthetize highly effective antitussive drugs requires the knowledge of physiology and pathophysiology of the cough reflex and a methodical readiness is also needed. It is impossible to study the effects of antitussive drugs on animals which do not cough or in healthy ones because the disease will alter the reactivity of their organism. The problems of the mentioned aspects have been discussed by Višňovský, Nosálová, Strapková, Bánovčin and Salátová [12, 23].

The papers that will be given by Tatár et al. [19, 20, 21] will deal with the effects on cough of hypoxia and hypercapnia. Respiratory insufficiency may be due to numerous respiratory tract diseases affecting the defence

reflexes of the airways. Kulíšek et al. will deal with the super-regulatory functions of cough and sneeze on breathing. The papers by Tomori, Frndová et al. [13, 22] intend to clear up the same problem. The mentioned authors have used computing techniques to solve the given tasks.

I am fully convinced that all the lectures will attract your attention. Let me wish the speakers and the audience a deep and agreeable scientific experience.

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TUSSIPHONOGRAPHIC ANALYSIS OF COUGH SOUND RECORDINGS PERFORMED BY SCHMIDT—VOIGT AND HIRSCHBERG AND SZENDE

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The cough sound records published by Schmidt-Voigt [9] and Hirschberg and Szende [1] were submitted to tussiphonographic analysis. It has been established that all the recordings of various types of cough sounds registered in airway diseases were of pathological character in the tussiphonographic recordings. It has repeatedly been confirmed that tussiphonography is a suitable means for screening of respiratory diseases.

Keywords: tussiphonography, voluntary cough, respiratory diseases screening.

On the basis of earlier experience [2, 3] we elaborated the method of tussiphonography [4, 5, 6, 7] that proved a convenient means for seeking out subjects suffering from airways diseases. The method is non-invasive. The examined patient or healthy subject is inspiring deeply on command and thereafter he is coughing once, voluntarily. The cough sound is registered via a microphone on tape-recorder and thereafter it is rescribed by an adequate recorder, by on- or off-line mode, graphically (Fig. 1). Under normal conditions the cough consists of one single double-sound. Its intensity is maximum 50 mm and its duration not longer than 0.45 s [6]. Under pathological conditions, however, the character of cough is changed, so that it is turning to a trebled, fourfold or multiple sound with markedly increased intensity and duration.

As to the literature, three authors have described and illustrated on records samples of pathological cough sounds. These were Schmidt-Voigt [9] and Hirschberg and Szende [1]. The aim of the present study was to evaluate the given records reflecting pathological conditions, by our own method.

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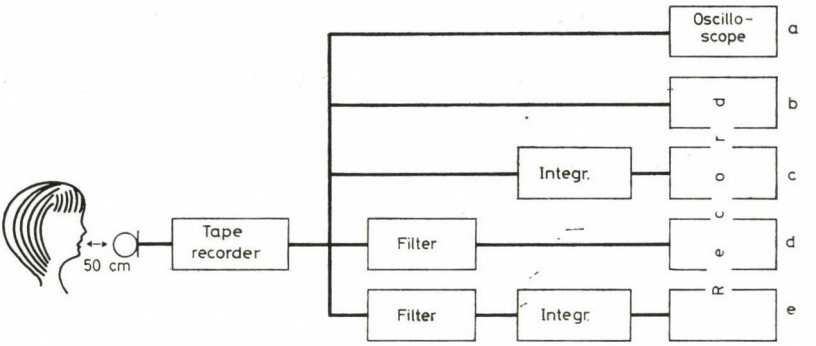

Fig. 1. Scheme of voluntary cough sound recording. The cough sound is observed by the oscilloscope (a), recorded by means of the multscriptor (b), integrated and recorded (e). The value of the integral characterizes the cough sound intensity

Methods

Schmidt-Voigt [9] registered on records 28 samples of pathological cough, under conditions of various types of bronchitis, lung tuberculosis, pertussis, aortic aneurysm, cardial oedema, etc. Hirschberg and Szende recorded in children the sounds of cry and cough by the means of tape recorder under similar conditions. The registered sound was analysed by spectrograph (Sona-Graph, Kay Electric Company, Type 6061—A). Their publication is giving 11 samples of cough, registered on record.

In order to analyze the sound, we proceeded by our routine method [5, 6]. The sound source (the tape recorder or the record player) was put at a distance of 50 cm from the microphone, connected with the tape recorder Sony (TC 133 CS). In that way we described the recording on paper by Mingograph Elema 81, at a rate of 100 mm· s^{-1}. The character, intensity and duration of cough were considered from single recordings. Similar mode of recording have recently been described in the literature [1, 7].

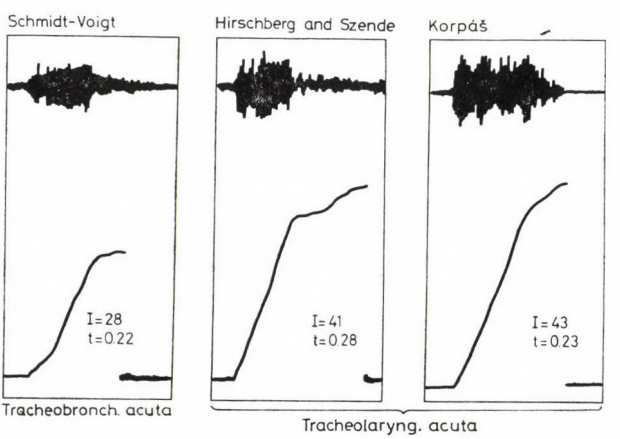

Fig. 2. Cough sound recording — above, and integrals — below, in slight airways diseases. I = the value of intensity, t = value of cough sound duration

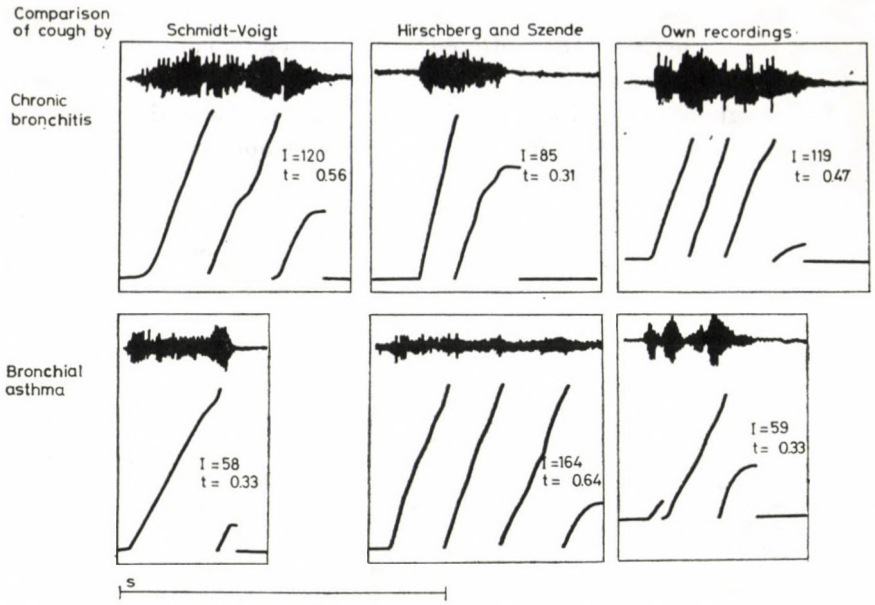

Fig. 3. Comparison of cough sound recordings in severe airways diseases. Description as above

Results

After we have applied our own mode of analysis, we could establish that all types of cough registered by Schmidt-Voigt [9] and by Hirschberg and Szende [1] were of a definitely pathological character.

Slight inflammatory diseases were marked by changes of the cough sound character only (Fig. 2). Beside changes of character chronic diseases have shown increased cough intensity and a delay in its duration (Fig. 2).

Discussion

The analysis of pathological types of cough, published by the mentioned authors, has confirmed the suitability of our method, because it unequivocally pointed to pathological types of cough sounds. As it is seen in the recordings, slight conditions could mostly be differentiated well from the severe ones.

Figure 2 shows that in the records b, d, f, the duration of cough was not delayed, in contrast to our expectations. This might be explained so that in these cases a chronic bronchitis or bronchial asthma in the quiet phase had occurred, but these conditions were not mentioned in the given publications [1, 9].

The sonagrams given by Hirschberg and Szende [1] were especially interesting for us because they offered a possibility of comparison.

In accordance with the recordings, even they have shown an increased number of cough efforts and also a delayed sough cound duration under pathological conditions, but the mentioned changes have not attracted the authors' attention.

After verifying our method we concluded that it was successful because it showed again that it could reliably be applied for screening of subjects suffering from respiratory tract diseases, providing possibilities to differentiate slight diseases from severe ones.

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TUSSIPHONOGRAM IN PROBANDS WITH CHRONIC OBSTRUCTIVE BRONCHITIS

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Tussiphonographic examinations were conducted in 21 probands admitted to hospital with chronic bronchitis, diagnosed by spirometry. Voluntary cough registered in subjects with obstructive chronic bronchitis appeared in the recordings as a marked multifarious sound, an increased mono-sound or a connected double-sound. The average value of cough sound intensity was increased to 244%, and the duration of cough was delayed to 77% when compared to normal values. No correlation was found between the changes observed in spirometric values, and values indicated by the tussiphonogram.

Keywords: tussiphonogram, chronic bronchitis.

In recent papers we have described a method of registration of the complete voluntary cough sound. The method was based on experience of Kelemen and Cséri [5, 6] and could be well used for differentiating healthy subjects from those suffering from respiratory tract diseases [8, 9, 15]. The present paper will deal with the question of changes in cough sounds, registered in patients with clinically defined chronic bronchitis.

Materials and methods

Twenty-one diseased probands with chronic obstructive bronchitis (16 males, 5 females) of 50 years mean age were examined in order to establish the above given cough parameters by sound records. They fulfilled the criteria required by WHO.

The voluntary induced cough sound was registered via a microphone on a SONY TC 133 tape recorder and rescribed by an Mingograph Elema 81 at a rate of 100 mm/s. We have registered the complex cough sound and then evaluated its character, intensity and duration. The finding of a single double-sound indicated the healthy state of the respiratory tract. If the sound was trebled, fourfold, or multiple it was considered to indicate an inflammatory disease of the airways.

Cough sound recording was evaluated by its intensity. It illustrated the amplitude integral given in millimetres. Values over 50 mm were regarded as pathological.

The cough sound duration was computed from the integrals. If these pointed to an, overlap of more than 0.45 s, they were regarded as pathological. To confirm the normal limits we compared them to mean $\pm$ S. E. values obtained in healthy probands [8, 9, 10, 15].

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Cough sound recordings were conducted in the course of four days, always at the same time. All the probands were examined by the devices Pneumotest-Junior Jaeger and AW-test Jaeger. We demterined the values for forced vital capacity (FVC), the one-second vital capacity (FEV_1), peak expiratory flow, maximum expiratory flow at 75%, 50% and 25% of vital capacity, and the resistance of airways (Raw), according to methods recommended in the literature [1, 11, 13, 14] and considered sufficient for confirmation of the diagnosis of chronic bronchitis [2, 3, 4].

Tussiphonographic findings were then compared to results obtained from examinations performed in 100 healthy probands (50 females and 50 males).

All probands were made acquainted with the process of examinations. In the course of four days they received no therapeutical procedures and drugs.

The order of examinations was arranged as that the probands were submitted to spirometric examination by the AW test and the Pneumotest-Junior. At the end they were asked to inspire deeply and immediately to perform a voluntary cough. The obtained values were evaluated statistically by means of a Hewlett Packard 41 C apparatus. Differences of $P < 0.05$ were regarded as significant.

Results

The record illustrating the registered voluntary cough sound in healthy probands consisted of one single double-sound. Its intensity was 28 ± 1.6 (SE) mm., its duration was 0.3 ± 0.01 (SE) s. Findings of this type were considered normal [9, 15].

Tussiphonographic examinations performed in patients have shown that almost all had a pathological cough sound. Predominantly, a striking multifarious sound, an increased mono-sound, or a powerful double-sound, interrupted in the first or in the second sound, was accompanied with a forcible noise (Fig. 1).

The intensity of the cough sound registered in bronchitics in the course of four days, were markedly increased (to 244%, 96.8 mm) when compared to values obtained in healthy probands, and the sound duration was delayed by 77% (0.54 s) (Fig. 2). The values given above were statistically significantly increased when compared to values obtained in the course of each single day in healthy probands. Four probands have shown a pathological character of the cough sound registered on record but these values were not increased.

Spirometric values observed daily indicated a chronic bronchitis of obstructive type [11, 13] in all the examined patients. Mean values obtained from the first to the fourth day for forced vital capacity were 73%, the one-second forced vital capacity was 57%, the peak expiratory flow was 66%, the maximum expiratory flow was at 75% VC 38%, at 50% VC 30%, at 25% VC 28% of the actual values. Airways resistance was increased by 36% ($0.47\ kPa \cdot l^{-1} \cdot s$).

In contrast, no correlation could be found between the character, intensity and duration of the voluntary cough sound nor in the values obtained by spirometry.

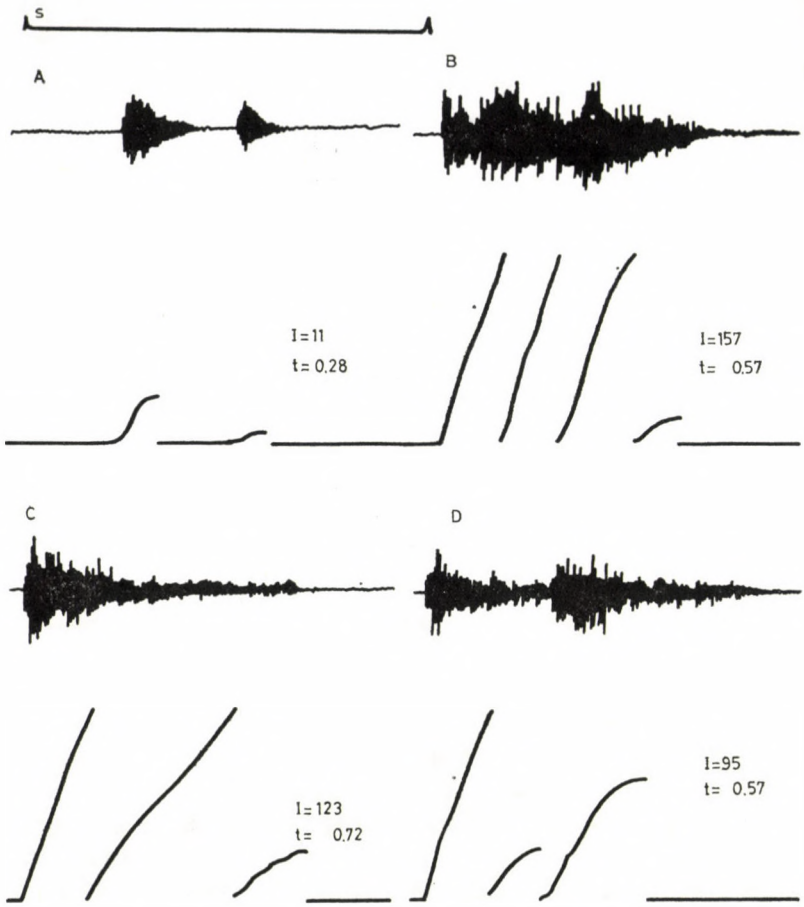

Fig. 1. A. Normal double-sound; B, C, D. Tussigrams from probands suffering from bronchitis. Traces from above to below: time in seconds, recording of the complex cough sound, recording of integral (indicating the magnitude of cough sound intensity in millimetres). The commencement and the end of the record illustrating the integral is pointing to the cough sound duration. The ciphers illustrate the determined values (I = intensity, t = duration in seconds)

Discussion

The results of tussiphonography have shown that probands suffering from obstructive chronic bronchitis displayed a pathological character of the cough sounds as well as of their intensity and duration. The findings were in good correlation with our experience [8, 9, 15]. The only exceptions were four probands whose cough sound character was pathological but the cough sound parameters were in the normal range. According to our experience, these

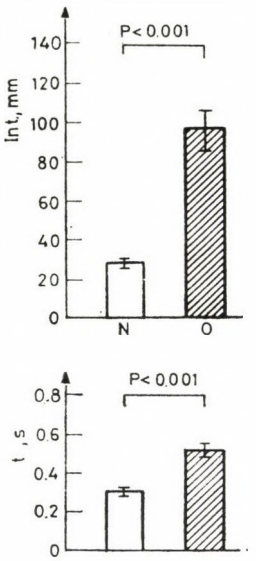

Fig. 2. Mean intensity (INT) and duration (t) of cough sound in healthy probands (plain columns), and of diseased ones (striped columns). The abscissa means SE

types of cough represent a mild type of airways disease [8, 9, 15], or else a chronic bronchitis in the state of quiescence. Spirometric examinations conducted in these patients pointed to pathological values.

The question of the mechanisms of the pathological cough sound has not been clarified. Still if the cough sound is conditioned by additional air in the airways, or even by vibrating adjacent tissues [7], then it is clear that under conditions of narrowed airways overflown with mucus and damaged lung tissues the normal cough sound must be changed. The changes observed in cough sounds have, however, not shown such alterations that would by themselves indicate the proper pattern of chronic bronchitis.

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MEASUREMENT OF AIRWAY RESISTANCE IN AWAKE CATS

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Airway resistance (Raw) measurement with continuous recording was studied in 10 awake cats. The method used was a transducer enabling to incorporate additive resistances by rotating blind. The signals were processed electronically. Part of the measuring device in a pneumotachographic transducer (measurement V') and V_T is obtained by integrating V'. Values for mean Raw were read in both parts of the breathing cycle.

The experiments have shown that the method could be used in awake cats. Values for mean $Raw_{insp.}$ were 1.3 ± 0.24 kPa·l^{-1}·s, $Raw_{exp.}$ of 1.43 ± 0.26 kPa·l^{-1}·s, end-inspiratory Raw was 1.44 ± 0.33 kPa·l^{-1}·s and end-expiratory Raw of 1.49 ± 0.31 kPa·l^{-1}·s.

Keywords: Airway resistance, awake cats.

Body plethysmography is the method most often used in human medicine. This method, however, as not very suitable for use in animals, because of difficulties of the technical equipment and the lack of cooperation required with the subject examined. This was why we decided to use airway resistance measurement by the additive method with continuous recording [3], which has proved to by very helpful.

Material and methods

Our study was performed in 10 awake cats (males and females) with a mean body weight of 2.61 kg (Table I). Tidal volume (V_T) flow (V'), mean inspiratory (mean insp. Raw) mean expiratory (mean exp. Raw), end-inspiratory (end-insp. Raw) and end-expiratory, (end-exp. Raw) airway resistances (Raw) were estimated and mean values were calculated.

The equipment required for measuring Raw by the additive method with continuous recording consists of a special pick-up tube and recording unit, made in the Respiratory Laboratory of Motol Hospital in Prague. Figure 1 (below) shows the scheme of the pick-up tube which consists of three parts: a flow pick-up tube which is a Fleisch type pneumotachograph, (V'), a mouth pressure (P_{mo}) pick-up, and a part with rotating orifice plate (whirling speed = 40 Hz). Periodic changes of the additive resistance corresponded well to the height of step-like

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Table I

Body weight and parameters of airway resistance (Raw) in awake cats

n (awake cats)	10
Body weight (kg)	2.61±0.18 (SE)
Mean insp. Raw (kPa · l^{-1} · s)	1.30±0.24
Mean exp. Raw (kPa · l^{-1} · s)	1.43±0.26
Mean values (kPa · l^{-1} · s)	1.36±0.25
End-insp. Raw (kPa · l^{-1} · s)	1.44±0.33
End-exp. Raw (kPa · l^{-1} · s)	1.55±0.30
Mean values (kPa · l^{-1} · s)	1.49±0.31

Mean values ± SE. n — number of cats.

changes, used for calculation of Raw in the first variant of the additive method (Fig. 1 above). The flow and mouth pressure signals are led to the recording unit which is able to show the actual value of Raw, pointing to the deviation from zero point. V_T is obtained by electronic integration of the flow signal V_T, V', Raw was registered by means of a Mingograph Elema Schönander device. Animals adapted to laboratory conditions before measurement were put into a linen bag. The rubber mask with the recording apparatus was put on the cats' face and V_T, V' and Raw were registered.

Results

During the experiments the cats were quiet and did not resist. After placing the rubber mask on their face, V_T, V' and Raw during 40—60 seconds were recorded.

Figure 2 shows an original record obtained in one of the cats. The above curve represents V_T, in the middle of the curve V' and the curve below means Raw. We evaluated the representative part of the record, when the

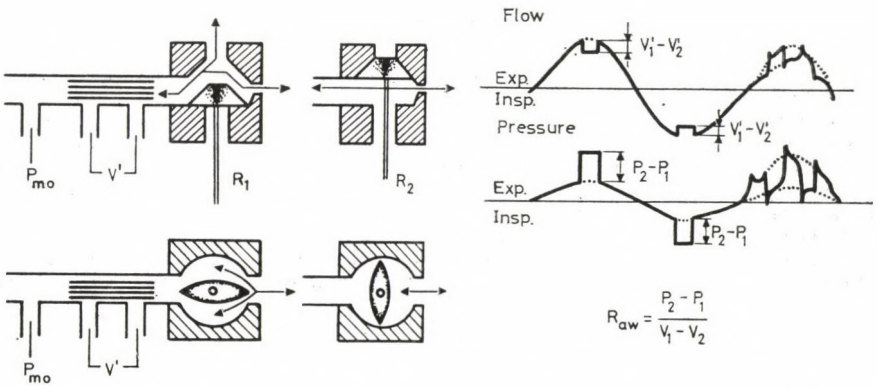

Fig. 1. Scheme of pick-up apparatus of the first (above) and second variant of additive method (below). On the right, a schematic drawing of flow and mouth pressure curve during alteration of added resistance

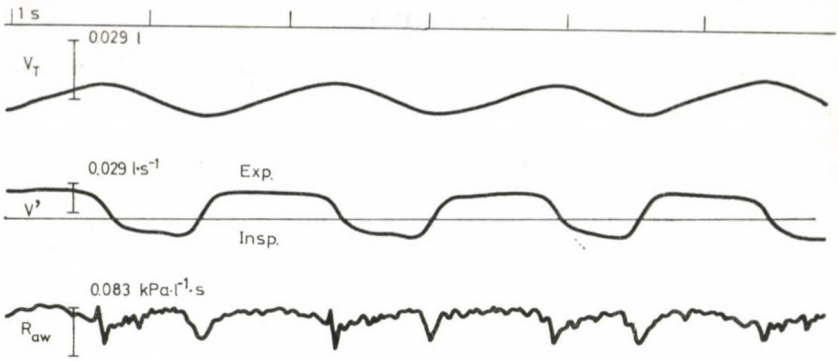

Fig. 2. Original record of tidal volume (V_T), flow (V') and airway resistance (Raw) during measurement in a cat

cat had become adapted to the mask equipped with the recording apparatus. Table I illustrates mean values of Raw. The mean insp. Raw was 1.3 ± 0.24, $kPa \cdot l^{-1} \cdot s$, mean exp. Raw was $1.43 \pm 0.26\ kPa \cdot l^{-1} \cdot s$, their mean was 1.36 ± 0.25 $kPa \cdot l^{-1} \cdot s$. The value of end-inspiratory Raw was $1.44 \pm 0.33\ kPa \cdot l^{-1} \cdot s$, end-expiratory Raw was $1.55 \pm 0.30\ kPa \cdot l^{-1} \cdot s$, their mean values were 1.49 ± 0.31 $kPa \cdot l^{-1} \cdot s$.

Discussion

The technique of measurements of Raw described in this study is analogous to measurements of the intrinsic resistance coming from an electrical source, via two different extrinsic sets, representing series of connected resistances. Kureš [2] presented in detail the theory and the first variant of the additive method. A similar principle of Raw measurement was recently described by Shaw et al. [4]. In accordance to Kureš [2], the given values of the additive method correlated well with those obtained by body plethysmography. Our results indicated values higher than those obtained in anaesthetized animals [5, 6]. The differences in comparison to the results given by Vrlík [6] were not significant.

Thus the additive method allows separated measurements of Raw in the course of inspiration and expiration. The examination is simple and does not last long and does not require any other breathing manoeuvre.

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PHARMACOLOGICAL ANALYSIS OF REACTIVITY CHANGES IN AIRWAYS DUE TO ACUTE INFLAMMATION IN CATS

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Our recent *in vitro* studies on airways smooth muscles of the cat with turpentine oil inflammation showed the occurrence of a contractile response of tracheal preparations and a significant increase in the isometric tension of lung strips to histamine application. This study was aimed to establish whether histamine H_2-receptors participated in the changed in vitro reactivity of the airways smooth muscles of cats suffering from experimentally induced airway inflammation. Pretreatment of control tracheal preparations, control and experimental groups of the lung strips by cimetidine did not change the character of the histamine response. Similarly, the amplitude of histamine relaxation, of the tracheal preparations partially contracted by carbachol was unchanged by experimental inflammation. Clemastine significantly shifted the histamine dose-response curves to the right in both groups of lung strips. However, significant differences in lung strip reactivity between control and experimental groups of cats were not eliminated. Our results do not support the role of histamine H_2-receptors in the pathologically increased airway reactivity to histamine in vitro.

Keywords: airway smooth muscle, experimental inflammation, histamine H_1- and H_2-receptors, cat.

Increased reactivity of airways smooth muscles to wide variety of pharmacological (e.g. methacholine, histamine, prostaglandin $F_{2\text{-alpha}}$) and physical (hyperventilation, inhalation of cold air) stimuli is one of the most characteristic yet poorly understood features of bronchial asthma [4, 5]. It also occurs in normal subjects shortly after a viral upper respiratory tract infection [14], influenza vaccination [22] and ozone inhalation [18] as well as in patients suffering from cystic fibrosis [6].

A number of workers have postulated mechanisms that contribute to the increased airways reactivity. These mechanisms include hyperreactivity of vagal reflex [17, 14, 25] and diminished reactivity to beta-adrenergic stimuli [28]. Based on some experimental observations, a hypothesis of a deficiency of histamine H_2-receptors in asthma was proposed by Chand [8].

Our recent in vitro studies on airway smooth muscles of cats with turpentine oil inflammation showed the occurrence of a contractile response of tracheal preparations and a significant increase of isometric tension of lung

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strips to histamine application [2, 3]. This study was aimed to establish whether histamine H_2-receptors participated in the increased in vitro reactivity of the airway smooth muscles of the cats suffering from experimentally induced airway inflammation.

Materials and methods

The experiments were conducted on 30 male and female cats weighing from 2.0 to 3.0 kg.

Model of airways inflammation. In this study we used the model of turpentine oil inflammation elaborated by Korpáš et al. [20] for studies of respiratory reflex mechanisms under pathological conditions [21]. The cats were anaesthetized with thiopental sodium (SPOFA). The cervical part of trachea was exposed, taking care not to damage the tracheal nerves and blood vessels. A cannula was then introduced into the cervical part of the trachea and through it 5 mg of turpentine oil aerosol was administered for 2 min to spontaneously breathing cats. The cannula was then removed and the incision was sutered. When the inflammatory process in the airways had fully developed i.e. 48 h after turpentine oil application, the inflammatory process in the airways was evaluated on the basis of gross changes in the tracheal mucosa and lungs of sacrificed animals. Part of the trachea and lung parenchyma was taken for histological examination.

In vitro method. The cats of control and experimental groups were anaesthetized with thiopental sodium and exsanguinated. Their thoracic cavity was then opened and the lungs were removed. A 3 mm wide ring was cut out from the trachea [7] and a lung parenchyma strip ($20 \times 3 \times 3$ mm) was cut from the diaphragmatic lobe of the lung, according to Lulich et al. [23]. The preparations were placed in an organ bath containing Krebs—Henseleit solution (NaCl 112.9, KCl 4.7, $CaCl_2 \cdot 2H_2O$ 2.8, $MgSO_4$ 0.5 $NaHCO_3$ 24.9 and glucose 11.1 $mol \cdot l^{-1}$) pH 7.4 at 37 °C saturated with 95% O_2 and 5% CO_2. After an equilibration period of 1 h, doses of histamine (SPOFA, expressed as final molar bath concentration) were added to the organ bath in a cumulative fashion. The histamine H_2-receptor antagonist cimetidine (SMITH, KLINE and FRENCH) and the histamine H_1-receptor antagonist clemestine (SANDOZ) were added to the organ bath 5 min before the addition of histamine. Relaxation responses to histamine were obtained from preparations partially contracted by carbachol (ACO LAKEMEDEL). On each smooth muscle preparation only one dose-response curve was determined. Mechanical responses were recorded isometrically, using a static-dynamic M 1000 tensometric apparatus, and registered by a MTA 175 KUTESZ linear recorder.

Statistics. Histamine responses were plotted as percentage of maximum force at highest concentration of the control group. Individual concentration-response curves were drawn by hand and ED_{50} was defined as the concentration of agent producing a half maximum response. Student's *t* test was used to test the probability of observed differences between means. Differences producing values of less than 0.05 were accepted as significant. In the Figures mean values $\pm$S. E. M. are presented.

Results

Trachea. Tracheal smooth muscle preparations from the control group of cats ($n = 9$) did not respond to histamine in doses of 10^{-7} to 10^{-3} $mol \cdot l^{-1}$. Pretreatment of the tracheal preparations ($n = 5$) by cimetidine (10^{-5} $mol \cdot l^{-1}$) did not change the character of the histamine effect. Figure 1 demonstrates the relaxation on histamine application of a tracheal preparation partially contracted by carbachol administration. In the control group ($n = 9$) the isometric tension achieved after carbachol administration ($5 \cdot 10^{-6}$ $mol \cdot l^{-1}$) was 4.8 ± 0.69 mN. Histamine in a concentration of 10^{-4} $mol \cdot l^{-1}$ relaxed the carbachol contraction to $62.1 \pm 9.9\%$ of its initial value. In the experi-

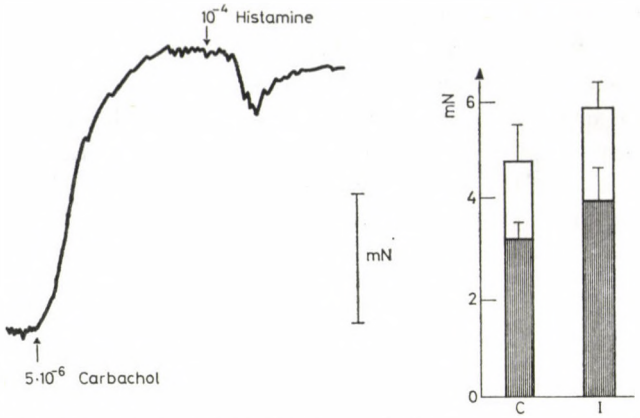

Fig. 1. Relaxation of the carbachol ($5 \cdot 10^{-6}$ mol·l^{-1}) contracted tracheal preparation on histamine (10^{-4} mol·l^{-1}) application (right part). Amplitude of histamine relaxation (open part of column) of the carbachol contraction (whole column) of the tracheal smooth muscle preparation from control (C) and experimental (I) animals (the left part)

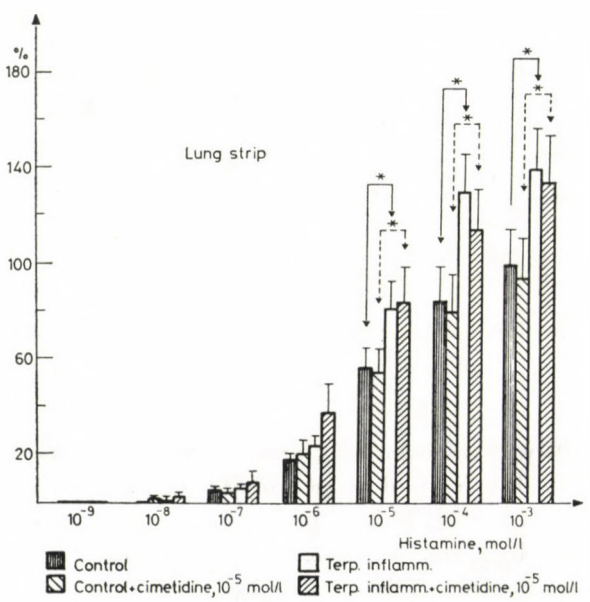

Fig. 2. Effect of cimetidine on histamine (10^{-9}—10^{-3} mol·l^{-1}) response of lung strips from control and experimental groups of cats

mental group (n = 6), no significant differences in mean isometric tension were achieved after carbachol administration (5.89 ± 0.6 mN), neither in amplitude of histamine were relaxations (to $68.1 \pm 5.1\%$) observed.

Lungs. Histamine (19^{-9}—10^{-3} mol·l^{-1}) produced a dose related contraction of the cat isolated parenchyma strip (Fig. 2). In the control group

(n = 8), the maximum isometric tension of the lung strips was 0.61 ± 0.1 mN. The ED_{50} value for histamine was 7.61 ± 0.4. 10^{-6} mol·l^{-1}. In strips, differences between control histamine responses and those obtained from cats suffering from airways inflammation, were evident. The maximum isometric tension of these strips was 0.86 ± 0.12 mN, a value statistically different ($p<0.05$) from that of the control group. The ED_{50} value of histamine (7.08 ± ± 0.38 · 10^{-6} mol·l^{-1}) was, however, unchanged. Cimetidine (10^{-5} mol·l^{-1})

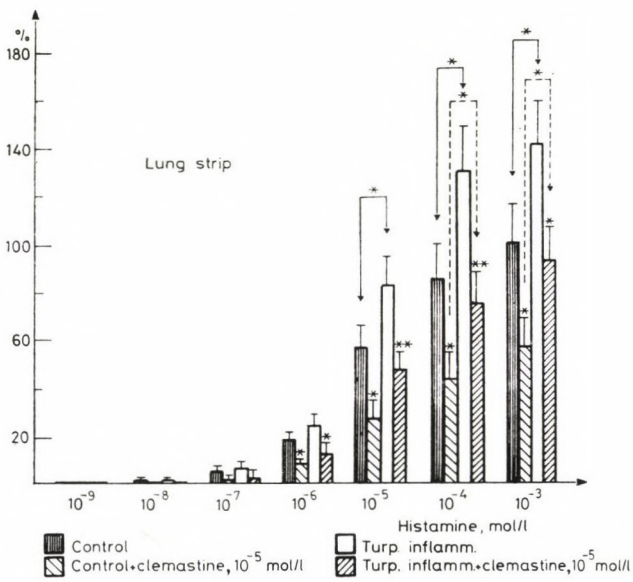

Fig. 3. Effect of clemastine on histamine response of lung strips from control and experimental groups of cats

did not significantly change the maximum isometric histamine tension of the control (0.57 ± 0.09 mN) and experimental (0.82 ± 0.13 mN) lung strip groups. Similarly, the ED_{50} values of histamine were in the control (7.5 ± ± 0.41 · 10^{-6} mol·l^{-1}) and experimental (7.3 ± 0.37 · 10^{-6} mol·l^{-1}) lung strips unchanged after cimetidine.

Figure 3 demonstrates the reactivity to histamine of lung strips in the control and experimental groups after pretreatment by clemastine in a dose of 10^{-5} mol·l^{-1}. Isometric tension in both the control (0.34 ± 0.08 mN) and experimental (0.56 ± 0.1 mN) groups was significantly ($p < 0.05$) decreased after clemastine. Clemastine significantly shifted to the right the histamine dose-response curves. The ED_{50} values for histamine were in the control and experimental lung strip groups 4.48 ± 0.37 · 10^{-5} mol·l^{-1} and 2.7 ± 0.33 · 10^{-5}· mol·l^{-1}, respectively.

Discussion

Histamine is a powerful bronchoconstrictor in many species including man. The effects, however, vary widely between species and between sites within the tracheobronchial tree. It is now well established that the airways smooth muscle of a number of species may contain two histamine receptor types: H_1-receptors, generally causing tracheobronchial constriction, and H_2-receptors, generally producing airway relaxation [15]. The overall airways response to histamine would therefore represent an algebraic sum of the two phenomena occurring simultaneously [16]. Tracheal preparations from healthy adult cats did not respond to histamine by change of tone [23, 9, 2], in agreement with the results reported in this paper. Chand et al. [11] suggested that the adult cat's tracheal muscle contains mainly, or only, histamine H_2-receptors. The presence of histamine H_2-receptors cannot manifest itself with relaxation because the cat tracheal smooth muscle preparations do not possess an internal tone [23]. Partially contracted tracheal smooth muscles of the cats were, however, relaxed by histamine [24]. In contrast to the trachea, the lung strips of the cat responded by contraction in a dose related manner [23, 19, 1].

Our recent in vitro studies on airways smooth muscles of cats with turpentine oil and staphylococcal inflammation of the airways showed contractile responses to histamine application in tracheal preparations [2, 3]. A similar effect was observed by Chand and Eyre [12] in experiments on bronchial smooth muscle preparations of sensitized cats. Rhesus monkeys suffering from common cold [10] and guinea pigs vaccinated with H. somnus [27] showed a diminution of the histamine induced relaxation. Chand [8] suggested that a histamine H_2 receptor deficiency or a physiological imbalance of histamine H_1 and H_2 receptors in the airways may, at least in part, explain the remarkable hyperreactivity to histamine in asthmatics. In this study we were not able to imitate the contractile histamine response of cimetidine treated tracheal preparations in the control group of cats. In addition, the histamine induced relaxation of tracheal preparations from cats with airways inflammation was not significantly changed as compared to the control group. On the basis of these experiments we suppose that the tracheal contractile response to histamine under pathological conditions was not due to a deficiency of histamine H_2-receptors. Under conditions of an inflammatory process of the airways the occurrence of histamine H_2-receptors may be responsible.

In previous experiments we observed a significant increase of isometric tension of the lung strips from cats with turpentine oil and staphylococcal inflammation on histamine application, as compared to the control group [2]. Cimetidine did not significantly change the histamine reactivity of lung strips from control and experimental groups. These results were in agreement with

in vivo experiments that attempted to demonstrate the modulation by specific H_2-antanogists of the histamine-induced or associated bronchospasm. The H_2-receptor antagonists cimetidine and metiamide have been reported to have no effect or to potentiate the histamine induced bronchospasm [13]. Neogrady and Bevan [26] reported no significant effect of cimetidine on the bronchial hyperreactivity or ventilatory function of asthmatic patients. Clemastine significantly shifted to the right the histamine dose-response curves in both the control and experimental groups. However, significant differences in lung strip reactivity between control and experimental groups of cats were not eliminated by clemastine. These results showed that the histamine-induced contraction of the lung strips was mediated through histamine H_1-receptors without a significant participation of histamine H_2-receptors. The results of our experiments with lung parenchyma strips did not exclude the role of histamine H_1-receptors under pathological conditions.

Further studies are needed to elucidate whether the increase in isometric tension under pathological conditions was caused by changes of the smooth muscle membrane H_1-receptors or else an indirect mechanism of the liberation of some endogenous mediators via histamine H_1-receptors was involved; the participation of some other receptor types could not be excluded, either.

Summary: This study was aimed to establish whether histamine H_2-receptors participated in the changed in vitro reactivity of the airways smooth muscle of cats suffering from experimentally induced airways inflammation. Pretreatment with cimatidine of control tracheal preparations and lung strips did not change the character of the histamine response. The amplitude of histamine relaxation of carbachol contracted tracheal preparations was unchanged by experimental inflammation. Clemastine significantly shifted to the right the histamine dose-response curves in both groups of lung strips. Significant differences in lung strip reactivity between control and experimental groups of cats were, however, not eliminated. These results do not support the role of histamine H_2-receptors in the pathologically increased airways reactivity to histamine *in vitro*.

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GABA-ERGIC MECHANISMS IN THE CENTRAL CONTROL OF COUGH

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The antitussive activity of gabalinoleamide (Gabalid® U CB) was studied in 40 non-anaesthetized cats. The antitussive action of the substance was compared to that of codeine (Codein Spofa). Cough was induced by mechanical stimulation using a chronic tracheal cannula. Single cough parameters were evaluated from changes in the side tracheal pressure. When gabalinoleamide was administered in a dose of 100 mg/kg b.w. intramuscularly all the studied parameters of cough showed a statistically significant decrease, only the intensity of maximum cough effort remained unaffected. Gabalinoleamide administered in the same dose induced a statistically significant decrease of respiratory frequency and breathing amplitude, and prolonged the cycle of breathing by delaying the expiratory phase. Higher doses (200 and 300 mg/kg b.w.) did not have an increased cough suppressing effect. The quality and quantity of cough parameters were similar after codeine and gabalinoleamide.

Keywords: experimental cough, breathing, gabalinoleamide, codeine.

Recent results of research in the fields of biochemistry, neurophysiology and neuropharmacology have pointed to the significant role of gamma-aminobutyric acid (GABA) as one of the most important inhibitory neurotransmitters of the mammal central nervous system (Allan and Johnston [1]). Its importance is mirrored by the fact that 30—50% of all synapses in the brain were to be GABA-ergic (Guidotti et al. [3]). Hedner et al. [4, 5] reported that activation of GABA-ergic mechanisms leads to respiratory depression, meanwhile GABA-neurones at different levels of the brain show different activity influencing the central respiratory areas. Since respiration and the cough centers are closely related, we studied the effect of GABA-ergic drugs exerted on the cough reflex.

Of the group of GABA-ergic substances we have chosen gabalinoleamide combination of the amide of the gammaamino-butyric acid and linolenic acid (Papadopoulou-Daifotis et al. [13]), facilitating GABA-ergic transmission. The substance has the advantage to penetrate across the haematoencephalic

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barrier. According to Vamvakides and Papadopoulou-Daifotis [13] the drug causes hypothermia and is an anticonvulsant, it prolongs sleep initiated by barbiturates, and interrupts the effect of amphetamine.

Materials and methods

Healthy awake cats weighing from 1500—2500 g were used in the experiments after one week quarantine. In this period the animals were given a normal diet (Kemény and Balázs [8]). Cough was induced by mechanical stimulation of airways, by the method of Korpáš et al. [9, 10]. Parameters of cough and breathing were evaluated on basis of the side tracheal pressure. Of the cough components, we evaluated the number of cough efforts, their intensity, the intensity of cough attacks during expiration and inspiration cough frequency and the intensity of maximum expiratory and inspiratory cough effort (Korpáš and Tomori [10]).

Statistical evaluation of results was performed by Wilcoxon and Wilcoxs rank test [13]. The values given in diagrams illustrate the average $\pm$S. E. M. values of single cough parameters. The significance of 5% is signed by one dot, the 1% significance by two dots. Gabalinoleamide (Gabalid U CB) was applied in doses of 100, 200 and 300 mg/kg body weight, i.m. Codeine dihydrophosphate (Codein Spofa) was used in a dose of 10 mg/kg b.w. intraperitoneally as a reference substance to which the antitussive activity of gabalinoleamide was compared.

Results

After gabalinoleamide was applied intramuscularly (i.m.) in a dose of 100 mg/kg b.w., i.m., a statistically significant decrease was observed in the number of cough efforts (NE) as well as in the intensity of cough attacks during expiration (IA^+) and inspiration (IA^-), both from the laryngopharyngeal (Fig. 1) and the tracheobronchial mucous area of the airways (Fig. 2). The character of decrease in the given cough parameters was very similar to that registered after codeine applied intraperitoneally in a dose of 10 mg/kg

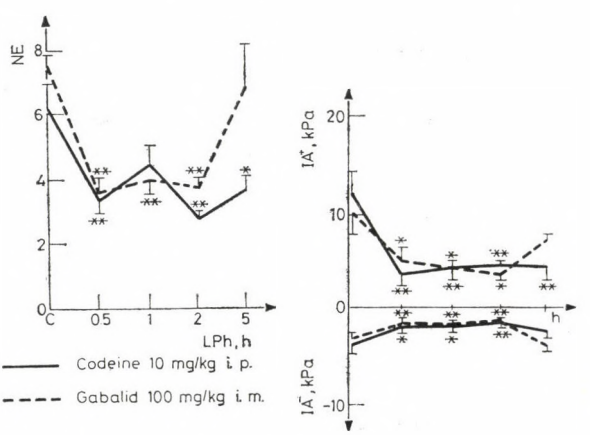

Fig. 1. Number of efforts (NE) and intensity of cough attack during expiration (IA^+) and inspiration (IA^-) from the laryngopharyngeal area (LPh) after application of codeine and gabalinoleamide C — control

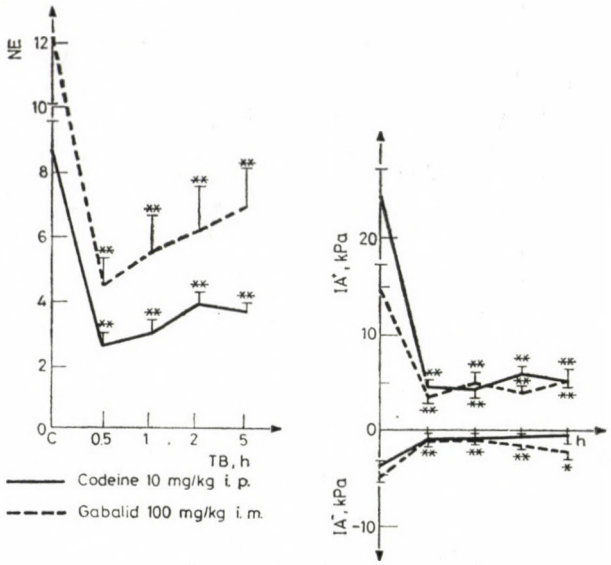

Fig. 2. Number of efforts (NE) and intensity of cough attack during expiration (IA^+) and inspiration (IA^-) from the tracheobronchial area (TB) after application of codeine and gabalinoleamide

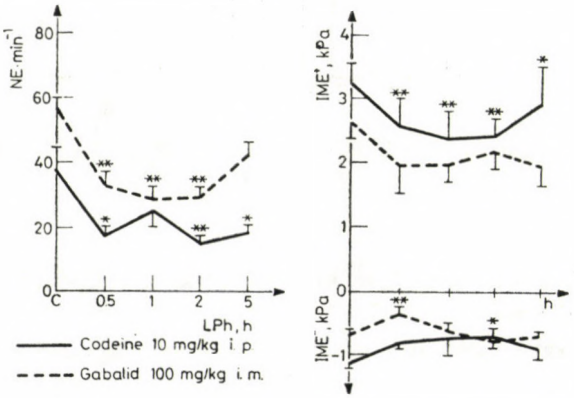

Fig. 3. Cough frequency ($NE \cdot min^{-1}$) and intensity of maximum cough effort during expiration (IME^+) and inspiration (IME^-) from the laryngopharyngeal area (LPh) after application of codeine and gabalinoleamide

b.w. Cough frequency ($NE \cdot min^{-1}$) was statistically highly significantly decreased from both the laryngopharyngeal (Fig. 3) and the tracheobronchial area (Fig. 4).

The intensity of maximum cough effort (IME) was the only parameter to resist the effect of gabalinoleamide; a statistically significant decrease of that component appeared in the 30th minute of the experiment.

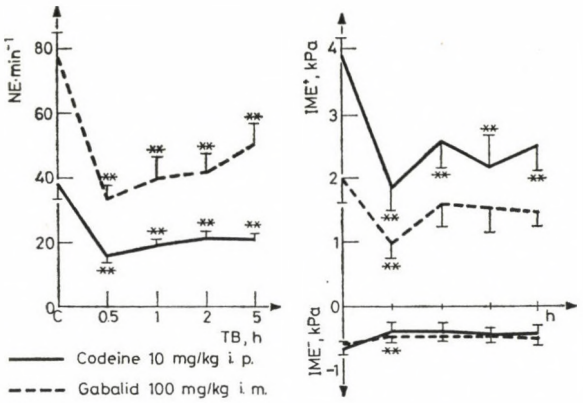

Fig. 4. Cough frequency ($Ne \cdot min^{-1}$) and intensity of maximum cough effort during expiration (IME^+) and inspiration (IME^-) from the tracheobronchial area (TB) after application of codeine and gabalinoleamide

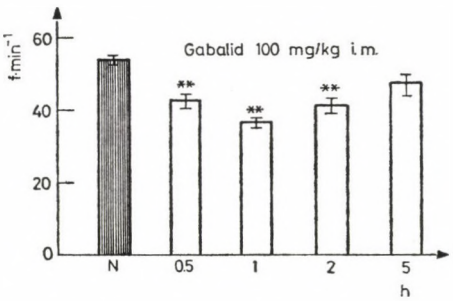

Fig. 5. Respiratory frequency after intramuscular application of gabalinoleamide in a dose of 100 mg/kg body weight

Trying to increase the cough-suppressive effect, we administered higher doses. Still, neither 200 mg/kg b.w. nor 300 mg/kg i.m. had a higher suppressive effect on the parameters studied.

When, however, gabalinoleamide was applied in a dose of 200 mg/kg b.w. i.m., very significant decrease of breathing frequency could be observed. The decrease began in the 30th minute of the experiment, and the values returned to normal in the 5th hour. As it is seen in Fig. 5, the amplitude of breathing decreased and the breathing cycle was prolonged, mainly by the delay of expiration.

Discussion

The results showed that gabalinoleamide suppressed all the studied cough parameters in healthy awake cats. The antitussive effect appeared after application of doses 7—8 times less than one LD_{50}. This seems to indicate

the possible participation of GABA-ergic mechanisms in the central control of cough. A comparison of the character of decrease of the single cough parameters after application of gabalinoleamide and codeine offers only theoretical possibilities for explanation of the mechanism of centrally acting cough suppressing substances by interaction with GABA-ergic receptors. Our suggestion is supported by Kuryama and Yoneda [11] who reported that GABA-ergic mechanisms do take the role of opiate effects. Besides, DeFeudis [2] found that if morphine was administered in a dose of 20—30 mg intraperitoneally, the activity of glutamic acid decarboxylase was increased, whereas the activity of aminotransferase (GABA-T) was inhibited. This fact pointed to the increased gammaamino-butyric acid contents in certain areas of the central nervous system. Since codeine is a morphine derivative, its cough suppressing effect could therefore be exerted on the level of gammaamino-butyric acid, but only further studies will decide this point.

Our results obtained in awake cats have shown that gabalinoleamide has a suppressing effect on the breathing frequency. The amplitude of breathing and the breathing cycle were prolonged by the delay of expirations. Our findings of a decrease in breathing frequency and the prolonged expiration correlate well to findings by Hedner et al. [4, 5, 6] and Lagercrantz [12], who also found a similar effect of gammaamino-butyric acid and muscimol, also in anaesthetized cats. According to these authors, the respiratory depression appearing after application of the given substances resulted from a direct activation of GABA-ergic receptors.

Application of gabalinoleamide in a dose of 100 mg/kg b.w. statistically significantly decreased all the studied cough parameters with exception of the intensity of maximum cough effort. When gabalinoleamide was administered in a dose of 200 mg/kg b.w., its cough suppressing effect was not augmented.

It is suggested that GABA-ergic mechanisms participate in the inhibitory effect acting on the cough centre. Since the character of the decrease in the studied cough parameters was similar after codeine and gabalinoleamide administration, this justifies the suggestion that GABA-ergic mechanisms might be involved in the mechanism of action of narcotic antitussive drugs.

Gabalinoleamide administered in a dose of 100 mg/kg b.w. induced a statistically significant decrease of breathing frequency. The amplitude of breathing was decreased, and the breathing cycle was prolonged, due to the delayed expiration.

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EFFECT OF HYPOXIA AND HYPERCAPINA ON THE AIRWAYS DEFENCE REFLEXES

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In experiments on 10 adult anaesthetized cats (pentobarbital 30 $mg \cdot kg^{-1}$ i.p.) the effect of stimultaneous hypoxia and hypercapnia was studied on the defence respiratory reflexes of the airways. Expiratory reflex and cough were elicited by mechanical stimulation of the airways mucosa, and the obtained values were evaluated on basis of the intrapleural pressure. Inhalation of the hypoxic-hypercapnic gas mixture (11% + 7% CO_2 in N_2) for 15 minutes led to a significant decrease of respiratory frequency, tidal volume and $PaCO_2$, while pHa and $PaCO_2$ also decreased significantly together with the intensity of the expiratory reflex and that of cough. Recent studies, showed that in the course of the effect of hypoxia (11% O_2) and of hypercapnia (5% CO_2), cough intensity decreased, but the change was not significant. The decrease of the intensity of respiratory defence reflexes under hypoxic-hypercapnic conditions might have been due to the changes of centrally controlling structures, or to the effector part of the reflex arc, resulting from fatigue of the respiratory muscles. The possible effect of anaesthesia exerting a significant influence on the intensity and character of airways defence reflexes could not be excluded.

Keywords: Expiration reflex, cough, hypoxia, hypercapnia, anaesthetized cats.

Significant airways defence reflexes may appear in the course of inflammatory and other respiratory tract diseases. Loudon and Shaw [6] have found that cough was more frequent in patients suffering from chronic bronchitis, and it was depending on the higher demand of clearance activity, producing a greater amount of mucus. Further it also depended on its lowered effectiveness. To obtain an effective cough effort accompanied with expectoration, several cough efforts have to be produced. The cough response during bronchoscopy was observed by Lemoine [5] who reported that in chronic bronchitis a rather high percentage of cases showed a weakening of cough in response to a tactile stimulus.

Respiratory tract diseases may lead to respiratory insufficiency, the sequelae of which are a lowered saturation of haemoglobin with oxygen, and a deficient removal of CO_2 from blood.

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In recent studies performed in anaesthetized cats it was found that inhalation of hypoxic and hypercapnic gas mixtures for 15 minutes affected the parameters of airways defence reflexes. Isobaric hypoxia (11% O_2 and 7% O_2 in N_2) decreased the intensity of cough and of the expiratory reflex, meanwhile a significant decrease of expiration reflex intensity could be observed at 11% O_2, and a weakening of cough intensity at 7% O_2 [10]. During hypercapnia (5% CO_2 and 10% CO_2 + 25% O_2 in N_2) a significant decrease of expiration reflex intensity could be found at 5% CO_2, as well as of cough intensity at 10% CO_2 [7]. The aspiration reflex did not show any significant changes even under conditions of hypoxic hypoxia and/or hypercapnia.

This paper gives results of our studies of the effects of simultaneous hypoxia and hypercapnia on expiratory reflex intensity and on cough parameters.

Methods

Ten adult cats (2.0—3.5 kg) of both sexes were anaesthetized with pentobarbital (Spofa 30 mg·kg^{-1}, i.p.). A polyethylene cannula was introduced into the trachea and its open end was connected to a respiratory circuit with a Fleisch No 1 resistance head [10]. An approximately constant inspiratory gas stream of 10 l·min^{-1} was maintained. In that way, an inspiratory-expiratory valve was created, without additional resistance for breathing. The hypoxic-hypercapnic mixture was produced so that air was mixed with N_2 and CO_2 in respiratory circuit. The levels of O_2 and CO_2 were checked by means of the oximeter Spirolyt Junkalor and Capnograph Godart. An intrapleural cannula was introduced into the 5th intercostal space, a pressure transducer was connected to it, and pleural pressure was measured. The left femoral artery was catheterized and arterial blood samples were analyzed immediately with Gas Check AVL 938. The animals were fixed in supine position, and a constant body temperature (about 38 °C) was maintained by means of a heated pad. Tidal volume (V_T), respiratory frequency (f) and minute ventilation (V_E) — were determined by the means of the Godart Pneumotachograph.

Airways defence reflexes were elicited by mechanical stimulation of the mucosa, according to Korpáš and Tomori [4], the intensity was evaluated by changes observed in intrapleural pressure. The expiratory reflex (ER) was induced by a silon fibre loop, touching the vocal cords. Cough was elicited by inserting the silon fibre repeatedly for 5 s into the tracheobronchial area. The intensity of ER illustrated the change of pressure, observed during one expiratory effort [3].

In cough, the number of efforts (NE), the number of efforts per minute (NE·min^{-1}), the intensity of maximum effort (IME), and the intensity of the coughing attack (IA) were evaluated [4]. The inspiratory and expiratory phases were analysed separately.

Parameters of ventilation, pH, blood gases and airways defence reflexes were studied in the course of inhalations of ambient air (control conditions). After an interval of 15 minutes, the above parameters were studied again in the 10th to 15th minute after the animals were exposed to inhale the hypoxic-hypercapnic gas mixture (11% O_2 + 7% CO_2 in N_2). The inhaled gases were heated approximately to 37 °C and then saturated with water vapour.

The statistical significance of the effect of hypoxic-hypercapnic conditions on V_T, f, V_E, PaO_2, $PaCO_2$ and the pH was evaluated by Student's *t* test. Airways defence reflex parameters were evaluated by Wilcoxon's signed rank test. Values of $p<0.05$ were regarded as significant.

Results

Changes in parameters of ventilation, pH and blood gases induced by acute inhalation of hypoxic-hypercapnic gas mixture were compared to control conditions. Results are illustrated in Table I. V_T, f, V_E and $PaCO_2$

Table I

Ventilation parameters: V_T — tidal volume, f — frequency of breathing, V_E — minute ventilation, pH and partial pressure of O_2 ($PaCO_2$) and CO_2 ($PaCO_2$) in arterial blood during hypoxic-hypercapnic gas mixture inhalation, compared to control values

	Ambient air $\bar{x} \pm SD$	11% O_2 + 7% CO_2 $\bar{x} \pm SD$
V_T (ml)	25.7±4.9	64.0±15.3
f (min^{-1})	32.3±8.3	63.8±9.9
V_E (l)	0.81±0.2	3.92±1.2
pHa	7.284±0.041	7.220±0.044
PaO_2 (kPa)	11.5±0.8	7.3±0.5
$PaCO_2$ (kPa)	3.5±0.6	4.8±0.3

Changes of all the parameters were at the level of significance, $p < 0.001$.

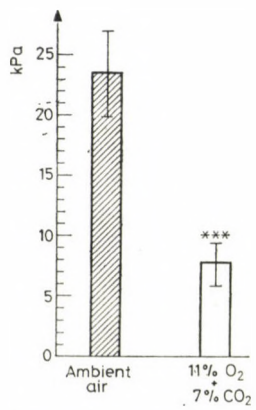

Fig. 1. Intensity of expiration reflex (ER) under hypoxic-hypercapnic conditions. ***: $p < 0.001$

showed a significant increase and also a significant decrease in pH and PaO_2 values.

Parameters of airways defence reflexes were significantly influenced by hypoxic-hypercapnic conditions. The ER (Fig. 1) was markedly weaker. Changes in single cough parameters are given in Fig. 2. Whereas the number of cough efforts during one attack (NE) did not change significantly, the number of cough efforts per minute ($NE \cdot min^{-1}$) was significantly increased. Of the parameters indicating the intensity of the cough response, the intensity of maximum effort (IME) and the intensity of cough attack (IA) decreased significantly during expiration, but on inspiration only IME was significantly decreased.

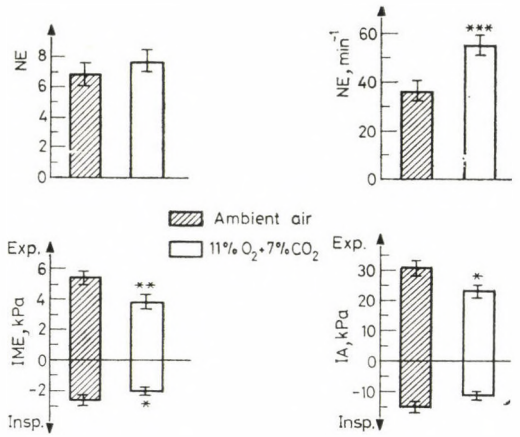

Fig. 2. Cough parameters: NE — number of efforts, Ne·min^{-1} — number of efforts per minute, IME — intensity of maximum effort, IA — intensity of attack under hypoxic-hypercapnic conditions. exp — expiration, insp. — inspiration *: $p < 0.005$, **: $p < 0.01$, ***: $p < 0.001$

Discussion

Isobaric hypoxia (11% O_2 in N_2) in anaesthetized cats increased significantly the frequency of breathing, but V_T did not change [10]. Marked increase of V_T and frequency appeared under the influence of hypoxia and hypercapnia (11% O_2 + 7% CO_2 in N_2) even under different PaO_2 values in the given groups of cats. Whereas PaO_2 decreased under the effect of hypoxia to 5.6 ± 0.3 kPa, PaO_2 decreased under the influence of hypoxia and hypercapnia to 7.3 ± 0.5 kPa only, because the oxygen gradient between the inspired air and alveolar air was reduced.

The significant decrease in intensity of the expiratory reflex under hypoxic-hypercapnic conditions can be explained by changes which may occur in central controlling mechanisms in the oblongata medulla, and also by the altered tension of the vocal cords.

Hypoxia and hypercapnia caused a significant decrease of the expiratory laryngeal resistance [1]. The vocal cords were only slightly tensed during expiration, so that when the silon fibre loop was inserted into the glottis, the vocal cords were less deformed causing a weaker stimulation of the expiratory reflex receptors.

The number of cough efforts (NE) during the attack did not change under conditions of hypercapnia associated with moderate hyperoxaemia [7] after the airways mucosa was stimulated constantly, NE became, however, significantly decreased under conditions of hypoxic hypoxia [10]. Until the number of cough efforts per minute had not changed under hypoxia [8], hyper-

capnia was shown to induce a significant increase in cough parameter [7]. Both cough parameters showed the same change under simultaneous hypoxia and hypercapnia, as we could observe it in cats under hypercapnic conditions. After comparing the results obtained in the three groups they indicated that afferent stimulations originating from central chemoreceptors had a different influence on the generation of cough efforts in anaesthetized cats than had the afferentation from peripheral chemoreceptors.

Inhalation of the hypoxic gas mixture (11% O_2) [10] and also of the hypercapnic mixture (5% O_2) [7] led to a non-significant decrease of expiratory cough intensity. Hypoxia and hypercapnia decreased the expiratory cough intensity significantly, even when the percentage decrease of cough intensity was not more stressed than the percentage decrease under conditions of hypoxia or hypercapnia. Fig. 2 shows that while the intensity of the cough attack decreased non-significantly during inspiration, the maximum cough effort decreased significantly. The difference can only be explained by effects that involve the maximum effort within one cough attack during inspiration, while the other efforts did not change significantly.

It is not clear how cough receptor activity would change under conditions of hypoxia and hypercapnia. One finding only is known, that the activity of irritant receptors, to which the cough receptors also belong, do not change when subjected to effects of hypercapnic gas mixture inhalations. To clarify why the cough is weakened under hypoxic hypercapnic conditions, requires further studies. It will then be possible to confirm whether the decrease of cough intensity is due to the degree of centrally acting structures or to a weakening of the effector part of the reflex arc by fatigue of the respiratory muscles.

We may conclude that the influence of hypoxia and hypercapnia together affects significantly the parameters of airways defence reflexes. The intensity of expiration reflex and cough decreased significantly. Beside factors explaining the cause of the changed reflex intensity a certain role must be attributed to anaesthesia, because of its important effect on the airways defence reflex control [9]. This suggestion is supported by our earlier findings indicating that the phase of acute isobaric hypoxia (11% O_2), the intensity of the respiratory reflex and of cough was increased in conscious cats [11], meanwhile it was decreased in anaesthetized cats [10].

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AGE- AND BODY-WEIGHT-DEPENDENT CHANGES IN THE REACTIVITY TO HISTAMINE OF THE AIRWAYS SMOOTH MUSCLES IN CATS

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The relation between age and body weight and the reactivity to histamine of the tracheopulmonary contractile tissues was studied is cats in vitro. The reactivity of the tracheal smooth muscle to histamine decreased with increasing age from the newborn (100%) and one-month-old (57%) to the adult (0%) cat, whereas the amplitudes of contractions of lung strips increased. The amplitudes in adult animals also increased moderately with increasing body weight.

Keywords: smooth muscle, trachea, lung strip, age, body weight, histamine, cat.

Some recent studies (e.g. 6) proved the usefulness of the cat in the field of pharmacology of smooth muscle. The physiology and pathophysiology of respiratory functions in the cat has been studied thoroughly (e.g. 7). For these reasons it was decided to choose the cat for pharmacological studies on the smooth muscle of the airways. To obtain the basic parameters it was necessary to find out the possible changes in reactivity in dependence of some physiological factors. Thus, the dependence of the reactivity to histamine of the airways smooth muscles was studied in vitro on the age and body weight of cats.

Materials and methods

The experiments were conducted in 9 newborn, 7 one-month-old, and 10 adult cats of both sexes. The adult cats weighted from 1.5 to 3.5 kg. They were anaesthetized with thiopental sodium (30 $mg \cdot kg^{-1}$) and exsanguinated. Their thoracic cavity was opened and the trachea and lungs were removed. A ring 3 mm wide was cut out from the cervical trachea and a lung perenchyma strip ($20 \times 3 \times 3$ mm) was cut out from the diaphragmatic lobe according to Lulich et al. [8]. The preparations were placed in an organ bath containing Krebs—Henseleit solution (NaCl 112.9, KCl 4.7, $CaCl_2 . 2H_2O$ 2.8, $MgSO_4$ 0.5, $NaHCO_3$ 24.9 and glucose 11.1 $mol \cdot l^{-1}$) pH 7.4 at 37 °C saturated with 95% O_2 and 5% CO_2. After 20 min. initial tension up to 5 g (trachea) or 4 g (lung strip) and after an adaptational interval of 30 min. at a working tension up to 2 g, histamine (Spofa) was added cumulatively to the organ bath. The various doses used for the final concentration in the organ bath (10^{-9}—10^{-3} $mol \cdot l^{-1}$) were always added in 0.2 ml volume. Mechanical responses were recorded isometrically using a static — dynamic TDA_3 tensometric apparatus and registered on a MTA 175 KUTESZ linear recorder.

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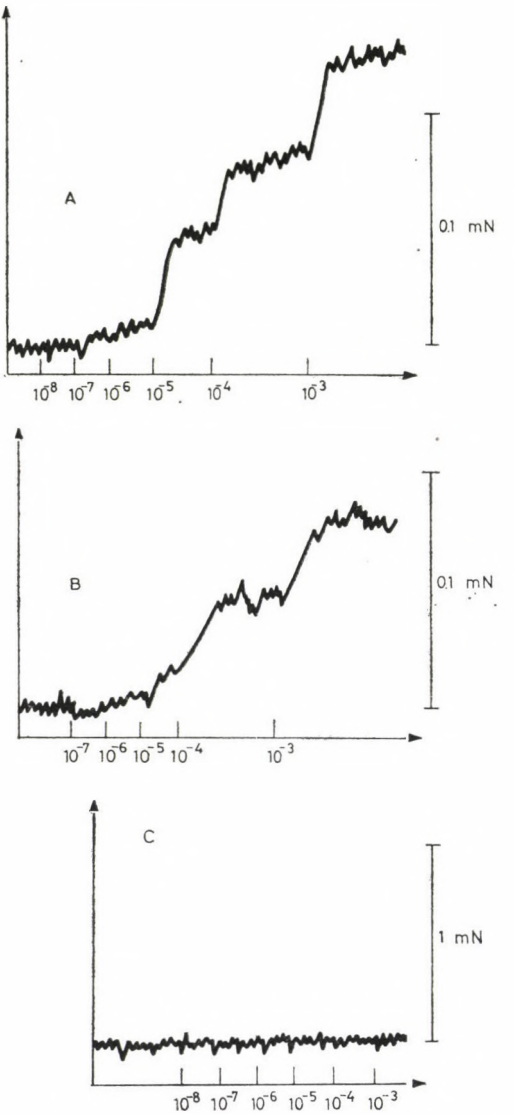

Fig. 1. Response to histamine of tracheal preparations from newborn (A), one-month-old (B) and adult (C) cats. Abscissa: concentration of histamine, mmol·l^{-1}

Results

Figure 1 shows recordings of tracheal preparations from newborn, one-month-old and adult cats giving responses to cumulative concentrations of histamine. Meanwhile preparations from the newborn and partially from the

one-month-old cats responded to histamine at a concentration of 10^{-5} mol·l^{-1} with contractions, while tracheal preparations of adult cats did not respond even after the highest concentration (10^{-3} mol·l^{-1}). The contractions were observed in all preparations from the newborn cats, but only in four of 7 preparations from one-month-old cats (57%) and in none of adult cats. Amplitudes of contractions observed in the preparations of newborn and one-month-old cats did not differ significantly.

The reactivity to histamine of the lung strip preparations of the newborn, one-month-old and adult cats is shown on Figures 2 and 3. All preparations responded with contraction above the concentration of 10^{-8} mol·l^{-1}, but the amplitudes of the contractions to the highest concentration (10^{-3} mol·l^{-1}) were in the newborns 28.6 $\pm$ 4.2% only, in the one-month-old cats 35.0 $\pm$ 3.2% of the amplitudes of contraction in the preparations from adult cats. The values of pD_2 for histamine were in the newborn lung strips 4.66 $\pm$ $\pm$ 0.09 and in the one-month-old 5.62 $\pm$ 0.09. Both values in comparison with the value of adult cats (5.32 $\pm$ 0.08) differed significantly ($p < 0.05$).

The dependence of the reactivity of lung strips from the adult cats on their body weight was tested separately in preparations from the left and the right diaphragmatic lobus, the results are given in Figure 4. The reactivity did not differ significantly. The body weight dependence was of moderate degree with the left lungs $r = 0.67$, and with the right lungs, $r = 0.62$.

Discussion

Changes in the reactivity of smooth muscle cells to substances of mediator or autacoid character are generally interpreted in relation to the changes of membrane receptors. So were observed the changes of adrenergic beta receptor, density in the smooth muscle of the airways of guinea-pigs [3, 4] as well as of the $alpha_1$ receptors of dogs [9]. Pandya [9] did not find any change in the sensitivity to acetylcholine.

There occur two types of histamine receptors in the smooth muscle of the airways [4]: H_1 for contraction and H_2 for relaxation. In dogs [10] and guinea-pigs [1] changes in histamine receptors were found which depended on the phase of ontogenesis. On the basis of the obtained results (Fig. 1) it could be suggested that in the course of ontogenesis the number of H_1 receptors of the smooth muscle cells decreased in the cat trachea. Brink et al. [1] observed similar changes in the tracheal smooth muscle of the guinea-pig. The higher reactivity and contractility of the lung tissue in older animals may reflect the growth of the pharmacologically active lung tissues in the course of ontogenesis. In the lung strips taken from one-month-old cats a significant increase of pD_2 values in comparison with those from newborn cats was

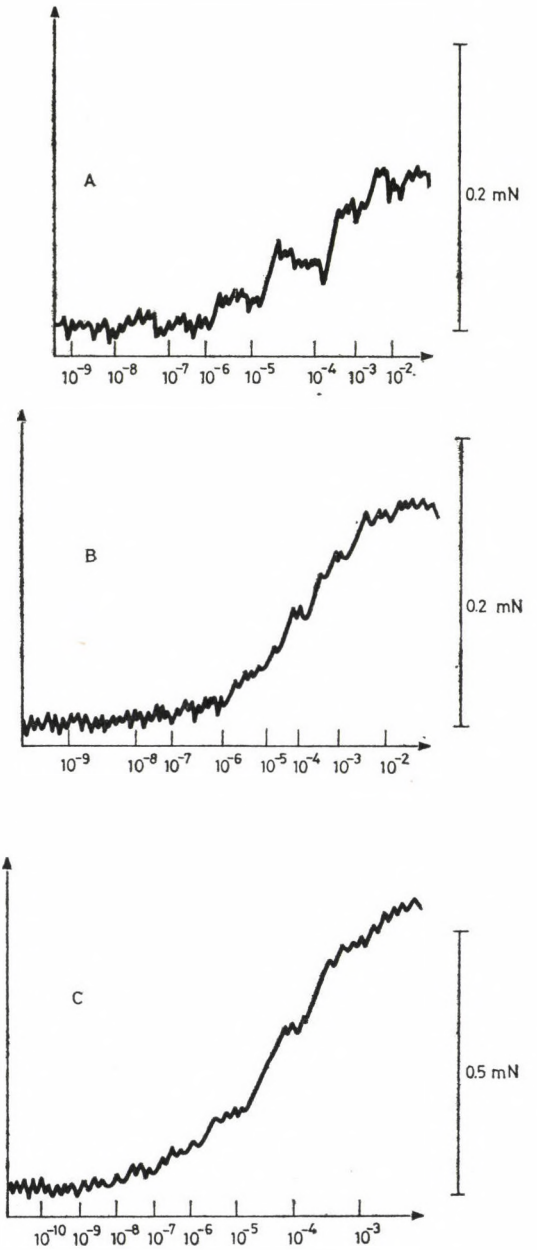

Fig. 2. Response to histamine of lung strip preparations from newborn (A), one-month-old (B and adult (C) cats. Abscissa: concentration of histamine, $mmol \cdot l^{-1}$

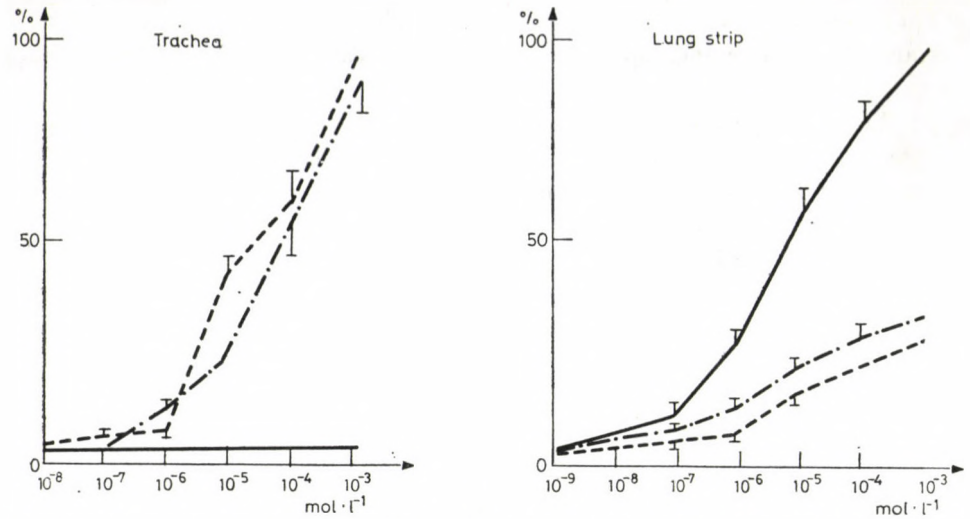

Fig. 3. Reactivity to histamine of tracheal and lung strip preparations from newborn (– – – – –), one-month-old (–·–·–·–·–) and adult (———) cats. Abscissa: concentration of histamine. Ordinate: contraction of the preparations in per cent of the maximum contraction of the adult group. Each value represents $\bar{x} \pm$ S. E. M.

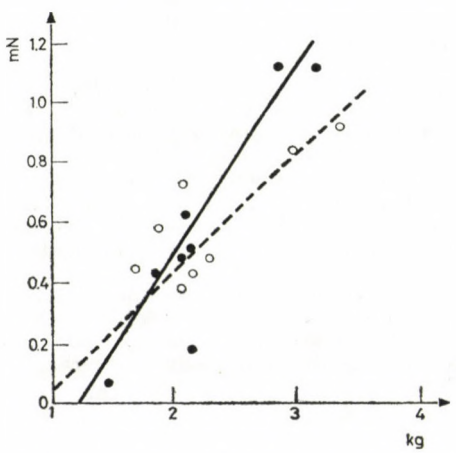

Fig. 4. Dependence of the reactivity to histamine of lung strips from adult cats on body weight of the animals. Abscissa: body weight, ordinate: contraction in mN after 10^{-3} mmol·l^{-1} of histamine. (———) preparations from right lung; (– – – – –) preparations from left lungs; solid line — correlation for right lungs; dotted line — correlation for left lungs

observed, with a partial decrease in the reactivity of adult cats. An analogous double-phase shift of the pD_2 values was observed in the reactivity to isoproterenol of the rabbit aorta by Hayashi and Toda [5]. The beta receptor activity gradually increased in their experiments from the 2nd to the 30th day with a consecutive decrease till the end of the 1st year of life. Brink et

al. [1], however, did not find any age-dependent change of the reactivity to histamine in guinea-pig lung strips, while the values of pD_2 of the tracheal smooth muscle decreased with increasing age.

The high percentage of pharmacologically active airways tissues may be the cause of the high reactivity to histamine in the lung strips of cats with higher body weight. The correlation is of moderate degree only, so one may suppose the body weight not being the factor essentially altering the reactivity of the animals used (e.g. between 2 and 3 kg in cats). Neither were these distinct differences in the reactivity of preparations between right and left lungs. This fact allows to take more preparations from the same animal simultaneously.

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EFFECTS OF ANTITUSSIVE DRUGS UNDER NORMAL AND PATHOLOGICAL CONDITIONS

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The antitussive effect codeine and l-propoxyphene have been studied in nonanaesthetized healthy cats and cats with respiratory tract inflammation elicited by undiluted croton oil. The drugs were administered intraperitoneally in doses of 10 and 20 mg/kg body weight. The antitussive effect was studied on the 4th day, after inflammation had set in. Cough induced in nonanaesthetized cats by mechanical irritation of the laryngopharyngeal and tracheobronchial areas was evaluated by changes of the lateral tracheal pressure. Experimentally induced inflammatory changes of the respiratory tract due to the antitussive activity of l-porpoxyphene were significantly reduced, but that of codeine had not changed at all.

Keywords: cough, codeine, l-propoxyphene, pathological conditions.

Cough represents one of the common and main symptoms of respiratory diseases of various aetiology. Clinical experiences have often shown that if a cough was present in respiratory tract inflammatory diseases it was usually of non-productive, irritant character causing exhaustion and general worsening of the patient's condition. In such case, the physician is forced to suppress the cough. According to Korpáš and Tomori [12] and to Hanáček [7] during airways inflammation induced in experimental animals, changes of quantity and quality of the cough reflex could be observed. Data are, however, scarce on the question whether the activity of drugs inducing cough reflex depression is changed under pathological conditions.

Most authors studied the antitussive activity of drugs in healthy subject [2, 3, 5, 6, 20]. This has made us to study the question whether experimentally induced inflammatory changes of the respiratory tract may affect the antitussive activity of some drugs in laboratory animals. Codeine, the most often prescribed antitussive and l-propoxyphene a synthetic morphine substitute were studied.

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Materials and methods

Adult non-anaesthetized cats of both sexes, weighing 1500—2500 g, were used in the experiments. According to the method described by Korpáš et al. [10] a chronic cannula was introduced into the animals' trachea for mechanical stimulation of the airways and measurement of side tracheal pressure. Single cough parameters were evaluated from pressure changes. Cough was induced by stimuli applied on the laryngopharyngeal and tracheobronchial area, using a 0.35 mm thick nylon fibre. We evaluated the number of cough efforts (NE), and the intensity of cough attacks during expiration (IA^+) and inspiration (IA^-).

Experimental inflammation of the respiratory organs was evoked by undiluted croton oil administered in gross-article aerosol under 0.6 atmosphere pressure via the chronic tracheal cannula. In the course of the experiment the animals were kept in conscious state. Inflammatory changes had manifested by days.

Values obtained before administration of the antitussive drugs illustrated the normal parameters, then cough was induced after 0.5, 1, 2, and 5 hours in conscious cats. If the substances were applied orally, many cats vomited so no exact doses could be ensured. Therefore, the drugs were applied intraperitoneally, codeine in a dose of 10 mg/kg b.w., and l-propoxyphene in the form of orotane salt in a dose of 20 mg/kg b.w.

Results are shown in the Figures. They illustrate the mean values of the cough parameters. The abscissa means $\pm$ S. E. M. The significance was evaluated in points by the rank test of Wilcoxon and Wilcox [21] 5% for one point ($P < 0.05$) and 1% for two points ($P < 0.01$).

Results

Figure 1 illustrates the number of efforts and the intensity of cough induced by stimulation of the laryngopharyngeal area, after codeine applied in a dose of 10 mg/kg body weight to healthy cats (solid line) and to cats with experimentally induced inflammation of the organs of respiration (broken line). Codeine induced a statistically significant decrease of the number of efforts and also of the intensity of the cough attack. Both changes could be observed both in healthy and test animals.

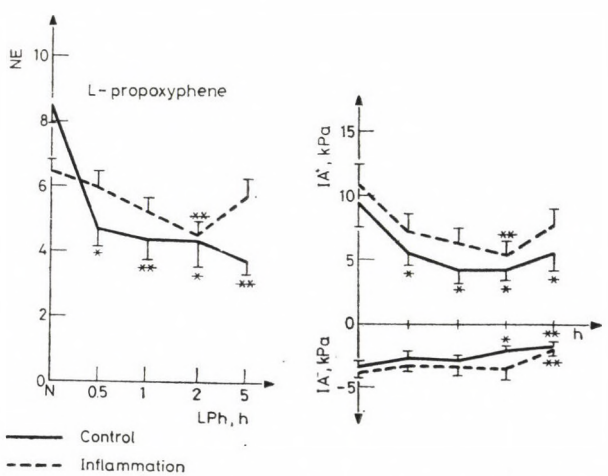

Fig. 1. Effect of codeine phosphate in a dose of 10 mg/kg b.w. on number of cough effors (NE) and intensity of cough attack during expiration (IA^+) and inspiration (IA^-) from the laryngopharyngeal area

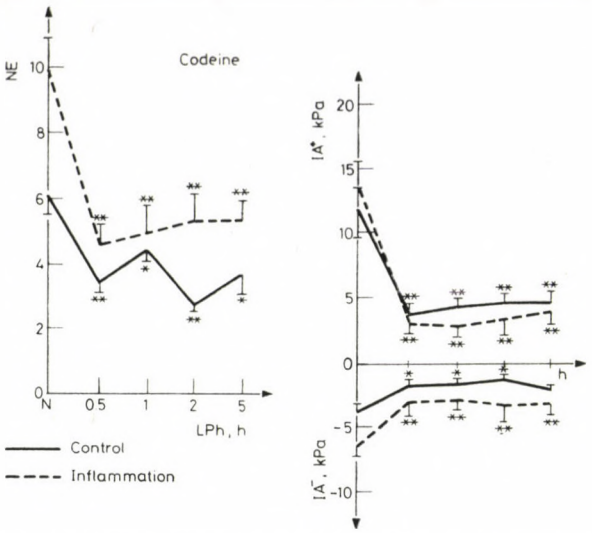

Fig. 2. Effects of codeine phosphate on cough parameters from the tracheobronchial area

Changes in cough efforts and in intensity of the cough attack observed in the tracheobronchial area are shown in Figure 2. It also illustrates the striking suppressive effect of codeine administered in the same dose to healthy and diseased animals, and the effect was not different in cats with airways inflammation.

After l-propoxyphene in healthy cats (solid line) a statistically significant decrease could be observed in the number of cough efforts and the intensity of cough attack, and these were mainly manifest during expiration. The antitussive activity of l-propoxyphene, however weakened in the diseased cats (broken line). Statistically significant changes were observed during the second hour of the experiment, when the cough was induced from the laryngopharyngeal area (Fig. 3).

L-propoxyphene in healthy cats had a more striking effect on cough evoked from the tracheobronchial area than on cough evoked from the laryngopharyngeal area. The antitussive activity was reduced under inflammatory conditions. They were statistically significant only in the latter case (Fig. 4).

Discussion

Cough is an important sign of inflammatory diseases of the organs of respiration. Its character and also its further patterns may change [12] and our aim was to clarify whether the activity of substances inducing suppression of the cough reflex would change under pathological conditions.

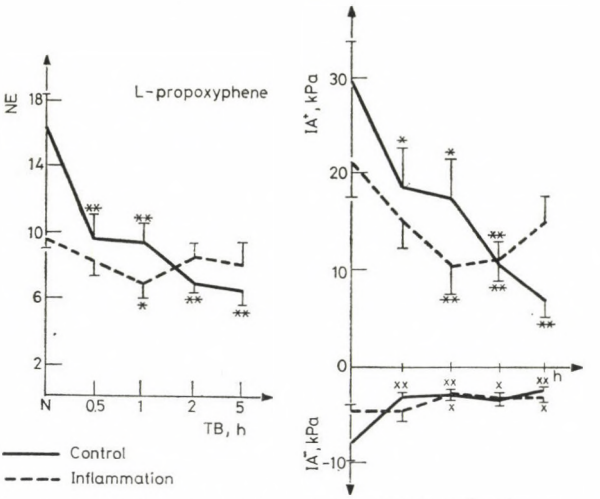

Fig. 3. Effects of l-propoxyphene orotane in a dose of 20 mg/kg b.w. on cough parameters from the laryngopharyngeal area

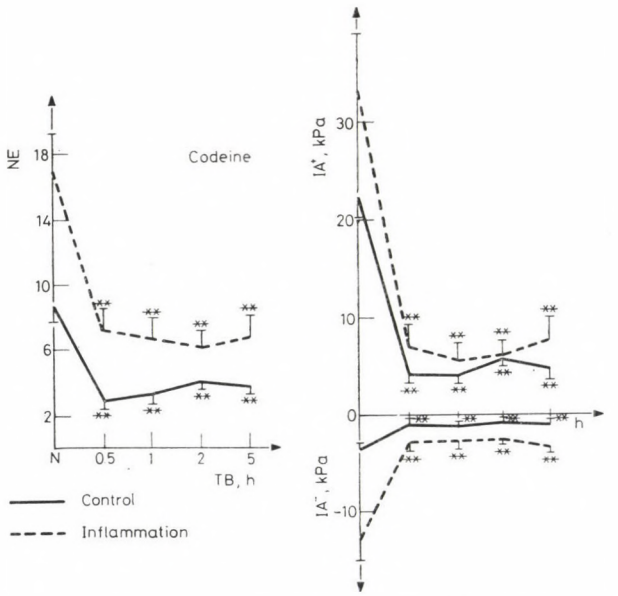

Fig. 4. Effects of l-propoxyphene orotane in a dose of 20 mg/kg b.w. on cough parameters from the tracheobronchial area

Codeine, the best known representative of morphine derivatives was studied in great detail [5, 18] and considered to be the best antitussive [13]. Its site of action is in the medulla oblongata. According to Salem and Aviado [17] its antitussive effect is in connection with the decreased sensitivity of the cough centre to impulses coming from the airways receptors.

L-propoxyphene is a laevorotatory isomer of propoxyphene, used in the form of various forms of salts (Contratuss®, Letusin®, Novrad®, Rava®, Regretos®, Sotorni®) [1, 4, 19]. The mechanism of its antitussive effect is in relation to its ability to influence central structures. Although l-propoxyphene has shown to possess locally acting anaesthetic effects [15], their role was demonstrated in some drugs with cough suppressing action, but in spite of this the antitussive activity of l-propoxyphene must be ascribed first of all to the central mechanism responsible for it [18].

Silvestrini and Maffii [19] compared the antitussive activity of l-propoxyphene to that of codeine. They found that their antitussive dose was approximately the same when applied in cough induced chemically or mechanically. L-propoxyphene was less effective in cough induced by electrical stimulation.

Calesnick et al. [1] induced cough in normal subjects and evaluated the number of cough efforts after administering l-propoxyphene and codeine, they found that l-propoxyphene was less effective than codeine.

The present results showed that both codeine and l-propoxyphene had a cough suppressing effect in mechanically induced cough but on whole the codeine had the stronger effect.

Further, our results pointed to the possibility of influencing the single types of cough in different ways. Both drugs had a stronger effect on cough induced from the tracheobronchial area. Ivanco et al. [8], Korpáš et al. [11] and Nosálová et al. [13] confirmed that cough coming from the upper airways is more resistant to drugs, and this might be in relation to the existing central mechanisms or structures controlling the type and quality of cough or also to effects elicited by drugs at these sites [9].

Nosálová et al. [14] studied the antitussive activity on models of respiratory tract inflammation evoked with croton oil and staphylococci. The results indicated that the antitussive effect of ruzozol, a peripherally acting substance, was less clearly defined, especially in laryngopharyngeal cough. Ruzozol was applied in aerosol form and the differences in its efficacy were explained by histological findings which showed that the airways were covered with mucus, and in places they were blocked with mucous plugs. Nosálová et al. [13] evaluated the antitussive effects under pathological conditions. In their studies they used glaucine, a substance belonging to the group of non-narcotic antitussives. Chemically and bacterially induced inflammatory changes of the organs of respiration studied in cats have not shown to be influenced with glaucine as to its ability to suppress the number of cough efforts and the intensity of cough attack during expiration.

We have attempted to complete the data on the antitussive effect of codeine and l-propoxyphene in healthy subjects [3, 5, 6, 16] and to establish the effect of both drugs under pathological conditions. The results obtained

pointed to different effects of the two drugs in healthy animals and in animals with experimental airways inflammation. Codeine caused no change of antitussive activity in chemically induced airways inflammation. In contrast, it l-propoxyphene markedly decreased the antitussive activity in experimentally induced airways inflammation. The difference might be attributed to the mechanism of effect of the drugs, or to some unknown factor. To confirm the suggestion, further studies will be required.

Codeine administered intraperitoneally to conscious cats in a dose of 10 mg/kg body weight had a better antitussive effect than l-propoxyphene administered in the same way in a dose of 20 mg/kg body weight on the parameters of cough. The effect of both the drugs was more marked in cough induced by mechanical stimulation of the tracheobroncheal area. Under conditions of chemically induced airways inflammation the antitussive activity of codeine was unchanged but the one of l-propoxyphene was reduced.

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PROTECTIVE EFFECT OF BEROTEC®, BERODUAL® AND ATROVENT® IN ASTHMATICS

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The protective effect of Berote®, Berodual® and Atrovent® (Boehringer-Ingelheim) were compared in asthmatics, before inducing airways obstruction with acetylcholine. Three groups (8 patients in each) were chosen. They were given two doses of the above drugs, from originally packed under pressure bottles, 30 minutes before acetylcholine inhalation. All the drugs provided sufficient protection against the induced acetylcholine obstruction of airways. No significant differences were found in protective effect.

Keywords: bronchodilating drugs, protective effects, spirometry.

A study was made in asthmatic patients to compare the protective effect of the drugs Berotec®, Berodual® and Atrovent® after a single administration.

Material and methods

The bronchodilating drugs Berotec®, Berodual® and Antrovent® (Boehringer, Ingelheim, GFD) were studied. One dose of Berotec® contains 0.2 mg of phenoterol, Berodual® represents a combined preparation the single dose of which consists of 0.05 mg phenoterol and 0.02 mg ipratropium bromide, Atrovent® consists of 0.02 mg ipratropium bromide. Their FEV_1 value was reduced by at least 30% on acetylcholine inhalation.

In the study, 24 patients with bronchial asthma were involved, 17 females and 7 males mean age, 34.7 years. They were divided into three groups, each consisting of 8 patients. In the first part of the study all patients were given liquid acetylcholine in 1% concentration to inhale for 10 minutes by means of the device TUR—USI 2 in an amount of 10 mg at least. If this amount of acetylcholine was not successfully inhaled, the inhalation was delayed for 2 to 3 minutes. If marked signs of airways obstruction appeared they were exposed to short-term inhalations of acetylcholine. Their condition was checked by spirometry. To relieve the respiratory tract obstruction, atropine in 5% concentration was inhaled for 10 minutes, by means of the device TUR—USI 4. All changes of FEV_1 values are shown in Fig. 1, after acetylcholine it was reduced by about 38% when evaluated by Student's *t*-test, which showed astatistically significant difference ($p < 0.001$).

The second part of the study was conducted one weak later, when they have inhalated. The patients were given to inhale acetylcholine for 30 minutes and then two doses of each. Berotic®, Berodual® gr Atrovent®. All spirometric examinations were performed by means of the devices Pneumocreen I and AW-test (Jaeger).

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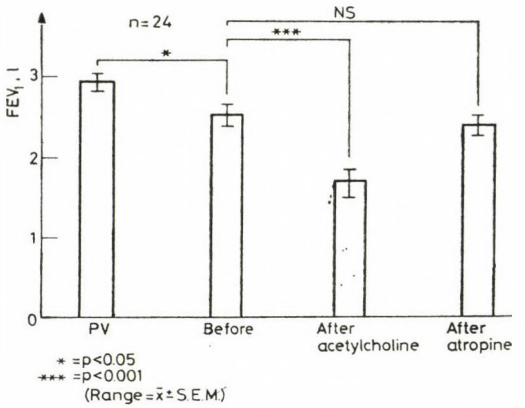

Fig. 1. FEV_1 in 24 asthmatic patients after acetylcholine inhalation. PV — Values predicted by Cherniack and Raber (1982). NS — non-significant changes

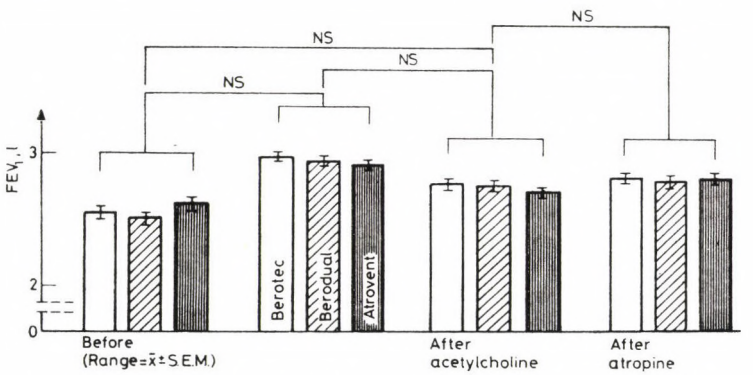

Fig. 2. FEV_1 in 3 groups of patients after Berotec®, Berodual® and Antrovent® administration, NS — non-significant change

Results

The results obtained are shown in Fig. 2. Each of the three columns represents one group of patients. As to the protective effect of the drugs no statistically significant changes could be found in any of the three groups. The value of FEV_1 was reduced by about 8 to 11% on the average but from the clinical point of view the reduction was not significant.

Discussion

Phenotherol and ipratropium-bromide were found to be convenient drugs for the treatment of reversible bronchial obstruction when taken in single doses or in combination [1]. If Berodual® was taken in combination, a reduced dose of phenoterol (0.05 mg) is recommended in contrast to the original drug Berotec® (0.02 mg).

Conclusion

The results obtained have confirmed that each of applied drug exerts a sufficient effect before induced respiratory tract obstruction, in good agreement to findings of other authors [3, 4].

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BRONCHODILATING EFFECTS OF BEROTEC®, BERODUAL® AND ATROVENT® IN ASTHMATICS

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The bronchodilatating effect of one single dose of Berotec®, Berodual® and Atrovent®, has been studied in 28 patients suffering from bronchial asthma. All three drugs had a beneficial bronchodilatating effect lasting for 300 minutes. There were no statistically significant changes between the drugs studied.

Keywords: bronchodilatating drugs, spirometry.

The bronchodilating effect of Berotec®, Berodual® and Atrovent® (Boehringer, Ingelheim) were studied and compared in asthmatics.

Material and methods

One dose of Berotec® contains 0.2 mg of phenoterol, Berodual® represents a combined preparation, a single dose of which consists of 0.05 mg phenoterol and 0.02 mg ipratropium bromide, Atrovent® consists of 0.02 mg ipratropium bromide. The drugs were compared in 28 asthmatics in the quiet state, 22 females and 6 males, of 32.1 years mean age and a mean history 5.6 years.

The asthmatics had inhalatory or mixed type of allergy, with slight or moderate obstructive ventilatory disorder with higher airways resistance (Raw).

The patients were submitted to examination for four days, when airways resistance, maximum breathing and the flow/volume curve were evaluated. Examinations were performed by the devices Pneumoscreen I and AW-test (Jaeger). Examinations started at 8.0 in the morning, functional examinations were made at 15, 30, 60, 120, 180, 240 and 300 minutes after the patients had inhaled a double dose of the given drugs. On a single day they were given placebo to inhale. The patients did not receive any drug affecting the bronchial lumen, and neither had they tea, coffee or cola, and were not submitted to inhalation for 12 hours before the examination.

Results were processed statistically, using Student's t test and the programming calculator HP 41 C.

Results

In Fig. 1 it can be observed that each of the three drugs Berotec®, Berodual® and Atrovent® caused a statistically significant bronchodilatation in all the measured parameters during the whole time of measurements, thus

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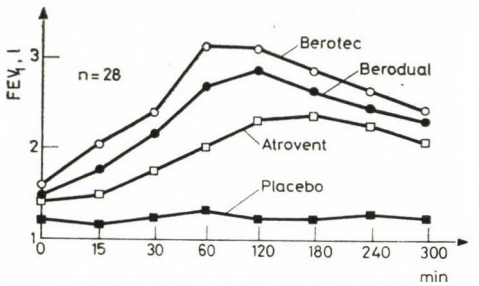

Fig. 1. Changes of FEV_1 after a single dose of Berotec®, Berodual®, Atrovent® and placebo in 28 asthmatics

at 5 hours at the 1‰ to 1% level of significance, showing a maximum in the 1st to 3rd hour after the drug's administration.

If the results were given in percentage, then the strongest bronchodilating effect was observed with Berotec®. In comparison, Beroudal® had a weaker bronchodilating effect, of about 15—20%, and Atrovent® an even weaker one, of about 20—40%. No other differences between the drugs were of statistical significance.

Blood pressure, pulse frequency and the subjects judgement were also checked. Blood pressure was not affected by any of the drugs. Pulse frequency became statistically significantly increased by Berotec® in the 1st and 2nd hour up to $90.min^{-1}$ on the average. Berodual® and Atrovent® caused no statistically significant change in pulse frequency.

The subjects said that they tolerated Atrovent® better than Berodual®. After administration Berotec® many patients had heart palpitation, tremor, tachycardia (see Table I).

Table I

	Berotec	Berodual	Atrovent	Placebo
	n = 28			
Headache	2	1	0	0
Nervousness	3	0	0	0
Tremor	8	2	0	0
Anxiety	0	0	0	0
Palpitation	2	0	0	0
Tachycardia	3	0	0	0

Conclusion

The patients displayed a statistically significant bronchodilatation after Berotec®, Berodual® and Atrovent® administrations during the whole time of 5 hours in the course of which measurements were performed. Atrovent® was

the drug that was tolerated best even when its percentage effect was 20 to 40%. Generally, when treated with overdoses patients claiming to have unpleasant feelings, may show a risk [1], but this was not observed with Atrovent®. That is why Atrovent® is especially suitable to be administered to patients suffering from cardiac diseases or hypertension [2, 3].

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THE TUSSIPHONOGRAM DURING ASTHMATIC ATTACK

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By tussiphonography the voluntarily induced cough sound was registered in the course of asthmatic attacks and in the state of quinescence. Evaluation of the recordings revealed that in the course of the asthmatic attack, the intensity of voluntarily induced cough sound was significantly decreased.

Keywords: voluntary cough, asthmatic attack, tussiphonography.

The aim of the present study was to establish whether the sound of voluntarily induced cough changed in the course of a dyspnoeic attack of asthma.

Material and methods

Sixteen probands, 12 females and 4 males of 29.1 years average age were chosen for the study. The duration of their disease was 4.6 years on the average. The patients fulfilled the clinical and functional WHO criteria of bronchial asthma. The sound of the voluntarily induced cough was registered according to Korpáš and Korpášová-Sadloňová [1] in the course of an asthmatic attack, and then in a 7 to 14 days period when the patients had no complaints for dyspnoic attacks. The patients were followed up after their ventilatory tests had given normal values during the period of relative quiescence. At the given time, the patients were submitted to complete therapy (drugs, climate and rehabilitation). The voluntary cough sound was registered on tape, so that later it could serve for registration processing on a device Mingograph Elema 81, at a paper rate of 100 mm $\cdot$ s^{-1}. Evaluation was done according to Korpáš and Korpášová-Sadloňová [1] where the cough sound was judged by its character (temporary physiological or pathological in type) and duration, intensity and the amplitude integral. Results were evaluated statistically by Student's *t*-test and a calculator HP 41 C.

Results

A sample of original cough sound record is shown in Figure 1. It is easy to note that the character of the sound had changed in the course of the attack, when compared to the recording performed in the quiet state. It was

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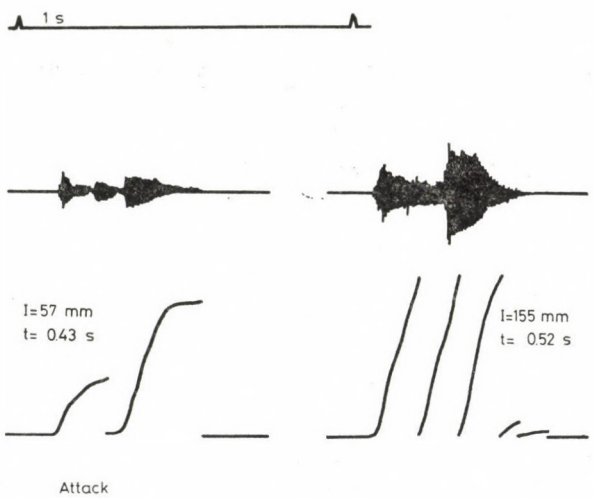

Fig. 1. Original record of cough sound obtained in 34-years old male asthmatic in the course of an attack, and one of the 7 days lasting state of quiescence. I = amplitude integral (intensity). t = duration of cough sound record in seconds

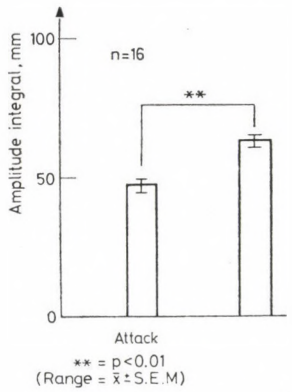

Fig. 2. Amplitude integral (intensity) of cough sounds of 16 asthmatics, in the course of attacks and in the state of quiescence

striking that the amplitude had fallen first, the amplitude integral became statistically significantly decreased ($p < 0.01$) during the attack, as it is shown in Figure 2. Figure 3 illustrates the fact that the duration of the registered cough sound did not change in the course of the asthmatic attack, when compared to the sound registered in the quiet state.

Discussion

The cough sound generated in the airways by the altered luminal airflow is turning to a turbulent one, the surrounding lung tissue is taking part in its generation (2). The airways lumen may be effected by changes occurring

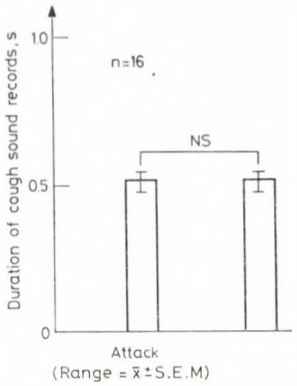

Fig. 3. Cough sound duration in 16 asthmatics in the course of attacks and in the state of quiescence. NS — non-significant

in the cough sound. We could confirm that the effects of bronchodilator drugs acting on voluntarily cough sounds do not exert changes [3]. Our aim was to determine whether the volunatarily induced cough sound would change, during a spontaneously appearing airways obstruction in asthmatics. The obstruction induced by acetylcholine showed a statistically significant decrease in the amplitude integral of voluntarily involved cough sounds [4] shown in the records.

Conclusions

The acute occurrence of an obstruction of the respiratory tract in asthmatics is due to changes appearing in the cough sound character, even if it remains pathological in type. In the course of a dyspnoeic asthmatic attack the value of the amplitude integral was statistically significantly increased, even if the cough sound duration remained unchanged.

Acknowledgement

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INFLUENCE OF SULPHUR DIOXIDE BREATHING ON DEFENSIVE REFLEXES OF THE AIRWAYS

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The influence of sulphur dioxide (SO_2) on cough and expiratory reflexes was studied in 22 anaesthetized (pentobarbital, 30 mg/kg i.v.) rabbits. The cough reflex (CR) was elicited by tracheobronchial mucous membrane stimulation, using a soft venous catheter. In order to induce the expiratory reflex (ER) irritation of the larynx was performed by a silon fibre loop. The strength of both reflexes was assessed from the interpleural pressure fluctuation before SO_2 breathing, mmediately after, and 30—120 minutes after SO_2 breathing had been stopped. The animals breathed SO_2 in 200—300 ppm concentration through a tracheal cannula. The influence of SO_2 on the direct dependence of lung inflation pressure magnitude (LIPM) on strength of the expiratory reflex (ERS) was tested.

It was found that elicitability and strength of the cough reflex decreased immediately after SO_2 beeathing had been stopped, and did not reach the preexposure level at the end of the experiment, i.e. 2 hours after discontinuation of SO_2 breathing.

The direct dependence of LIPM on ERS in unaffected rabbits [10] was abolished immediately after SO_2 breathing had been stopped but was present again 30—60 minutes later, and did not differ significantly from the control values.

The results give evidence of the strong depressive influence of SO_2 on the defensive reflexes of the airways in rabbits.

Keywords: cough reflex, expiratory reflex, sulphur dioxide, rabbit.

Sulphur dioxide is well known as one of the gases exerting a strong irritative influence on the mucous membrane of the airways. It has been well documented that SO_2 induces morphological [1, 5, 9] and functional [6, 8, 14, 15] changes in the airways. For example, results due to its influence are damaged ciliary cells alveolar macrophages changed mucus secretion, mucociliary clearance, and many others. Little is known about SO_2 influence on respiratory defensive reflexes which are important and powerful mechanisms of airway clearance. Our previous experiments showed that SO_2 is a strong stimulus inducing cough in animals, but spontaneous cough was stopped after the animals had breathed SO_2 for 5 minutes or longer. We suppose therefore that the second effect couldbe attributed to an adaptation of the cough receptors to the influence of SO_2.

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It seems that the depressive effects of air pollutants acting on the lung defensive reflexes, play an important role in the formation of chronic respiratory diseases.

The purpose of the present paper was to determine the influence of short-term SO_2 breathing on the artificially induced cough reflex, and on the direct dependence of long inflation pressure between the strength of the expiratory reflex.

Methods

The experiments were carried out in 22 adult male and female New Zealand white rabbits weighing 2.4—3.8 kg, under pentobarbital anaesthesia (30 mg/kg, i.v.). Anaesthesia was maintained at the appropriate level by adding supplementary doses of the anaesthetic, if necessary. Catheters were tied into the right femoral artery of some animals and the femoral vein. Interpleural pressure was measured by a transducer EMT 34, and manometer EMT 31 Elema Schönander, connected to the interpleural catheter. The animals breathed through the caudally-directed tracheal cannula. The cough reflex was induced by mechanical stimulation of the tracheobronchial mucous membrane by a soft venous catheter. To induce the expiratory reflex, we stimulated the larynx by a silon fibre loop (0.4 mm fibre diameter, 5 mm loop diameter). To study the influence of SO_2 on dependence between LIPM and ERS, the lungs were inflated by pressure 0.5, 1.0 and 1.5 kPa. SO_2 mixture was made in a polyethylene bag, mixing air and SO_2. The SO_2 concentration of the mixture ranged between 500—800 $\mu g/m^3$ (200—300 ppm). For SO_2 administration, a stream of gas mixture was drawn across the rabbit trachea, and adjusted so that the pressure at the tracheal cannula was atmospheric, and that in the polyethylene bag was slightly positive. The animals breathed the SO_2 mixture for 10—15 minutes. Stable body temperature (38.0—38.5 °C) was maintained in the course of the experiments.

The arrangement of experiments was as follows. First the control values of cough and expiratory reflexes were obtained. Then the animals breathed the SO_2 mixture. Immediately after they had stopped to breath SO_2, the cough reflex was elicited. Then we tested the influence of SO_2 on the direct dependence between LIPM and ERS. These procedures were done 30 to 120 minutes later, too. Differences in elicitability and strength of both reflexes at the given times of the experiments were assessed using Student's t test.

Results

SO_2 breathing caused decreases in both cough reflex elicitability and cough reflex strength in rabbits (Fig. 1). Elicitability of the cough reflex was decreased to 46% of control values considered as to be of 100% immediately after SO_2 breathing, and was restored 30—120 minutes later to 64% of the control. If we managed to elicit the cough reflex under these conditions, its strength, expressed in number of cough efforts in one cough attack (NE), intensity of maximum cough effort (IME) and intensity of cough attack (IA), were substantially decreased when compared with control values (100%). The NE decreased to 20%, IME to 20—36%, IA to 6—10% of control values, immediately after SO_2 breathing had been stopped. The parameters mentioned did not reach the control value, 30—120 minutes later.

The above-mentioned changes of cough reflex strength are illustrated in absolute values in Fig. 2. All quantitative cough parameters, except of NE

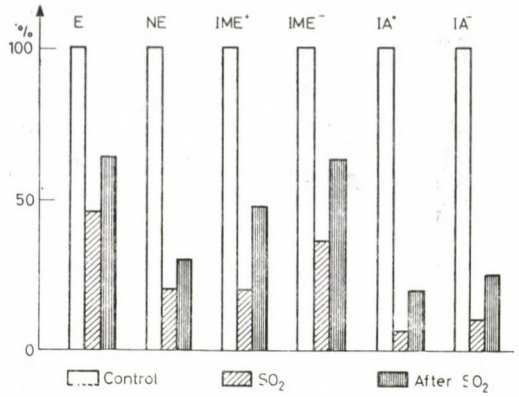

Fig. 1. Parameters of tracheobronchial cough reflex before SO_2 breathing (first columns), immediately after SO_2 breathing was stopped (second columns) and 30—120 minutes later (third columns) expressed in percentage. E — elicitability of cough reflex, NE, IME, IA = cough reflex strength parameters, + = expiration values, — = inspiration values

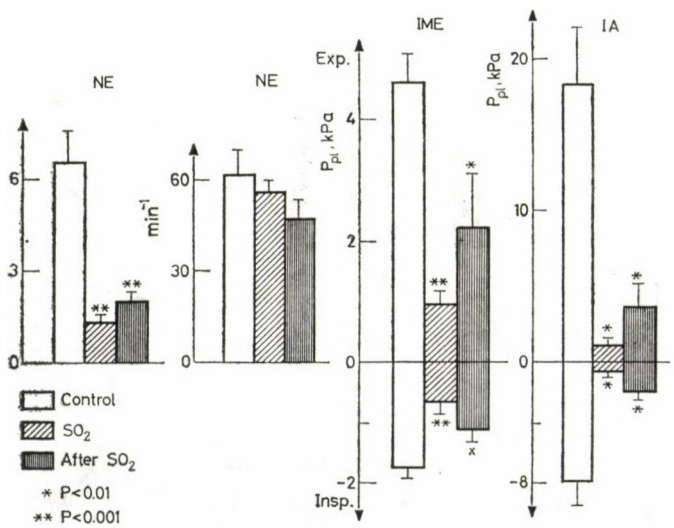

Fig. 2. Mean values of strength of tracheobronchial cough reflex before SO_2 breathing (first columns), immediately after SO_2 breathing was stopped (second columns) and 30—120 minutes later (third columns) expressed in absolute values. Stars above the columns mean significance between experimental values and control ones. Vertical lines above columns = S. E. M.

per minute, were significantly decreased immediately after SO_2 breathing compared to counted values. There was a tendency to increase the cough reflex parameters 30—120 minutes later, but they were significantly decreased below the preexposure values.

Recently we have shown [10] that in rabbits the ERS increased linearly with the increase of LIPM (Fig. 3). In the present experiment we found that

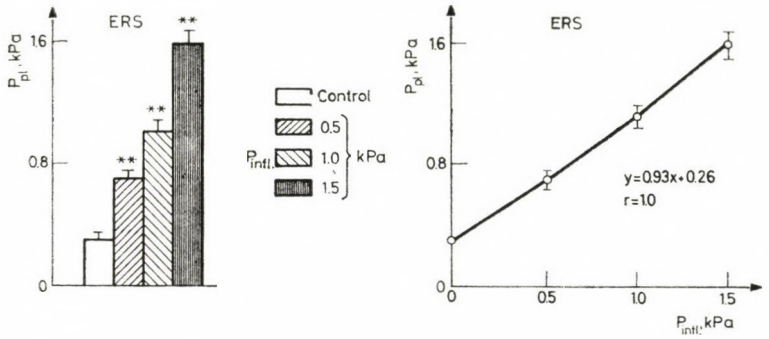

Fig. 3. Dependence between lung inflation pressure and strength of expiratory reflex in rabbits. P_{pl} — interpleural pressure, P_{infl} — lung inflation pressure. Vertical lines above the columns = S. E. M. Stars above the columne = significance $P < 0.001$ between control values and experimental ones. Y = equation, expressing linear dependence, r = correlation coefficient. Vertical lines above columns = S. E. M.

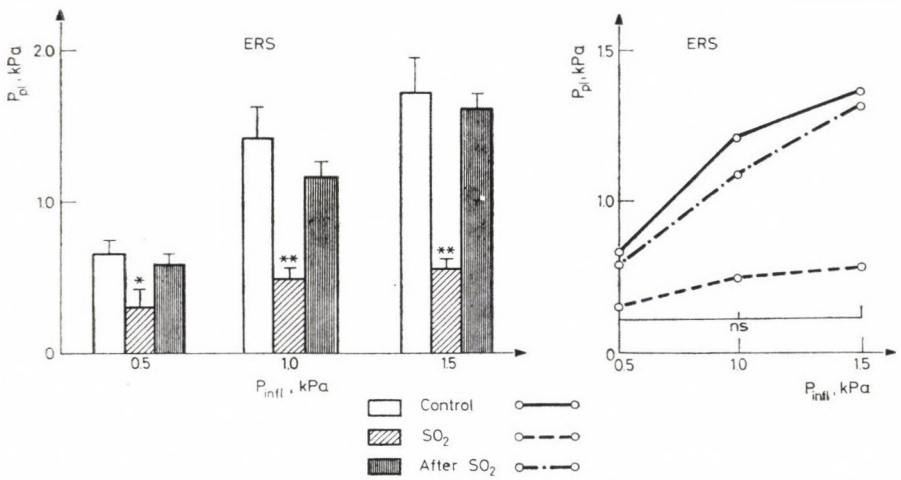

Fig. 4. Influence of SO_2 breathing on dependence between lung inflation pressure and strength of expiratory reflex in rabbits. * = $P < 0.05$, ** = $P < 0.01$, ns = non-significant difference. Other descriptions as in Fig. 3

the given dependence was abolished immediately after SO_2 breathing had stopped (Fig. 4, at right). The strength of the expiratory reflex decreased significantly after SO_2 breathing, at all three inflation pressures, when compared to control values (Fig. 4 at left). Under these conditions, the slope of the dependence was lower then before SO_2 breathing. There were no significant differences in the ERS values obtained at the three inflation pressures 30—60 minutes later.

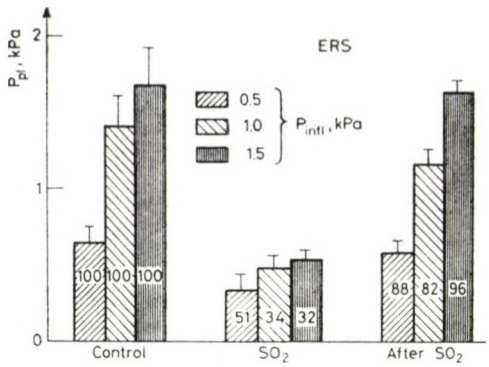

Fig. 5. Influence of SO_2 breathing on dependence between lung inflation pressure and expiratory reflex strength in rabbits, expressed in percentage

If the ERS values obtained before SO_2 breathing were considered to be 100%, then the ERS obtained immediately after SO_2 breathing had stopped reaching only 32—51% of control values at corresponding inflation pressures. 30—60 minutes later, the ERS reached 82—96% of control values (Fig. 5).

Discussion

The present experiments have confirmed our earlier findings [12] concerning the depressive influence of SO_2 on respiratory defensive reflexes. Sant' Ambrogio et al [13] obtained similar results with the difference that we achieved a higher percentage of successful stimulations to cough after SO_2 breathing (46%—2.4%). The difference could be ascribed to the different dose of anaesthetics we gave: 30 mg/kg body weight, while Sant' Ambrogio et al. gave 40 mg/kg body weight.

Interpretation of the results obtained is difficult. We agree with Sant' Ambrogio et al. [13] in that the cough reflex inhibition appearing after SO_2 breathing could be attributed to the slowly adapting stretch receptors (SASR). Despite the evidence by Davis et al. [6], and Sant' Ambrogio et al. [13] of the slight effect of SO_2 breathing on the rapidly adapting stretch receptors (RASR) considered to be cough receptors, we still suppose that changes of their properties may have an important role in the inhibition of the elicitability and strength of the cough reflex. Our view is based on indirect evidence. One of them is the fact that the Hering-Breuer inflation reflex (HBIR) one of the indices of the slowly adapting stretch receptor activity, recovered earlier after SO_2 breathing than the elicitability and strength of the cough reflex. Another indirect evidence is the elicitability of cough from the larynx and the tracheobronchial tree after SO_2 breathing. Elicitability of laryngeal cough was far less

inhibited than the elicitability of tracheobronchial cough. The explanation of the difference given by Sant'Ambrogio et al. seems to be too sophisticated and we propose a more reasonable one. In some way, SO_2 must also change RASR function, and not only the one of SASR. Our suggestion is supported by the results demonstrating the influence of SO_2 breathing on the dependence between LIPM and ERS. The direct dependence was abolished immediately after SO_2 breathing, but 30—60 minutes later it was present again, and it did not differ significantly from the control values. Elicitability and strength of the cough reflex induced from the tracheobronchial tree were inhibited much longer. It is therefore reasonable to propose that the abolition of the direct dependence was due to inhibition of SASR by SO_2. According to Cohen [4], Bianchi and Barillot [2], von Euler [7] and others, it is known that SASR activity facilitates the expiratory neurones in the medulla, oblougata and the expiratory muscles [3]. If the activity of SASR is blocked by SO_2, than its facilitation is absent, and this is the reason for abolition of the mentioned dependence. 30—60 minutes later the SASR had already recovered, HBIR was present again, and the dependence did not differ from the control values.

The results of both experiments showed the important role of SASR in regulation of the intensity of cough and expiratory reflexes. It seems that SO_2 may play a negative role in airway clearance. It may be considered to be not the only one causing a strong cough reflex, but under certain conditions it may cause a decrease of sensitivity of the peripheral part of the defensive reflexes of the airways too. That fact may cause an increased cough reflex threshold as well as a deterioration of airway clearance. It seems that not only SO_2, but many other air pollutants (e.g. ozone) have a similar influence. One has to suppose that the mentioned mechanism may come into play even when SO_2 concentration in the inhaled air is much lower than that used in our experiment, if it was breathed for a long time, and associated with some other negative factors. It could well be one of the mechanisms participating in the formation and persistance of chronic bronchitis.

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COMPUTER-ASSISTED EVALUATION OF SOME RESPIRATORY PARAMETERS DURING VOLUNTARY COUGH

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Three different microcomputers proved to be useful in processing of pressure, flow and volume signals during cough and other respiratory manoeuvres. In young healthy volunteers, values of forced vital capacity, peak expiratory flow and flow at 0.1 s measured during cough were smaller in women than in men due to their smaller body surface and FVC. Compared to forced expiration a sudden and vigorous cough effort, in spite of its shorter duration, resulted in an increased mean flow rate and in similar peak flow reached by expulsion of a smaller volume. Analysis of flow-volume curves indicated higher flow-rates at the beginning (5—10% FEV) and to the end of cough expulsion (65—95% FEV) than in forced expiration. The volume, peak flow and flow 0.1 s from the beginning of inspiration and expiration were practically the same in 5 successive cycles of voluntary cough performed by maximum effort. The mechanisms involved in cough expiration are more automatic than the mechanisms of cough inspiration. A personal computer can be useful for monitoring the increase in PEP, PIP, PEF, PIF, FEV, FIV and in maximum resistance as well as the decrease in compliance observed during cough compared to quiet breathing.

Keywords: cough, forced expiration, flow-volume curve, microcomputers, lung function tests.

Our complex study of cough and other respiratory reflexes performed in experimental animals (10) required a verification of some characteristics of voluntary cough in men applying lung function tests (5—9, 11—13). This contribution presents the results of three series of investigations intended to compare some indices of forced expiration and voluntary cough effort, to analyse some parameters of an attack of voluntary cough, and to evaluate the changes in respiratory mechanics during voluntary cough. At the same time we shall analyse our experience with application of three simple computer-assisted methods, similar to techniques used for respiratory measurements (1—3, 6, 11).

Methods

1) Comparison of forced expiration and voluntary cough effort using a desk calculator

Determination of forced vital capacity (FVC) and various parameters from the flow-volume curves (F-V) based upon measurement of the flow and volume expelled by maximum voluntary effort mostly involves off-line data processing (2). In order to study F-V curves

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we recorded the airflow using a pneumotachograph (Pneumoscreen Jaeger) and stored the data to the memory of *desk calculator EMG 666B* (8 kB). Sampling of data from an interval of at least 3 s at 120 Hz by a monolitic 11-bit analogue-to-digital converter (C 520 D) requires 360 addresses. The program considers both the resting voltage of the flow transducer and the calibration, and permits measurement of changes exceeding the noise level.

After data acquisition the calculator starts automatic data processing and computes various *parameters from the F-V curve.* At first the time integral of all flow values, i.e. the volume of the expelled air is determined. From these data various parameters such as maximum volume of the expelled air (FVC), peak expiratory flow (PEF), time of expulsion (T_e), mean airflow ($\dot{\bar{V}}$), forced expiratory volume in one and two seconds (FEV_1 and FEV_2) etc. can be computed [4].

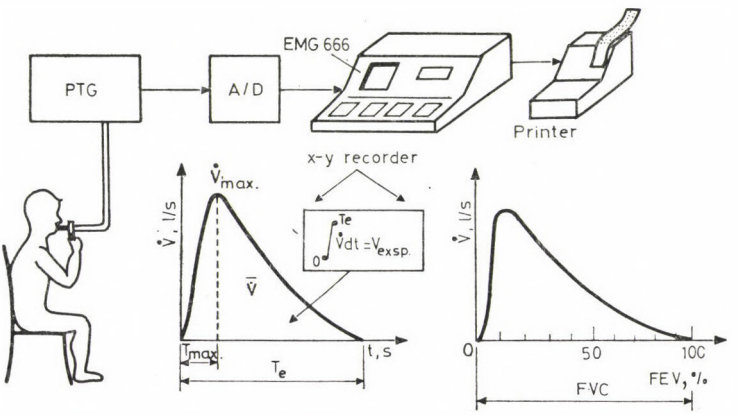

Fig. 1. Investigation of flow-volume curve. PTG — pneumotachograph, A/D — analog-to-digital converter, desk calculator EMG 666 B, printed and graphical illustration of the results by x—y recorder as flow-time and flow-volume curves

In order to compare more precisely the efficacy of ventilatory manoeuvres in forced expiration and in cough, the instantaneous flow at 25%, 50% and 75% FEV (75%, 50% and 25% FVC) was measured. In addition, the airflow after expulsion of each 5% portion of volume (at 5%, 10%...95% FEV) was measured together with the volume expelled in each 5% of the duration of the processes. The calculated values and the computed results were printed for the protocol. The airflow signal was plotted by an X—Y recorder as flow-time and flow-volume curves (Fig. 1).

2) Analysis of cough attacks by microcomputer

A microcomputer ŠEMS 02 (Research Institute for Computer Techniques, Žilina) designed for teaching purposes was used for automatic analysis of cough attacks in volunteers. A simple program was employed for computing various selected respiratory parameters from the flow signal detected by a pneumotachograph, connected to an AD input of the microcomputer. Sampling of data (200 Hz) was organized by a program written in Assembler, covering signal detection from an interval of 16 s and data deposition to the memory (64 kB). Following storage of data the microcomputer delivered at command the separate measured values to the desk calculator for reprocessing and monitoring the results (printing, x—y plotting, graphical illustration, etc.).

The program for the desk calculator comprised analysis of five successive inspiratory-expiratory cycles (breaths or coughs). In each cycle PEF and PIF, time of their completion (T_{max} and T_{min}), time of inspiration (T_i) and expiration (T_e) were determined. By integration of the flow signal the volume of forcefully inspired or expired air (FIV and FEV) as well as various volume contingents could also be measured.

3) Analysis of respiratory mechanics by personal computer

A personal computer Sinclair ZX—81 was adapted and used for evaluation of changes in respiratory mechanics during cough in volunteers. The computer was supplemented by a 4-channel analog input and a 2-channel analog output. The sampling rate could be chosen by a program written in Assembler. A 2-channel AD converter (8 bits-256 levels) was used with sampling 200 values in a second for each channel separately. In addition to airflow detected by a pneumotachograph, the pressure in the oral or nasal cavity was measured by an electromanometer (LDP 105, Tesla). The signals (± 1.5 V) were sampled in parallel and the data stored alternatively in a memory (4 kB) with the usual sampling time of 10 s. The shape of the input signals, the computed results and the graphical informations were monitored on a TV screen. The operating program of the computer was stored on commercial magnetic tape (Fig. 2).

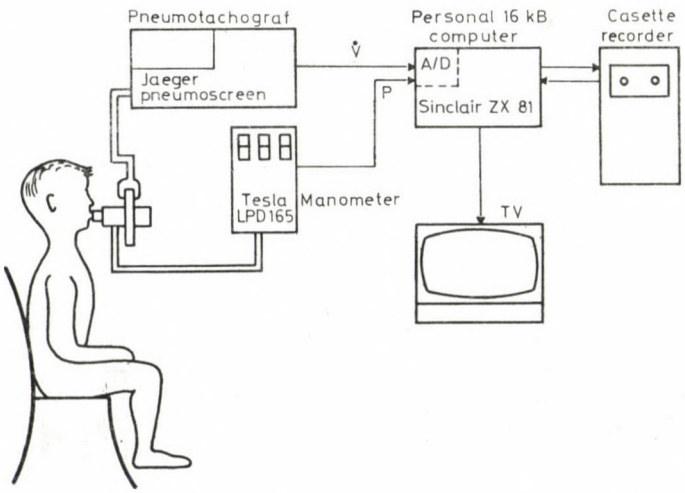

Fig. 2. Schematic illustration of the investigation of pressure, flow and volume signals using a personal computer

From the detected signals we have identified three complete inspiratory-expiratory cycles produced by three successive processes: quiet breath, cough and the following breath. In all three processes the following parameters were determined:

1) Total respiratory resistance (R) during inspiration and expiration, respectively, estimated as the highest value calculated from the pressure and flow.

2) Compliance (C) during inspiration and expiration evaluated as a mean from the ratio V/P during the relevant respiratory phase (except zero values).

3) Correlation coefficient (r) for regression analysis of the resistance and compliance.

4) Minimum and maximum values of pressure and flow in the relevant respiratory phase (P_{min}, P_{max}, $\dot{V}_{min}$, $\dot{V}_{max}$).

5) Volume of air ventilated during the relevant phase of breath and cough, obtained by integration of the flow signal.

6) Time of the inspiratory and expiratory phases (T_i and T_e).

The mentioned parameters were calculated off-line. Signal detection and storage of data were controlled by a program written in Assembler permitting real time sampling and recording. A 10 s interval was chosen for signal detection, but the time could be prolonged up to 90 s (memory 16 kB). Program for data processing was written in Basic. Although being slower, it permitted illustration of detected signals in a simplified form, allowing elimination of artifacts, selection of typical records, general analysis of the shape, etc. During calculation, the pressure-flow loop indicating the resistance and the pressure-volume loop monitoring compliance could be followed on the TV screen. A digitized description of the detected signals could also be displayed.

The calculation comprised the following steps:

1) Detect the first inspiration ($\dot{V} < 0$).

2) Evaluate inspiration to the first expiration ($\dot{V} > 0$) using the mentioned algorithm.

3) During calculations depict pressure-flow and pressure-volume loops.

4) After completion of the respiratory cycle (inspiration-expiration) monitor the computed values on command.

5) On demand evaluate another cycle (step 2) till the next inspiration or to the end of the signal.

Results and discussion

1) Cough compared to forced expiration

The results of investigation in 37 healthy 20—22 years old medical students in sitting position with a nose-clip indicated that FVC and PEF measured in non-smokers *during cough* are higher in men than in women (Fig. 3). The values computed for the body surface area and FVC (FVC/surf. and PEF/FVC), respectively, were similar in both sexes. Higher FVC and PEF values obtained during cough in young males compared to women correspond to their larger body surface areas and FVC, respectively. Three

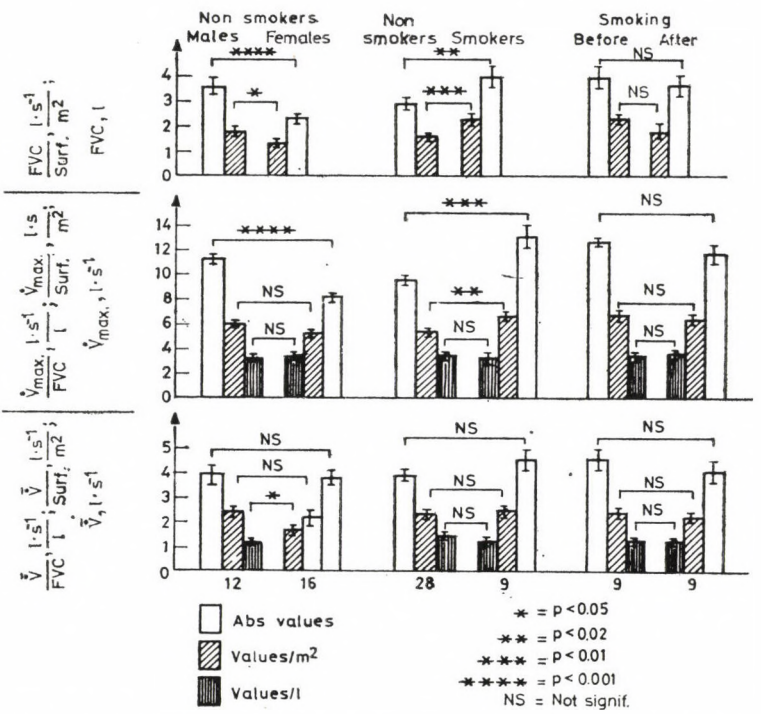

Fig. 3. FVC and peak expiratory flow ($\dot{V}_{max}$) measured during cough in absolute values and after correction for body surface area and FVC in non-smokers (men and women), smokers compared to non-smokers, and before and after smoking a cigarette. Means ± SEM, n = number of cases

minutes after smoking a cigarette there was no change in FVC, PEF and mean flow-rate obtained during cough.

Values of FVC and PEF measured *during forced expiration* in the same 19 females (3.832 $\pm$ 0.145 l and 7.801 $\pm$ 0.564 $l \cdot s^{-1}$) did not differ ($p > 0.05$) from the reference values given by Knudson et al. [9]. In 18 males the values of FVC and PEF measured during forced expiration (5.730 $\pm$ 0.160 l and 11.584 $\pm$ 0.732 $l \cdot s^{-1}$) were higher ($p < 0.01$) than the reference values.

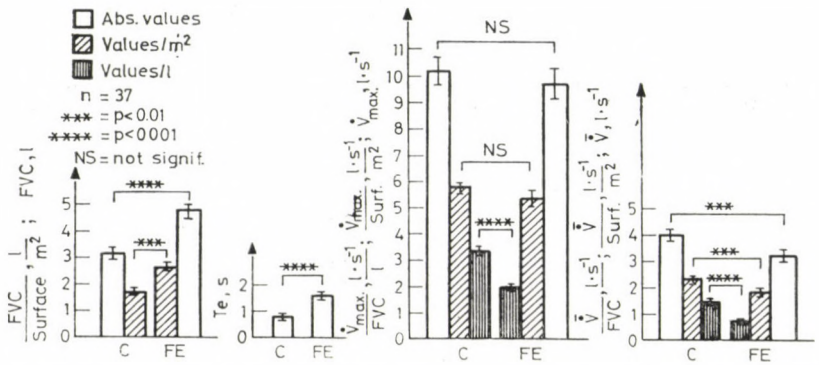

Fig. 4. Comparison of FVC, $\dot{V}_{max}$, expiratory time (T_e) and mean airflow ($\bar{\dot{V}}$) during cough (C) and forced expiration (FE). Other symbols as in Fig. 3

Comparison of cough and forced expiration according to various parameters in the same persons are given in Fig. 4. Compared to forced expiration the sudden and vigorous cough effort in spite of its shorter duration attained higher values of mean airflow and equal PEF. The volume expelled during cough given in absolute values or calculated for the body surface area was smaller than in forced expiration probably because of air-trapping. Therefore the peak expiratory flow calculated for the expired volume is higher in cough.

The mean values of *flow-volume curves* measured in the same persons were higher at the beginning and at the end of the cough effort (5—10% and 65—95% FEV, respectively), than in forced expiration (Fig. 5). Nevertheless, PEF and the midexpiratory effort-independent flow rates were similar in both cases. The higher initial airflows obtained in the compressive phase of cough could be ascribed to additional expulsion of a volume from the larger airways due to their dynamic compression resulting in so-called cough transient [8, 12, 13]. Higher flows at expulsion of the final portions of the lung volume in cough than in forced expiration could be explained by sudden decompression of the airways.

The semiautomatic data processing performed by the desk calculator can be used for investigation of forced expiratory efforts in clinical practice as

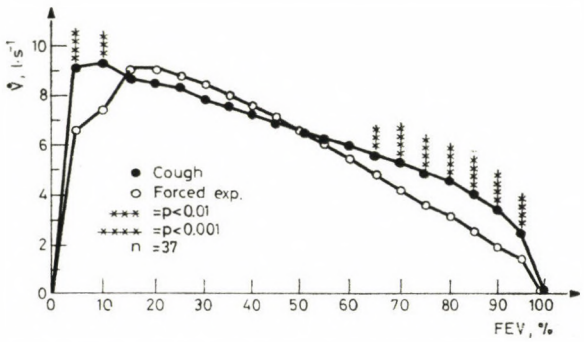

Fig. 5. Comparison of flow-volume curves during cough and forced expiration in the same volunteers. Mean values of flow at particular percents of FEV. Other symbols as in Fig. 3

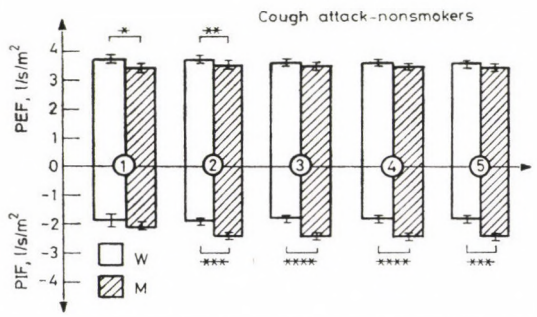

Fig. 6. PEF and PIF in 5 successive cycles of voluntary cough in men and women. Other symbols as in Fig. 3

well as in experimental animals, applying proper transducers. Compared to commercial lung function test systems equipped with microprocessors this technique has various advantages such as its lower cost, easy adaptability of software, and wide applicability in many medical fields, and especially in research.

2) Analysis of cough attacks

Cough attacks were investigated in 44 healthy 20—22 years old medical students, 20 men and 24 women. *The mean values of FEV* did not differ significantly ($p > 0.05$) in five successive cough efforts. Similarly as indicated in the first series of investigation, the FEV values were higher in men than in women (except the first and second effort). After calculating FEV for body surface area the differences between men and women disappeared except in the fourth effort — $p < 0.05$. Mean values of peak flow (Fig. 6) indicated that in all 5 cycles performed by maximum effort both *PEF* and *PIF* were practically the same ($p > 0.05$). The ratio of peak flows (PEF/PIF) was 1.61, 1.42, 1.46, 1.47, and 1.46 for men, and 1.98, 1.94, 2.04, 1.95 and 1.95 for women.

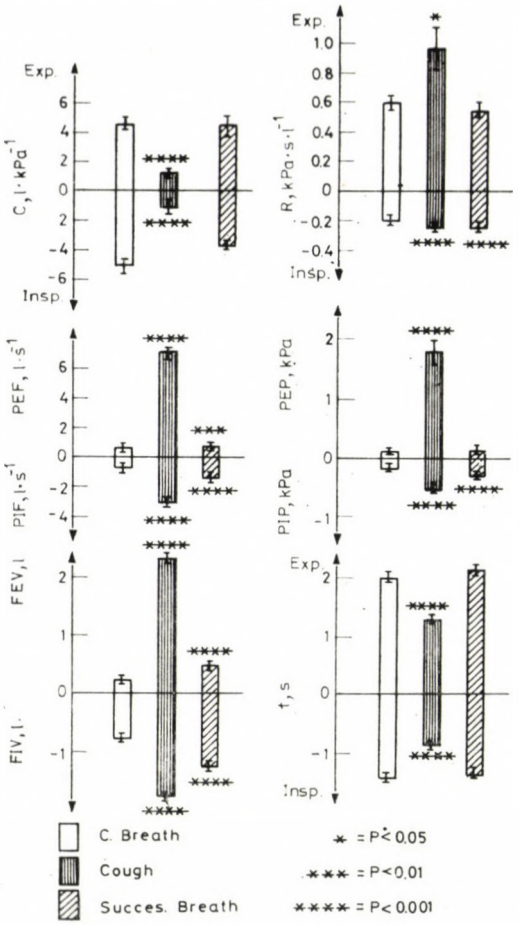

Fig. 7. Maximum respiratory resistance (R), compliance (C), peak expiratory (PEP) and inspiratory pressure (PIP), peak expiratory (PEF) and inspiratory flow (PIF), volume of the inspired (V_{insp}) and expired air (V_{exp}), time of inspiration (T_i) and expiration (T_e) in quiet breath, cough and successive breath

Flow values achieved 0.1 s from the beginning of expiration — $\dot{V}_{0.1\,exp}$ were in all 5 cough efforts practically the same and did not differ in men compared to women. $\dot{V}_{0.1\,insp}$ was more variable (the first cough inspiration was smaller than the other four ones, both in men and women). These results and the measurements of some time characteristics suggested that inspite of voluntary initiation the mechanisms involved in the cough expiratory effort are more automatic compared to the initial cough inspiration, which depended more on training, voluntary involvement, etc.

This technique proved to be valuable especially for its readiness to rapid computation and providing the results soon after signal detection. In our

investigations the separate steps (preparations and calibrations, measurements and calculation) were controlled manually, but it can be done also automatically by a single command. Such an approach is advantageous for routine work. The possibility to keep the data in a form of calculated and printed results or as a collection of the detected signals is also useful. The calculations can be performed also by a microcomputer software. For practical reasons (more complex periphery) and implemented programs we have used a desk calculator in our investigations.

3) Analysis of respiratory mechanics

During verification of the method 14 investigations gave mean values as follows (Fig. 7). *The compliance* of the respiratory system was lower both in inspiratory and expiratory phases of cough compared to the preceding and following breaths ($p < 0.001$). *The maximum resistance* was higher in expiratory than in inspiratory phase during all three processes (cough, preceding and following breaths). In addition, this resistance was higher during cough expiration than during the preceding and following expiration ($p < 0.05$). *Peak values of pressure* in the inspiratory and especially in the expiratory phase of cough were higher than during the preceding and following breaths. The first inspiration after the cough had still increased peak pressure. As a result of these high pressures the *peak inspiratory and expiratory flows* were also increased in cough and in a smaller degree also during the following breath. *The inspired and expired volumes* indicated similar changes. The inspiratory and expiratory phases were shorter during cough. Compared to control breath (1.43) the expiratory-inspiratory time ratio changed little in cough (1.47) and the following breath (1.61).

Our experience with computer-assisted evaluation of various respiratory parameters emphasized the importance to specify the right place and the correct method for the pressure measurement. Another problem was the selection of a suitable algorithm for the calculation of parameters such as, e.g. resistance and compliance.

We have used the Sinclair ZX—81 personal computer *in two regimes*. In a slow regime the calculations and the curves could be monitored continuously on a display, which was advantageous. The time required for data processing could be reduced by programs written in Assembler. In the quick regime only the final results of calculations and the graphical illustrations were at disposal but in a short time. According to our experience, personal computers proved to be useful also in such an untraditional sphere of application as mechanics of breathing in humans. The use is simple and the program language is not pretentious, allowing effective interventions also for operators with no programming knowledge.

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BULBAR RESPIRATORY ACTIVITY DURING DEFENSIVE AIRWAYS REFLEXES IN CATS

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Experiments in anaesthetized nonparalyzed cats indicated that mechanical stimulation of the airways and a longitudinal split of the brainstem cause marked alterations of the regulatory function of the bulbar respiratory neurones. The resulting changes in breathing and in defensive airways reflexes depend on intactness of the relevant structures of both halves of the "respiratory centre" and their interconnections.

Keywords: respiratory neurones, cough, respiratory reflexes, split brainstem, control of breathing.

Central neuronal structures and mechanisms involved in the defensive airways reflexes have been studied only sporadically. Cough and sneeze attacks seem to result from activation of special bulbar centres [5, 13], their localization and functional relation to the respiratory rhythm generator (RRG) are still confused. According to another view, specific functions involved, e.g. in the control of breathing are not localized in circumscribed centres, but regulation is done by different interconnected plurifunctional subpopulations of neurones in the lower brainstem [17]. In order to analyse more precisely the character and the central mechanisms of defensive airways reflexes, two electrophysiological methods such as microelectrode recording of the electrical activity of bulbar respiratory neurones and a split of the brainstem were used.

The aim of the present study was to investigate the changes in activity of bulbar respiratory neurones during cough and other respiratory reflexes, and to study how these reflexes were affected by longitudinal section of the brainstem.

Material and methods

The experiments were performed on 27 spontaneously breathing cats anaesthetized with either alpha-chloralose (60 mg/kg intraperitoneally, — 1st group) or sodium pentobarbitone (40 mg/kg i.p.-2nd group).

In the first group the activity of bulbar respiratory neurones was recorded and analysed during cough, sneeze, aspiration and expiratory reflexes elicited by mechanical stimulation

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of the respective area of the airways [14]. Single unit activity was recorded from 23 inspiratory (I) and 35 expiratory (E) neurones from the region of nucleus ambigous in the ventral respiratory group of the medulla where the hypothetical cough centre is located [13]. The respiratory neuronal activity was recorded parallely with the diaphragmal electromyogram and the pleural pressure, as indicated and evaluated in more detail elsewhere [10].

Results and Discussion

The results indicate that in defensive airways reflexes such as cough and sneeze there is a marked increase in maximum and mean frequencies of discharge both in I and E neurones compared to the control breathing. In cough (Fig. 1) the inspiratory phase is characterized by a marked rise in

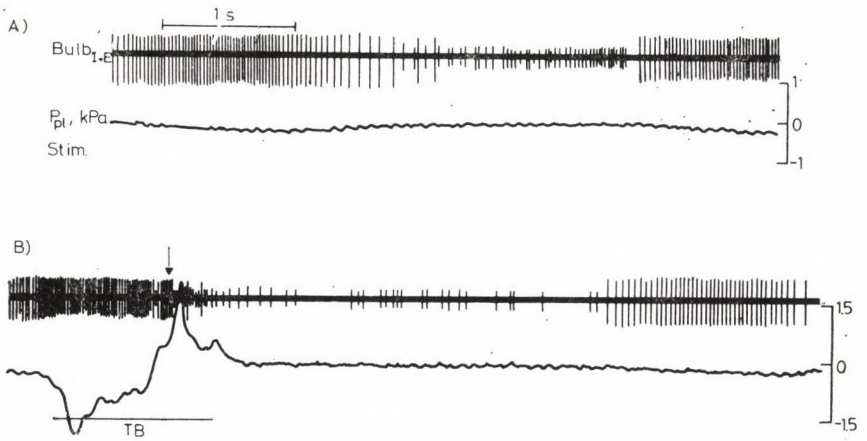

Fig. 1. Activity of bulbar inspiratory (large spikes) and expiratory (small spikes) neurones ($Bulb_{I+E}$) and pleural pressure (P_{pl}) during cough (record B) elicited by mechanical stimulation of tracheobronchial mucosa (TB) compared to control breathing before cough (record A). Recruitment of latent expiratory neurone during cough is indicated by arrow

frequency of discharge in I neurones. In 20 cough tests there was no recruitment of activity in latent I neurones. This finding is in agreement with the statement that adequate inspiration can be provided in cough by an increase in the frequency of I neurones discharging only during spontaneous inspiration [6]. In contrast to the cough in sneeze and aspiration reflexes where the inspiratory efforts are faster and stronger, new I neurones recruite as a rule.

In the aspiration reflex (Fig. 2) stimulation of the nasopharyngeal mucosa elicits short bursts of high-frequency discharge in at least two I neurones with different amplitude.

During the compressive and expulsive phases of cough (Fig. 1) and expiratory reflex (Fig. 3) and in sneeze there is a marked increase in frequency of spontaneously firing E unit, together with a recruitment of another spon-

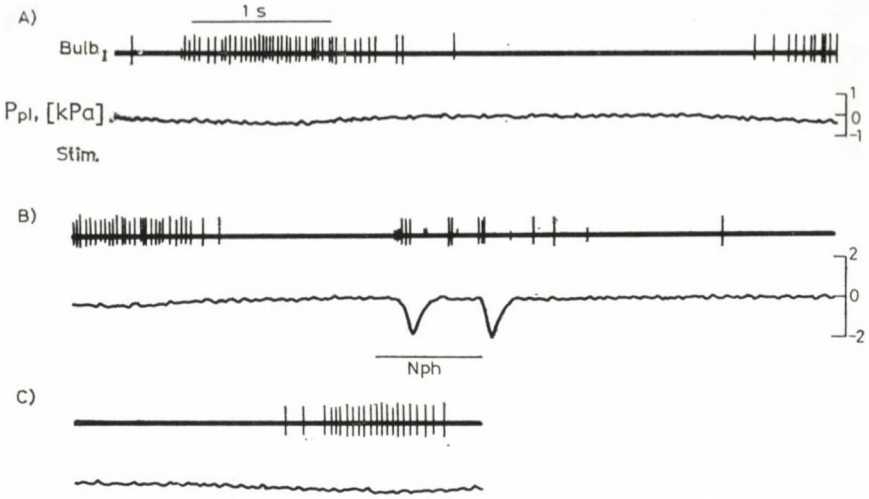

Fig. 2. Bulbar inspiratory neuronal activity—$Bulb_I$ (spontaneously firing—large spikes, and recruited—small spikes) and pleural pressure (P_{pl}) during aspiration reflex (record B) elicited by mechanical stimulation of nasopharyngeal mucosa (NPh) compared to control breathing before (record A) and after the reflex (record C)

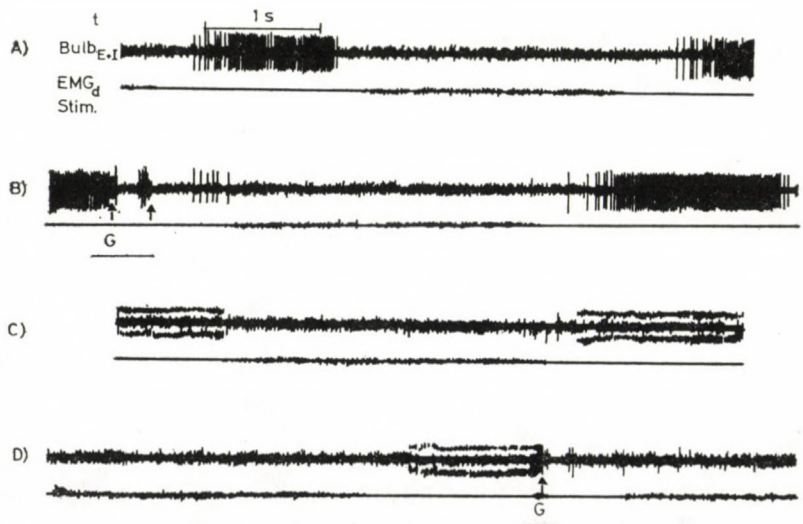

Fig. 3. Activity of bulbar expiratory (large spikes) and inspiratory (small spikes) neurones ($Bulb_{I+E}$) and diaphragmal electromyogram (EMG_d) during expiratory reflex (records B and D) elicited by mechanical stimulation of glottal area (G) compared to control breathing (records A and C). Recruitment of latent expiratory neurone is indicated by arrows

taneously not firing E neurones. The recruited E neurones for their higher threshold were not firing during spontaneous expiration or in the second part of expulsive phase in cough, sneeze and expiratory reflexes.

The central mechanism of active expiration in cough, sneeze and expiratory reflex are not clear, newertheless the regular recruitment of E neurones suggests that there are in the ventral respiratory group numerous neurones activated during respiratory acts comprising active expiration. We could

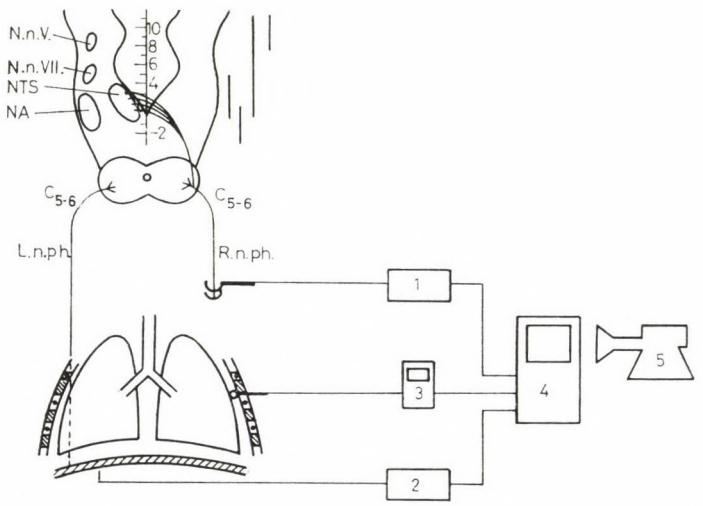

Fig. 4. Schematic illustration of the experimental procedure. Electroneurogram in the right transected phrenic nerve (R.n.ph.), electromyogram of left innervated hemidiaphragm and pleural pressure (P_{pl}) continuously recorded by means of preamplifiers (1, 2), electromanometer (3), oscilloscope (4) and a camera (5). On the left, brainstem structures: N.n.V. — nucleus n. trigemini, N.n. VII. — nucleus n. facialis, NTS — nucleus tractus solitatiurs, NA — nucleus ambiguus, and on the right the length of the split in mm from the level of the obex

not find any group of neurones activated "specifically" only in some kind of reflex. Similar attempt to localize the "vomiting centre" was unsuccessful [15]. Therefore the special character of the defensive airways reflexes seem to result from different organization of their afferent pathways and interneuronal connections in the brainstem, as well as from their participation in the proceeding respiratory phase (inspiration—expiration) at the moment of stimulation.

In order to study systematically both the airways reflexes and control mechanisms of breathing, we have introduced and modified the method of brainstem split [7, 8, 9, 12]. To this purpose in the second series of experiments on 10 anaesthetized cats a longitudinal midline section extending from 2 mm caudal to 11 mm rostral to the obex was performed (Fig. 4). The localization of section interrupting the connection between the left and right side was

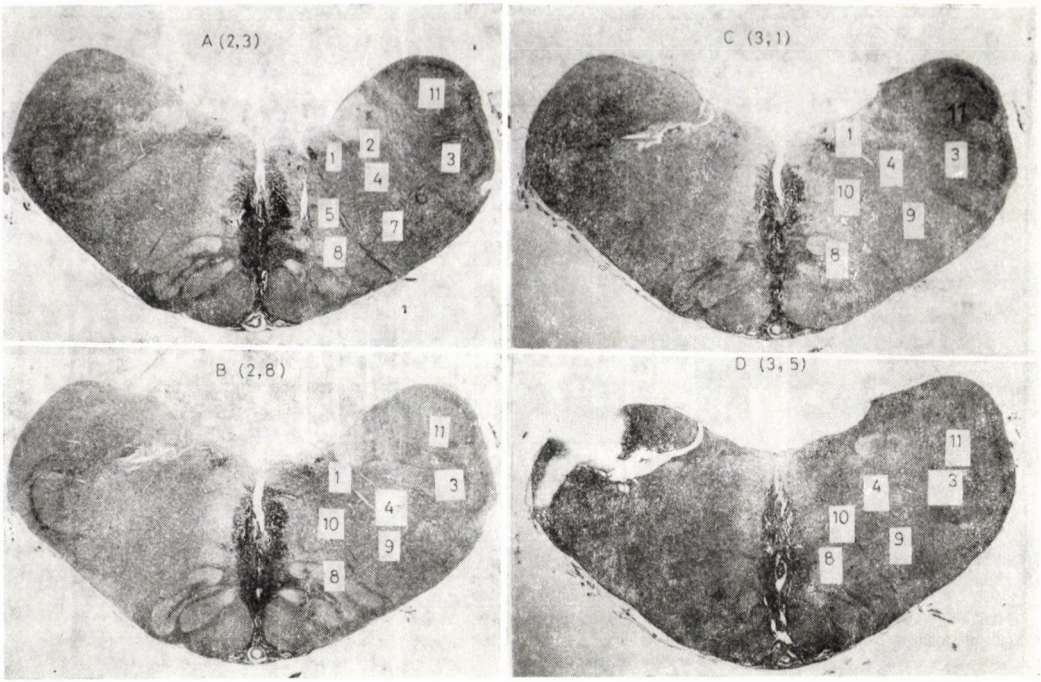

Fig. 5. Histologic cross sections through medulla oblongata in cat with midline split. Capitals-A, B, C, D correspond to the levels (2.3 mm, 2.8 mm, 3.1 mm, 3.5 mm) rostral to obex. Numbers denote neural structures (according to Berman): 1. Nc. n. hypoglossi, 2. Nc. tract. solitarii medialis, 3. Nc. spinal. trigeminalis, 4. Lateral segmental field, 5. Nc. reticularis paramedialis, 6. Nc. ambiguus, 7. Nc. reticularis lateralis, 8. Nc. olivaris, 9. Nc. retrofacialis, 10. Gigantoscellular segmental field, 11. Nc. cuneatus

verified histologically (Fig. 5) according to stereotaxic coordinates for cats [3]. The electroneurogram of the right transected phrenic nerve (C_{5-6}), the electromyogram of the left innervated hemidiaphragm and the pleural pressure were recorded continuously by an oscilloscope and a camera.

The effect in cats on normal rhythmogenesis of brainstem sections was studied extensively [1, 2, 8, 9, 11, 16, 18, 19], but we could not find references to cough and other airways reflexes in these conditions. Recent results have indicated that splitting of the brainstem in paralyzed and artificially ventilated cats produces an arrest of electrical activity in both phrenic nerves, in bulbar I and E neurones as well as in vagal efferent nerves under normocapnic conditions ($PaCO_2 = 32 - 33$ torr, $PaO_2 = 102$ torr, pH = 7.33). Hypercapnia ($PaCO_2 = 55$ torr, $PaO_2 = 280$ torr, pH = 7.2) gradually restored the neuronal activities [9].

In our experiments on nonparalyzed cats a split of the brainstem extending from 2 mm caudal to 2.5–3 mm rostral to the obex (group IIa) caused transient apnoe (18.0 $\pm$ 3.7 s) followed by restoration of spontaneous respiration. The restored breathing indicated a lower frequency (65% of the control)

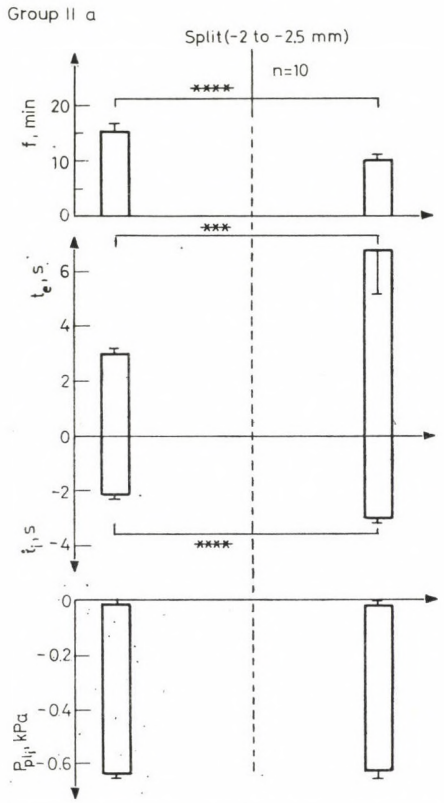

Fig. 6. Frequency of breathing (f), inspiratory (t_i) and expiratory time (t_e) evaluated from phrenic ENG and diaphragmal EMG and maximum pleural pressure in inspiration (P_{pli}) before and after brainstem splitting (left and right). Means $\pm$ SEM from 10 cats. Asterisks indicate the level of significance: *** — $p < 0.01$; **** — $p < 0.001$

prolonged but synchroneous activity of both sides, longer expiratory phase and nonsignificant changes in pleural pressure compared to control breathing (Fig. 6).

A split of the brainstem extending from 2 mm caudal to 3—11 mm rostral to the obex (group IIb) induced a permanent arrest of respiration characterized by disappearance of both the phrenic and diaphragmal activities, and the pleural pressure variations. It appeared that prolongation of the section above 3 mm rostral to the obex invariably resulted in terminal apnoea (Fig. 7-D compared to B and C). The nucleus n. facialis (nucl. VII) and nucleus n. retrofacialis (nucl. r. VII) are located in this area which may according to results in rabbits, play a specific role in normal respiratory rhythmogenesis [12].

I neurones in the area of nucl. r. VII are linked to contralateral respiratory neurones of the ventral respiratory group [4]. Section of these connections

to nucl. r. VII, nucl. n. VII and other higher structures plays a dominant role in respiratory arrest. This may result from an increase in the threshold of neurones to CO_2 [1], from a direct damage of the neuronal circuit of RRG or else from a decrease of general excitability due to reduction in "critical mass" of respiratory neurones [12, 18]. The area extending 2.5—3 mm rostral

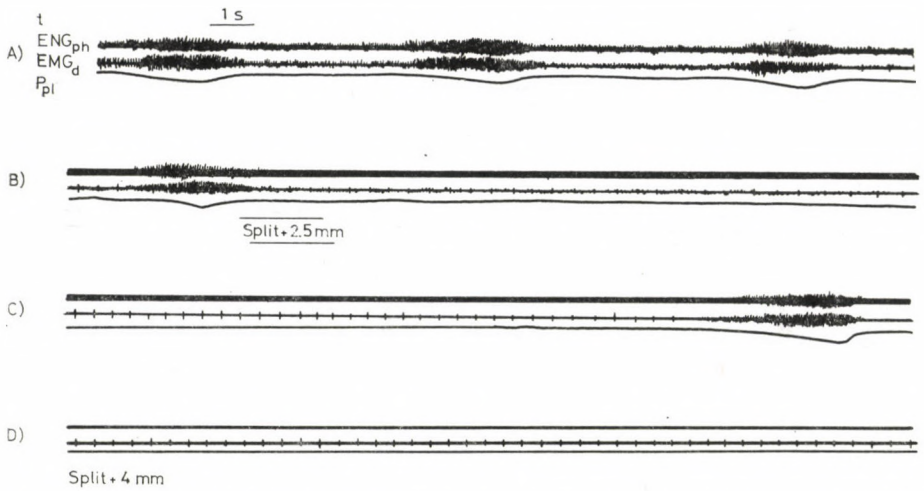

Fig. 7. Right phrenic electroneurogram (ENG_{ph}), left diaphragmal electromyogram (EMG_d) and pleural pressure (P_{pl}) after a split extending from 2.5 mm rostrally (records B and C) and 4 mm rostrally to the obex (record D) compared to control breathing before section (record A)

to the obex, 2—2.5 mm from the midline and 3—4 mm below the dorsal surface of the medulla is supposed to be critical for the neurogenesis of gasping [19, 20]. The generator of gasping is probably linked to normal RRG because a midline split in this region in cats eliminates gasping and restores the periodic respiratory rhythmicity [18]. In our experiments on brainstem-split cats (groups IIa and IIb) there was no gasping before the terminal apnoea.

In our experiments in nonparalyzed cats after a brainstem-split extending from 2 mm caudal to 2.5—3 mm rostral to the obex, breathing persisted in contrast to the respiratory arrest observed in paralyzed and artificially ventilated cats. It seems therefore that structures localized above the mentioned regions are of vital importance for periodic respiratory rhythmicity in cats. It is assumed that in paralyzed cats bilateral phrenicotomy and successive vagotomy would eliminate the afferent signals from the pulmonary stretch receptors as well as from the denervated diaphragm, intercostal and other muscles which may be important for normal RRG.

Brainstem-spliting markedly affected also the elicitability and character of cough, aspiration and expiration reflexes. Results with sections extending from 2 mm caudal to 2.5—3.0 mm rostral to the obex (group IIa) indicated

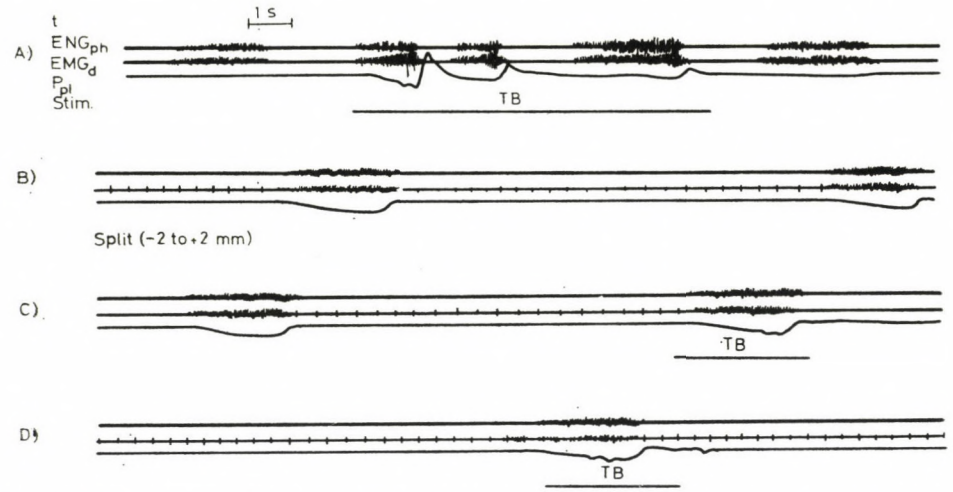

Fig. 8. Right phrenic electroneurogram (ENG_{ph}), left diaphragmal electromyogram (EMG_d) and pleural pressure (P_{pl}) during mechanical stimulation of tracheobronchial mucosa (TB) after brainstem split extending from 2 mm caudal to 2 mm rostral to the obex (records C and D) compared to control breathing after the split (record B) and cough before the split (record A)

that mechanical stimulation of the tracheobronchial region in no animal from among the 10 tested ones elicited electrophysiological or ventilatory signs of cough (Fig. 8 C and D). The pleural pressure records indicated only slight alterations in ventilation, which cannot be regarded as coordinated cough efforts. Stimulation of the glottal region also failed to elicit signs of the expiratory reflex, while mechanical stimulation of the nasopharynx nevertheless elicited the aspiration reflex in a weak form (Fig. 9B). Prolongation of the split above 2.5—3.0 mm rostral to the obex eliminated not only the breathing (C), but also the aspiration reflex (D).

Figure 10 summarizes the effects on defensive airways reflexes of a shorter split in cats (group IIa). After the split there were no cough and expiration reflexes, but the aspiration reflex persisted. It was characterized by both a shortening of the inspiratory phase (1.9-times) and a decrease of maximal inspiratory values of pleural pressure (2.45-times). The persistence of the aspiration reflex (though halved) suggests crossing projections from the structures localized more than 3 mm rostrally to the obex. Elicitability of the aspiration reflex in spite of the split indicated that in addition to chemical stimuli such as hypercapnia [9] strong afferent impulses from the nasopharynx may also be effective.

The absence of cough and expiration reflexes in splitbrainstem cats with persistent spontaneous breathing may be explained by disruption of interconnections of structures involved in these reflexes, localized in the

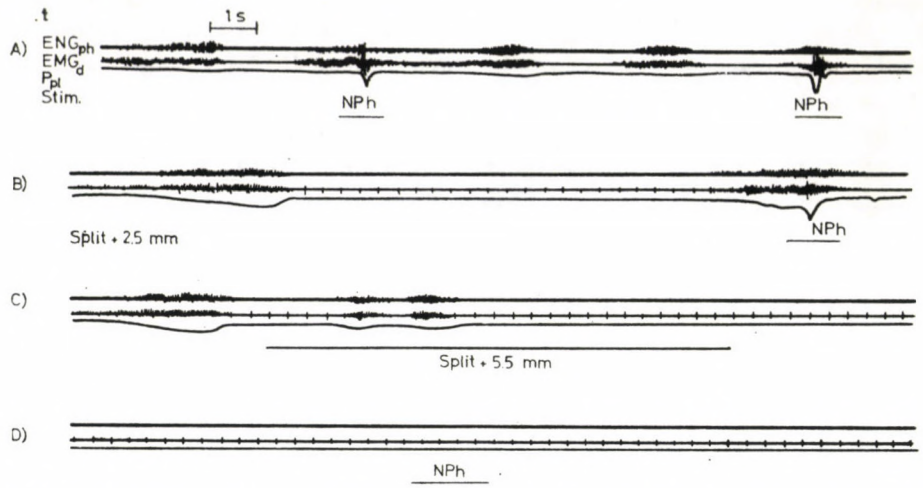

Fig. 9. Right phrenic electroneurogram (ENG_{ph}), left diaphragmal electromyogram (EMG_d) and pleural pressure (P_{pl}) during mechanical stimulation of nasopharyngeal mucosa (NPh) after a split extending from 2.5 mm (record B), and 5.5 mm rostral to the obex (records 3 and D), compared to control breathing and aspiration reflexes before the split (record A)

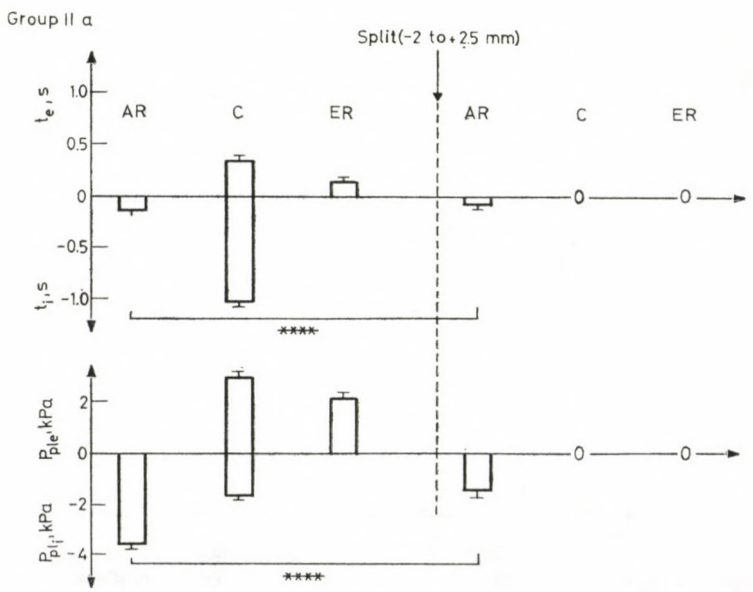

Fig. 10. Inspiratory (t_i) and expiratory time (t_e) evaluated from phrenic ENG and diaphragmal EMG and maximum pleural pressure in inspiratory (P_{pli}) and expiratory phase (P_{ple}) of airways reflexes before and after a brainstem split. Means ± SEM for aspiration reflex (n = 20), cough (n = 10) and expiratory reflex (n = 10). Other marks as in Fig. 6

area from 2 mm caudal to 2.5—3 mm rostral to the obex. On the contrary, cough and sneeze may be elicited in spontaneously breathing rabbits even after a split of the brainstem extending from 2 mm caudal to 11 mm rostral to the obex [21]. For technical reasons we could not determine whether a strong chemoreceptor drive such as hypercapnia or hypoxia could restore the missing cough and other expulsive processes in cats. This requires further investigation.

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SPECTRAL ANALYSIS OF RESPIRATORY PARAMETERS IN DEFENSIVE RESPIRATORY REFLEXES

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A detailed analysis of respiratory signals in normal breathing and defensive respiratory reflexes was performed to obtain maximum information that can be used for modelling of respiratory processes. Physiological interpretation of the problem is difficult and requires further measurements to achieve convincing conclusions. We suggest therefore that the search for appropriate methodological backgrounds should contribute to further clarification of the problems concerned with reciprocal linkage of the respiratory signals. Appropriate attention must be paid to investigations aimed at explaining the regulatory mechanisms of breathing.

Keywords: Probability density function, power and cross spectrum, transfer function, correlation and cross correlation, spectral analysis of cough.

We have attempted to obtain maximum information from analysed signals during normal breathing and defensive respiratory reflexes. In the present study the registered signals of pleural pressure and tracheal airflow were subjected to detailed analysis.

Probability density function, power spectrum, cross spectrum, transfer function, autocorrelation and cross correlation of the recorded signals were determined. Analysis of the mentioned signals during normal breathing and the defensive respiratory reflexes such as cough, sneeze, aspiration and expiration reflexes, was performed by dual-channel digital spectrum analyser TR 9405. Because of the wide spectrum of problems we present detailed analysis of these signals only during cough.

Materials and methods

Studies on respiratory mechanics were performed on cats weighing 2.8 kg (cat A) and 2.5 kg (cat B) under general anesthesia (Pentobarbital Spofa 40 $mg \cdot kg^{-1}$ i.p.). Body temperature was kept constant (37 °C) since temperature influences quiet breathing, and that affected the spectral densities [7]. Some reflexes may be depressed and some increased even by the same anaesthesia [3, 4].

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Mechanical stimuli were applied in order to induce cough from the tracheobronchial area. A nylon fibre 0.4 mm thick was introduced into the animals airways' through an injection-needle pricked into the tracheal cannula until it achieved the areas of the carina and large bronchi [5, 6]. Stimulation was performed for 1—3 seconds.

Time signals in pleural pressure and tracheal airflow were followed on a six-channel osciloscope (OPD 600, TESLA) at simultaneous acoustic control and recorded by means of an eight-channel tape recorder (EPI 2 B, SCHLUMBERGER). For better orientation these signals were recorded simultaneously on four-channel recorder with unidirectional rectifier (MULTISCRIPTOR 9400, HELLIGE) at necessary speeds.

Static and dynamic properties of both measured chains were verified.

Results and discussion

Time signals of pleural pressure and tracheal airflow in the cough used for analysis were chosen in their typical course.
Analysis of the recorded signals:

1. Probability density function

Probability density function is a fundamental function representing probability characteristics of periodic and impulse signals in a defined interval. It represents a probability at which a time series signal exists within a certain range of amplitude.

Figure 1 demonstrates the probability density of pressure signals (Pa) and airflow signals (Pb) during the cough. The cough is characterized by division of the probability density of trimodal shape characterization, typical for the given reflex, and it is also significantly different if compared to waveforms of guiet breathing and other analysed reflexes.

Normal breathing is characterized so that the histograms of both signals have a slightly bimodal shape.

2. Power spectrum

Power spectrum is used in typical securum frequence and is expressed by technical Unit (V^2). It is a real function having only amplitude information, it has no imaginary part and therefore has no phase information.

Figure 2. The power spectra of both signals during cough are approximately of the same shape with limited power to higher levels of frequency, with exponential function and with sectional frequency 4.0 Hz in both spectra. Markedly increased power during cough is observed especially in the low frequency band [8].

3. Cross spectrum

Cross spectrum is useful to determine the amplitude and phase information for common frequency components of two signals. At each frequency, the amplitude indicates the product of magnitudes of two signals, and the

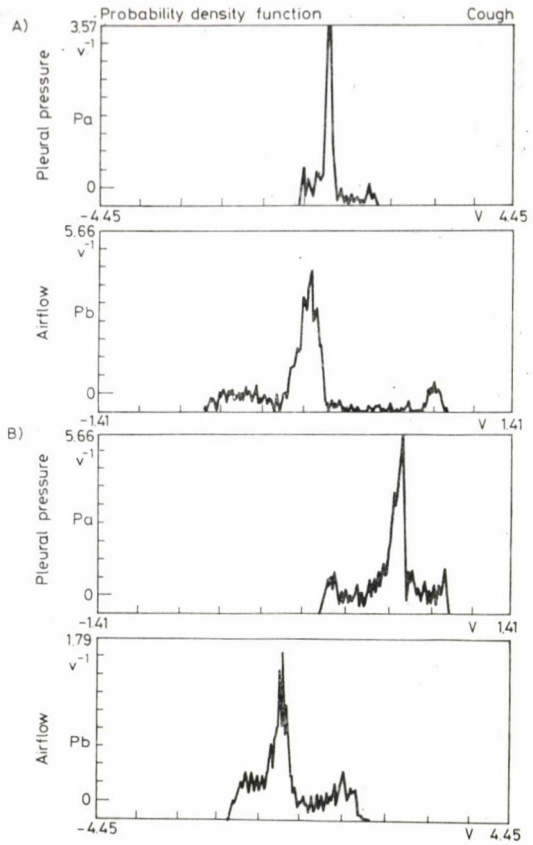

Fig. 1. Probability density function of pleural pressure signals (Pa) and tracheal airflow signals (Pb) during cough in two cats (cat A and B)

phase value indicates the relative phase difference between two signals. The cross spectrum corresponds to cross correlation function expressed in the frequency domain, and it is applied to measurement of the delay time, as is the case with cross correlation function.

Figure 3. The cross spectrum confirms the homogeneity of the spectra of pressure and flow signals during cough. Beside noisliness of 50 Hz and 150 Hz, there is no other significantly linked component in these spectra.

4. *Autocorrelation*

On an irregular signal varying with time, a strong correlation may be found between two points with relatively small time difference (γ). However, the correlation between those points may be weakened as the time

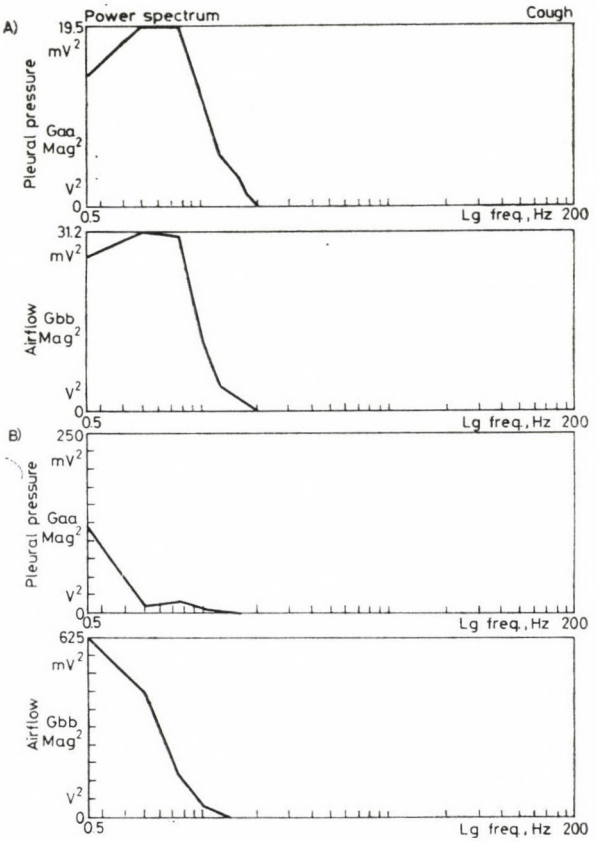

Fig. 2. Power spectrum of pleural pressure signals (Gaa) and tracheal airflow signals (Gbb) during cough in two cats

difference increases. If a periodical signal is contained in an irregular signal, the correlation between two points will be strengthened at a regular interval.

Figure 4. Autocorrelation functions of pressure signals in cough have a typical shape, a sudden drop of the autocorrelation function from $\gamma = 03. —$ — 0.4 s to Raa from 0.7 — 0.5 with a following increase to 0.9 — 1. The flow signals have a striking drop within the interval linkage depending on γ. The autocorrelation functions were decreased to zero even at $\gamma = 0.2 — 0.4$ s.

5. *Cross correlation*

Cross correlation function is used to determine the correlation between two points on each of the two signals which are apart from each other by time difference γ. It is used to determine velocity or distance by delay time measurement or to determine propagation paths of signals.

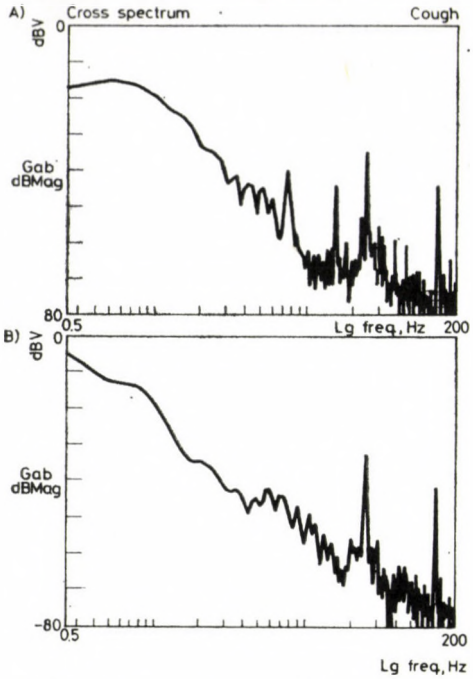

Fig. 3. Cross spectrum of pleural pressure and tracheal airflow signals (Gab) during cough in two cats

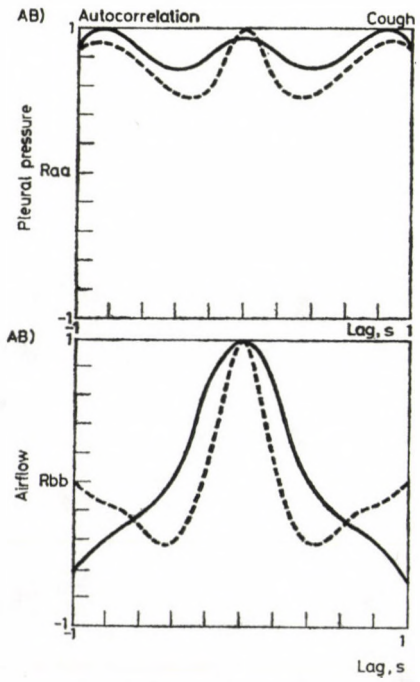

Fig. 4. Autocorrelation function of pleural pressure signals (Raa) and tracheal airflow signals (Rbb) during cough in two cats

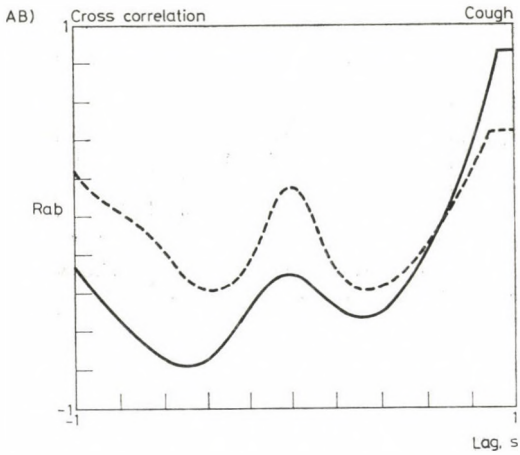

Fig. 5. Cross correlation function of pleural pressure and tracheal airflow signals (Rab) during cough in two cats

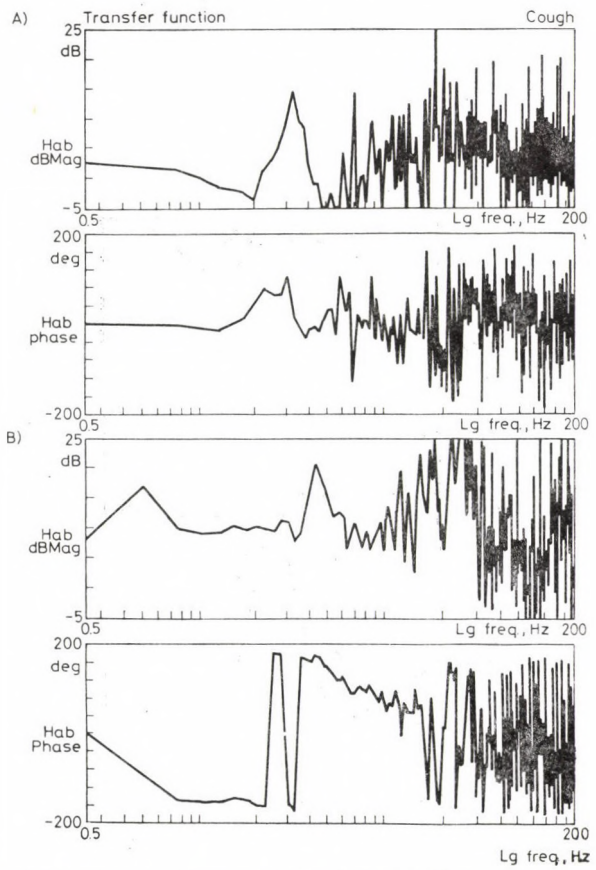

Fig. 6. Transfer function in cough. The transfer function (Hab) provides the frequency response of a system in terms of input (pressure)—output (airflow) relationship

Figure 5. Linkage of pressure and flow signals expressed by their cross-correlation function indicates a particular dependence on the time lag γ. The signals are linked weakly at $\gamma = 0$ and increased linkage is seen at $\gamma = -0.4 - 0.5$ s and at $\gamma = +0.3$ s after a significant linkage has been achieved at $\tau = +0.9$ s (even Ras $= 0.44 \div 0.86$).

6. Transfer function

Transfer function provides the frequency response of a system in terms of input—output relationship (in our case, pressure-flow). It contains amplitude and phase information.

Figure 6. The transfer function sensitively indicates linkage of both signals in the frequency domain. In the cough amplitude transfer characteristics have a similar shape and a typical course of this reflex. A similarity can be found in the relation to the actual amplitude transfer characteristics given for normal breathing and also for defensive respiratory reflexes.

During normal breathing the transfer can be regarded as practically unchanged until the frequency that corresponds to the second harmonic breathing frequency. The transfer characteristics in the frequency range up to the second harmonic of breathing frequency in their amplitude characteristics show a course that can be approximated by a delay network of the first or second—order [1, 2]. Phase characteristics cannot be given in general. Amplitude transfer characteristics seem to emphasize the type of "individuality" of the studied object.

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CHANGES IN MECHANICS OF BREATHING DURING EXPERIMENTAL COUGH AND SNEEZE IN ANAESTHETIZED CATS

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Pleural pressure, airflow and tidal volume during experimental cough and sneeze elicited by mechanical stimulation of the tracheobronchial and nasal mucous membranes were investigated in fifty anaesthetized cats (pentobarbital, 40 mg/kg i.p.). Pressure-volume, pressure-flow and flow-volume relations were studied during these expulsive processes. In comparison to quiet breathing there was a decrease in dynamic lung compliance in both respiratory tract reflexes ($p < 0.001$), especially in their expiratory phases. As compared to quiet breathing, the total work of breathing was significantly increased ($p < 0.001$) in cough (20 times) as well as in sneeze (13 times). The total lung resistance increased markedly ($p < 0.001$) in both cough and sneeze compared to quiet breathing. In these expulsive processes there was also a high "cough index" (resistance calculated from the peak flow and instantaneous pressure). The flow-volume curve in cough, in contradistinction to sneeze, indicated a significantly reduced airflow of the end of expiration (at 85% of the expired volume), demonstrating a concomitant bronchoconstriction.

Keywords: breathing mechanics, lung compliance, lung resistanc e, cough index, flow-volume curve.

In the last 20—30 years there is a tendency in both research and clinical practice to perform functional examinations of the respiratory system, mainly from the aspect of breathing mechanics. These function tests are intended to measure the changes in pressure, airflow and tidal volume occurring during breathing in the lungs, chest and airways. After a complex analysis of the physiology and pathophysiology of cough and other respiratory reflexes [3] it was necessary to interpret these processes also from the point of view of breathing mechanics.

Materials and methods

The experiments were performed on fifty cats in pentobarbital anaesthesia induced by a dose of 40 mg/kg given intraperitoneally. The cough and sneeze reflexes were elicited by mechanical stimulation of the tracheobronchial and nasal mucosa. Tracheal airflow, tidal volume and pleural pressure were recorded simultaneously and stored on magnetic tape under visual control on an oscilloscope (Fig. 1).

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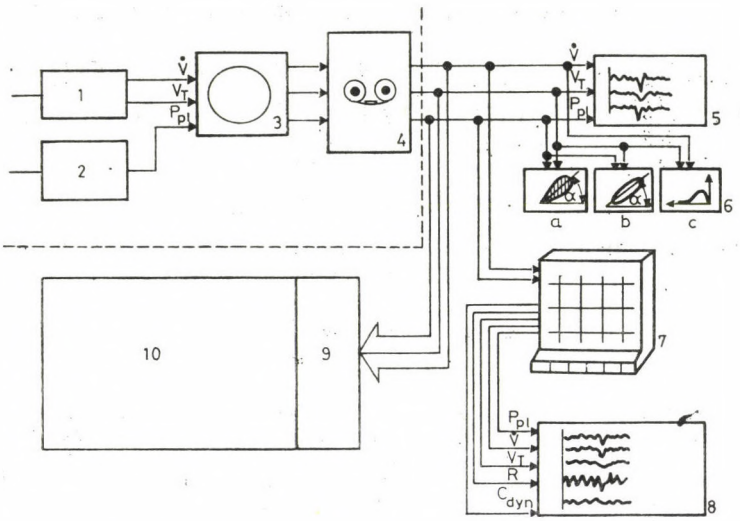

Fig. 1. Block scheme of method: 1 — Pneumotachograph, 2 — Manometer, 3 — Oscilloscope, 4 — Magnetic tape, 5 — Direct writer, 6 — X—Y plotter, 7 — Analogue computer, 8 — Recorder, 9 — Analogue-digital converter, 10 — Digital computer, $\dot{V}$ — airflow, V_T — tidal volume, P_{pl} — pleural pressure, C_{dyn} — dynamic lung compliance, R_L — total lung resistance

The information from the tape was later replayed and recorded either continuously on a 4-channel recorder as parallel traces of the pressure, flow and volume changes or plotted on an x—y recorder as separate loops of inspiratory-expiratory cycles in three different combinations. The slope of the pressure-volume loop or tg_{alfa} indicates the dynamic lung compliance. The work of breathing can also be calculated by planimetry of the whole area of this loop. The slope of the pressure-flow loop or $cotg_{alfa}$ indicates the total lung resistance. The expiratory and in some cases also the inrpiratory part of the flow-volume curve can be recorded. For a more complex analysis of the methods and results, see ref. [4].

Results

Figure 2 illustrates the changes in dynamic compliance during defensive airways reflexes in comparison with control breathing. The lung compliance decreases in the inspiratory phase of strong respiratory processes such as cough and sneeze, probably because of overdistension of the lungs at values close to the total lung capacity. These decreases are even more significant in the expiratory phase of cough and sneeze. The total work of breathing, calculated from the area of pressure-volume loop, is significantly increased during cough and sneeze. It reaches 20 to 13 times higher values than during quiet breathing (Fig. 3). Figure 4 illustrates the mean values of total lung resistance during control breathing and in both phases of cough and sneeze. Compared to the control breath, the total lung resistance is higher in the expiratory phase of these reflexes, but not significantly higher in their inspiratory phases. It is also evident that the total lung resistance is significantly

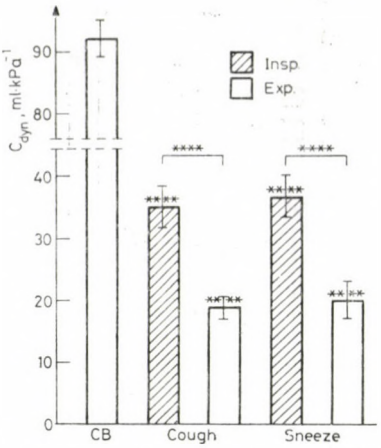

Fig. 2. Dynamic lung compliance (C_{dyn}) in control breath (CB), and in inspiratory (cross linked columns) and expiratory (blank columns phases of cough and sneeze. Means ± SEM. **** — $p < 0.001$

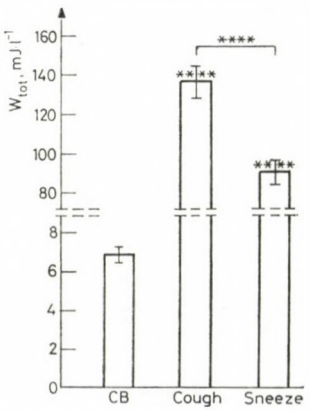

Fig. 3. Total work of breathing in control breath (CB), and in cough and sneeze. Means ± SEM. ****, — $p < 0.001$

higher during the expiratory than the inspiratory phase of these expulsive processes.

The mean values of the so-called "cough index", which is the resistance calculated from the peak flow and the corresponding pleural pressure, are illustrated in Figure 5. This "cough index" is much higher in cough and sneeze than during quiet breathing.

Figure 6 shows the expiratory parts of flow-volume curves obtained during expulsive processes in a group of cats. The flow-volume curve of cough effort, contrary to that of sneeze, indicates a significantly reduced airflow at the end of expiration (at 85% of forced expiratory volume), demonstrating a concomitant bronchoconstriction.

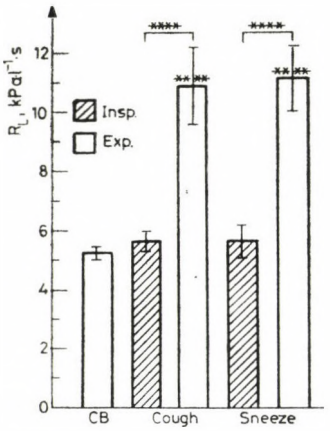

Fig. 4. Total lung resistance (R_L) in control breath (CB), and in inspiratory (cross linked columns) and expiratory (blank columns phases of cough and sneeze. Means ± SEM. **** — $p < 0.001$

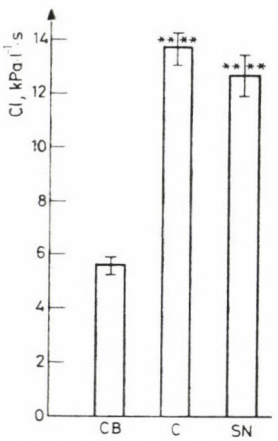

Fig. 5. "Cough index" (CI) in control breath (CB), and in cough (C) and sneeze (SN). Means ± SEM. **** — $p < 0.001$

Discussion

Parallel recording of pleural pressure, tracheal airflow and tidal volume on magnetic tape followed by plotting the obtained pressure-volume, pressure-flow and flow-volume curves on an x—y recorder and an off-line evaluation of data proved to be useful for complex analysis of breathing mechanics in animal experiments [2]. The present results showed that in cough and sneeze, marked changes occur in the mechanics of breathing.

The considerable decrease of lung compliance during cough and sneeze, especially in their expiratory phases, is caused mainly by the changes in lung volume. Strong and frequent cough and sneeze attacks by the decrease of

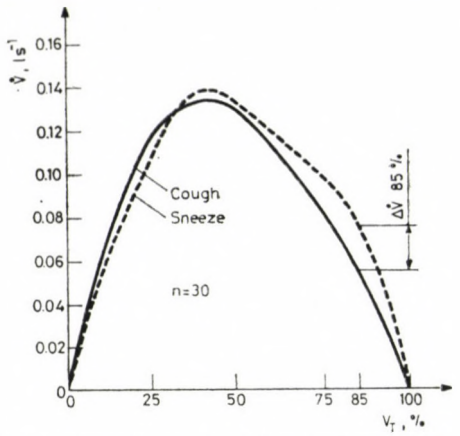

Fig. 6. Expiratory part of the flow-volume curve in cough (solid line) and in sneeze (dashed line). On the ordinate the forced expiratory volume is given in percentage

lung compliance may precipitate the development of atelectasis and respiratory distress in some pathological situations. Assessment of the work of breathing has a practical importance, first of all in cough. Because the total work of breathing in cough is 20 times higher than during quiet breathing, the energetic expenses caused by frequent and exhausting cough attacks may become significant. Therefore, it is necessary to suppress the ineffective and chronic cough using antitussive drugs.

Total lung resistance during the inspiratory phase of cough and sneeze remains at the level of control breathing. On the contrary, during the expiratory phase of these expulsive processes there are high increases in the total lung resistance. which are caused first of all by dynamic compression of the airways and in cough also by bronchoconstriction. Besides estimation of the total lung resistance, it is useful to calculate the "cough index", which is regarded as a reliable indicator of the effectiveness of cough [1, 5].

Evaluation of the flow-volume curves showed that the tracheal airflow at 85% of the forced expiratory volume is significantly lower in cough than during a sneeze. This fact confirms the existence of reflex bronchoconstriction developing during cough, especially in the peripheral airways [3].

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THE RELEVANCE OF STUDYING RESPIRATION IN SMALL LABORATORY ANIMALS

(CHAIRMAN'S INTRODUCTION)

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Animal experimentation has become an established scientific tool. This paper is not meant to go into the ethics of experiments on animals, it starts from the assumption that some experiments have full acceptance and, assuming the proper guide-lines of work with animals are observed, give no reason to objections. Typically, in respiratory physiology, those parts of research that could not be realized on man, have been performed on animals like the dog or the cat. Most of our knowledge about the mechanics of breathing, ventilation-perfusion relationships and control of breathing has been obtained in these animal species. In those fields of research, however, where groups of subjects rather than individuals are important, it is not feasible to work with large animals. This is especielly true for experiments designed in the fields of pharmacology and pathophysiology.

The interindividual variability is important not only for clinically active physicians but also for experimenters who want to ascertain not only one aspect of the functional relationships or one aspect of a drug effect, as it may be observed in a single organism, but who must look for the variance of the studied phenomenon. This is true for normal functions studied by physiologists, or drug effects in healthy individuals. It becomes much more prominent under pathological conditions. The situation in a diseased organism is much more complex than in a healthy one. Therefore, pathophysiological studies need to take into account a great variability. This is also true for pharmacologists who ought to test drug effects not only in healthy but also in diseased subjects.

This is typically done by providing models of certain diseases both for pathophysiological and pharmacological research. Disease modelling in animals can never aim at a complete replica of a human disease. This must be so because

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any disease in man is modified by his higher nervous activities, by the psychosomatical relationships and also by his conscious and rational attitudes, learning, etc. Thus we may study in the animal model just some, though assumably basic, aspects of any disease with the appreciation that in man the conclusions must be verified.

Even so, animal models of disease provide an exquisite tool for obtaining invaluable knowledge for medical research. As mentioned above, even an identical stimulus or procedure will provide in a group of animals a whole range of responses due to their interindividual differences. To be able to take them into account, large groups are needed. For such a purpose small laboratory animals are best suited. Therefore, the laboratory rat has become the most widely used experimental animal.

Once the research in respirology has included also pathophysiology and pharmacology apart from physiology, the question of the use of the rat has become pressing. Using small animals has not only its advantages but also means some problems. The problems can be listed as follows.

1. Can the rat, an animal phylogenetically distant from man, provide useful information when used as a disease model? The answer has been given above. We cannot aim at producing a complete "human" disease in any kind of animal, even if it is phylogenetically closer to man than the rat.

2. Some basic aspects of breathing may be different in the rat and man. This is especially true for the mechanics and control of breathing. It must be taken into consideration before applying the results of rat experiments to man. On the other hand, knowledge of some specific and well described difference (e.g. large compliance of the chest wall or strong vagal control over resting breathing of the rat) may stimulate comparison with, for instance, the human newborn.

3. The fact that the rat is small calls for special techniques. Miniaturization of clinical equipment sometimes is easy (body plethysmograph for the rat), sometimes difficult (blood sampling, some pressure measurements, etc.). Therefore, it has become important that laboratories which use the rat for respiratory studies should exchange experience not only in the form of results — as published in scientific periodicals — but also with special respect to the methodology. It has been suggested that an attempt be made to provide an effective exchange of experiences.

Unfortunately, the laboratories which have systematically used the rat for respiratory studies for many years, are scattered throughout the continents (Table I). The original idea to organize a meeting of interested scientists had to be rejected because of financial reasons. An alternative solution has been proposed, namely to collect papers from these laboratories and have them published in the Supplement of a physiological journal. Such a publication would undoubtedly provide a good basis for extending future cooperation.

Table I

Some laboratories with long-lasting interest in rat respirology

Belgium

Dept. of Respiratory Diseases
Academic Hospital
Ghent

Czechoslovakia

Institute of Pathophysiology
Faculty of Paediatrics
Charles University
Praha

German Democratic Republic

Dept. of Experimental Therapy
Research Institute for Tuberculosis
Berlin-Buch

Great Britain

Dept. of Experimental Medicine
Medical School
Sheffield

Hungary

National Korányi Institute
for Tbc and Pulmonology
Budapest

Japan

Dept. of Physiology
School of Medicine
Chiba University
Chiba

New Zealand

Dept. of Physiology
Medical School
University of Otago
Dunedin

U.S.A., Indiana

Lilly Research Laboratories
Greenfield

U.S.A., Indiana

Lilly Research Laboratories
Indianapolis

U.S.A., Kentucky

College of Pharmacy
Lexington

U.S.A., Massachusetts

Biomedical Engineering
Boston University
Boston

U.S.A., Massachusetts

Dept. of Anesthesia
Harvard Medical School
Boston

U.S.A., New Mexico

Lovelace Inhalation Toxicology
Research Institute
Albuquerque

U.S.A., New York

Brookhaven National Laboratory
Upton
Long Island

U.S.A., Washington

Pulmonary Division
Veterans Administration Hospital
Seattle

Conclusions

Rat respirology is an important part of research especially for pathophysiology and pharmacology of breathing. International exchange of methodological experience should be advanced.

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LUNG FUNCTION TESTS IN SMALL EXPERIMENTAL ANIMALS BY MEANS OF A SIGNAL PROCESSING SYSTEM

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For the analysis of the airflow velocity and oesophageal pressure signals of anaesthetized spontaneously breathing small animals (rats and guinea pigs), a signal processing program package was developed for a multiprocessor system (Electronic Measuring Gears Co., Budapest). 4—10 respiratory cycles were analysed in a signal series and the arithmetic mean value was used to increase the accuracy of the method. Since standardization of the respiratory parameters of small animals has not yet been specified, the normalization of volume values per 100 cm^2 body surface is proposed.

Keywords: lung function tests, standardization of respiratory parameters, signal processing.

Small animals (rat, guinea pig) are widely used in experimental medicine. Determination of their respiratory parameters is not easy. To perform such experiments, improvement of analogue signal sources is required. Technical difficulties are caused not only by the quantitative determination of the ex/inspired gases of changing temperature and composition, but also by the fact that the magnitude of airflow velocity and respiratory volume is lower by about 2.5 order of magnitude, whereas the respiratory frequency is higher by about 1 order of magnitude in small animals than in humans. Sensitivity of the measuring technique is represented by the units of some respiratory function parameters (Table I). Furthermore, it seems more useful to analyse signals serials of respiratory cycles instead of one cycle. To satisfy these demands, a signal processing system was created (Electronic Measuring Gears Co. (EMG), Budapest, 5500/6 Universal Signa lAnalyser) for which program routines were developed.

Methods

The experiments were carried out in supine rats and guinea pigs anaesthetized with pentobarbital (50 mg/kg i.p.). Airflow velocity and oesophageal pressure were estimated according to the procedure shown in Figure 1. The signal processing system is demonstrated in Fig. 2, and 3. Steps of signal processing:

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Table I

Units of parameters

Frequency	Hz
Pressure (P_{PI} P_{RES}, P_{oe})	Pa
Airflow velocity ($\dot{V}$)	$ml \cdot s^{-1}$
Volume (V, V_T, V_{min})	$ml \cdot (100\ cm^2$ body surface$)^{-1}$
Elastance (E_L)	$Pa \cdot ml^{-1}$
Resistance (R_{dyn}, R_L)	$Pa \cdot s \cdot ml^{-1}$
Work (W_{RES}, W_{VISC})	$\mu W \cdot ml^{-1}$
Loss of heat	$\mu J \cdot ml^{-1} \cdot s^{-1}$

The accuracy of frequencies measured depends on the sampling frequency, whereas sensitivity (1 bit) was 0.16 $ml \cdot s^{-1}$ for airflow velocity and 5.9 Pa for oeasophageal pressure.

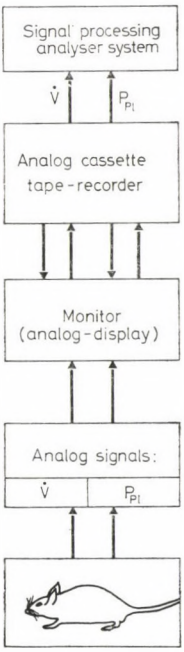

Fig. 1. Data acquisition. Pressure changes proportional to airflow velocity, and oesophageal pressure measured in the symmetric loops of the π-formed glass tubes placed into the trachea of anaesthetized and spontaneously breathing animals were measured by a capacitive differential manometer built in a modified MEDICOR RM-01 respiratory monitor. The linear segment of the pressure-difference—flow relationship was used to estimate airflow velocity. Signals obtained during the experiment were displayed on a multi-channel MEDICOR VM 61/A visualizer and recorded on an analogue tape recorder (Rolitron type MCAM 4, 4-channel, frequency response: DC-625 Hz). After the experiments, data stored on cassette were rechecked on a monitor (visualiser) and cycles selected for analysis were fed into the signal processing system (Major characteristics of the system are shown in Figure 2)

a) signal series selected for analysis was fed in the analyser and checked qualitatively by eyes. Prior to detailed analysis, environmental parameters (air pressure, room temperature, humidity) and calibration factors should be specified.

b) the following parameters were determined in each respiratory cycle:

1. time parameters: respiratory frequency, quotient of the duration of inspiration (T_{in}) and the duration of a total cycle, average time of expiration;

2. ex/inspiratory airflow peak velocity (PEF, PIF);

3. Tidal volume (V_T) normalized to 100 cm^2 body surface, determination of respiratory minute volume and inspiratory volume normalized to duration of inspiration (V_T/T_{in});

4. inspiratory mid-volume, inspiratory airflow acceleration and velocity of oesophageal pressure-change;

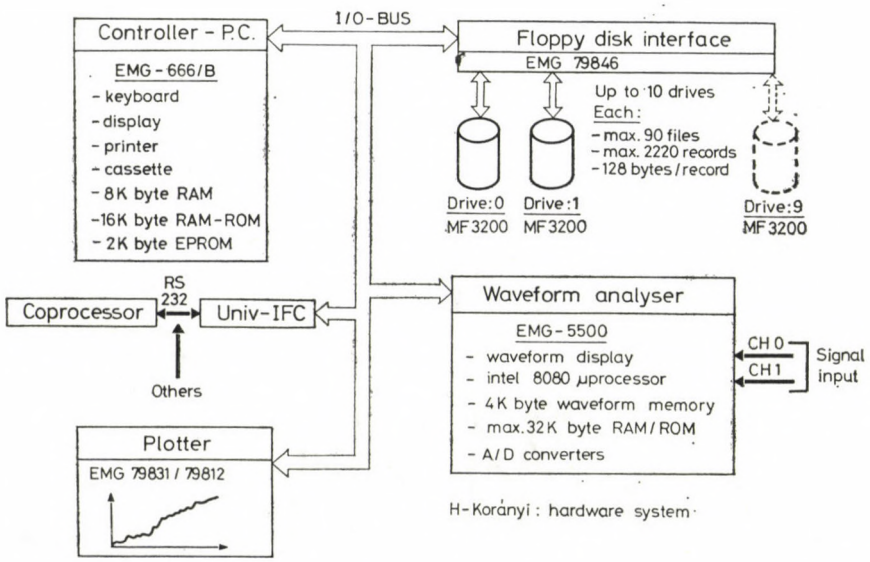

Fig. 2. Signal Processing hardware system. In the analyser an 8-bit converter was used as an analogue to digital converter and the sampling frequency was generally 200 Hz. Size of the signal serial selected for analysis was 1 Kbyte per channel. Signals stored in the memory of the analyser directly or signals stored in the EMG-666/B personal computer by means of handlers, as a function of time or as loops, can be drawn on an X—Y plotter .Results of analysis can be printed by a mini-printer or stored on floppy discs (Electronic Measuring Gears Co., Budapest, Hungary)

5. effective elastance and effective resistance, the second order component of resistance;

6. resistive pressure component (which would require the application of a more complicated model) is derived according to the Mead-model;

7. resistive and viscous component of respiratory work and loss of heat (work dissipated as heat);

8. dynamic resistance, i.e. the quotients of difference of two neighbouring pressures and that of velocity points, which is composed of derivatives and for this reason it is noisy. The frequency components of this time serial were determined by using FFT (Fast Fourier Transformation).

c) in a signal serial selected for analysis, generally 4—10 respiratory cycles were analysed and to improve the accuracy, the arithmetic mean value of parameters calculated for each respiratory cycle was given (depending on the experimental conditions, respiratory cycles may vary in a certain time interval, as shown in Figure 4).

d) after printing, signals found in the analysed serial and belonging to peak volume cycle were drawn by an X—Y plotter (Figure 5).

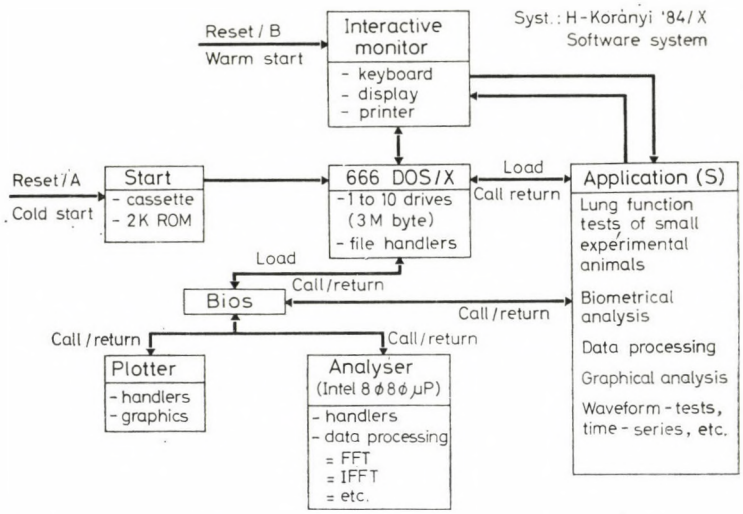

Fig. 3. Software. The system is controlled by a special purpose program "Korányi Software" The program packages are integral parts of the system providing an interface between the user and the hardware system. When a user command is detected, the changes required are initiated by the interactive software system. Tipically in the activation one or more programs, 90 files, 2220 records, 128 bytes/record can be stored on each disc unit. This structure gives a possibility to store signals and identification codes of each animal having several treatments in different files

Fig. 4. Respiratory parameters in function of time. Airflow velocity ($\dot{V}$), pleural pressure (i.e. oesophageal pressure, P_{Pl}) without smoothing, after a sampling frequency of 200 Hz. Respiratory volume (V) is integrated from airflow velocity by digital (trapezoid) method. Resisative pressure (P_{RES}), shown in the lower part of the Figure, is derived from pleural pressure and the elastance, according to the Mead-model. The Figure demonstrates the effects of a slight narcosis in the rat: respiratory cycles differ significantly

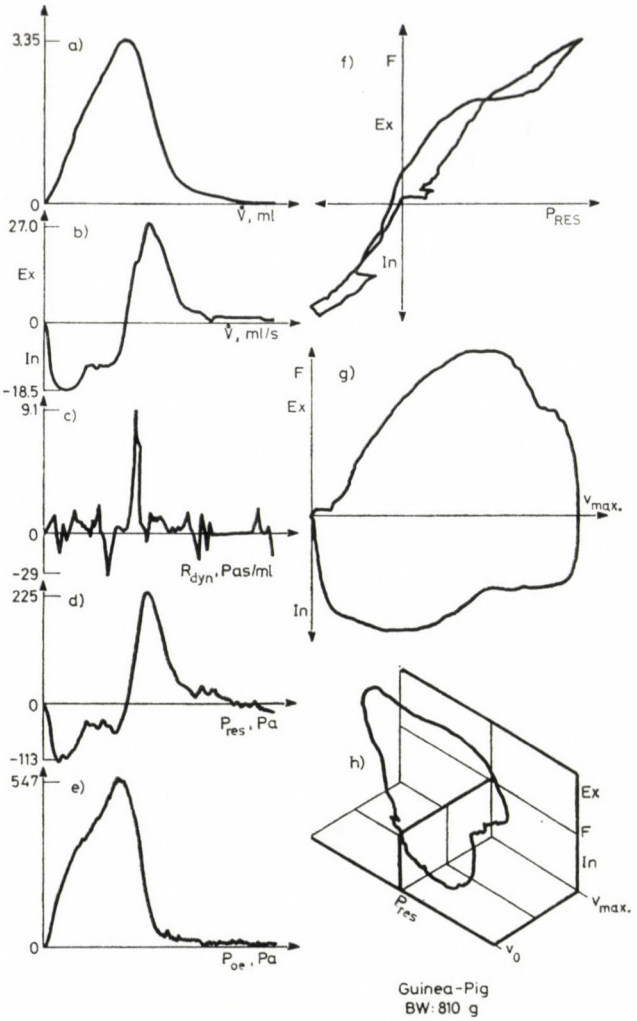

Fig. 5. Results of analysis. Plotting of cycle (lasting for 0.93 s) belonging to the peak respiratory volume in the selected serial of signals analysed: a) respiratory volume (V), b) airflow velocity ($\dot{V}$), c) dynamic resistance, d) resistive pressure, e) oesophageal pressure. Abscissa is the time-axis in Figures. a—b—c Figures are normalized to a given area. In further parts: f) airflow velocity as function of resistive pressure, g) airflow velocity as function of respiratory volume, h) axonometric projection of airflow velocity, volume and resistive pressure loops

Results and discussion

Qualitative and quantitative accuracy of the analysis is determined by the transducers and analogue to digital converters. Similarly to Mortola and Noworaj [4], symmetrically cut glass π-formed tubes and a differential manometer as pneumotachograph were used in the experiments. The capacitive differential manometer, (Medicor, Budapest) showed the linear frequency response

ranging over several decades of Hz. The level of 0—255 of the analogue to digital converter meant an accuracy of 0.4% of full scale, satisfying the requirements of the measuring technique. To store airflow velocity and oesophageal pressure signals 2×1024 bytes were available. Depending on the breathing frequency, the sampling rate was selected (generally 200 Hz were used) to have at least 5 to 10 respiratory cycles (more than 100 data points per one cycle) to minimalize the error of digital algorithms.

Standardization of respiratory function parameters of small animals has not been specified. So, the values were normalized to 100 cm^2 body surface (Table II), similarly to metabolic values.

Table II

Tidal volumes related to body mass and body surface

Body mass g	Body surface** cm	V_T**	V_T***	V_T****
200	315	1.365	0.682	0.433
300	412	1.705	0.568	0.413
400	499	2.045	0.511	0.410
500	580	2.385	0.477	0.411

* = body surface (cm^2) = $9.2 \sqrt{(\text{body mass (g)})^2}$

** = estimated tidal volumes based on the regression equations $Y = 0.685 (\pm 0.192) + 0.0034 (\pm 0.0005) \times$, where Y = tidal volume and X = body mass (n = 55).

*** = ml · (100 g body mass)$^{-1}$

**** = ml · (100 cm^2 body surface)$^{-1}$

Table III shows some transformed respiratory function mean values found in the literature. These data did not differ significantly from our measured values.

An Intel 8080 microprocessor (μP)-based analyser and a Personal Computer involved in the signal processing system could be operated in parallel during analysis (co-operative, divided intelligence). So, as long as the calculator recognized the signals and performed cycle-parameter determinations, the analyser counted the function of airflow velocity and resistive pressure power-density spectra, on the basis of which — in certain cases — some information could be obtained on bronchial resistance. A typical example is shown in Fig. 6 (parameter values measured are listed in Table IV). The physiological explanation of spectral analysis should be explored by further experiments.

Based on values of variation coefficients of parameters of lung function tests (Table IV), a relatively high reproducibility of frequency as well as the volume parameters can be predicted.

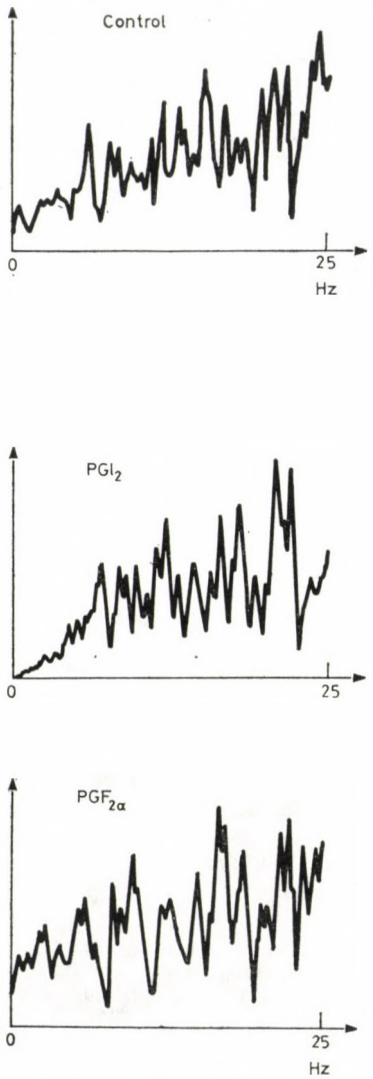

Fig. 6. Amplitude-spectra of airway dynamic resistance. Fourier transformations of digitally fltered (low pass filter) dynamic resistances found in experiments descrıbed in Table IV

Table III

Some respiratory function data of rats
(mean values)

Author	n	Body mass, g	Frequency min^{-1}	V* $ml \cdot min^{-1} \cdot 100\ cm^{-2}$	R_L** $Pa \cdot s \cdot ml^{-1}$	E_L** $Pa \cdot ml^{-1}$	Narcosis
Diamond and O'Donnell (1977)	32	233	115	46.3	25.5	392	urethan
Johanson and Pierce (1972)	12	402			43.2	516	
Palecek et al.	8M	258		30.0	32.4	178	urethan +
(1967)	12F	195		29.7	38.2	280	allobarbital
Palecek (1969)	10	220	104	39.4	49.0	265	halothane
Yokoyama et al.	6M	368			22.6	280	urethan +
(1984)	6F	248			23.5	251	chloralose
Hedner	17		83	49.3			enflurane
(1983)***	21		114	52.5			halothane
	16		93	47.0			pentobarbital
	15		132	58.1			urethan
Debreczeni	16	242	75	31.8	18.9∅	201	pentobarbital
	31SHR	213	78	31.9	13.6∅	224	pentobarbital

* = Calculated per 100 cm^2 body surface based on mean values, ** = Expressed in SI, based on mean values, *** = Values calculated per 100 g body mass, M = male, F = female SHR = spontaneously hypertensive rats, ∅ = effective resistance.

Table IV

Respiratory effect of PGI_2 *and* $PGF_{2\text{-alpha}}$ *in rats**

	Control	PGI_2	$PGF_{2\text{-alfa}}$	VC** (per cent)
Number of cycles analysed	6	6	4	—
Frequency	1.35	1.45	0.57	24
Tidal volume	0.34	0.34	0.51	18
V_T/T_{in}	0.99	1.05	1.15	29
Minute ventilation	27.7	29.3	17.3	26
Elastance	156	170	156	21
Resistance	10.7	11.1	18.1	41
Viscous work/cycle	59	66	184	40
Resistive work/cycle	44	64	179	49
Heat loss	14	1.2	4.0	44

* = The rats had a body mass of 310 g · 10μg · kg^{-1} PGI_2 and 100 $\mu g \cdot kg^{-1}$ $PGF_{2\text{-alfa}}$ were injected intravenously and the analogue signals were stored. The results of dynamic resistances measured 5 minutes after treatment, are shown in Figure 6.
** = Variation coefficients based on analysis of experiments performed in 16 rats.

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ALGORITHMIC ERRORS OF LUNG FUNCTION TEST ANALYSIS

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Nowadays lung function parameters will be determined by digital data processing algorithms. Minimal sampling frequency characterizing analogue-digital convertion is given by Shannon's law.

Integration is a typical operation in data processing. The dynamical errors of generally used integration algorithms are much influenced by the sampling frequency. Theoretical examinations show unambiguously that using Simpson's rule the condition $6f_{signal} \leq f_{sample}$ has to be fulfilled to keep the amplitude error of integration lower than 1%. This means that sampling frequency will be determined by both the signal's spectrum and the structure of the data processing system.

Keywords: Amplitude spectrum, Shannon's law, Rectangular integration, Trapezoidal integration, Simpson's rule

Presently many different diagnostic instruments are used in clinical and experimental pulmonology. An intelligent data processing system analyzing the primary measuring results is the common characteristic of all modern sophisticated instruments.

The aim of this paper is to analyze the characteristics of the most often used mathematical operation, which is integration.

Analyzing method of different structures

Generally it is true that correct development of data processing algorithms is based on primary knowledge. A data processing system should be calibrated obviously in a way that its dynamical error should be negligible compared to the error of the measuring system. This means that the error of parameter estimation is fundamentally determined by the error of the measuring system.

Figure 1 shows the general block diagram of the measuring and data processing system. And now let us examine the theoretical relations of determining the parameters of the economically realizable A/D conversion.

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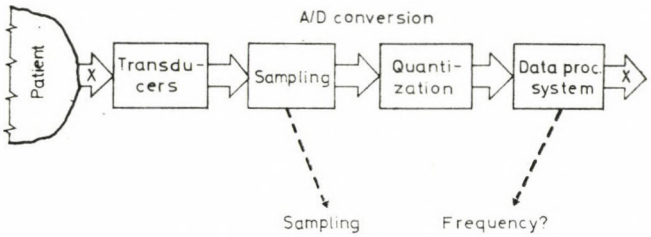

Fig. 1. **General block diagram of the measuring and data processing system**

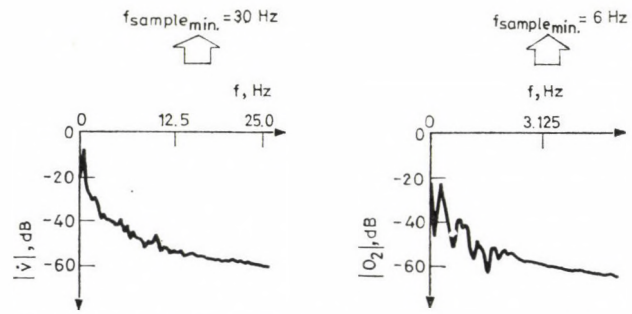

Fig. 2. **Amplitude spectrum of flow and oxygram patterns (1)**

Sampling frequency will be determined by the spectrum of the input signs on one hand and by the types of algorithms realized in the data processing system on the other.

Two typical amplitude spectra can be seen in Fig. 2. The main part of the spectrum of the flow pattern was below 15 Hz and that of the oxygram below 3 Hz [1].

The following conclusions can be drawn from these frequency distributions:

— Shannon's law for sampling is fulfilled, if the sampling frequency is twice as large as the spectrum.
— Using Shannon's law it will be sure that the sampled signs will not be distorted in information, therefore the original signs are reproducible.
— Shannon's law does not tell anything about the structure of the data processing system, therefore further examinations are necessary.

Many lung function parameters can be determined by applying integrator algorithms. Integration can be carried out as rectangular summation, trapezoidal summation and by Simpson's rule.

The performed analysis has the following boundary conditions:

— Shannon's law for sampling should be fulfilled.
— Quantization should be ideal.

Figure 3 shows the physical operation of the above mentioned algorithms for integration and the amplitude and phase transfer characteristics. The arguments of the transfer functions contain the angular frequency of the examined signal and the sampling time. This means that the amplitude and phase characteristics of the transfer functions can be represented in the function of the angular frequency, if T is constant. This method makes it theoretically possible to compare the operation of algorithms having different structures.

The error of algorithms is obviously given by their deviation from the ideal integration algorithm.

Transfer characteristics of the ideal integrator are shown in Fig. 4. In order to make comparison easier, it is useful to choose unit sampling time. Amplitude characteristics of the integrator algorithms get only within a nar-

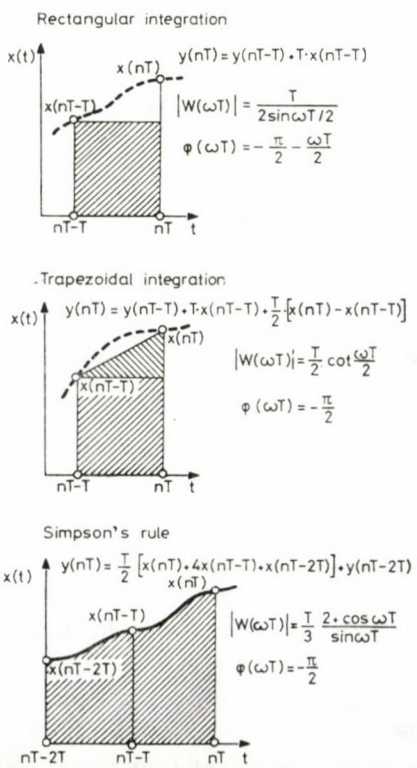

Fig. 3. Generally used integration algorithms (2)

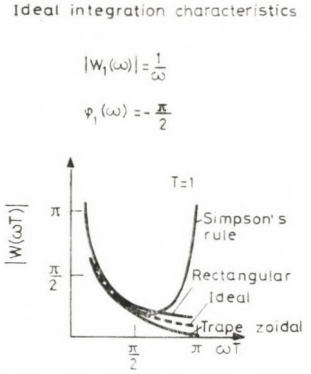

Fig. 4. Comparison of amplitude characteristics of different integration algorithms

E_A / ω	RECT	TRAP	SIMPS
$\frac{\pi}{6}$	1.1 %	−2.3 %	0.04 %
$\frac{\pi}{3}$	4.7 %	−9.3 %	0.7 %
$\frac{\pi}{2}$	11.0 %	−21.0 %	4.7 %
$\varphi(\omega T)$	not ideal	ideal	ideal

$\omega T \leq \frac{\pi}{3}$; $2\pi f T \leq \frac{\pi}{3}$; $f_{sample} = \frac{1}{T} \geq 6f$

Amplitude error function

$$E_A = \frac{|W(\omega T)| - |W_1(\omega T)|}{|W_1(\omega T)|}$$

Phase error function

$$E_\varphi = \frac{\varphi(\omega T) - \varphi_1(\omega T)}{\varphi_1(\omega T)}$$

Fig. 5. Results of the developing process

row range near to the ideal characteristic. If the signal's angular frequency is increasing, the dynamical error of integration will also increase. Phase error can be seen in the case of rectangular integration, but the phase characteristics of trapezoidal integration and Simpson's rule are ideal.

Interpretation range of the characteristics is $\omega T = \pi$, because Shannon's law for sampling is fulfilled only within the range $\omega T \leq \pi$.

Methods and results

Development of algorithms is just the inverse of the analyzing process. This means that knowing the spectrum of the input signal, sampling frequency has to be determined, where the amplitude and phase error of the given algorithm is still acceptable. It is a practical question whether the sampling frequency determined this way can be realized, because processors have finite operation time.

Figure 5 shows the relation between the development and the results. Amplitude and phase errors were determined by the above mentioned relations. Table 1 contains the results of calculation in the case of different angular ferquencies. It can be seen that Simpson's rule has the best characteristics.

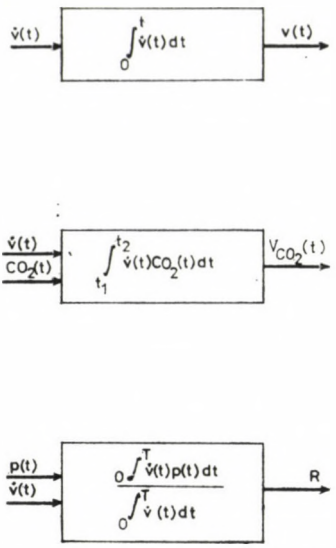

Fig. 6. Some typical applications in lung function tests

Its amplitude error is 0,7% in the case of $\omega = \pi/3$. This brings about a satisfactory result in nearly all practical cases. Therefore sampling frequency has to be at least six times higher as the signal's spectrum. This means that the minimum sampling frequency should be 90 Hz for lung function applications. Therefore the choice of $f_{\text{sample}} = 100$ Hz is suitable and easy to realize. It is dangerous to use Simpson's rule within the range $\omega T \geq \pi/2$, because the algorithm's amplitude error is suddenly growing here. Therefore the input sign's spectrum has to be limited. High-frequency noises must be eliminated. This can be realized by using a lowpass filter.

Some typical examples for data processing can be seen in Fig. 6:

— Making a spirogram from flow pattern data,
— Determination of the time function of carbon dioxide production based on the flow pattern and capnogram,
— Determination of airway resistance and other parameters of lung mechanics.

In order to choose the right sampling frequency in the case of using data processing methods of other structures, a similar theoretical study has to be made.

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RESPIRATORY MECHANICAL IMPEDANCE IN THE RAT

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The forced oscillatory impedance of the total respiratory system (Z_{rs}) was measured in seven anaesthetized, paralysed rats weighing 351 $\pm$ 55 g. Tracheotomy was performed, and the animals were placed in the supine position in a body box. Pseudo-random pressure variations between 0.5 and 10 Hz were applied around the chest. Central airflow was measured with a heated screen pneumotachograph. Total respiratory resistance (R_{rs}) and elastance (E_{rs}), corrected for the impedance of the tracheal cannula, were markedly frequency-dependent: R_{rs} fell from 37.3 $\pm$ 19.1 kPa $\cdot$ l^{-1} $\cdot$ s at 0.5 Hz to 17.6 $\pm$ 4.4 at 2 Hz and 10.3 $\pm$ 3.3 at 10 Hz; the corresponding E_{rs} values were 453 $\pm$ 14, 594 $\pm$ 90 and 713 $\pm$ 104 kPa $\cdot$ l^{-1}, respectively. This indicates that in the frequency range encompassing spontaneous breathing rates the classical resistance-intertance-compliance model provides an inadequate description of the respiratory mechanics in the rat.

Keywords: Forced oscillations, resistance, elastance, frequency-dependence of impedance.

The forced oscillation technique has been widely used for measuring the mechanical impedance of the total respiratory system in healthy subjects and in patients with obstructive pulmonary disease. The original method of DuBois et al. [4], which used single-frequency sinusoidal oscillations, was improved by sophisticated versions applying multiple-frequency excitation with random noise [10] or pseudo-random signals [11], allowing thereby a rapid assessment of respiratory resistance and reactance over a wide range of frequencies. The impedance data obtained according to these methods show characteristic frequency-dependences, different in health and disease, and offer material for the structural identification of respiratory mechanical models.

Data are, however, scarce on the oscillation mechanics of animals, especially of rats. The only study performed in rats with forced oscillations was reported by Jackson and Watson [6]. Their measurements covered the 20 to 90 Hz frequency interval; thus, the lowest frequency investigated was at least ten times higher than the spontaneous breathing rate of the rat. The

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resistance was found to be independent of frequency between 20 and 40 Hz, but it then increased slightly towards higher frequencies. One of the most striking results of Jackson and Watson was that the 10 kPa·l^{-1}·s plateau of R_{rs} amounted to less than half the pulmonary resistance alone, obtained in previous studies [3, 8, 13]. The use of different set-ups, as well as subtraction of the endotracheal tube impedance in one study [6], cannot sufficiently explain this discrepancy. It is more likely that the R_{rs} values above 20 Hz are by no means representative of those measured at spontaneous breathing rates, i.e. there might be a significant negative frequency-dependence of R_{rs} at lower frequencies not investigated by Jackson and Watson [6]. This hypothesis is supported by a recent study [5] where in relaxed apnoeic subjects R_{rs} was some three times higher at 0.25 Hz than at 5 Hz. A fall in R_{rs} was also demonstrated between 2 and 8 Hz in paralyzed bonnet monkeys [16], and above 4 Hz in dogs [7].

It was therefore studied whether there was a significant decrease in R_{rs} in normal rats over the frequency range encompassing spontaneous breathing rates. Since we had previously found that in breathing animals the superposition of external oscillations did not give reliable estimates of impedances below 10 Hz, we measured Z_{rs} in paralyzed rats with their artificial ventilation suspended.

Methods

Seven male rats weighing 351 ± 55 g were studied. The animals were anaesthetized intraperitoneally by 30 mg/kg sodium pentobarbital (Nembutal, Ceva, Paris). Tracheotomy was performed and a 35 mm long, 2 mm ID polyethylene cannula was introduced into the trachea. The animal was placed in the supine position into a 1.6 l plexiglass body box, which was closed after paralysis of the rat with 4 mg pipecuronium bromide (Arduan, Richter, Budapest) injected intraperitoneally. 180 ml/min minute ventilation was maintained with a small animal respirator (KUTESZ, Budapest).

Figure 1 shows the schematic arrangement of the experimental set-up. The pressure fluctuations in the body box were produced by a loudspeaker 30 cm in diameter (HA 31/1008,

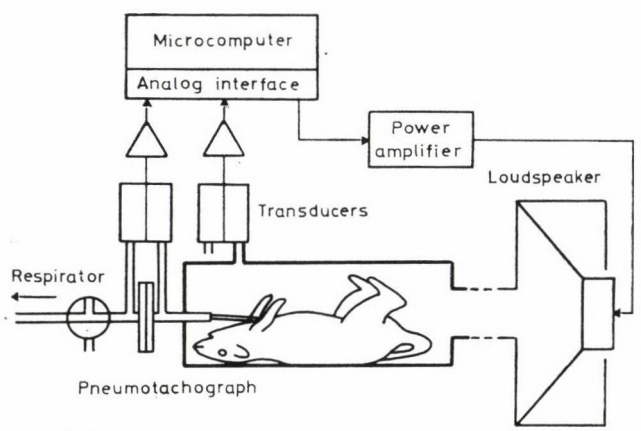

Fig. 1. Schematic representation of the experimental apparatus

Videoton, Székesfehérvár), the membrane of which was made leak-free with a silastic layer. The loudspeaker was driven by a microcomputer-generated pseudo-random signal (U) with 0.5—10 Hz bandwidth and 4 s repetition time, allowing 0.25 Hz frequency resolution. The power spectrum of U monotonously decreased from unit value at 0.5 Hz to 0.25 at 10 Hz; the peak-to-peak value of the resulting box pressure was kept within the limits of $\pm$ 0.2 kPa.

The difference between the box pressure and ambient pressure (P) was sensed by a Validyne MP-45 ($\pm$ 0.5 kPa) transducer (Validyne Engineering Corp., Northridge, California). An identical transducer measured the pressure drop between the side-ports of the cylindrical (3 mm ID) heated screen pneumotachograph. The linearity error of the flowmeter was below 2% at 2 ml $\cdot$ s^{-1}, which was never exceeded by the oscillatory flow signal ($\dot{V}$). The frequency responses of the devices measuring P and $\dot{V}$ were calibrated together, using as a known mechanical impedance [14] a 5 l glass bottle: at 10 Hz the measured impedance agreed with the theoretical value within $\pm$ 1% in magnitude and $\pm$ 0.5° in phase.

The signals of P, $\dot{V}$ and U were bandpassed between 0.1 and 10 Hz, sampled at a rate of 32 s^{-1} and fed into a special-purpose microcomputer during the 16 s intervals of oscillatory measurements. Preceding these intervals, the respirator was disconnected from the animal by a three-way valve which, at the same time, opened the central airways of the rat to ambient pressure through the pneumotachograph and a short segment of connecting tube. Thus, the impedance of the latter parts of the equipment (Z_e) was added to the respiratory impedance ($Z'_{rs} = Z_{rs} + Z_e$). In each animal 4 measurements were made, between which ventilation was resumed for at least 2 min. After the experiment, when the animal was killed with an overdose of sodium pentobarbital and disconnected from the tracheal cannula, Z_e was measured separately. To avoid the effects of non-linearity, this was done by adjusting approximately the same flow level as that maintained during the measurement of Z'_{rs}.

The impedance was computed according to the indirect procedure described elsewhere [2, 5]. In short, Z'_{rs} was estimated as

$$Z'_{rs} = \frac{G_{PU}}{G_{\dot{V}U}}$$

where G_{PU} and $G_{\dot{V}U}$ are cross-power spectra between P and U, and $\dot{V}$ and U, respectively; Z_e was determined analogously. To assess the reliability of the individual impedance estimates, the coherence functions

$$\gamma^2_{PU} = \frac{|G_{PU}|^2}{G_{PP}G_{UU}}, \quad \gamma^2_{\dot{V}U} = \frac{|G_{\dot{V}U}|^2}{G_{\dot{V}\dot{V}}G_{UU}} \quad (0 \leq \gamma^2_{PU}, \gamma^2_{\dot{V}U} \leq 1)$$

were also computed, where G_{PP}, $G_{\dot{V}\dot{V}}$ and G_{UU} are autocorrelation spectra of P, $\dot{V}$ and U, respectively. The value of coherence is 1 if there is a noise-free, linear relationship between the signals, and decreases in the presence of non-linearities and extraneous noise [2, 10].

Results

Impedance and coherence data obtained in a single 16 s measurement are shown in Figure 2. The impedance is displayed in terms of the real (R'_{rs}) and imaginary (X'_{rs}) parts, i.e. resistance and reactance, respectively. The decrease of R'_{rs} with increasing frequency is most prominent in the low-frequency interval and then tends to disappear towards higher frequencies. X'_{rs} is negative (elastic) throughout the interval studied and seems to be inversely proportional to frequency. It can also be seen that the resistance of the equipment (R_e), including that of the tracheal cannula, the pneumotachograph and the connecting tubes, is constant and comprises a significant proportion of the total resistance measured. The inertive reactance of the equipment (X_e) increases linearly and becomes comparable with the total reactance at higher frequencies.

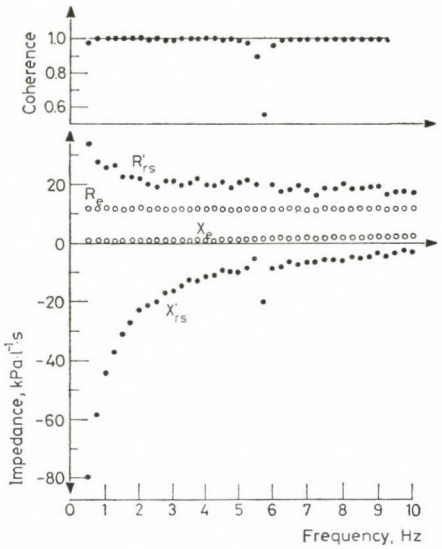

Fig. 2. Results of an individual measurement. R'_{rs} and X'_{rs} (closed circles) represent the real and imaginary parts, respectively, of the measured total impedance. The components of the equipment's impedance (R_e and X_e, open circles) correspond to the resistance and reactance of the tracheal cannula, pneumotachograph and connecting tubes. Also shown are the values of coherence ($\gamma^2_{\dot{V}U}$) between the driving signal (U) and airflow ($\dot{V}$)

Of the two coherences, γ^2_{PU} was greater than 0.99 at all frequencies in each measurement. In contrast, there was a fall in $\gamma^2_{\dot{V}U}$ between 5 and 7 Hz, indicating a powerful heart-frequency noise component in the flow signal, the fall coinciding with deviations in the values of R'_{rs} and X'_{rs}. At other frequencies, $\gamma^2_{\dot{V}U}$ always exceeded 0.97, a level at which the impedance values can be accepted [10]. However, after averaging of the 4 impedance spectra obtained in each animal, the mean value of Z'_{rs} at the critical frequencies no longer deviated much from the overall course of the impedance. Thus, all impedance values from each animal were retained when the mean impedance data for the group were calculated.

Figure 3 shows the mean $\pm$SD impedance values for the seven animals. Subtraction of the constant R_e enhanced the frequency-dependence of the real part: R_{rs} fell from 37.3 $\pm$ 19.1 k·Pal^{-1}·s at 0.5 Hz to 17.6 $\pm$ 4.4 at 2 Hz and 10.3 $\pm$ 3.3 at 10 Hz. The course of the imaginary part shows that the respiratory system behaves as a viscoelastic one, although from the X_{rs} curve it is not clear whether or not the elastance was frequency-independent. We therefore computed effective elastance as $E_{rs} = 2\pi f X_{rs}$, where f is frequency. The mean $\pm$SD values of elastance for the group are shown in Figure 4: E_{rs} increases from 453 $\pm$ 74 kPa·l^{-1} at 0.5 Hz to 594 $\pm$ 90 at 2 Hz and 713 $\pm$ 104 at 10 Hz.

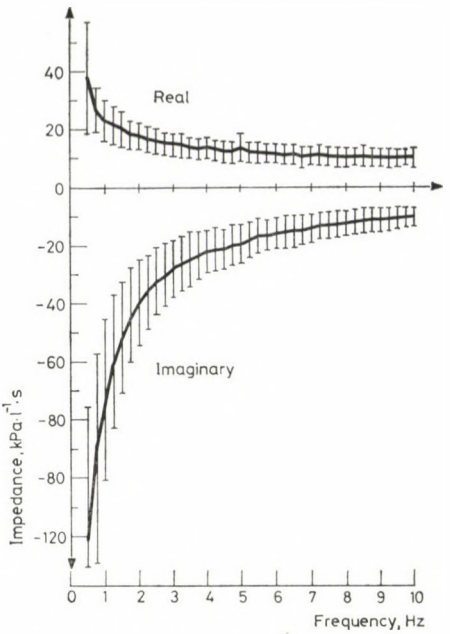

Fig. 3. Mean values of the impedance of the respiratory system (Z_{rs}) for the group (N = 7). Bars denote ± SD

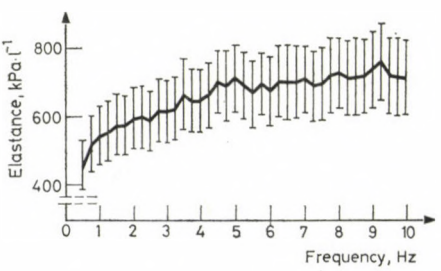

Fig. 4. Mean values of the effective elastance of the respiratory system (E_{rs}) for the group (N = 7). Bars denote ± SD

Discussion

To our knowledge, this was the first study to provide data on the frequency-dependence of total respiratory impedance in the rat over the frequency interval containing the rate of spontaneous breathing. Before paralysis the animals breathed at about 1.5 Hz with a pattern rich in harmonics, and the oscillatory signal would have had to be kept low to avoid non-linear effects;

the unfavourable signal-to-noise ratios would therefore have excluded the collection of reliable impedance data. Accordingly, the measurements were carried out under neuromuscular blockade, during 16 s interruptions of artificial ventilation. Under these circumstances, the impedance of the chest wall is presumably lower than that during the periodic activity of respiratory muscles, and the total impedance might have been underestimated. Nevertheless, it seems unlikely that the frequency-dependence of Z_{rs} was significantly altered in the relaxed state.

Our results can be compared with those of Jackson and Watson [6], although there are methodological differences between the two studies. For instance, Jackson and Watson measured spontaneously breathing animals in a consequently higher frequency interval (20—90 Hz), where the negative frequency-dependence of R_{rs} was completely lacking; at higher frequencies, there was even a slight increase in resistance. Further, owing to the inertial properties of the respiratory system, which are more important at higher frequencies, they could observe zero-crossing (resonance) of X_{rs} at 39 Hz. In spite of the different frequency intervals permitting the manifestation of different mechanical properties, our 10 Hz values agreed remarkably well with those obtained from a resistance-inertance-compliance (C_{rs}) model in the above study [6], where on average $R_{rs} = 10.3$ kPa·l^{-1}·s and $E_{rs}(=1/C_{rs}) = 703$ kPa·l^{-1} were determined.

Below 10 Hz, however, the present study revealed a pronounced increase of R_{rs} towards lower frequencies: at 0.5 Hz R_{rs} was 3.6 times higher than at 10 Hz. Even so, our mean value at 2 Hz (17.6 kPa·l^{-1}·s) was still considerably lower than the pulmonary resistance measured by Diamond and O'Donnell [3] during spontaneous breathing at a rate of 115 ± 21 min^{-1} (26 ± 7 kPa·l^{-1}·s), by Johanson and Pierce [8] at 108 ± 29 min^{-1} (44 ± 16 kPa·l^{-1}·s,) and by Paleček [13] at 104 ± 11 min^{-1} (50 ± 12 kPa.l^{-1}.s). In addition, the contributions of the pulmonary and chest wall compartments to the total resistance of the rat are not known. If, similarly as in normal subjects [5], the chest wall resistance comprises the major proportion of R_{rs} and is almost entirely responsible for its frequency-dependence at low frequencies, then the pulmonary component of our R_{rs} values would be even farther from the pulmonary resistance values reported previously [3, 8, 13]. The reasons for this discrepancy are unclear; (1) one explanation may be that we subtracted the impedance of the tracheal cannula, and thus our Z_{rs} values corresponded to that seen at a point 13—15 mm above the tracheal bifurcation. (2) The mean weight of our animals considerably exceeded the weights in two of the studies [3, 13] mentioned above, but was somewhat less than that in [8]; hence an explanation based on the inverse relationship between body size and Z_{rs} would not be convincing. (3) A further study would be needed, combining the forced oscillation technique with classical transpulmonary measurements during spon-

taneous breathing, to see whether non-linear phenomena involved in tidal breathing but absent during low-amplitude oscillations might clarify this inconsistency.

The negative frequency-dependence of R_{rs} was accompanied by an opposite, though more moderate, change in E_{rs}. As noted above, our E_{rs} values at 10 Hz corresponded well with that obtained between 20 and 90 Hz [6], while our 0.5 Hz value ($C_{rs} = 2.21$ ml·kPa^{-1}) approached the static total compliance (2.3 ml·kPa^{-1}) determined by Diamond and O'Donnell [3] and that which may be inferred from measurements of Johanson and Pierce [8]. Hence, our findings offer a link between static and dynamic total compliances that are rather conflicting in man, too [5]. The only study performed at frequencies falling in our interval (40 to 320 min^{-1}) was that of Diamond and O'Donnell [3], who failed to find any systematic change in C_{rs}, including the static values, with frequency. This is a striking observation, because at the same time they did observe a marked difference between static (3.4 ± 1.7 ml·kPa^{-1}) and dynamic (2.5 ± 0.8 ml·kPa^{-1}) pulmonary compliances. Previous studies have shown that under static conditions the chest wall structures of the rat are more compliant than the lung [3, 8], but nothing is known about the dynamic behaviour of the rib cage-diaphragm compartment. Therefore, similarly as in the partitioning of R_{rs} into pulmonary and chest wall components, the contributions of the respective compartments to the total elastance and its frequency-dependence remain to be clarified.

In conclusion, the frequency-dependent behaviour of the total respiratory impedance which we observed in the 0.5 to 10 Hz frequency interval in anaesthetized and paralyzed rats is inconsistent with the classical three-parameter (resistance-inertance-compliance) modelling of the respiratory system. The most conspicuous changes occurred at the lowest frequencies, around the spontaneous breathing rates: R_{rs} decreased with increasing frequency, while E_{rs} increased throughout the frequency range, in spite of the neglected inertial effects that tend to decrease the effective elastance progressively. If this frequency-dependence is of pulmonary origin, then the bronchial compliance acting in parallel with the peripheral part of the respiratory system [9], the inhomogeneity of parallel lung units [12], or collateral ventilation [1] may be suggested as underlying mechanisms. Alternatively, parallel inhomogeneity of the rib cage-diaphragm compartment [4, 5, 10, 11, 15] can also cause such frequency-dependent behaviour.

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COMPLIANCE, BLOOD GASES, AND ACID-BASE BALANCE IN GUINEA PIGS ARTIFICIALLY VENTILATED WITH DIFFERENT IPPV PATTERNS

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In order to estimate optimum parameters for arfificial ventilation of adult guinea pigs, the effect of four hours intermittent positive pressure ventilation (IPPV) was studied using different tidal volumes (V_T), respiratory frequencies (f), and minute volumes ($\dot{V}_e$). Total compliance was measured by placing the animal in a whole body plathysmograph, the arterial blood gases, pH and base excess by catheterizing the carotid artery.

In Series I 9 guinea pigs were ventilated at parameters adapted to the spontaneous breathing pattern ($V_T = 2$ ml, $f = 70$ breaths $\cdot$ min^{-1}). This ventilatory pattern resulted in severe disorders in compliance, gas exchange, and acid-base balance.

In Series II 3 different V_T (2, 6, 10 ml) were studied by changing f so that V_e was kept constant. The results demonstrated a favourable effect of slow and deep ventilation upon lung mechanics and oxygenation.

In Series III 3 different $\dot{V}_e$ (300, 250, 200 ml $\cdot$ min^{-1}) were tested using a constant $\dot{V}_T = 10$ ml. Optimum parameters for artificial ventilation of adult guinea pigs were: $\dot{V}_T = 10$ ml and $f = 20$ breath — min^{-1} which resulted is stable compliance, good O_2-saturation, normocapnia and normal acid-base balance.

Keywords: intermittent positive pressure ventilation, guinea pig, compliance, gas exchange, low frequency ventilation.

Several studies have shown that artificial ventilation is frequently followed by disorders in lung mechanics, gas exchange, and acid-base balance [2, 6, 12, 13, 15, 19, 22, 24]. Prevention of these failures requires the employment of a carefully selected ventilatory pattern.

In guinea pigs, however, there does not exist such a definite standard, and different parameters were applied by various investigators [5, 9, 11, 14, 21, 23]. As the literature shows, adult guine pigs are ventilated at tidal volumes (V_T) ranging between 3 [9] and 10 ml [14], and at frequencies (f) between 8 [21] and 72 [9] breaths per minute. This inconsistency induced us to study systematically the effect on lung mechanics, gas exchange, and acid-base balance of varying V_T, f, and minute volumes (V_e) in artificially ventilated guinea pigs.

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Material and methods

A total of 44 animals were used, their body weight ranging between 500 and 900 g. After anaesthesia, tracheal cannulation, and muscle paralysis intermittent positive pressure ventilation was carried out using a constant ventilatory pattern for 4 hours. A time-cycled, pressure controlled ventilator was used at a constant I: E-ratio of 2 : 3. An arterial catheter was inserted into the carotid artery. Every 30 minutes arterial blood samples were taken and pH, pCO_2, pO_2, and base excess were measured. In order to record continuously the total compliance, the animal was placed into a volume-constant body plethysmograph with small openings for the arterial and venous catheters, endotracheal tube, and the calibration system.

Series I. Artificial ventilation adapted to the spontaneous breathing pattern

Physiological respiration of the adult guinea pig is performed by a mean V_T of 2 ml, a mean f of 70 breaths per minute, and a mean I: E-ratio of 2 to 3 [1, 10, 17, 18]. A group of 9 animals was artificially ventilated at these parameters.

Series II. Effect of various tidal volumes and ventilatory frequencies

Three different ventilatory programmes were tested

— $V_T = 2$ ml, $f = 150$ breath $\cdot$ min^{-1}, $n = 3$
— $V_T = 6$ ml, $f = 50$ breath $\cdot$ min^{-1}, $n = 6$
— $V_T = 10$ ml, $f = 30$ breath $\cdot$ min^{-1}, $n = 6$

each at a $\dot{V}_e$ of 300 ml.

Series III. Effect of various minute volumes

The ventilatory programme of Series II

— $\dot{V}_e = 300$ ml $\cdot$ min^{-1}, $f = 30$ breath $\cdot$ min^{-1}, $n = 6$

was compared with the programmes

— $\dot{V}_e = 250$ ml $\cdot$ min^{-1}, $f = 25$ breath $\cdot$ min^{-1}, $n = 5$

and — $\dot{V}_e = 200$ ml $\cdot$ min^{-1}, $f = 20$ breath $\cdot$ min^{-1}, $n = 6$

each at a $\dot{V}_T$ of 10 ml.

Results and discussion

Series I. Artificial ventilation adapted to the spontaneous breathing pattern

Figure 1 shows the alteration of the compliance in our experiments. In the first 15 minutes already the distensibility of the lung-thorax-system showed a dramatical reduction to about one third of the initial value. After 2 hours ventilation the pressure which is necessary to insufflate 2 ml of air rose up to 1 kPa. Blood gas values also showed a progressive deterioration. An uncompensated respiratory acidosis developed at pCO_2 values of about 12 kPa and a decrease of pH to about 7.0. Oxygen saturation of arterial blood diminished to about 50%. Although for many species it is usual to adapt artificial ventilation to the physiological breathing pattern [3, 4, 16, 20, 24], artificial ventilation of guinea pigs at such parameters is accompanied by severe disorders in lung mechanics, gas exchange, and acid-base balance.

Series II. Effect of various tidal volumes and ventilatory frequencies

Figure 2a shows alteration of the compliance during ventilation. A favourable effect of a slow and deep ventilation upon loung mechanics is demonstrated. Using a V_T of 10 ml instead of 6 ml, guinea pigs showed a significantly higher compliance.

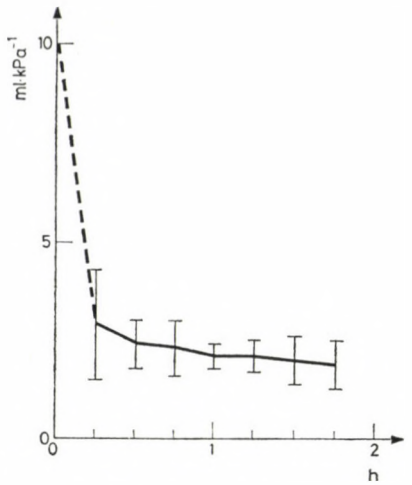

Fig. 1. Alteration of total compliance during artificial ventilation adapted to spontaneous breathing pattern. $V_T = 2$ ml, $f = 70 \cdot min^{-1}$. Means and standard error

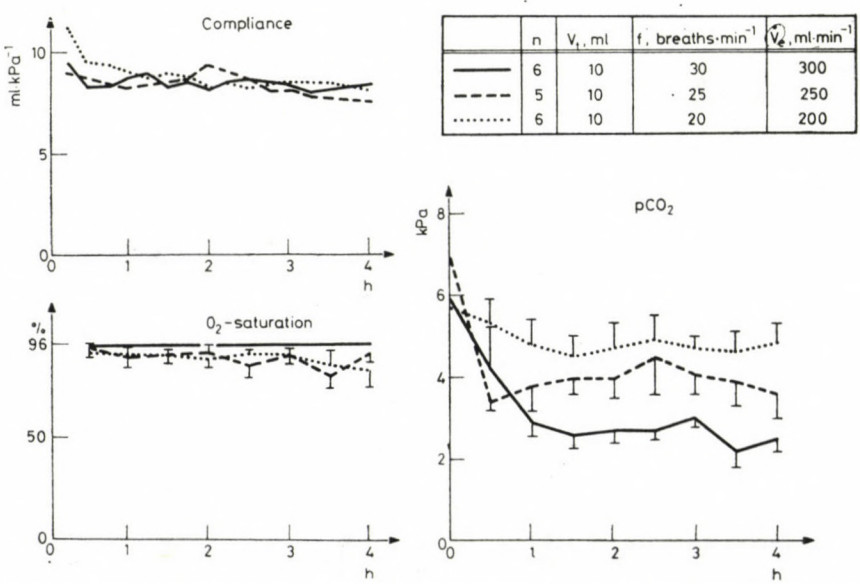

Fig. 2. Influence of rate and depth of ventilation on compliance (2a), oxygen saturation (2b) and CO_2-elimination (2c) at various time intervals after onset of artificial ventilation. Means and standard error. The standard error is not calculated for the 3 experiments at $V_T = 2$ ml

In Figure 2b the development of oxygen saturation is shown. Complete oxygen saturation during the entire ventilation period was found to require a V_T of 10 ml. At the end of a ventilation period of 4 hours at 6 ml V_T an arterial oxygen saturation of 90% seemed to be satisfactory. In contrast dramatic hypoxaemia resulted from a ventilation at 2 ml V_T.

The pCO_2-alteration is demonstrated in Figure 20. Only when using the smallest V_T of 2 ml developed a global insufficiency of lung function with severe hypercapnia, caused by the complicance disorder and by the large dead-space volume.

On the other hand, there was no considerable difference between ventilation at 6 or 10 ml V_T. With both volumes, a remarkable hypocapnia with pCO_2-values between 2 and 4 kPa occurred.

Series III. Effect of various minute volumes

In all experiments at V_T of 10 ml we observed a stable compliance (Figure 3a) which was independent of f. Oxygen saturation (Figure 3b) showed good results in all three experimental groups although a complete oxygen saturation occurred only with 300 ml·min^{-1}. CO_2-elimination showed, however, a clear dependence on the $\dot{V}_e$ (Figure 3c). While a $\dot{V}_e$ of 300 ml was accompanied by pCO_2-values between 2 and 3 kPa, and a $\dot{V}_e$ of 200 ml by values between 3 and 4 kPa, a $\dot{V}_e$ of 150 ml resulted in normocapnia.

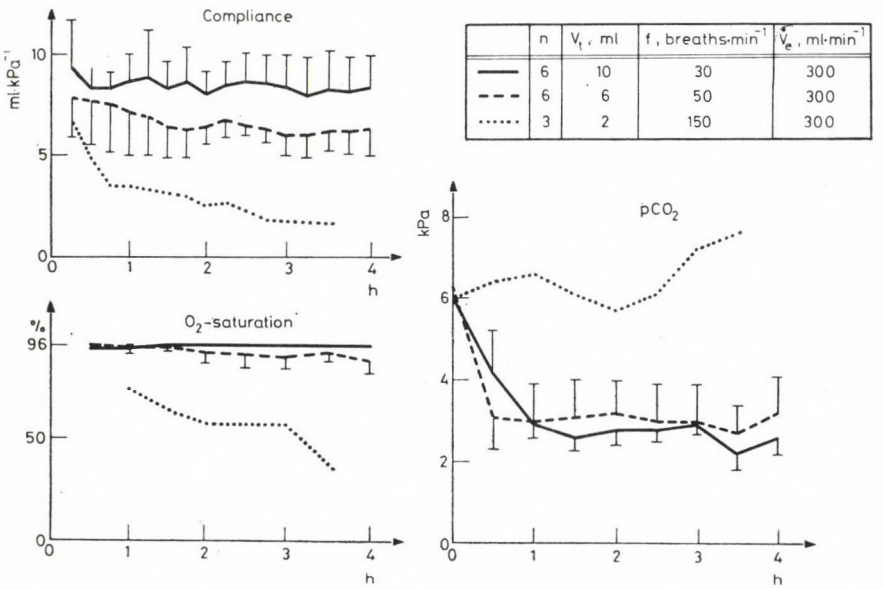

Fig. 3. Influence of V_e on compliance (3a), oxygen saturation (3b) and CO_2-elimination (3c) at various time intervals after onset of artificial ventilation. Means and standard error. In the diagrams, the standard error was neglected

Thus, we found optimum parameters for artificial ventilation of adult guinea pigs at a V_T of 10 ml and an f of 20 breaths per minute. These parameters ensured good O_2-saturation, normocapnia, normal acid-base balance, and stable compliance. Comparing these exprimentally obtained parameters with the spontaneous breathing pattern of guinea pigs, the similarity of minute volumes was remarkable. The essential difference consisted in a five-fold larger V_T. Such a low frequency ventilation was recommended by several authors for humans, too [7, 8]. In guinea pig, however, only a duplication of V_T was necessary. Thus, atelectasis and hypoxaemia occurring under artificial ventilation at physiological parameters, may be prevented by increasing the V_T [2, 13].

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A NEW CONVENTIONAL AND HIGH-FREQUENCY VENTILATOR FOR SMALL ANIMALS

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A pressure limited, time controlled ventilator has been designed especially for studies on experimental animals with severe respiratory distress syndrome (SRDS). Inspiration: Expiration (I : E) ratio (1 : 99—99 : 1) and frequency can be changed independently. Frequency ranged from 1 to 199/min in conventional ventilation (CV), while in high-frequency jet ventilation (HFJV) from 1 to 30 Hz. The gas delivery system consists of 3 magnetic valves (inspiration, expiration and HFJV, respectively) to ensure superposition of CV with HFJ or to use them separately. A monitoring unit switches off inspiration gas sources during HFJV if intratracheal pressure exceeds the alarm threshold. The device has been used in the following animal models: premature newborn rabbits with surfactant deficient lungs, emphysematous rats and guinea pigs as well as dogs and rabbits with SRDS due to lung lavage. Ventilation was most effective with an I : E ratio of 4 : 1 during pressure controlled CV, whereas during HFJV optimum gas exchange could be maintained with an I : E ratio of 1 : 4 and a frequency of 15 Hz in beagle dogs and 10 Hz in rabbits, respectively.

Keywords: high-frequency jet ventilation, pressure controlled conventional ventilation, animal experiments

For the standardization of gas exchange during lung investigations we had to find a solution for artificial ventilation of animals between mice and dogs in size, but we knew only few companies who marketed volume controlled respirators for small animals. As we were familiar with some advantages of pressure controlled, time cycled ventilation [4, 5] we decided to develop not an electromechanically or a pneumatically working but an electronically controlled unit for magnetic valves. It had to meet two additional demands, *viz.* it had to be suitable for conventional mechanical (CMV) as well as for high-frequency jet ventilation (HFJV), and that the inspiration/expiration ratio should be changeable independently from any desired frequency in a wide range.

Methods

The main parts of the device are the keyboard, the timers for CMV as well as for HFJV, the power amplifiers for the magnetic valves and the pressure alarm unit (Fig. 1).

Every time runs independently from the others and can be controlled separately by the keyboard on the front panel. For CMV, frequency ranged from 1 to 199/min and for HFJV

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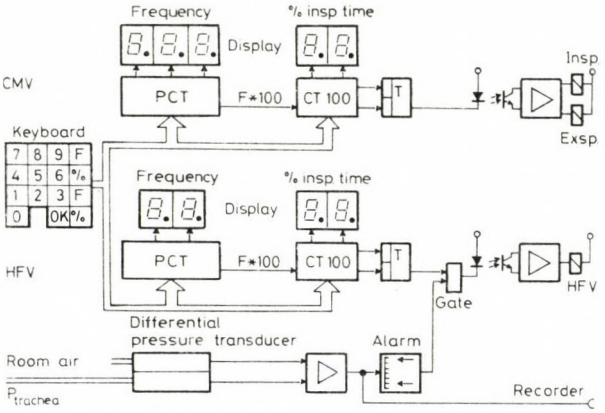

Fig. 1. Block circuit diagram of the electronical control unit: CMV = conventional mechanical ventilation; HFV = high-frequency ventilation; PCT, CT 100 = programmable timer for frequency and inspiration time ⊓⊔ = magnetic valves switching the gases to and from the animals

from 1 to 99 Hz (i.e. 60 to 6000/min). Now we use a maximum of 30 Hz on account of the upper frequency limit of the magnetic valves used for periodically interrupting the high pressure gas source during HFJV. The timers for the intervals of inspiration time are identical and able to produce I : E ratios from 1 : 99 till 99 : 1. The magnetic valves switching the gas to and from the animal during CMV are controlled inversely, that means only one of them is left open during inspiration or expiration while the other one is closed. The central part of the pressure-alarm unit consists of a differential pressure transducer, part of the chamber is left open to the environment, the other one is normally used for pressure monitoring in the endotracheal tube or in the airways with an air-filled catheter.

If pressure oscillations during HFJV exceed an upper alarm threshold the high pressure inspiratory gas source is interrupted in order to protect the airways and lungs from barotrauma.

The device has been used in several experiments with different aims.

Rats with papain induced emphysema were ventilated in a tank respirator (Fig. 2). The negative pressure in the tank during inspiration was generated via inspiration valve MV-I by a suction pump. Expiration takes place passively switching the tank via the expiration valve MV-E to the environment. The oxygen tension of inspired and expired gas was measured by a micro Clark electrode with a 6 μm teflon membrane. Thus, the response time of the electrode was lowered to less than one second. Breathing frequency was set to 16/min and inspiration time to 40%.

Results and discussion

In the first example (Fig. 3) variances between the individuals in the groups should be reduced by artificial ventilation to detect even small changes of ventilation inhomogeneity. Multiple breath oxygen washin and washout tests were performed to calculate parameters for inhomogeneity of lung ventilation. For comparison with untreated controls, driving pressure, frequency and I/E ratio were kept constant in this study. In the next example it was tested whether adequate ventilation could be ensured with pressure controlled CMV in very small animals with severe infantile respiratory distress

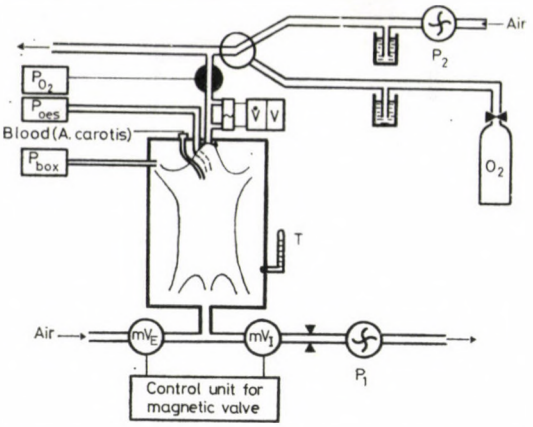

Fig. 2. Experimental setup for rats during artificial ventilation in a tank respirator (with permission from (4)) P_1, P_2 = pumps; O_2 = oxygen supply T = temperature; $\dot{V}/V$ = pneumotachograph; P_{O_2} = Oxygen analyser; P_{oe} = oesophageal pressure; P_{box} = negative driving pressure; MV_I, MV_E = magnetic valves for inspiration and expiration; control unit = frequency 1 — 199/min, I : E ratio 1 : 9—9 : 1

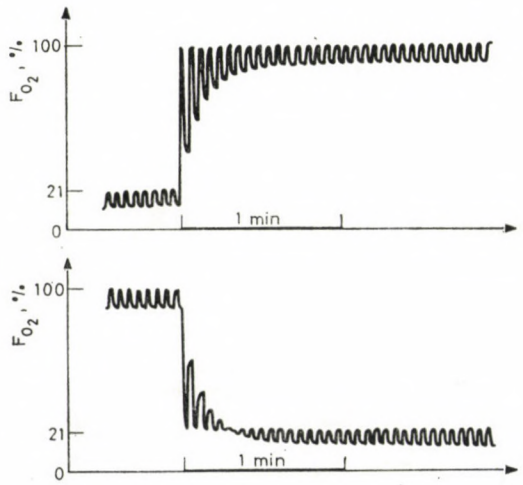

Fig. 3. Oxygen washin (upper) and washout (lower) recordings for calculation of parameters of ventilation inhomogeneity (with permission of (4))

syndrome (IRDS). We investigated premature newborn rabbits delivered on day 27 of gestation with an IRDS due to surfactant deficit (Fig. 4).

The fetuses were tracheostomized, paralyzed and placed into a multi chamber constant pressure bodyplethysmograph. Driving pressure was adjusted to about 3 kPa and measured between the magnetic valves MVI and MVE for inspiration and expiration. Flow and volume were obtained from each animal one after another. If desired, positive endexpiratory pressure (PEEP) could be added.

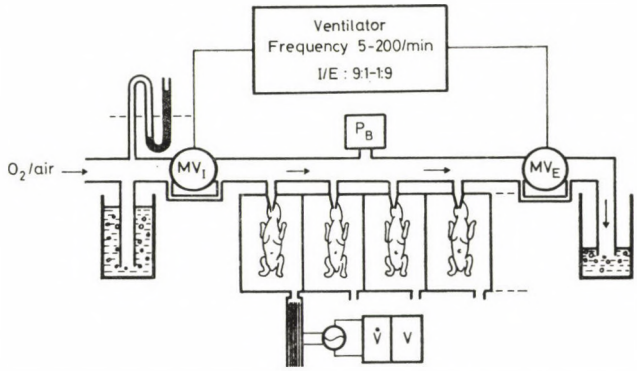

Fig. 4. **Multichamber constant volume body plethysmograph and ventilator setup for newborn rabbits (modified from (5))**

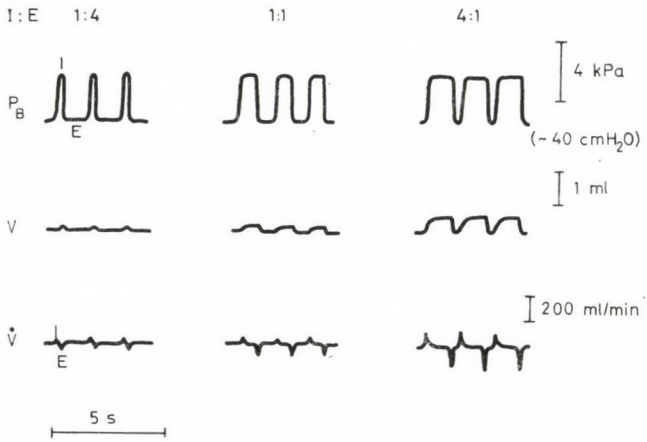

Fig. 5. **Recordings of driving pressure (P_B), flow ($\dot{V}$) and volume (V) obtained from a premature newborn rabbit ventilated with a constant driving pressure of 3.5 kPa, a frequency of 40/min, and different inspiration:expiration ratios (I : E)**

Under constant driving pressure and at a frequency of 40/min, the influence of inspiration time on lung ventilation was studied (Fig. 5). At 1 : 4 I : E-ratio only very low ventilation appeared. With the often used 1 : 1 E-ratio an adequate ventilation could not yet be obtained. But if inspiration time was prolonged to 80%, that means an I : E-ratio of 4 : 1, immediately after the inspiration the highest tidal volume V was obtained and remained on a volume plateau during further inspiration. This is caused by the shortened expiration that did not let expiratory flow decrease to zero so that the surfactant deficient lungs could not collapse. These few examples with small animals may point out the benefit of pressure-controlled, time-cycled CMV especially during treatment of severe respiratory disorders by artificial ventilation.

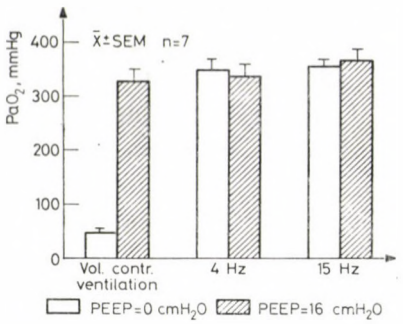

Fig. 6. Arterial oxygen tension (PaO_2) in seven beagle dogs after lung lavage (3), ventilated with and without positive endexpiratory pressure (PEEP) by volume controlled ventilation and high-frequency jet ventilation at 4 and 15 Hz. Note that during volume controlled ventilation without PEEP no adequate oxygenation could be obtained

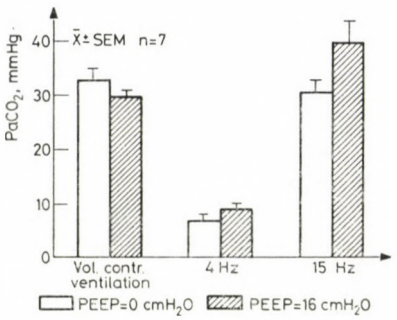

Fig. 7. Arterial carbon dioxide tension during the study shown in Fig. 6. With high-frequency jet ventilation, hyperventilation (at 4 Hz), or normoventilation (at 15 Hz) could be obtained

In order to answer the question whether HFJV was suitable for maintaining an optimum gas exchange even in adult animals with strong respiratory disorders, it was tested in comparison with conventional ventilation.

Seven beagle dogs with experimentally induced adult respiratory distress syndrome (ARDS) were used (Fig. 6). ARDS was obtained by lung lavage [3]. The surfactant system of the lung was severely damaged so that volume controlled ventilation without PEEP could not maintain adequate oxygenation, whereas HFJV or volume controlled ventilation with 16 cm H_2O PEEP maintained the PaO_2 at a level of more then 320 mm Hg (Fig. 6).

We could obtain any desired CO_2 clearance during HFJV, depending only on the frequency used. We found a nearly linear relationship between frequency and arterial CO_2-tension at constant driving pressure and ventilation. The blood gas values were reproducible for hours so that we could easily obtain hypo-, normo- or hyperventilation (Fig. 7).

One of the most delicate problems in treating ARDS by conventional ventilation is the protection from barotrauma. Many authors reported about a reduction of intrapulmonary pressures during HFJV [1, 2, 6, 7, 8]. In the last example we want to demonstrate the influence of frequency on pressure amplitude in the airways and on gas exchange in rabbits.

During HFJV of adult rabbits with the same lung lavage induced ARDS, at constant inspiration time of 20%, the influence of frequency was studied on intrapulmonary pressure and gas exchange (Fig. 8). Because of

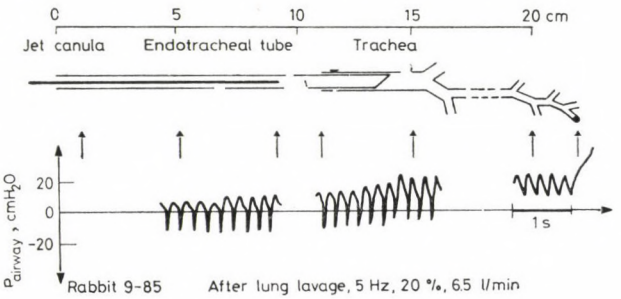

Fig. 8. Pressure oscillations in the tracheal tube and in the airways during high-frequency jet ventilation, measured by a Millar Micro-Tip Catheter Pressure Transducer PC 340. Arrows indicate fixed locations for pressure monitoring; the wedge position inside the bronchial tree is marked by a closed circle (right part of Figure). Pressure oscillations 2 cm before represent peak- and mean airway pressure and AUTO-PEEP (see text)

the smaller size of airway diameters, pressure oscillations in the tracheal tube and in the airways were measured by a Millar-Micro-Tip-Pressure-Transducer. Caused by additional entrained air, negative peaks on the pressure appeared up to —15 cm H_2O proximal of the tip of the jet cannula. Within 2 till 4 cm in front of the tip of the jet cannula these negative peaks disappeared and more and more positive oscillations arose until the tip of the pressure transducer had reached the wedge-position. The highest oscillations 2 cm before the wedge-position were taken as reference values for the whole lung. The remaining positive pressure at the end of any expiration is called "AUTO PEEP" because it is a self controlled PEEP between in- and expiratory gas stream and lung mechanic parameters.

At frequencies ranging from 3 to 20 Hz, AUTO PEEP and peak-pressures were estimated keeping mean airway pressure between 13 and 16 cm H_2O nearly constant (Fig. 9). Two results shown in Fig. 9 are remarkable: peak airway pressures and the amplitude of the pressure oscillations decreased drastically with increasing frequency. We therefore concluded that lungs jet ventilated with higher frequencies are better protected from lung injuries by barotrauma. The influence of increasing AUTO PEEP on oxygenation is

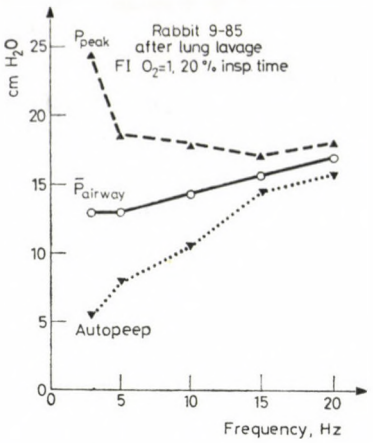

Fig. 9. Frequency dependence of peak- and mean airway pressure and AUTO-PEEP during high-frequency jet ventilation of a rabbit with severe respiratory distress syndrome. Mean airway pressure was kept nearly constant

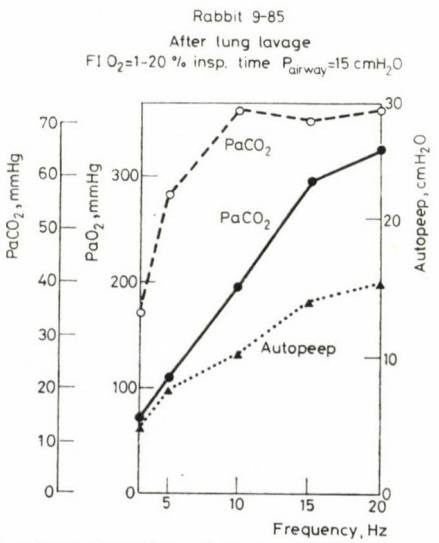

Fig. 10. Blood gases and AUTO-PEEP level during high-frequency ventilation in the same animal as in Fig. 9

shown in Figure 10. Although the animals were ventilated with pure oxygen, the PaO_2 decreased clearly at lower frequencies. We interpreted this results as an influence of AUTO PEEP in ARDS lungs. Because of the loss of surfactant, the lungs became unstable and collapsed during expiration at low AUTO PEEP-levels. At low frequencies even high peak pressures were not able to recruit such areas. We again found a linear relationship between frequency and CO_2 washout.

Conclusions

If investigators use animals with healthy lungs, only to maintain sufficient gas exchange, every kind of artificial ventilation is useful. If cardiovascular studies are to be done or experiments on injured lungs are to be performed, the influence of pressure controlled ventilation with long inspiration time or HFJV is the lower the smaller the compliance of the lungs.

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LUNG MECHANICS AND BLOOD GASES IN PREGNANT GUINEA PIGS

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Lung resistance and compliance were measured in early pregnant, late pregnant and non pregnant guinea pigs. Lung resistance was increased, lung compliance was decreased in pregnancy.

A new finding was the increase of chest wall compliance in the course of pregnancy. In order to obtain a sufficient gas exchange during artificial ventilation, pregnant animals have to be ventilated with a higher frequency or a higher pressure than non pregnant controls.

Keywords: Lung and pregnancy, lung resistance. lung compliance, chest wall compliance, artificial ventilation.

After sheep the guinea pig is used most frequently in experimental perinatal research. The main reason for this are the likeness in structure and function of the placenta compared with the human placenta and the large size of the fetuses of this small species.

Sometimes a 100 g fetus is found in a 500 g mother, while the weight of a mature rat fetus is only 5 g. To get physiologic responses in the fetus the gas exchange has to be adequate and stable. In the anaesthetized animal these conditions are best attained by artificial ventilation. Measurements in human pregnant women showed that three important parameters influencing the gas exchange were changed,

1) increased impedance of the respiratory system,
2) increased metabolism of mother-fetus complex,
3) increased PO_2 and decreased PCO_2 to attain a high gradient between mother and fetus or vice versa, respectively.

If the conditions are similar in the pregnant guinea pig, one should choose different ventilatory patterns in pregnant than in non pregnant animals in order to achieve optimum gas exchange. Not only hypoventilation but also hyperventilation of the mother is dangerous for the fetus.

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Methods

The animals were 24 non pregnant control (499 ± 67 g), 14 early pregnant (530 ± 89 g), and 11 late pregnant (601 ± 93 g) guinea pigs.

First we measured lung mechanics and blood gases during spontaneous breathing in order to obtain basic values. After urethan anaesthesia (1.32 g/kg i.p.) we introduced a carotid catheter, a tracheal tube and a saline filled oesophageal catheter and placed the animals into a body box. 10 minutes after adaptation we measured flow and volume pneumotachographically and pressure during spontaneous breathing. 100 μl arterial blood as drawn for measuring pO_2 pCO_2 and pH.

Thoracic gas volume was determined by body plethysmography according to the technique of Palecek [4] in order to calculate specific resistance and compliance. Then the animals were paralyzed with pancuronium bromide (0.4 mg/kg i.p.) and ventilated in the body box used as a tank respirator with a negative pressure of 1 kPa and a frequency of 8, 16, 24, 32 and 40/minute, each frequency o for 6 minutes. At the beginning of each period, three sights were applied. During spontaneous breathing lung resistance and dynamic compliance were obtained according to the method of Neergaard and Wirz [1, 2]. During artificial ventilation we measured the "quasi static" lung, chest wall and total compliance at a frequency of 8/minute and determined the blood parameters at each frequency.

Results

As in the case of pregnant women, in pregnant guinea pigs ventilation was increased and PCO_2 was decreased at spontaneous breathing (Fig. 1, upper part). Because of the stability of frequency the ventilatory increase was realized exclusively by an increase of the tidal volume.

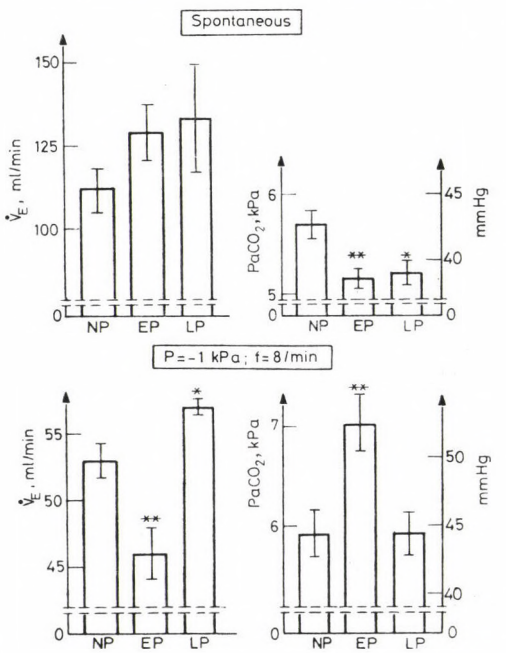

Fig. 1. Upper part: Mean and S. E. M. of ventilation ($\dot{V}_E$) and $PaCO_2$ in non-pregnant (NP) early pregnant (EP) and late pregnant (LP) guinea pigs during spontaneous breathing. Lower part: Mean and S. E. M. of $\dot{V}_F$ and $PaCO_2$ in NP, EP and LP guinea pigs during artificial ventilation

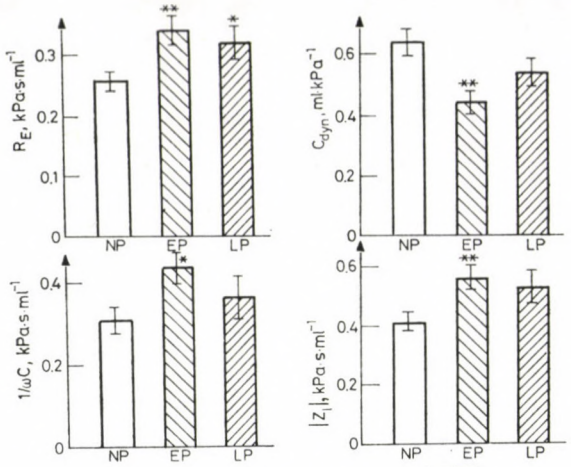

Fig. 2. Expiratory resistance (R_E), dynamic compliance (C_{dyn}), reactance ($1/\omega c$) and amount of lung impedance ($|Z_I|$) in non-pregnant (NP), early pregnant (EP) and late pregnant (LP) guinea pigs during spontaneous breathing (mean ± S.E.M.)

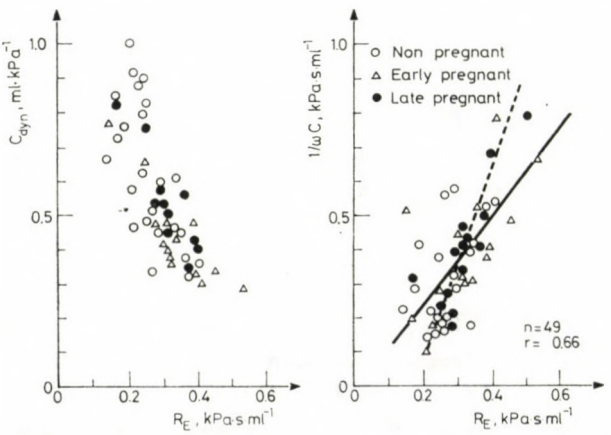

Fig. 3. Left: Relationship between expiratory resistance (R_E) and dynamic compliance (C_{dyn}) Right: Relationship between expiratory resistance (R_E) and reactance ($1/\omega c$)

Lung resistance was increased and dynamic compliance was decreased in both pregnant groups, more so in early pregnancy than in late pregnancy (Fig. 2). Logically the amount of lung impedance was also increased. There was a hyperbolic relationship between resistance and compliance and a linear relationship between resistance and reactance ($r = 0.66$, Fig. 3).

The thoracic gas volume was equal in the three groups, so that the relationships of specific resistance and compliance between the groups were the same. During artificial ventilation, lung compliance was decreased in pregnant animals whereas chest wall compliance was increased (Fig. 4).

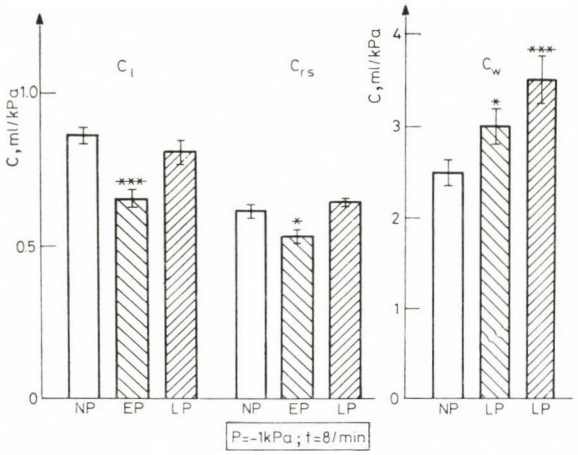

Fig. 4. "Quasi static" lung compliance (C_l) total compliance (C_{rs}) and chest wall compliance (C_w) in non-pregnant (NP), early pregnant (EP) and late pregnant (LP) guinea pigs during artificial ventilation

The total compliance of the respiratory system was decreased in early pregnancy and increased slightly in late pregnancy. If we use the same ventilatory pattern (P = —1 kPa, f = 8/min) then an increased PCO_2 results in early pregnancy, whereas the PCO_2 in the late pregnant group is comparable with that of the controls (Fig. 1, lower part).

Discussion

At a frequency of 8/min, ventilation is too low in pregnant animals, since pregnant subjects demand physiologically a low PCO_2.

On the basis of PCO_2 and PO_2 values at increasing frequencies and a given ventilatory pressure of —1 kPa (Table 1) we propose the following

Table I

PaO_2 *and* $PaCO_2$ *values* (mean ± S.E-; kPa) *in non pregnant* (n = 24), *early pregnant* (n = 14) *and late pregnant* (n = 11) *guinea pigs during spontaneous breathing and during artificial ventilation* (pressure —1 kPa) *at different frequencies*

	Non pregnant		Early pregnant		Late pregnant	
	PaO_2	$PaCO_2$	PaO_2	$PaCO_2$	PaO_2	$PaCO_2$
Spontaneous	9.69±0.85	5.69±0.65	9.67±1.19	5.15±0.40	9.87±1.01	5.20±0.39
8/min	8.97±1.57	5.93±1.11	7.65±1.75	7.03±0.97	8.65±1.77	5.93±0.68
16/min	12.37±1.39	4.45±0.67	11.68±1.97	5.51±1.29	13.19±1.24	4.51±0.77
24/min	13.76±2.11	3.21±0.64	12.80±1.99	4.05±0.96	14.79±1.32	3.43±0.84
32/min	14.32±1.49	2.57±0.49	13.72±2.33	3.21±0.75	14.95±0.91	2.77±0.67
40/min	14.77±1.41	2.17±0.51	14.16±2.63	2.48±0.80	15.92±0.92	2.08±0.49

frequencies for artificial ventilation:

controls, 10/min,
early pregnancy, 20/min,
late pregnancy, 15/min.

In earlier experiments performed in healthy controls and during asthmatic attacks we found a strong hyperbolic relationship between resistance and compliance. In the present study it has been shown that this was valid also for pregnant guinea pigs. The change of lung mechanics in pregnancy concerns only the amount of impedance and not the phase angle or the time constant.

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EFFECT OF ENZYME INDUCER FLUMECINOLUM (ZIXORYN®) ON AMINOPHYLLINE METABOLISM IN RATS

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The effect of the enzyme inducer flumecinolum, m-trifluoromethyl-alpha-ethylbenzhydrol (Zixoryn®), on aminophylline metabolism was examined in rats. Aminophylline plasma levels were determined by HPLC. Aminophylline $T_{1/2}$ was 2.85 hours ($r = 0.9353$) in the pretreated group and 3.75 hours ($r = 0.9471$) in the untreated control group. Flumecinolum was found to accelerate the elimination of aminophylline and the effect became significant 3.92 hours after the administration of aminophylline.

Keywords: enzyme inducer flumecinolum, m-trifluoromethyl-alpha-ethyl-benzhydrol (Zixoryn®), aminophylline metabolism.

Aminophylline is of paramount importance in the therapy of bronchial asthma. It is metabolized in the liver where it is decomposed via demethylation and oxidation by the hepatic mixed-function oxidase system [4] (Figure 1). According to literary data, the plasma half-life of aminophylline could be reduced from 3.5 hours to 2.6 hours by phenobarbital and from 3.5 hours to 0.8 hours by 3-methylcholantrene [13]. Considering that the patients smoke, take sedatives (phenobarbital, meprobamate, diazepam) and drink alcoholic beverages, which all possess enzyme inducing effects [3, 8, 6], it seemed to be of interest to examine the effect of flumecinolum, m-trifluoromethyl-alpha-ethyl-benzhydrol (Zixoryn®), on aminophylline metabolism under experimental conditions.

Material and methods

Animal experiments

The experiments were carried out on Wistar rats of both sexes, 253.9 ± 21.8 g mean body weight. To develop enzyme induction, 0.50 g of flumecinolum, suspended in 1 ml of Triton X-100, was administered orally by a gastric tube in a dose of 100 mg/kg daily for 5 days (pretreated group). On the 5th day both the pretreated and the untreated control group

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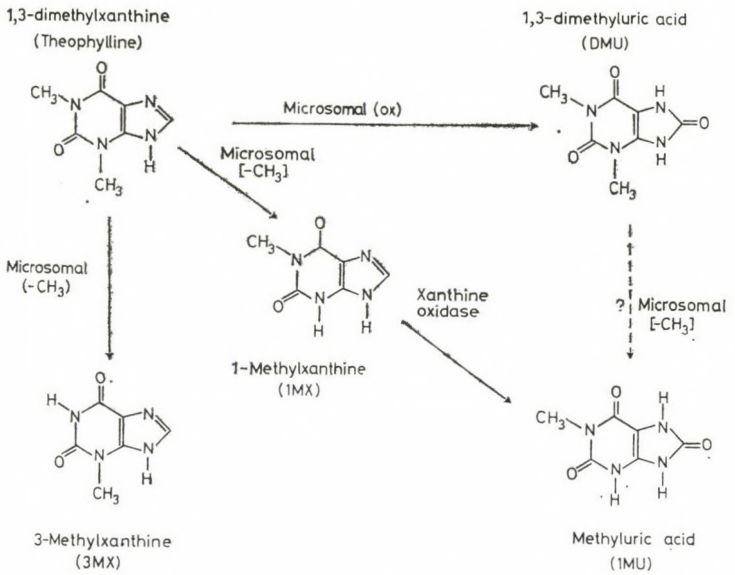

Fig. 1. Biotransformation of theophylline in man (Grygiel et al. (1))

received intraperitoneally 40 mg/kg aminophylline (Diaphyllin venosum 4.8% inj.®). Blood samples were collected 0.5—2—4—7—12 hours after the aminophylline-administration and the heparinized plasma samples were stored at —20 °C until HPLC measurements.

HPCL measurement

10 μl concentrated HCl was added to 240 μl plasma and the mixture was mixed. The protein-free plasma was extracted with 4.0 ml organic solvent (chloroform : isopropanol, 20 : 1) to which 5 μl 8-chloro-theophylline (internal standard) was added prior to extraction. The mixture was Vortex-mixed for 30 seconds then centrifuged at 2500 r.p.m. for 20 minutes. 2.0 ml of the organic phase was evaporated, the residue was dissolved in 125 μl absolute methanol and 20 μl was submitted to HPLC determination (11.). Device: Model 2010 HPLC Labor MIM, Budapest, Hungary; column: Lichrosorb® 7 RP-18; room tempearture; eluent: 10 parts acetonitrile, 10 parts methanol, 80 parts phosphate buffer, pH 4.85; flow rate: 1 ml/minute; detection at 273 nm; 0.2 A, chart speed: 1 cm/minute. The chromatographic procedure separated satisfactorily aminophylline from its relative compounds, i.e. caffeine, theobromine, 8-chloro-theophylline (Figure 2). This was achieved by altering the composition and pH of the eluent phase [11]. When the acetonitrile content was decreased under 10%, the elution sequence of the xanthine compounds remained unchanged but their retention times were prolonged resulting in more straddle of the peaks which decreased the accuracy of evaluation. When pH was changed, retention time of the components was the same except for 8-chloro-theophylline the retention time of which decreased rapidly on raising the pH, so its elution site could be adjusted almost at will. pH 4.85 was adopted as the peak of the internal standard followed that of aminophylline by about one minute. In the above conditions, retention times of the individual components relative to 8-chloro-theophylline were: theobromine 0.52, aminophylline 0.76, caffeine 1.37 (Fig. 2).

Evaluation

A calibration curve was plotted by measuring such a plasma series where the aminophylline concentration was standardized to 2.5—5—10—15—20 μg/ml. After elution from the chromatographic column, the retention times of aminophylline peaks were related to that of 8-chloro-theophylline and the quotients obtained were plotted against the previously adjusted standard plasma concentration of aminophylline (Fig. 3).

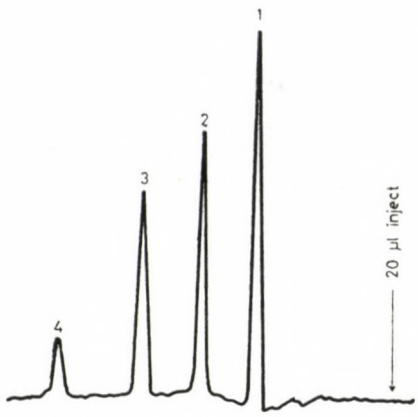

Fig. 2 HPLC separation of theobromine (1), theophylline (2), 8-chloro-theophylline internal standard (3), and caffeine (4). Equipment: Labor MIM Model 2010 HPLC, eluent: 10 parts acetonitrile, 10 parts methanol, 80 parts phosphate buffer, pH 4.85, total elution time 6 minutes

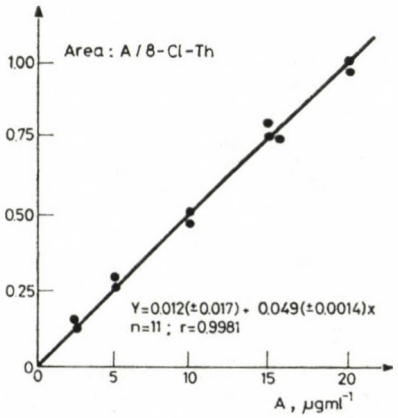

Fig. 3. Calibration curve. A = aminophylline, 8-ch-th = 8-chloro-theophylline, internal standard

Chemicals

All were of analytical garde
Aminophylline, theobromine and caffeine were bought from E. Merck, Darmstadt, GFR; 8-chloro-theophylline from Chinoin Budapest; Diaphyllin venosum 4.8% inj.®, flumecinolum from Chemical Works of Gedeon Richter Ltd. Budapest; chloroform from Fine Chemicals Co., Budapest; isopropanol, methanol, acetonitrile, Triton X-100 (alkyl-phenyl-polyethylene-glycol) from Reanal Budapest.

Statistical analysis

Log concentration vs. time regression curves were plotted for both the pretreated and the untreated control groups and between-run comparison were carried out by *t* test [7, 2, 5].

Results

The experiments were performed on 43 rats in the pretreated and 46 rats in the untreated control group. Results are shown in Figure 4. Comparison of the regression curves showed that, as the result of enzyme induction, aminophylline elimination was accelerated and the effect became significant 3.92 hours after its administration.

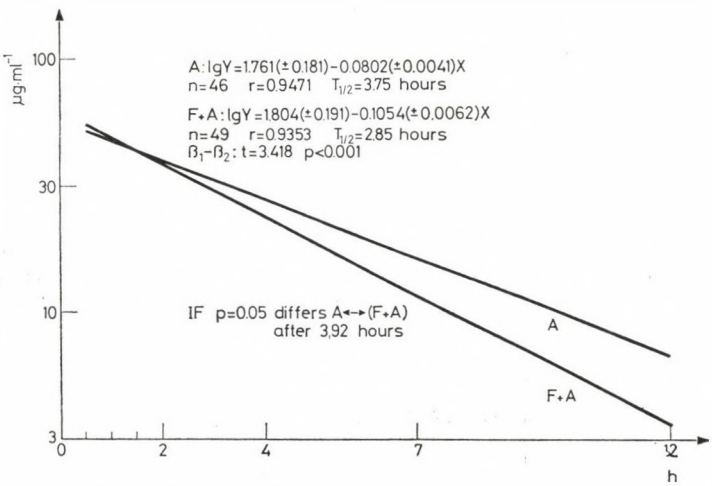

Fig. 4. Effect of enzyme inducer flumecinolum on aminophylline elimination in rats. F = flumecinolum, A = aminophylline. After 5-day pretreatment, elimination rate of aminophylline became significant 3.92 hours after its administration

Discussion

Flumecinolum stimulates the hepatic microsomal oxidase system, at an intensity equal to that of phenobarbital [10]. Flumecinolum is well-absorbed in the gastro-intestinal tract, its clearance also begins immediately but is rather slow, so it can be detected in the blood even after 96 hours. Its metabolites appear in 90—95% in the bile [10, 12]. Flumecinolum develops its effect in one hour for meprobamate, 1—6 hours for hexobarbital, but in 18 hours for other compounds (10), it enhances the elimination rate of drugs and there is no age-limit for its use. Thus, Korányi and Boross [9] reported its beneficial effect in neonatal jaundice.

It was concluded that under experimental conditions, flumecinolum accelerated the elimination of aminophylline. This may explain why aminophylline levels of patients comsuming enzyme inducer agents such as

cigarettes, drugs and alcoholoic beverages falls soon under the therapeutic range resulting in an increased frequency of asthmatic attacks in the morning. Due to narrow therapeutic margins of theophylline and its derivatives, an overdosage of these drugs occurs frequently in both children and adults. The treatment of acute theophylline intoxication consists of the prevention of further absorption : ipecacuanic vomitation, absorption with active charcoal, purgation with sodium sulphate. These methods leave untouched the elimination rate of absorbed theophylline. Based on the results presented, flumecinolum may be expected to prevent the unwanted side effects of methylxanthines in overdosage.

Acknowledgement

We thank assistant Mr. T. Nagy for drawing the figures. This work was supported by the grant "Bronchoconstrictor and bronchodilator prostanoid receptors" of the Hungarian Ministry of Health (Code: 02/-09-535/1981—85).

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GRAPHOMETRIC ANALYSIS OF COUGH TACHOGRAM

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The tachogram shows that the flow of cough is neither continuous nor steady. Within a cough, 5 different phases can be differentiated: 1) acceleration, 2) initial fast decleration, 3) intermediary decleration, 4) terminal fast decleration, and 5) the flow-pause. Only in the course of phases 1) and 3) is the air flowing out from the lungs. In cough series the peak-flows of subsequent coughs are growing smaller and smaller. This decrease follows the form of an isosceles hyperbola. A cough can be short or elongated. From the flow-phases the 1-st, 2-nd and 4-th ones are of fairly stable duration. The wide deviation of the intermediary deceleration shows that this phase makes it possible to elongate voluntarily the cough.

Keywords: cough tachogram, cough phases, intervals of cough phases, peak flow rates of cough.

Tachography is a method suitable for the registration of very high flow changes during very short periods of coughing. The curve arising this way, the tachogram (tg), is a complex periodical code-system, the formal characteristics of which can be described, measured, classified, evaluated, explained, and compared with our former data.

Materials and methods

The examinations were done by 5 colleagues of our laboratory. By them 58 bouts of cough and within these 426 single coughs were registered simultaneously on 2 equipments, the plethysmograph of Fenyves Co (Basel, Switzerland), and a polygraph type NEK-3 (Zwönitz, GDR). The latter is a direct drawing ECG-equipment, connected electrically to the plethysmograph. The x-axis was set on at 20 mm/s on the plethysmograph and at 100 mm/s on the polygraph. On the y-axis of the plethysmographic tg 10 mm amplitude meant a flow of 500 ml/s. One channel of the polygraph was set so that its 50 mm wide band could contain the highest amplitude of the first cough. Mathematical analysis was performed by calculating average values and deviations, furthermore by calculating the squared average differences. A graphomorphological description of the cough tg and its characteristics have been given in some of our former papers [5, 6].

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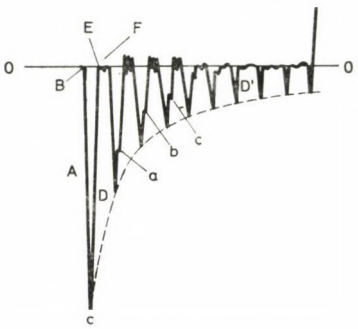

Fig. 1. Tachogram of a cough-series drawn by plethysmograph. Abbreviations: A = accelerating branch, B = onset of cough, C = peak-flow, D = unbroken deceleration phase of first cough, D' = unbroken decleration arising repeatedly at end of coughseries, E = end of deceleration, F = flow-pause between two coughs, a = break, b = plateau-formation, c = rebound on the decelerating branch. The line marked 0——0 is the baseline of flow and the dotted line connects the points of peak-flows

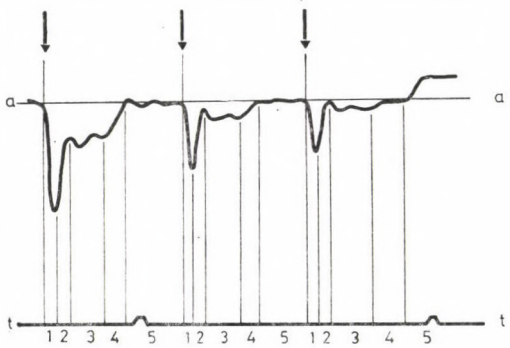

Fig. 2. Polygraphic registration of part of a cough-series. bl. = baseline, t = time. The perpendicular lines indicate the flow-phases on the tg, their numbers are given on basis of the 5 phases of cough. The arrows mark the onset of each cough

Results

On the tg of the cough-series (Fig. 1) each cough shows an accelerating and a decelerating branch. The first starts with an abrupt movement from the baseline of flow, making a sharp change of direction in a nearly perpendicular line. The other one, in contrast to the above, may show a great variety as it may display breaks, plateaus and rebounds. This branch becomes again an unbroken straight line by the end of the cough-series. The end of the decelerating branch is the point where it reaches the baseline of flow. From this point the curve oscillates by small waves on both sides of the baseline as far as to the onset of the accelerating branch of the next cough. The spiked point between the two branches of the tg corresponds to the cough's peak-flow.

These characteristics can be studied in detail on simultaneously gained polygraphic registrations (Fig. 2). On the basis of the above, a single cough

consists of 5 different phases, viz.: 1) acceleration, 2) initial fast deceleration, 3) intermediary deceleration, 4) terminal fast deceleration, and 5) the flow pause.

On the cough-series the tendency can clearly be seen that the size of the acceleration is decreasing. Inspecting the tg of a bout of coughs as a time-flow curve, we have the impression that the peak-flow values can be found on the arc of an isosceles hyperbola (Fig. 1, dotted line). It is also visible that the peak-flows are occurring in almost equal periods. If we consider the peak values following one another in a full cough-series as single parts of the series, our presumption can be justified arithmetically. Let the value of the first cough's peak-flow be "c" and

$$\text{“}c_1, c_2, c_3, \ldots c_n\text{”}$$

the series of peak-flows within a cough-series. Our hypothesis now can be formulated as follows:

$$c_1 : c_i \approx 1 : i \qquad (i = 2, 3, \ldots, n),$$

in other words, the value of the second, the third, the n-th value of that of the first peak-flow. As a control, we have determined the squared average differences between "$c_1 : ci$" and the hypothetic "$1 : i$", i.e. the σ-value.

$$\sigma = \sqrt{\frac{1}{n-1} \cdot \sum_{i=2}^{n} \left(\frac{c_1}{c_i} - \frac{1}{i}\right)^2}.$$

It is seen that the squared average differences between the hypothetic values (σ) do not reach 0.1 and vary by 0.05. On the basis of the above, the values of the peak flows of subsequent coughs in a cough-series correspond with good approximation to

$$c, \frac{c}{2}, \frac{c}{3}, \ldots, \frac{c}{n},$$

i.e. the peak flow values are found on the arc of an isosceles hyperbola.

On polygraphic registrations we measured the duration of full single coughs, and within this that of the flow phases of a cough. Taking into consideration the average values and their standard deviations, the average duration of each cough is fairly stable. From the point of view of the flow phases, the duration of the first, the second and of the fourth one is the most stable. Reproducibility is very good in the 1-st and 2-nd phases, and it is good in the 4-th one. The duration of the intermediary deceleration cannot be measured separately but in the first two cough-types, and while their average values are rather near, their deviations are fairly wide. The duration of the flow-pause could be measured one by one in the first 3 cough-types. The values fell between 0.22 and 0.27, but their standard deviations were also wide

Table I

Duration of the phases of the 4 cough-types and of the full coughs in s^{-2}, and their standard deviations

Types of cough flow		Duration of flow-phases, s^{-2}					Duration of a full cough
		1.	2.	3.	4.	5.	
Complete	$\bar{x}$	6.73	3.41	14.76	7.97	26.63	59.48
	SD	1.03	1.15	7.04	1.79	8.72	14.11
Quasicomplete	$\bar{x}$	3.71	3.94	15.19	5.77	25.56	54.17
	SD	0.68	0.45	8.77	2.22	10.77	11.11
Incomplete-I.	$\bar{x}$	3.04	3.64	19.80		21.68	48.16
	SD	0.28	0.44	3.38		6.44	4.12
Incomplete-II.	$\bar{x}$	3.15	3.59	39.67			46.41
	SD	0.43	0.48	11.31			8.42

(Table I). Valuable information can be obtained from the duration of full single coughs if the probands are considered. It could clearly be seen that the cough of one of the tested persons was elongated (Fig. 3). The histogram of the full time of a cough made on basis of the duration of the single coughs shows a bipartite distribution: the first peak is at 0.47 s, while the second one at 0.74 s (Fig. 4).

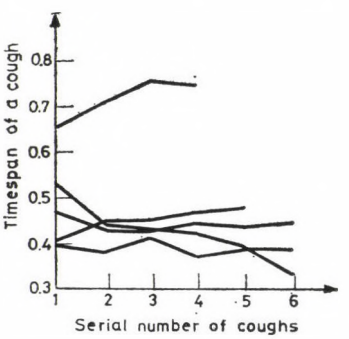

Fig. 3. Duration of the first sixs coughs. Each curve mean another proband

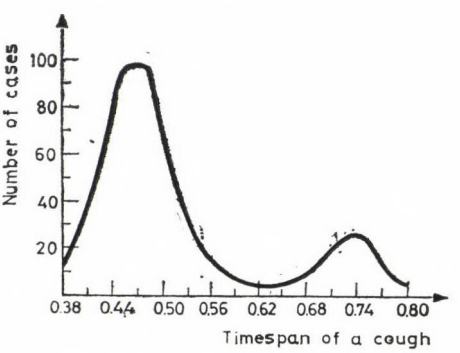

Fig. 4. Histogram of the full time of a cough

Discussion

We can only partially accept Odder's opinion [11] that if a cough has started it is already far beyond the voluntary control. This is supported only by the short and stable duration of the 1-st, 2-nd and 4-th phases, but the intermediary deceleration can voluntarily be elongated.

The role of the glottis has not been decided. Tomori [13] describes in detail the actions in the glottis arising during a cough. According to Widdicombe [14] that glottic closure is an integer part of the cough-reflex, but it is not necessary for it. We, on the other hand, could not show glottic closure only in complete and in incomplete-I. type (44.1%), while this could not be found in the quasi-com$_1$lete and incomplete-II types. According to Ross et al. [12] glottic closure occurs approximately 0.2 s earlier than the expulsion while we have measured a much shorter period, i.e. 0.02—0.03 s.

A particular form of the cough-flow on the tg is the peakflow, and its series. The figures given in various publications fall between wide limits, 3.3 and 11.2 l/s. These deviations originate from the fact that the authors did not deal with cough-series of identical number and under identical circumstances. In cough-series the peak-flows are showing a decreasing tendency. Langlands [9] stated that the decrease was "proportional". Our findings, on the other hand, verify that this decrease corresponds to the rule of an isosceles hyperbola. Berger and Nolte [1] have done another graphometrical analysis. In addition to the peak-flow they computed the average-flow ("zeitgemittelte Atemstromstärke") and the "flow-quotient". The peak flow is not a separate phase of the expulsion but it means merely the end of the acceleration, and is appearing some hundredth of a second earlier than the maximum of thoracic pressure [10]. Its size is volume-dependent, i.e. the larger the inspiratory volume at the starting of the cough, the larger the flow produced [2, 3]. The initial fast deceleration, called by Jones and Clarke [4] "post-peak phase", is the consequence of the fact that thoracic pressure is still increasing even after the peakflow [3, 9, 10] and by that the airways are compressed [4]. This dynamic compression on the tg corresponds to the initial fast deceleration. The post-peak collapse of the airways alters the flow: against the high flow of acceleration a lower one is arising. This is called by Langlands [9] "plateau flow-rate", and by Dayman [2] "critical flow". This flow-phase corresponds to the intermediary deceleration in our nomenclature. The terminal fast deceleration means that within a given thoracic air volume the coordinated expiratory muscular activity is capable of a given volume output and if this is done there is no possibility for further outflow although a certain part of the vital capacity has remained in the lungs.

Analysing the tussiphonograms, Korpáš and Korpášová-Sadloňová [7, 8] found that the duration of cough is 38% longer in 88% of the patients suffering

from bronchitis than in healthy subjects. On basis of our results we conceive the elongation of cough-time as follows. While from the two real flow-phases of a cough the time of acceleration is stable, that of the intermediary deceleration is less stable, moreover, it may even be five times longer, presumably the time of intermediary deceleration is elongated in patients suffering from chronic bronchitis and as such, the productivity of the cough is increased.

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DEVELOPMENT OF DRUG TACHYPHYLAXIS IN ASTHMATIC PATIENTS TREATED WITH BETA-ADRENERGIC DRUGS

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The development of drug tolerance in two groups of asthmatics treated with beta-adrenergic bronchodilators was studied by respiratory function methods. During one-year treatment with conventional therapeutic doses of selective beta-2-receptor stimulant aerosols, none of the patients showed subsensitivity to the bronchodilatory effect of terbutaline. Dose-response curves were plotted upon the inhalation of salbutamol in two-week intervals in patients treated with betamimetics. There was no decrease in the airways beta-adrenergic receptor function as compared to the untreated control group. The results show that prolonged treatment with therapeutic doses of inhaled beta-adrenergic bronchodilators does not result in drug tachyphylaxis, which is in accord with the clinical experience that there is no loss of effect of the preparations during their continuous administration.

Keywords: asthmatic patients, terbutaline, lung function tests, drug tolerance.

At the end of the sixties, in England, and later elsewhere there was a sudden increase in asthma mortality which coincided with the introduction of the beta-adrenergic bronchodilator aerosols [26].

Drug resistance, also referred to as tolerance, tachyphyalix, subsensitivity or refractoriness, appears as progressive, often prompt loss of the excitability of the cell by repeated exposition to beta-adrenergic catecholamines.

Numerous clinical studies have suggested that the excessive administration of beta-adrenergic bronchodilators may lead to the development of refractory state [16, 30]. Choo Kang described a reduction in the therapeutic effect of repeated salbutamol inhalation by asthmatic patients [4], whereas Patterson found a marked rebound effect, i.e. bronchoconstriction, in one fourth of the patients after prolonged isoproterenol infusion [23]. Nelson [21] and Jenne [15] experienced a decreased bronchodilatory effect after the permanent administration of oral beta-adrenergic agents. Similar observations were reported by Plummer [25] and Holgate [14]. Results of many in vitro

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experiments also agree with the aforementioned observations [5, 6, 7, 9, 11, 12, 18].

In contrast to these findings, Svedmyr [28] and Larsson [17] found no decrease in the effect of beta-agonist treatment after several years, arriving at the conclusion that beta-2-receptor stimulant bronchodilators preserve their therapeutic efficacy even after prolonged treatment. Results of Chervinsky and Bolinkoff [3], Brandon [2], Harvey and Tattersfield [13], Peel and Gibson [24] also failed to verify the development of drug resistance.

The reason of the conflicting results is not clear; the difference between the methods by which the receptor functions were studied, the severity of the asthmatic symptoms and the various treatment modalities might have played a role. The majority of asthmatic patients exhibit marked spontaneous airways caliber variations which may be related to climatic conditions, air pollution, upper airways infections, etc. These differences may mimic the development of drug tolerance. In tests performed in vitro it was shown that both in healthy and asthmatic individuals the degree of impairment in beta-adrenergic receptor function depended on the concentration of the beta-agonis applied [7] and the route of the administration [29]. In our present study we tried to answer whether the prolonged administration of conventional therapeutic doses of beta-adrenergic bronchodilatory aerosols modifies their bronchodilatory effect and whether a clinically significant tolerance develops against beta-agonist agents.

Materials and methods

Two groups of out-patients with clinically stable, perennial bronchial asthma were studied. The reversibility of their airways obstruction was tested by 0.4 mg of fenoterol which caused in each case an increase in FEV_1 higher than 20%.

Group I. Twelve patients (8 men, 4 women, aging 28—52, mean age 37 years). Their case history included asthma with a duration of 1 to 13 years.

Prior to examination all patients had been taking beta-adrenergic bronchodilatory aerosols and in addition, five patients had been given disodium chromoglycate (Intal, Fisons), whereas four patients had had inhalatory steroid treatment. In all patients, the treatment had included also theophylline preparations (tablets, suppositories). Duration of the observation was one year. Depending on their condition during this time they inhaled 1.5—2.5 mg (6—10 doses) of terbutaline (Bricanyl, EGIS) per day. There was no notable change in their accompanying medication. Respiratory function tests were performed on the first day and subsequently in the 3rd, 6th, 9th, and 12th month of the trial. Twelve hours prior to respiratory function tests, no terbutaline aerosol or other drugs were allowed to take, except for theophylline. At 8.00 a.m. a respiratory function test was carried out, which was repeated after 1/4, 1/2, 1, 2, 3 and 4 hours.

Group II. Sixteen patients (10 women, 6 men, aging 19—47, mean age 31 years) in the initial phase of bronchial asthma, not receiving any antiasthmatics, yet. The patients were divided into two groups for the duration of the 14 day long investigation period. Eight patients inhaled 4×0.2 mg of salbutamol (Salbutamol aerosol Polfa) per day, whereas 8 patients were not given beta adrenergic drugs but one tablet of Diaphyllin in case of dyspnoe. Dose response

curves were plotted with inhaled salbutamol on the first day, i.e. prior to beginning of the therapy, and thereafter on the 14th day of the trial. At 8.00 a.m., after a 12 hour period without any medication, the resting respiratory function tests were performed, then the patients inhaled 100, 200, 400 μg of salbutamol at 20 min intervals. Measurements were performed 20 min after the inhalations and then in every hour over a 4 hour period.

For both groups, the respiratory function tests were performed by a Medicor, Type SP 21 spirometer. FVC and FEV_1 were determined. Each measurement was done twice and the higher value was accepted. FEV_1 values were evaluated.

Statistical analysis: Within-run comparison was counted by unpaired *t* test, dose-response curves were plotted by regression analysis. Figure 4 shows the intercepts of the regression equations, the SD-values of the intercepts, regression coefficients, SD-values of regression coefficients and the correlation coefficients. Between-run regression coefficients were compared by the paired *t*-test.

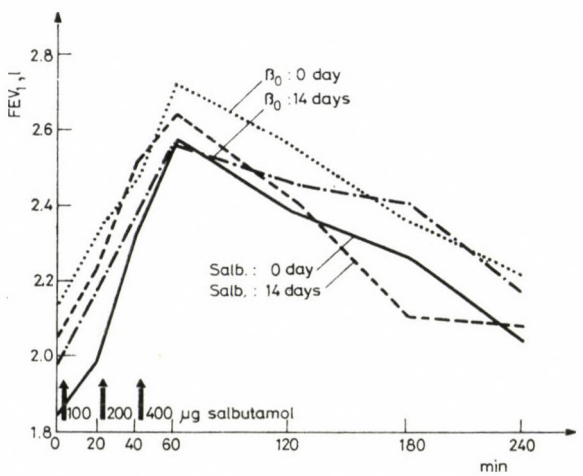

Fig. 1. Changes in FEV_1 values after the inhalation of 0.5 mg terbutaline by patients with asthma continuously treated with therapeutic doses of beta-adrenergic drugs

Results

In Group I, during the 12-month-treatment there was no decrease in the bronchodilatory effect of terbutaline. According to the respiratory function tests carried out at 3-month intervals, resting FEV_1 values increased by more than 20% in each case after inhaling 0.5 mg of terbutaline. Peak effect ensued 1 hour after the inhalation at the beginning of the study and in the 3rd and 6th month, whereas after 30 minutes in the 9th and 12th month (Fig. 1). These values measured prior to terbutaline inhalations and peak values representing the maximum bronchodilatory responses differed significantly at each examination time ($p < 0.001$). The former values started to decrease from the 3rd month on, while the peak values already from the beginning until the 9th

month, both values, however, still being higher than the respective initial values in the 12th month (Fig. 2).

In Group II, the effect of inhaled salbutamol on asthmatic patients treated and untreated with beta-adrenergic bronchodilator agents did not change significantly during the 14-day follow-up period, moreover, it rather increased at the end of the 2nd week (Fig. 3). In the dose-dependent changes of FEV_1 there was no significant difference ($p > 0.05$) between the two groups either on the first or on the 14th day (Fig. 4). There was also no significant difference between the FEV_1 values belonging to an instant salbutamol dose of the same group measured on the 1st and 14th day ($p > 0.05$).

Discussion

According to the results, prolonged treatment with conventional doses of inhaled beta-adrenergic bronchodilators does not result in the development of tolerance, which is in agreement with the clinical experience that there is no loss of therapeutic efficacy of these preparations, even during their continuous administration.

Several authors maintain that, while investigating tachyphylaxis, there is a need for an at least 2-week washing-out period to exclude a possible tolerance at the beginning of the trial [22]. According to Weber, in experiments where there was no wash-out period, subsensitivity could not be verified. As for his results, the subsensitivity developed within a few days after the beginning

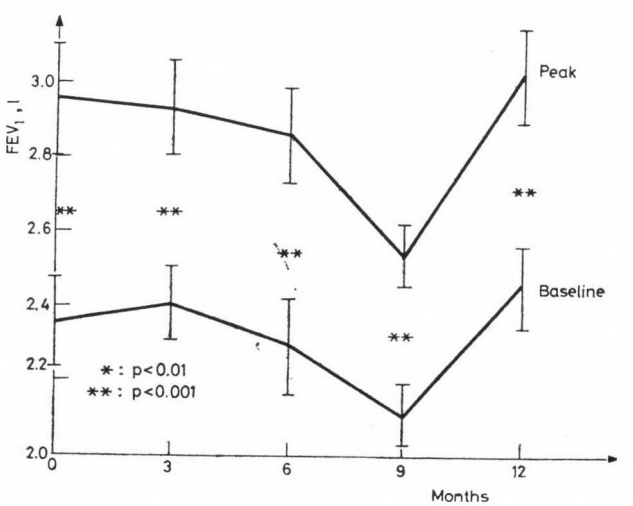

Fig. 2. FEV_1 baseline and peak values measured after the inhalation of 0.5 mg terbutaline in asthmatic patients continuously treated with conventional therapeutic doses of beta-adrenergic bronchodilators (mean values ± SD)

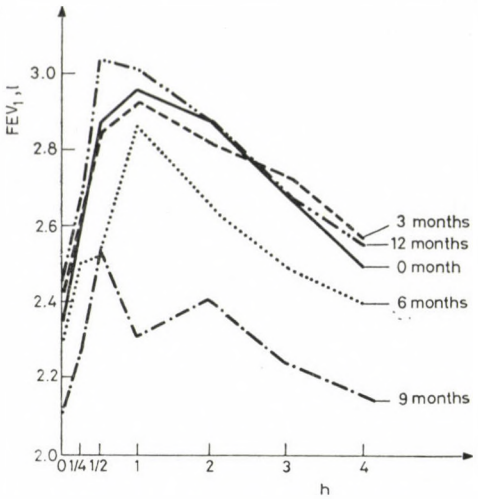

Fig. 3. Dose-dependent changes of FEV_1 values after inhaling 100, 200 and 400 μg of salbutamol, respectively, by asthmatic patients untreated with beta-adrenergic drugs (BO) and treated with conventional therapeutic doses of salbutamol (Salb.)

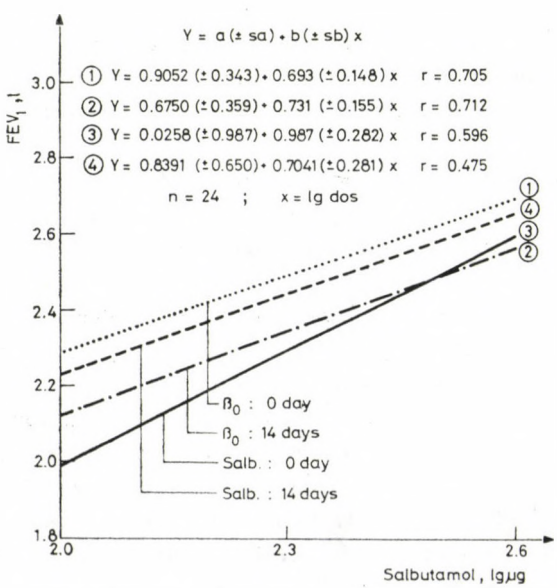

Fig. 4. Changes of FEV_1 values as the function of the logarithm of the administered salbutamol dose in asthmatic patients untreated with beta-adrenergic drugs (BO) and treated with conventional therapeutic doses of salbutamol (Salb.)

of beta-agonist treatment and persisted for at least 7 days after stopping the medication [31]. Nelson [19, 20] and Boney [1] showed that subsensitivity was not specific for certain beta-agonists, could be induced by any of the drugs and then demonstrated by another one in the same patient. Consequently, they proposed all kinds of beta-adrenergic bronchodilators to be withdrawn at least 2 weeks prior to the examination of tachyphylaxis. In contrast, Svedmyr [28] has suggested that the presence of a slight tolerance at the beginning of the examination is of rather academic importance if a control group is instituted and the follow-up period is satisfactorily long, i.e. at least one year.

The patients of Group II were given beta-adrenergic treatment for the first time in their life. At the end of the 2-week therapy, the development of tolerance was established by dose-response studies; the results showed that there was no fall in the bronchodilatory effect of the beta-adrenergic aerosol. The degree of bronchodilatation in response to salbutamol in the untreated asthmatic control group did not differ from that of the treated group. After prolonged beta-agonist pretreatment, the patients of Group I might have developed some subsensitivity at the beginning of the trial, however, at the time of the repeat test, i.e. after one year, the response of bronchial function to terbutaline did not change significantly.

There was a tendency in the basal FEV_1 values to decrease up to the 9^{th} month, the differences between the FEV_1 values measured at the start and in 9^{th} month being only 250 ml. Asthmatics show spontaneous variations in basal values for ventilation, probably as a result of prevailing climatic conditions, the variations in air pollution and, eventually, epidemics of upper respiratory tract infections. Such variations can mask the development of tolerance.

Similar results were reported by Peel and Gibson [24] who could not find significant changes either in the airways response or in the histamine sensitivity of the bronchi, after a 4-week-treatment with 4×200 μg of inhaled salbutamol.

Conolly, studying the development of drug tolerance by checking the airways response, experienced a decrease in the S_{gaw} value in the course of a continuous salbutamol treatment, which was attributed to the drug tachyphylaxis involving the central airways. However, the parameters characterizing mainly the small airways function ($MEF_{40\%}$) were not significantly affected. This phenomenon was explained by the effect of the beta-agonist agent being deposited in the central airways and reaching a high local concentration. On the other hand, during oral and parenteral administration, when the active substance is transported uniformly but in a lower concentration to the airways smooth muscle cells, no subsensitivity was found either in the central or in the peripheral airways [29].

In severe asthmatic attack, parenteral administration of a beta-adrenergic agent is of therapeutical benefit even if the patient previously inhaled large doses of betamimetics. This common clinical observation is explained by the assumption that the aerosol causes subsensitivity mainly in the central airways and leaves the sensitivity of the peripheral airways untouched [8].

Nelson [21] and Jenne [15], examining the clinical importance of beta-adrenergic bronchodilator subsensitivity, have concluded that the impairment of receptor function in these cases is partial rather than total. Doses of adrenergic bronchodilators used in the clinical practice is mostly limited by their side effects. Bronchodilatation obtained by the conventional therapeutic-doses does not represent the maximal bronchial response, i.e. the effect can be increased with the dose of the drug [10]. In the course of long-term treatment the side effects are fading [27]. These data make it probable that the loss of bronchodilatory effect, owing to the development of subsensitivity, can be reversed by increasing the dose [21].

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SERUM ANGIOTENSIN CONVERTING ENZYME VALUES IN CHRONIC OBSTRUCTIVE PULMONARY DISEASES

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Angiotensin converting enzyme (ACE) is stored in the endothelium. Its activity depends — among others — on the O_2-concentration of the blood. Aim of the study was to examine the serum ACE values in chronic obstructive lung diseases (bronchial asthma, chronic bronchitis, lung fibrosis etc.). At the time of blood sampling, blood-gas tensions and respiratory function parameters of the patients were also determined. On the basis of the blood-gas parameters and SACE $x + SD$ and $x - SD$ values, obtained from the normoxic-normocapnic group, the patients could be divided into sub-groups. In contrast to data in the literature increased enzyme levels in response to hypoxia could be found only in patients suffering from a pulmonary disease associated with severe tissue damage.

Keywords: Serum angiotensin converting enzyme, chronic obstructive pulmonary disease, blood-gases, acid-base balance, lung function parameters.

In each mammalian species so far studied the angiotensin converting enzyme (ACE), an exopeptidase bound to the membrane, proved to be one of the components of the luminar surface of the vascular endothelium [5]. It was also detected in the lungs [7], kidney [19], brain [24] and other organs, and ACE activity was found even in human alveolar macrophages [11]. ACE, also referred to as kininase (E. C. 3. 4. 15. 1) or dipeptidyl-carboxy-peptidase, is a part of the renin-angiotensin-aldosterone system. In the lungs, ACE converts biologically inactive angiotensin I to vasopressor angiotensin II [18] by removing a dipeptide, but also inactivates the potent vasodilator bradykinin. Many other peptides are also metabolized by ACE, such as Leu- and Met-enkephaline [8] and Substance P [25]. ACE activity appears also in the blood which is proportional — among others — to blood O_2-concentration; data in the literature shows that serum ACE activity is in good correlation with the enzyme level of the pulmonary tissue.

Liebermann was the first to report [14] that serum ACE (SACE) level of patients with active sarcoidosis is significantly higher than that found in healthy subjects. Since then many other reports have been published finding higher enzyme activity in Gaucher's disease [15], silicosis [10], asbestosis [10],

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while in certain other diseases, e.g. primary lung carcinoma or pneumonia [1] slightly decreased enzyme levels could be measured. SACE activity was also found to be lower in patients with bronchial asthma [22, 23] as compared to healthy individuals [14]. Similar changes were found in children with bronchial asthma [3].

The aim of the present paper was to study the relationship between the SACE-values and blood-gas and other respiratory function parameters in patients with chronic obstructive pulmonary disease.

Materials and methods

Fifty-three patients with chronic obstructive pulmonary disease were studied. Respiratory function tests and blood-gas tension determinations were carried out at the time of bood sampling. SACE values were determined by the method of Cushman and Cheung [6], using a hippuryl-histidyl-leucin (Calbiochem) substrate analogue. Fifty μl of serum was incubated in 0.1 M pH 8.3 phosphate buffer at 37 °C for one hour. The test solution contained 0.3 M NaCl and 50 mM hippuryl-histidil-leucin peptide. The blood samples with a total volume of 250 μl were kept in a Vibroterm water-bath continuous gentle shaking. The reaction was stopped by adding 250 μl of 1 M HCl; after 10 min the hippuric acid was extracted with 1.5 ml ethyl-acetate. The samples were separated by centrifugation at 3—4000 rpm for 20 minutes. One ml of the organic phase was evaporated at 100—120 °C in a Block-Term thermostate. After the evaporation of the organic solvent the residue was dissolved in 3 ml of distilled water and the extinctions were measured by a Spectromom 190 spectrophotometer (Hungarian Optical Works, MOM, Budapest, Hungary) at 228 nm. The concentration of the hippuric acid thus released was determined by the Cushman-Cheung equation using the calibration curve. The results were expressed in U/l. One unit of ACE activity is equal to one μM of hippuric acid released in one minute in 1 l of serum. Blood-gas tensions were determined by a Il Micro 413 blood-gas analyzer. Respiratory function parameters were determined by a Medicor MS-11 pneumotachograph. Blood-gas tensions were expressed in kPa, corresponding to the SI system.

Selection: The patients were selected on the basis of their blood-gas tensions. *Group I:* normoxic-normocapnic patients (O_2-tension $>$ 10 kPa, CO_2-tension between 4.6—6.0 kPa). *Group II:* hypoxic-normocapnic patients (O_2-tension $<$ 10 kPa, CO_2-tension between 4.6—6.0 kPa). *Group III:* hypoxic-hypercapnic patients (O_2-tension $<$ 10 kPa, CO_2-tension $>$ 6.0 kPa). Statistical analysis of the results was performed by the unpaired *t*-test.

Results

Results are shown in Table I and Fig. 1. The SACE-values for *Group I* where blood-gas parameters were within the physiological range amounted to 26.4 $\pm$ 8.3 U/l ($\bar{x} \pm 1$ SD), which was in good correlation with the results by Rømer [20] who found SACE-values of 24.4 $\pm$ 6.2 U/l in 116 healthy controls. Patients of *Group II* could be divided into two sub-groups, based on the upper limit $\bar{x} + 1$ SD obtained in normoxic-normocapnic patients: mean SACE-value for *Group IIa* was 39.2 which was higher than the upper limit of Rømer's normal range (12.0—36.8 U/l). Nine patients belonged to this sub-group who suffered from generalized bilateral pulmonary tuberculosis, lung fibrosis, etc. The diagnosis in 21 patients of *Group IIb* was mostly bronchial asthma and chronic bronchitis. Using the limit x—1 SD measured in normoxic-normocapnic patients, hypoxic-hypercapnic patients of *Group*

Table I

*SACE, blood gas and lung function parameters, (mean values ± SD), *deviation in % from normal values*

Group		n	m	fm	Blood-gas parameters ($\bar{x} \pm$ SD)			
					age (years)	pO_2 (kPa)	pCO_2 (kPa)	pH
1		11	6	5	51±17	10.1±0.6	4.6±0.5	7.40±0.05
2	2a	9	7	2	57±19	7.9±1.0	5.3±0.4	7.38±0.04
	2b	21	15	6	56±15	8.4±0.8	5.2±0.5	7.39±0.06
3	3a	4	3	1	70±8.0	7.6±1.6	6.9±1.0	7.36±0.02
	3b	14	3	11	55±13.6	6.7±1.5	8.0±1.0	7.38±0.04

Group		n	m	fm	Enzyme level ($\bar{x} \pm$ SD)	Lung function parameters*		
					SACE U/l	FVC (l)	$FEV_{1.0}$	MTT
1		11	6	5	26.4±8.3	24.4±15.2	50.4±40.0	59.3±40.3
2	2a	9	7	2	39.2±3.6	32.0±21.3	47.4±31.5	77.3±55.8
	2b	21	15	6	22.7±6.3	29.3±17.5	47.5±25.0	81.5±69.5
3	3a	4	3	1	14.5±1.0			
	3b	14	3	11	24.2±5.8			

m = male
fm = female

III could also be divided into two sub-groups. Enzyme activity was 14.5 ± 1.0 U/l in 4 patients in *Group IIIa*, which proved to be rather low, notwithstanding that it was in the normal range [20]. These patients had the highest mean age and the lowest blood pH values. Diagnosis in these cases was chronic bronchitis in two patients, emphysema and fibrosis in one patient each. Surprisingly, SACE-values of *Group IIIb* were almost equal to that of the normoxic-normocapnic patients, i.e. 24.2 ± 5.8 U/l in 14 patients.

Lung function parameters could not be determined in every group of patients, due to the old age (Group IIIa) and the poor general health. Significant difference between FVC- and FEV_1-values could not be detected in the normoxic-normocapnic and hypoxic-normocapnic patients. Significant differences ($p < 0.05$) were found, however, in the MTT-values between Groups I and IIa (see Fig. 2).

Discussion

The question how blood-O_2 tension affects the serum ACE level opened a Pandora's box in the literature. Gould and Goodman [9] and Mattioli et al. [16] reported the enzyme level-increasing effect of hypoxia. Árvay

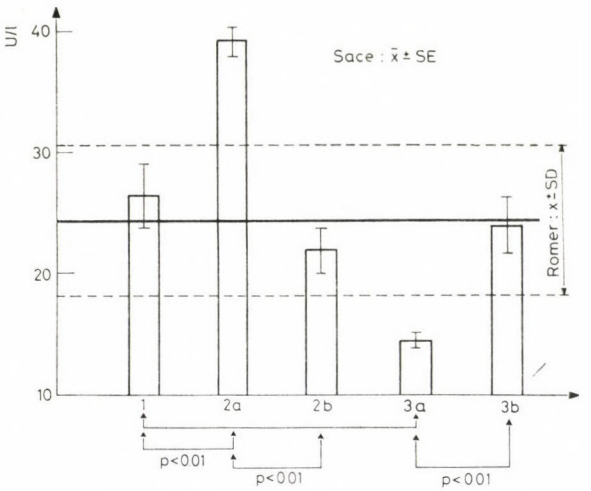

Fig. 1. SACE values (mean ± 1 SE) of patients with chronic obstructive lung disease. Groups shown in Table I

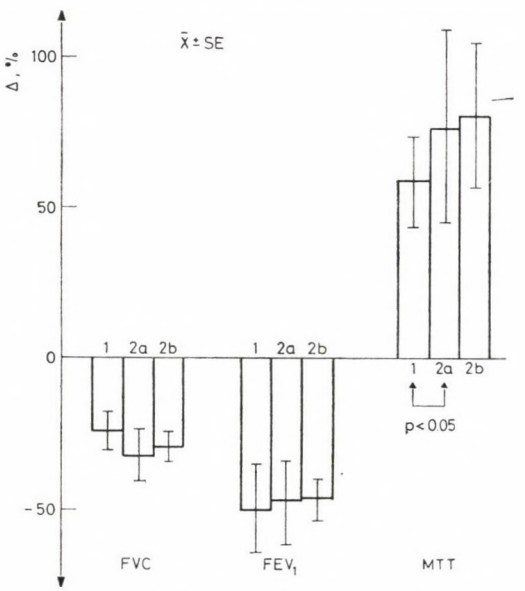

Fig. 2. Lung function parameters of patients with chronic obstructive lung disease. Data expressed in % of normal value. Abbreviations: FEV_1, Forced expiratory volume in the first minute FVC, Forced vital capacity MTT, Mean transit time

et al. [4] found that serum levels of the enzyme increased during physical exercise in healthy adults and children, whereas it decreased in patients with bronchial asthma [2]. Leuenberger et al. [12] found that in dogs acute hypoxia decreased the conversion of angiotensin I to angiotensin II which indicated

decreased enzyme activity. On the other hand, acute hyperoxia or changes in blood acid-base balance had no measurable effect on the conversion of angiotensin I in dogs [13]. Milledge [17] reported that hypoxia decreased ACE activity within 10 minutes in humans. Stalcup et al. [21] found that ACE activity could be inhibited by hypoxia in endothelial cell cultures, however, this result has not yet been supported by repeated experiments.

The present study revealed that SACE activity in patients with hypoxic-normocapnic pulmonary diseases with serious parenchymal damage increased as compared to patients with a primarily bronchial disease (chronic bronchitis, bronchial asthma) where SACE-values were normal. In the hypoxic-hypercapnic group — as shown by a few measurements in elderly patients — SACE activity decreased. This fall of activity could be explained, in addition to the impaired gas-exchange, by the severe pulmonary disease and/or the decreased metabolic activity associated with the elderly age. This assumption was supported by the finding that SACE activity of younger hypoxic-hypercapnic patients (Group IIIb) was normal.

Similar ventilation disturbances could be detected by the respiratory function tests in the normoxic-normocapnic and hypoxic-normocapnic groups (Groups I, IIa, IIb), whereas an increase in the enzyme activity was detected in patients suffering from any disease with severe pulmonary parenchymal damage.

By virtue of these results SACE activity is increased in chronic conditions associated with lung parenchymal damage, while enzyme activity may be reduced in the elderly and, finally, there was no relationship between the blood-gas tension parameters and enzyme levels.

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POWER SPECTRAL DENSITY OF BREATHING PATTERN IN PATIENTS WITH CHRONIC OBSTRUCTIVE LUNG DISEASE

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Newsom Davis and Stagg [4] stydying the interrelationship of the volume and time components of individual breaths in healthy resting man described a significant correlation between mean tidal volume (V_T) and inspiratory time (T_I) r = 0.704. The correlation between mean T_I and expiratory time (T_E) was lower, r = 0.381. Evaluation of these relationships and of the power spectral density of the breathing pattern was the aim of the present study. For breath by breath analysis we calculated power spectral density and cross correlations of V_T, T_I and T_E. We found a significant correlation between V_T and T_I in 9 patients with global respiratory insufficiency (RI) (mean r = 0.52) and 7 patients with partial RI (mean r = 0.56). The correlation between T_I, T_E was lower, in 9 patients with global RI (mean r = 0.21) and 7 patients with partial RI (mean r = 0.35). The results of boths groups did not differ from healthy subjects in power spectral density of the breathing pattern and in correlations of V_T and T_I as well as T_I and T_E.

Keywords: breathing pattern, power spectral density, chronic obstructive lung disease, respiratory insufficiency.

Study of the interrelationship of the volume and time components of individual breaths in healthy resting man revealed a significant correlation between tidal volume and inspiratory time (mean r = 0.704). The correlation between inspiratory time and expiratory time was lower (mean e = 0.381). Evaluation of these relationships in patients with chronic obstructive lung disease was the aim of the present study. To follow the slow rhythmical changes of the breathing pattern, autocorrelation functions, cross correlation functions and power spectral densities (O. —0.20 Hz) of inspiratory time, expiratory time and tidal volume were computed. We found a singificant correlation between tidal volume and inspiratory time in 9 patients with global respiratory insufficiency (mean r = 0.52) and 7 patients with partial respiratory insufficiency (mean r = 0.56). The correlations between inspiratory time and expiratory time were lower in 9 patients with global respiratory insufficiency (mean r = 0.21) and 7 patients with partial respiratory insufficiency (mean r = 0.35). The results in both groups did not differ from those of healthy

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subjects in the correlations between tidal volume and inspiratory time, and between inspiratory time and expiratory time as well as in power spectral densities of the breathing pattern.

In a previous study using the method of spectral analysis the rhythmicity of inspiratory time, expiratory time and tidal volume in healthy man was described [6, 7, 8]. The aim of the present study was to examine whether the rhythmical oscillations of the breathing pattern were altered in patients with chronic obstructive lung disease, and to determine the interrelationship of volume and time of individual breaths in our patients. Newsom, Davis and Stagg [4], studying the interrelation of volume and time components of individual breaths in healthy resting man described a significant correlation between tidal volume and inspiratory time (mean $r = 0.704$). The correlation between inspiratory time and expiratory time was lower (mean $r = 0.381$)

Materials and Methods

In 16 patients we examined pulmonary functions by spirometry, body plethysmography and the blood gases from arterialized capillary blood. For breath by breath analysis, 3 minutes lasting spirometric records were taken under resting conditions. Autocorrelation and cross-correlation functions of inspiratory time, expiratory time, inspiratory volume and expiratory volume were computed. The power spectral densities were calculated from the autocorrelation functions using the Hanning spectral window [1, 2].

Results

The patients with chronic obstructive lung disease were divided in 2 groups: 7 patients with chronic obstructive lung disease and with partial respiratory insufficiency (mean pO_2, 8.6 kPa), and 9 patients with chronic obstructive lung disease and global respiratory insufficiency (mean pO_2, 6.5 kPa). In both groups pulmonary functions had changed typically but the group with global respiratory insufficiency displayed much more pronounced obstructive signs than the group with partial respiratory insufficiency. In patients with global respiratory insufficiency the breathing pattern is markedly altered, minute ventilation is decreased (mean, 9.8 l), tidal volume is also decreased (mean, 468 ml), while breathing frequency is increased (mean, 22.6 cpm), and inspiratory time as well as expiratory time are shortened (mean 0.8 s and 1.9 s, respectively), in agreement with the finding of Sorli et al. [9] and Milic-Emili [3].

The autocorrelation functions of inspiratory time, expiratory time, inspiratory volume and expiratory volume show the occurrence of different frequencies in power spectra. Figure 2 indicates power spectral density of inspiratory time, expiratory time, inspiratory volume and expiratory volume in

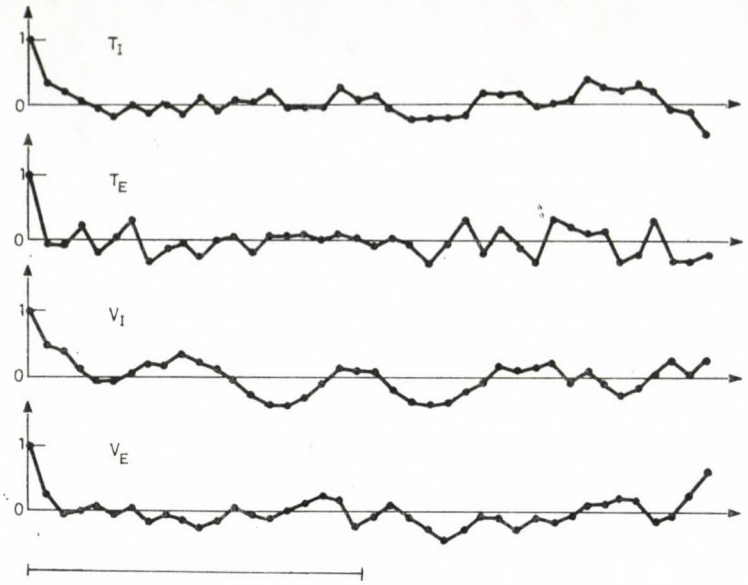

Fig. 1. Autocorrelation functions of inspiratory time (T_I), expiratory time (T_E), inspiratory volume (V_I) and expiratory volume (V_E) in patient L. A. with chronic obstructive lung disease and respiratory insufficiency. The abscissa shows the lag, the line is 60 sec. The ordinate shows the value of the autocorrelation coefficient which varied from +1 to −1. The autocorrelation functions show different periodical changesi n breathing pattern

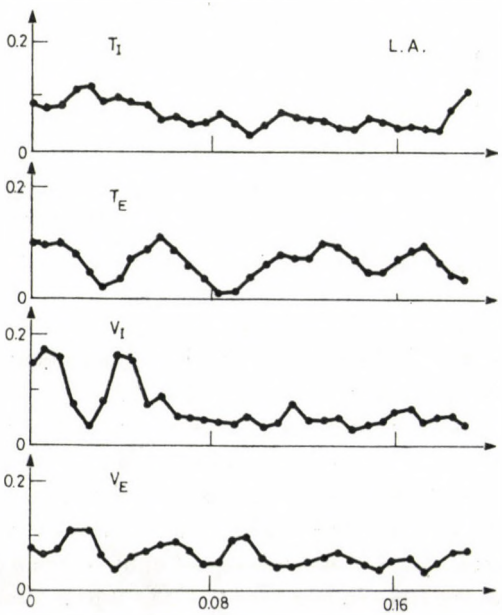

Fig. 2. Power spectral density of inspiratory time, expiratory time, inspiratory volume and expiratory volume in patient L. A. with chronic obstructive lung disease and global respiratory insufficiency. The abscissa shows the frequency in Hz, the ordinate the relative power. There are different frequency peaks in power spectra of inspiratory time, expiratory time, inspiratory volume and expiratory volume

patient L. A. with chronic obstructive lung disease and global respiratory insufficiency. There are, different frequency peaks in the power spectra of inspiratory time, expiratory time, inspiratory volume and expiratory volume.

Autocorrelation functions and power spectral densities of inspiratory time, expiratory time and tidal volume show different rhythmic changes in the same patient and they also differ among all patients.

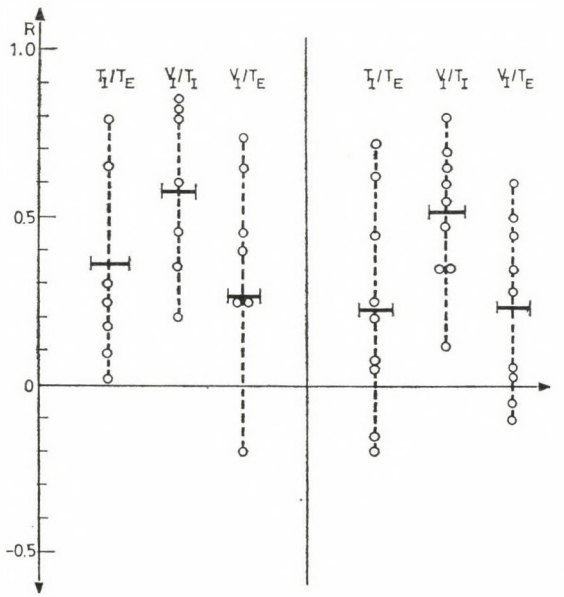

Fig. 3. Correlation coefficients between inspiratory time and expiratory time, between tidal volume and inspiratory time, between tidal volume and expiratory time. At left, in patients with partial respiratory insufficiency, at right, in patients with global respiratory insufficiency. The mean correlation coefficients are shown by horizontal lines

To study the correlations between tidal volume and time components of breaths we calculated correlation coefficients for inspiratory time and expiratory time, for tidal volume and inspiratory time, for tidal volume and expiratory time. Figure 3 shows these correlation coefficients in both groups of patients where on the left are the results of patients with partial respiratory insufficiency and on the right the values of patients with global respiratory insufficiency. We found significant correlations between tidal volume and inspiratory time (mean $r = 0.52$) in 9 patients with global respiratory insufficiency, and mean $r = 0.56$ in 7 patients with partial respiratory insufficiency. The correlations between inspiratory time and expiratory time were lower, mean $r = 0.21$ in 9 patients with global respiratory insufficiency and mean $r = 0.35$ in 7 patients with partial respiratory insufficiency.

We computed the cross-correlation functions of inspiratory time and expiratory time, of tidal volume and inspiratory time, of tidal volume and expiratory time. Figure 4 shows the findings in patient L. A. who had chronic obstructive lung disease and global respiratory insufficiency. There are marked positive deflections at zero line in cross-correlation functions between tidal

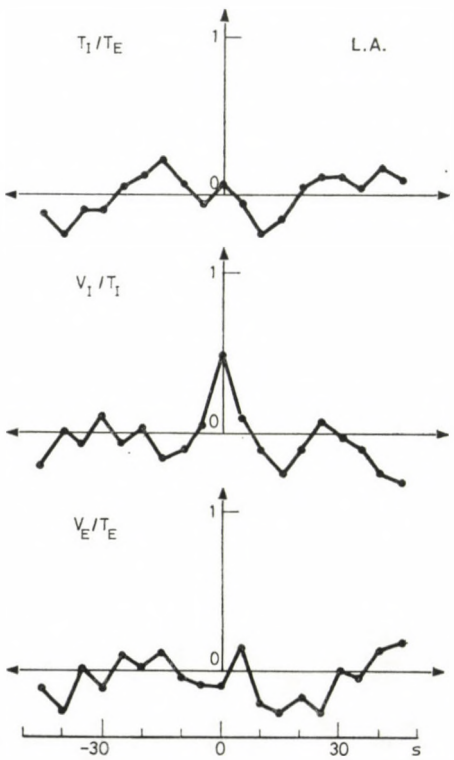

Fig. 4. Cross-correlation functions of inspiratory time and expiratory time, of tidal volume and inspiratory time, of tidal volume and expiratory time in patient L. A. with global respiratory insufficiency. On the abscissa the lag in seconds, on the ordinate the crosscorrelation coefficient. There is a significant correlation between tidal volume and inspiratory time

volume and inspiratory time, in contrast to cross-correlation functions between inspiratory time and expiratory time and between tidal volume and expiratory time. To compare the results of the patients we calculated the averaged cross-correlation functions of volume and time components of breaths for both groups. In Fig. 5 we can see the averaged cross-correlation functions between inspiratory time and expiratory time, between tidal volume and inspiratory time and between tidal volume and expiratory time in patients with partial respiratory insufficiency. The functions of tidal volume and inspiratory time

show a pronounced correlation between both components. In Fig. 6 we see the averaged cross-correlation functions between inspiratory time and expiratory time, between tidal volume and inspiratory time, between tidal volume and expiratory time in patients with global respiratory insufficiency. There are no differences between the two groups, so it could be concluded that the

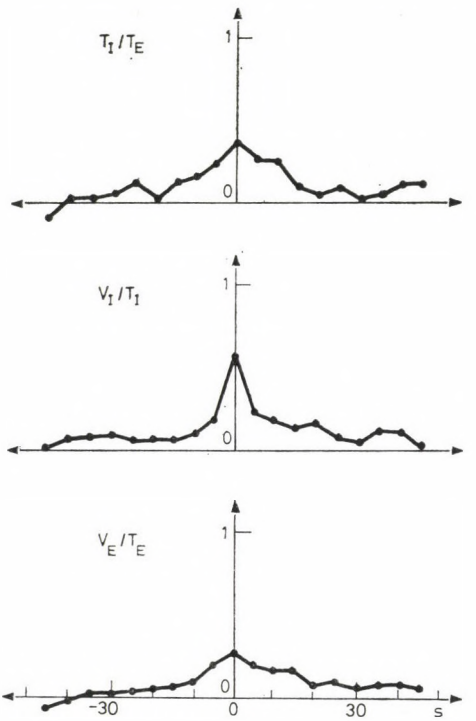

Fig. 5. Averaged cross-correlation functions between inspiratory time and expiratory time, between tidal volume and inspiratory time, between tidal volume and expiratory time in the group with partial respiratory insufficiency. The correlation between tidal volume and inspiratory time is significant statistically

correlation between tidal volume and inspiratory time was significant in patients with chronic obstructive lung disease and respiratory insufficiency. The correlations between inspiratory time and expiratory time as well as between tidal volume and expiratory time were less marked.

Discussion

Studies of the breathing pattern performed in the last decade revealed that chronic obstructive lung disease with respiratory insufficiency is accompanied by changes of the breathing pattern [3, 9]. These and other results [5]

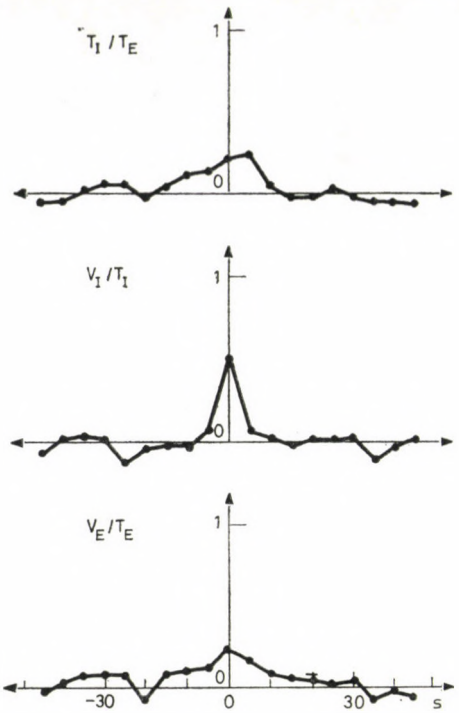

Fig. 6. Averaged cross-correlation functions between inspiratory time and expiratory time, between tidal volume and inspiratory time, between tidal volume and expiratory time in the group with global respiratory insufficiency. These results do not differ from those obtained in patients with partial respiratory insufficiency

indicated that the control of breathing is altered in diseases of the respiratory system. Thus it could be expected that the slow respiratory rhythm of the breathing pattern would also change. Our results, however, failed to support this hypothesis. The patients with chronic obstructive lung disease and respiratory insufficiency, and changes of pulmonary functions and breathing pattern have not altered the character of slow oscillations of the inspiratory time, expiratory time and tidal volume. Neither were the correlations of volume and time components of breaths different from those of healthy subjects. These results demonstrate the relative stability of the central control of respiration in patients with chronic obstructive lung disease and respiratory insufffciency.

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INFLUENCE OF SYMPATHETIC WITHDRAWAL ON EARLY POSTINFARCTION ARRHYTHMIAS

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In this work we studied the incidence and characteristics of early postinfarction arrhythmias in rabbits with sympathetic denervation. The electrical and haemodynamic changes were analyzed.

Three groups of animals submitted to coronary occlusion were studied. A control group of normal animals (I), another group of animals injected with practolol prior to occlusion (5 mg/kg i.v.) (II) and a third group of animals in which stellate ganglia were surgically excised prior to occlusion (III).

In the control group incidence of early postinfarction arrhythmias was 60% (12 of 20) while in Groups II and III incidence was only 25% (3 of 12 rabbits).

The influence of excision or pharmacological blockade of sympathetic system and their role in the genesis of the early arrhythmias is discussed.

Keywords: arrhythmias, myocardial infarction, autonomic nervous system.

It is well established that sympathetic stimulation or ablation alters vulnerability to ventricular fibrillation in experimental conditions. Sympathetic stimulation reduces fibrillation threshold while sympathectomy is protective and elevates fibrillation threshold [2, 5, 6, 8, 9, 14, 16, 17]. These responses are maintained in the presence of acute coronary occlusion in experimental animals. Occlusion itself reduces fibrillation threshold and superimposed sympathetic stimulation results in further reduction, while sympathectomy increases fibrillation threshold to or near to precoronary occlusion levels [1, 8, 10].

The use of beta-blocking drugs for control of arrhythmias in the acute infarction period also supports the important role of sympathetic activity in the genesis of arrhythmias in ischaemic heart disease [18—20]. As the relative role of sympathetic nervous system in the genesis of ventricular arrhythmias is still uncertain in acute myocardial infarction, the present study was undertaken with the aim to define the contribution of sympathetic activity to the development of ventricular arrhythmias in acute myocardial ischaemia in the rabbit.

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Methods

Animal preparation:

Male and female adult rabbits (2.0—2.5 kg) were anaesthetized with sodium pentobarbitone (40 mg/kg, intravenously). Tracheostomy was performed and artificial ventilation with room air was supplied by means of a respiratory pump. Cannulation of a carotid artery and jugular vein were also performed. The carotid cannula was connected to a pressure transducer Elema Schonander EMT 23 to give the measurement of blood pressure on one channel of a Mingograph 81 recorder. Electrodes for simultaneous recording of EKG leads, I, II and III were placed on the animals legs.

While the animal was ventral surface upwards, the exposure of the heart through a mid-line thorectomy and occlusion of the left ventricular artery with a silk thread were performed. The control studies were carried out in 20 animals, coronary occlusion was always performed after control records of heart rate, blood pressure and EKG leads I, II and III. After coronary occlusion recordings were made at 5—10—30 and 60 min.

A second group of experiments was performed in 12 animals, of the same weight as those of the control group. The anaesthetics and surgical procedure were the same as in the control group but prior to coronary occlusion a single dose of practolol (5 mg/kg) was injected via the jugular vein, and coronary occlusion was performed 30 min after the injection.

A third series of experiments was carried out in 12 rabbits, of the same weight on those the other two groups. The anaesthetics and surgical procedures were the same as in series I with the following modification: both stellate ganglia were carefully dissected and excised 15 min before the coronary occlusion and specimens were studied by light microscopy. All the cardiovascular variables were continuously monitored. The data were analyzed by student's paired t-test. The criterion for statistical significance was $p < 0.05$.

Results

Coronary occlusion was followed by electrocardiographic S—T segment changes in 11 animals in the control group. Two min after the occlusion there was a significant decrease in heart rate and blood pressure (Fig. 1). Subsequently, 12 of 20 animals (60%) exhibited ventricular arrhythmia characterized by unifocal premature ventricular beats, ventricular beats, ventricular fibrillation.

The time to onset of the arrhythmia was quite consistent, 6.9—8.5 (mean 7.75) min after coronary occlusion, while the duration was 6.5 min.

In the group of 12 animals injected with practolol half an hour before the coronary occlusion, heart rhythm disorders a rose within 8.9—17.4 (mean 13) min, after the occlusion, the duration was 7.6 min, but the incidence was only 25%, i.e. 3 out of all rabbits. The type of arrhythmias was similar as in the control animals.

In animals in which ganglionectomy was performed before occlusion, the incidence of heart rhythm disturbances was 3 out of 12 (25%), the same as in the group treated with practolol.

Arrhythmias in ganglionectomized animals appeared within 12.5—18.8 (mean 15.6) min after the occlusion and lasted less than two min (1.8 min). We have not observed ventricular fibrillation in this group of animals.

The histological samples prepared from animals subjected to removal of the stellate ganglia were identified by light myoroscopy as sympathetic

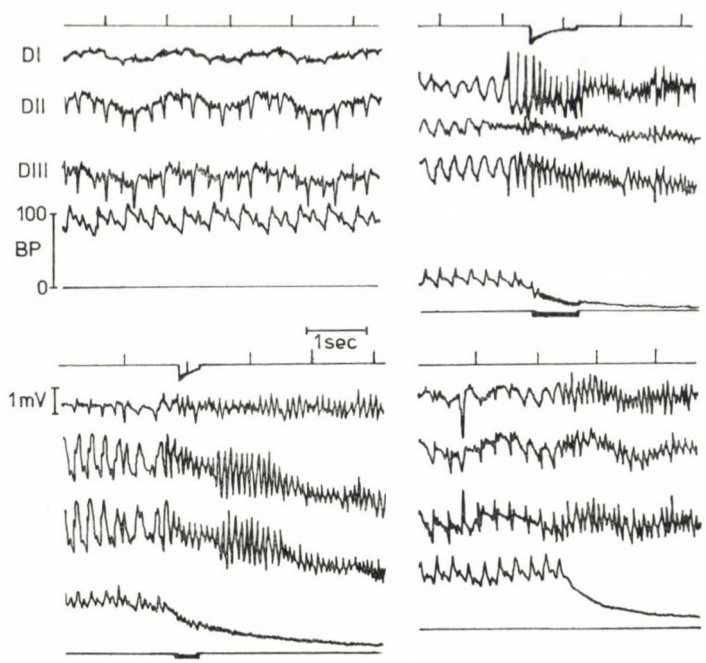

Fig. 1. Postinfarction arrhythmias in rabbits. Electrocardiographic (DI, DII and DIII, standard leads) and arterial blood pressure (B. P. calibration: 0—100 mmHg) features after acute coronary occlusion. Upper traces: ventricular fibrillation and ventricular premature beats. Upper, right and lower traces, ventricular premature beats, and ventricular fibrillation

Table I

Changes in heart rate after coronary occlusion in control and in sympathectomized animals. All values are shown as mean ± S.E.

	Before occlusion	After occlusion			
		5′	10′	30′	60′
Control n = 20	284 ± 10.3	250 ± 11.7*	250 ± 10.4*	255 ± 10.8*	260 ± 7.9*
Practolol (5 mg/kg iv) n = 12	237 ± 7.2**	212 ± 11.5	218 ± 8.9	215 ± 8.5	208 ± 7.9
Stellectomy n = 12	230 ± 7.4**	225 ± 3.9	227 ± 4.5	228 ± 5.6	218 ± 6.4

* $p < 0.05$ compared with its own control
** $p < 0.05$ compared with values before occlusion

ganglia. Heart rate changes evoked by coronary occlusion in all animals are summarized in Table I. The heart rate in the control group dropped from 284 to 250 beats/min 5 minutes after occlusion, and this significant reduction lasted for 1 hour. In the second group of animals the heart rate was 237

beats/min, after the injection of practolol and before coronary occlusion. This value was significantly lower than the heart rate values in the control group.

During the experiment, a continuous tendency to further reduction was observed though the changes did not reach statistical significance.

In the third group of animals heart rate decreased from 265 to 230 beats/min after stellectomy and persisted practically constant during the whole experiment.

Changes in blood pressure evoked by coronary occlusion are summarized in Table II. Blood pressure values showed similar changes in the three groups and there were not significantly different from each other. In the control series blood pressure was 93/74 mmHg, in the animals injected with practolol 95/78 mmHg, and in stellectomized animals 91/70 mmHg.

Table II

Changes in blood pressure after coronary occlusion in the three groups of animals. All values are shown as mean ± S.E.

	Before occlusion	After occlusion			
		5′	10′	30′	60′
Control n = 20	93/74 ± 4.5	77/59 ± 3.4*	79/60 ± 3.9*	70/49 ± 5.5*	68/49 ± 5.7*
Practolol (5 mg/kg iv) n = 12	95/78 ± 78 ± 4.2	70/51 ± 4.7*	71/52 ± 4.6*	70/50 ± 6.6*	66/47 ± 6.0*
Stellectomy n = 12	91/70 ± 3.6	86/64 ± 3.3	84/64 ± 3.3	82/62 ± 2.6	84/60 ± 3.1

* $p < 0.05$ compared with its own control

Discussion

In our previous papers we have reported on the influence of vagal tone on the vulnerability of the infarcted myocardium to ventricular fibrillation [7]. In this work interest was focused on the role of the sympathetic division of the autonomic nervous system in the incidence and characteristics of post-infarction arrhythmias.

We have found that blockade of beta-adrenergic receptors with practolol results in a drastic decrease of the incidence of early arrhythmias after acute coronary occlusion. This suggests that sympathetic activity may be responsible for the initiation and maintenance of electrical instability after myocardial infarction. Similar results were obtained by other authors [3, 14, 15, 19].

In our experiments blockade of the sympathetic beta-adrenoreceptors with practolol prevented the cardiac effects of the increased adrenergic dis-

charge after coronary occlusion and progressively antagonized the cardiac arrhythmias provoked by coronary occlusion. Marshall and Parrat (1976) compared different agents in equivalent beta adrenoreceptor blocking doses and found that practolol was of benefit in the ischaemic myocardium as demonstrated by electrocardiographic criteria, lactate measurements and myocardial consumption data. The beneficial effect of practolol was also observed in our experiments [13]. It is well established that sympathetic stimulation or ablation alters the vulnerability to ventricular fibrillation in experimental conditions [10]. Stimulation reduces fibrillation threshold while sympathectomy is protective and increases fibrillation threshold. Fowlis and collegues [4] have also reported that withdrawal of cardiac sympathetic drive in dogs significantly reduces the duration of ventricular tachycardia induced by coronary occlusion. In our experiments we have found that removal of both stellate ganglia decreases the incidence of cardiac arrhythmias to the same degree as in animals injected with practolol. This confirms the fundamental role of the sympathetic division of the autonomic nervous system in the incidence and characteristics of early postinfarction arrhythmias.

The heart rate decreases significantly after bilateral ganglionectomy and before coronary occlusion and remains on the same value during the whole experiment. There is electrophysiologic evidence showing a reflex increase of cardiac sympathetic outflow after coronary occlusion [12]. This is in accord with the slowing of heart rate we have found in our experiments.

We conclude that the sympathetic division of the autonomic nervous system plays a significant role in the incidence and characteristics of early postinfarction arrhythmias, and suppression of cardiac sympathetic drive by means of beta-blocking agents or by surgical sympathectomy protects the infarcted heart from early postinfarction arrhythmias.

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RADIOLOGICAL INVESTIGATION OF ALTERATIONS IN THE RAT FEMUR AFTER LONG-TERM IMMOBILIZATION

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The right hind limb of female rats was immobilized by plaster cast. Following 8 weeks of immobilization the alterations were compared with either the contralateral extremity of the same rat or with the extremities of non-immobilized animals. The femurs were investigated by microradioscopic, morphometric, densitometric and atom absorption methods. The results obtained have unequivocally shown that long-term immobilization by plaster cast leads to development of osteoporosis in the rat. Even though no considerable loss of calcium could be demonstrated when calculated in terms of unit layer thickness quantitative analysis of the bone revealed a high degree of osteopenia.

Keywords: immobilization, osteoporosis, radiomorphometry, microradioscopy, densitometry.

Immobilization is the best experimental model for inactivation-induced osteoporosis. In the present study, immobilization was performed by plaster cast, in contrast with the more popular method of denervation. The pathomechanism of the developing osteodystrophy was investigated by complex radiological methods developed and successfully used by our team [7, 8]. On the one hand, the methods have proved to be appropriate for investigating the possibility of using various procedures in order to diminish or prevent bone lesions. On the other hand, the present methodology might serve in the future as a model of bone lesions developing in the state of weightlessness [13].

Materials and methods

The right hind limb of 16 female Wistar rats was immobilized by plaster cast. The left legs of these animals and the legs of three untreated animals served as control. Eight weeks after application of the plaster cast the animals were bled and the femurs prepared. The cleaned femurs were placed side by side and the bones of the control and those of the experi-

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mental animals exposed on the same film. The fine-grained industrial film (Agfa Gewaert) was magnified and, thus, certain parts of the bones could properly be analyzed. Microradioscopic, densitometric and morphometric investigations were carried out on the film.

The theoretical basis of the morphometric and densitometric measurements is demonstrated in Fig. 1. The methodology itself was discussed in detail previously (6, 7, 8). After X-ray exposures the femurs were incinerated and the Ca^{++} and Mg^{++} contents of the ash determined by using the atom absorption method. The values obtained were calculated for one femur.

Results

Microradioscopy showed striking lesions in the immobilized extremity. The number of spongiosa trabeculae as well as the territory occupied by them decreased considerably in both the distal and proximal epi- and metaphyses. The cortical substance of the diaphysis was thinner and its density lower than the control value (Fig. 2).

Radiomorphometry revealed a diminution of mineralized bone substances (osteopenia) even quantitatively (Table I). At the level of the measurement in the middle third of the bone, both the periosteal and the endosteal diameters were considerably smaller, while the decrease of the endosteal

Table I

Immobilization-caused morphometric alterations in rat femur

Group	L	D	d	$D-d$	$\frac{D-d}{D}$	D^2-d^2	$\frac{D^2-d^2}{DL}$	$\frac{D^2-d^2}{D^2}$
Right $\bar{x}$	38.09	3.46	2.49	1.07	30.2	6.55	0.046	51.4
$n = 5 \pm SD$	0.40	0.20	0.16	0.06	1.2	0.68	0.004	1.98
Left $\bar{x}$	38.64	4.20	2.83	1.37	32.6	9.57	0.058	54.4
$n = 5 \pm SD$	0.50	0.09	0.12	0.12	2.8	0.85	0.005	3.83

diameter was slighter. The total thickness of the compacta, calculated from the differences of the two above-mentioned diameters, exhibited a 30% diminution. The other parameters also, indicated significant alterations ($p < 0.001$); the change in the square compacta index, indicative of a loss of territory, was the most pronounced one.

Densitometry (Table II) performed at the bisecting point of the diaphysis ("a" level in the Fig. 1) and referred to unit mineralized layer, failed to show a considerable loss of calcium. In contrast, the number of spongiosa trabeculae and the density value (μ^+) representing their calcium content were considerably decreased in the distal metaphysis.

Atom absorption investigations (Table III) showed no considerable decrease in the Ca^{++} and Mg^{++} content of bone ash. The Ca^{++} content, referred

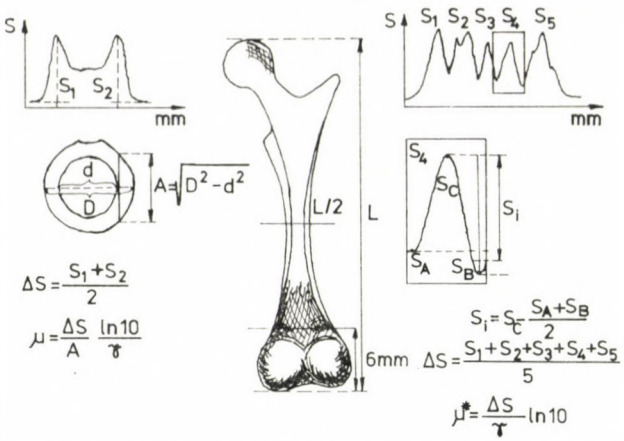

Fig. 1. Scheme of morphometric and densitometric measurements. D = Periosteal diameter; d = Endosteal diameter; S_1, S_2, S_3 . . . = The extreme peaks measured; S = Averaged value; A = Plain of measurement of cortical substance; D = Plain of measurement of spongiosa; γ = Degree of film hardness; μ = Density per unit layer thickness; μ^+ = Dimensionsless relative number, proportional with calcium content

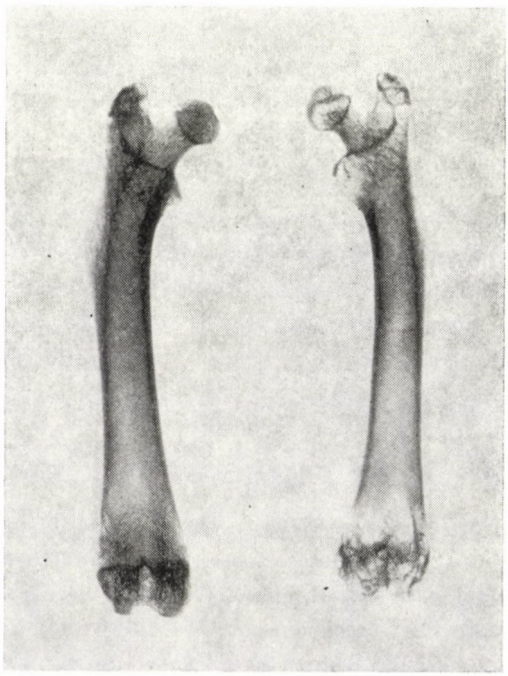

Fig. 2. Magnified X-ray picture of control (left) and immobilized femur (right). (M = 3x.) Control femur shows normal bone structure. The number of spongious trabeculae and the area occupied by them are decreased in the proximal epi-metaphyses of the immobilized femur. The cortical substance became thinner than normal along the full length of the diaphysis

Table II

Immobilization-caused densitometric alterations in rat femur

Group	Cortical		Spongiosa	
	μ l/mm	S. maximum layer thickness A (mm)	Number of trabeculae	μ^+
Right $\bar{x}$	0.54	2.9	13.8	0.037
$n = 5 \pm SD$	0.035	0.2	2.0	0.006
Left $\bar{x}$	0.59	2.7	22.8	0.055
$n = 5 \pm SD$	0.039	0.2	1.4	0.003

to 1 mg of bone mass was decreased slightly and a similar slight increase was seen in the Mg^{++} content. When, however, the Ca^{++} and Mg^{++} contents referred to the total weight of the immobilized femurs were compared with the controls, a marked and highly significant decrease could be observed ($p < 0.005$).

Table III

Immobilization-caused alterations in Ca^{++} and Mg^{++} content of rat femur

Group	Ca^{++}		Mg^{++}	
	Ash, μg/mg	Total, mg	Ash, μg/mg	Total, mg
Immobilization $\bar{x}$	317.60	92.6	5.04	1.74
$n = 5 \pm SD$	3.26	5.2	0.05	0.08
Control $\bar{x}$	324.40	135.4	4.75	3.02
$n = 5 \pm SD$	2.75	7.5	0.05	0.12

Discussion

The different results unequivocally proved that immobilization by plaster cast leads to osteoporosis (osteopenia) in the rat. The lesions are severe in the spongiosa, which finding corresponds to the results of Izawa et al. [9]. Numerous trabeculae were destroyed or thinner than normal. According to radiodensitometric measurements, no considerable loss of calcium could be demonstrated when referred to unit layer thickness, a finding corroborated by atom absorption data. In addition, quantitative analysis of the bone also demonstrated osteopenia, resulting from structural degradation of the bone and corresponding to the radiological criteria of osteoporosis.

The lesions developed might be interpreted as consequences of the lack of mechanical stimuli securing the equilibrium of bone reorganization. The stretching and pressing forces transmitted by muscles and tendons to the bone and periosteum are absolute prerequisites for the reorganization of the bone [5, 12, 14]. Decrease in these stimuli leads to the inhibition of bone reorganization and to an enhancement of bone resorption. These processes lead to a considerable decrease of the total mass of the bone, the decrease of which is proportional to the duration of immobilization. Due to the structural reconstruction not even the remaining bone substance is of whole value from a mechanical point of view, a phenomenon demonstrated also by parallel histological investigations [4]. In addition, the lack of mechanical load may lead to disturbances in blood supply resulting in local metabolic disturbances and, consequently, the hormonal sensitivity of the bone cells might also be modified. The parathyreoid gland may play an important role in the enhancement of resorption [1, 2, 3] as suggested by the histological findings of osteoclastosis and increased osteocyte-osteolysis. Furthermore, the impaired formation of the ground substance and the inhibition of consequent calcification may be also figure participated in the pathomechanism [10, 11].

Summing up, it may be concluded that immobilization disturbs the state of metabolic equilibrium between bone, muscle, cartilage and soft tissues. As a consequence, the hormonal factors leading to osteolysis will be strenghtened and the mechanical stimuli of bone formation abolished. The lesions developed may be easily demonstrated by radiological methods. A quantitative evaluation is also possible, and this is of utmost importance in the evaluation of the extent of the lesions and for planning attempts to influence the unfavourable condition.

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SKELETAL MUSCLE BIOPSY STUDIES OF CARDIAC PATIENTS

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Eleven patients diagnosed and treated for congestive cardiomyopathy (COCM) of unknown aetiology, and another 10 patients, with congestive alcoholic heart muscle disease (ACOCM) were studied. Muscle biopsy samples were obtained from the vastus lateralis (VL) and the gastrocnemius (G) muscles. In part of the sample muscle the fibre pattern was classified by means of ATPase activity staining, a technique based on the pH lability of the fibres concerned. Fibre typing and area measurements were carried out by light microscope. The other part of the sample was used as muscle homogenate of which the Ca^{2+}-activated ATPase activity as well as citrate synthetase (CS) and aldolase activities were measured.

No significant difference was found in these enzyme activities between the two groups of patients.

The proportion of the slow twitch (ST) fibres in the VL, mainly in the patients with ACOCM, was lower as compared to data for healthy subjects. A similar tendency was revealed for G.

In both muscles tested, the area of ST fibres was smaller in the ACOCM group. The fast twitch (FT) fibre area proved to be slightly different in the two groups of subjects tested.

Occurrence of degenerative signs in the histological tests was higher in the ACOCM than in the COCM group.

It was concluded that differences in the skeletal muscles of patients with ACOCM and COCM may primarily account for the alcoholism. The disease of the heart muscle has little effect on the function of skeletal muscle. Even so, a low amount or lack of physical activity may have an unfavourable influence on the skeletal muscles of patients with heart muscle disease.

Keywords: fiber area, fiber types, enzymes activity, cardiac patients, alcoholism.

In the vast majority of cases, the cause of congestive cardiomyopathy is rather obscure [15, 28]. However, a possible contribution of certain factors to the development of heart muscle disease is frequently assumed. The relationship between heart muscle disease and alcohol consumption has been documented both in experimental and clinical investigations [6, 25]. Still, the ultimate underlying mechanism is unknown [15].

Histological and biochemical investigations in the mentioned heart diseases have, in general been carried out independently. Whether or not the heart muscle disease has a deteriorating effect on skeletal muscle, or is there

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any common causal factor between them is an unanswered question. The effects of alcohol consumption can be traced in the skeletal muscles even in subjects with a healthy, normal heart [30, 46, 19].

The tibialis anterior muscle of chronic alcoholics and control subjects was studied by Langohr et al. [41]. In these patients atrophy of the FT fibre and an unaltered proportion of FT to ST fibres was found. The glycolytic, glycogenolytic and the oxidative enzyme activities were lower in these patients than in the control subjects. Squared cells as well as fibre type grouping were found and interpreted as the signs for the initiation of motor denervation [41].

Faris et al. [22, 23] suggested that alcoholic myopathy in the proximally situated muscles was due to the effect of alcoholic neuropathy. Slavin et al. [54] and Brumbach [8] reported on damage to the FT fibres induced by chronic alcoholism in both humans and experimental animals. Martin et al. [43, 44] and Kiessling et al. [38] observed a selective atrophy of the FTb fibres.

Patients with myopathy caused by alcohol consumption and healthy controls have been compared by Kiessling et al. [38] using samples from VL. The activity of triose-P-dehydrogenase and citrate oxidase as well as the mitochondrial enzymes were found to be lower in the patients, but no difference was found in the fibre type ratio.

The purpose of the present study was to clarify the differences in the histochemical and/or enzyme activity patterns of the skeletal muscles of patients with heart disease, and also the differences, if any, in the skeletal muscles of patients with cardiomyopathy of different origin.

Materials and methods

Patients for the study were diagnosed and treated at the Hungarian Institute of Cardiology. 11 patients with congestive cardiomyopathy (COCM) of unknown origin were examined by invasive and non-invasive procedures. Another 10 patients were classified as congestive alcoholic cardiac disease (ACOCM). Criteria for this classification were,

1) Existence of congestive heart muscle disease,

2) Absence of any additional heart muscle damaging factor which otherwise could have been identified,

3) On the basis of the history of long term and excessive consumption of alcohol [14].

Descriptive data regarding the subjects are presented in Table I.

Table I

	COCM (n = 11)	ACOCM (n = 10)
Mean age, years	39.4	41.8
Mean duration of the disease, years	5.5	4.5
Mean body weight, kg	72.2	81.9
Mean body height, cm	170.6	171.5

Informed consent on the nature and risk of the experiment was obtained from of the subjects for the study.

Muscle biopsy samples were obtained from the vastus lateralis (VL) and gastrocnemius (G) muscles. A 4.5 mm Bergström needle was used for sampling [5]. The samples were taken at the midpoint of the length of the respective muscle at a depth of 1.0—1.5 cm beyond the fascia.

Samples, weighing 20—50 mg, were divided into two parts. One part of the sample was embedded in Tissue Tek II and frozen in liquid isopentane-nitrogen system, and stored at —30 °C. Cross sections of 10 microns width were then cut at —20 °C. The rest of the sample was homogenized and used for enzyme activity assays.

Muscle fibre type classification was carried out according to descriptions [7]. Preincubations were performed at pH 4.6, 4.3, and 10.3, respectively, and followed by staining for the Ca^{2+} — activated ATPase activity at pH 9.4.

Percentual distribution of the muscle fibres was determined by counting 200—700 fibres [50].

The fat components were stained with Sudan III, while haematoxylyn was used for the determination of the cell nuclei.

Areas of muscle fibres was calculated by the smallest and largest diameters of 30 fibres per type.

The muscle was homogenized in a Potter homogenizer in ice for a minute, in 100 volumes of 0.5 mol KCl. This homogenate was used for ATPase activity assay. We defined the myosin ATPase activity, activated by Ca^{2+} (E.C. 3.6.1.8.) according to Colowick and Kaplan [10], and the anorganic phosphate according to Eibl and Lands [18]. The results were given in μmol Pi $\times$ g wet weight^{-1} $\times$ minute^{-1}.

Citrate synthetase (E.C. 4.1.3.7.) activities were measured according to Colowick and Kaplan [11]. The muscle homogenate was centrifuged (4000 r.p.m.: 10 min) and the supernatant was used for studies. The results were given in μmol DTNB $\times$ g wet muscle weight^{-1} $\times$ minute^{-1}.

Aldolase (E.C. 4.1.2.7.) activities were measured according to Bergmeyer and Bernt [4] in the supernatant of muscle homogenate. The final dilution was made with 12 volumes of phosphate buffer (0.05 mol, pH 7.4).

Results were given in U $\times$ g wet muscle weight^{-1} $\times$ min^{-1}.

Results

The aldolase activity in the muscle homogenates of both VL and G was found to be almost identical in the two groups of subjects studied (Table II). Somewhat higher CS activity in the G than in the VL group was found but the intergroup difference was not significant (Table II).

The Ca^{2+}-activated myosin ATPase activity tended to be higher in both muscles of the COCM group, but the intergroup difference between VL and G was not significant (Table II).

The ST% was significantly lower in the VL of the ACOCM than in the COCM group patients (Table III). Comparing the values obtained for patients with those reported for untrained (Table IV) and trained healthy subjects (Table V), ST% in the ACOCM patients was significantly lower. Similarly, the ST% in the G was lower in the ACOCM than in the COCM patients (Table VII).

Mean cross-sectional area of the ST fibres in both VL and G of the ACOCM patients was smaller than in the COCM group (Fig. 1). The mean area of the FT fibres differed very slightly between the two groups of subjects. In both VL and G area of the FT fibres was found to be smaller as compared to the ST fibres.

Table II

Key enzyme activities measured in muscle homogenate

M. vastus lateralis Group		ATP-ase	CS	Aldolase
COCM	$\bar{x}$	9.98	7.00	22.40
	SD	3.00	1.90	5.80
ACOCM	$\bar{x}$	9.02	7.86	22.90
	SD	2.60	1.88	5.40

M. gastrocnemius Group		ATP-ase	CS	Aldolase
COCM	$\bar{x}$	12.50	10.37	18.70
	SD	6.50	3.20	5.32
ACOCM	$\bar{x}$	8.98	9.60	23.20
	SD	5.10	2.50	5.00

ATP-ase μmol Pi$\times$g wet weight$^{-1}\times$min^{-1}
Citrate synthetase (CS) μmol DTMB$\times$g wet weight$^{-1}\times$min^{-1}
Aldolase U$\times$g wet weight$^{-1}\times$min^{-1}

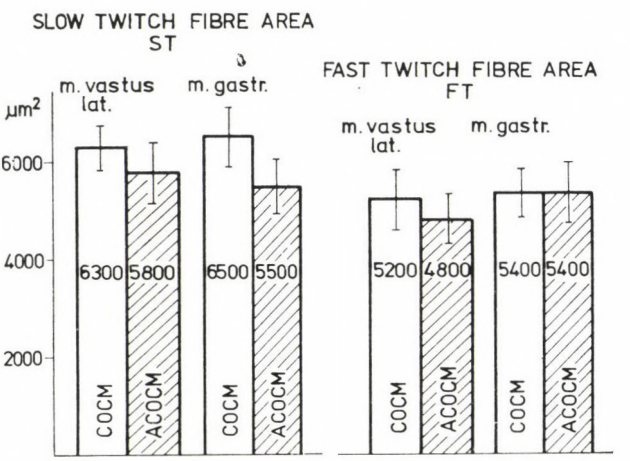

Fig. 1. Muscle fibre area of m. vastus lateralis and m. gastrocnemius in COCM and ACOCM patients

Table III

ST percentage in m. vastus lateralis measured in heart muscle patients subjects compared with sedentary and physically active

Subjects	No	ST%
COCM	11	43.9 ± 3.71
ACOCM	8	37.3 ± 2.58
Physically active (data in literature)	150	49.5
Sedentary (data in literature)	260	45.9

Table IV

Percentage of ST fibres in m. vastus lateralis in sedentary and untrained subjects 20—50 years of age. Review of the literature

Age, years	No. of subjects	ST% mean	Reference
20—23	5	39.0	Andersen and Henriksson [1]
44	11	35.4	Bylund et al. [9]
23.6	5	44.8	Costill et al. [12]
23—32	8	45.6	Edström and Ekblom [16]
20—25	6	53.0	Eriksson et al. [20]
20—41	9	55.0	Eriksson et al. [20]
22—41	4	58.0	Eriksson et al. [20]
21—28	4	49.0	Eriksson et al. [20]
24—30	12	36.1	Gollnick et al. [27]
31—52	14	43.9	Gollnick et al. [27]
	5	50.6	Gollnick et al. [26]
	6	56.9	Edström and Nyström [17]
23	8	43.8	Green et al. [29]
22—35	5	40.8	Inbar et al. [33]
20—29	11	40.5	Larsson et al. [40]
30—39	10	36.8	Larsson et al. [40]
40—49	8	48.2	Larsson et al. [40]
48	23	53.1	Lindgärd et al. [42]
22—35	9	32.1	Örlander et al. [48]
20—29	11	34.0	Örlander et al. [47]
30—39	16	45.0	Örlander et al. [47]
40—49	11	50.0	Örlander et al. [47]
	4	35.5	Prince et al. [49]
	10	44.0	Thorstensson et al. [60]
34	8	46.2	Viitasalo and Komi [63]
	5	52.0	Nygaard and Nielsen [45]
	15	50.0	Nygaard and Nielsen [45]
30	11	43.0	Sylven et al. [56]
	6	69.0	Sjögaard [53]
Total	260	45.9	

Table V

Percentage of ST fibres in m. vastus lateralis in physically active subjects 20–50 years of age. Review of the literature

Age, years	No. of subjects	ST% mean	Reference
29–40	6	32.2	Gollnick et al. [27]
19–25	10	49.6	Eriksson et al. [20]
19–33	8	41.6	Inbar et al. [33]
25.3	10	43.1	Jacobs et al. [34]
19–34	11	48.7	Jacobs et al. [35]
18–28	7	41.3	Jacobs et al. [35]
21–29	12	48.0	Jacobs et al. [35]
	9	68.3	Komi [39]
23	50	47.0	Tesch and Larsson [58]
	16	54.0	Tesch and Wright [59]
28	11	71.0	Sylven et al. [56]
Total	150	49.5	

Table VI

Percentage of ST fibres in m. gastrocnemius in sedentary and untrained subjects 25–50 years of age. Review of the literature

No. of subjects	ST% mean	Reference
4	57.8	Jennekens et al. [36]
6	50.2	Jaohson et al. [37]
11	52.6	Costill et al. [13]
4	51.3	Andersen and Kroese [2]
2	63.0	Edström and Nyström [17]
6	60.2	Gollnick et al. [26]
18	60.0	Henriksson et al. [31]
7	57.8	Houston et al. [32]
Total 58	56.6	

Occurrence of histological degenerative symptoms was as high as 27% (COCM) and 87% (ACOCM). These signs were considered more serious in the ACOCM group where fibre splitting, fibre breaking, angular cells and fat accumulation could also be observed.

Discussion

Muscle biopsy samples were obtained from the vastus lateralis (VL) and gastrocnemius (G) muscles of 21 patients. Half of the patients suffered from congestive cardiomyopathy of alcoholic origin.

The aldolase activity (Table II) in the muscle homogenates of the alcoholic and other cardiomyopathic subjects was approximately the same. The VL of alcoholic patients under hospital care, the tibialis anterior muscle of chronic alcoholic patients, and the VL of patients with rhabdomyolysis have been studied by Kiessling et al. [38], Langohr et al. [41] and Martin et al. [43], respectively. They concluded that the glycolytic enzyme activities were lower in the alcoholic subjects than in the controls. One of the reasons for this phenomenon could be the atrophy of FT fibres. A linear relationship between the change in glycolytic enzyme activity and the shift in the area ratio of the FT to ST fibres was pointed out by Langohr et al. [41]. It was suggested that the atrophy of the whole muscle might be the result of the decrease in the size rather than in the number of fibres. The present findings are in good agreement with the above observations since the cross-sectional area of the FT fibres was basically the same in the two groups of patients. Also, no significant difference was found in the glycolytic enzyme activities in patients representing the two groups. Martin et al. [43] suggested that alcohol consumption results in a reduced level of physical activity which hence does induce an atrophic process in the FT fibres (Fig. 1).

Kiessling et al. [38] indicated that the oxidative enzyme activities in the alcoholic patients were not significantly lower than in the controls. In addition, there was no significant difference in the oxidative enzyme activity of the tibialis anterior muscles [41] and there was no significant difference in maximum oxygen uptake between normal and alcoholic subjects, as reported by Kiessling et al. [38]. According to this finding no difference was revealed in the enzyme activity of CS (Table II).

It was pointed out earlier that both the Ca^{2+}- and Mg^{2+}-activated ATPase activities were two to three-times higher in the FT than in the ST fibres [51, 61]. The difference was attributed to the isoenzyme contents of the muscle fibres of varying ATPase activity [24]. Taylor et al. [57], Thorstensson et al. [60, 61] and Vándor et al. [62] found a positive relationship between FT% and the ATPase activity. The ATPase activity of VL and G tended to be lower in the ACOCM group (Table II) notwithstanding the fact that FT% was higher in this group of patients (Fig. 1). Although the mean FT area in both ACOCM and COCM groups was small (Fig. 1) it may be assumed that the isoenzymes with higher activity were missing or damaged in the ACOCM patients.

Interestingly, a lower ST% was found in the VL of the ACOCM than in the COCM patients (Table III). Örlander (personal communication) directed our attention to the difference in fibre composition between healthy physically active and sedentary people: those who led a physically active life (Table V) showed a higher proportion of ST fibres in the VL than did sedentary subjects (Table IV). Data in the literature (Table IV, V) proved this only in trends.

Between sedentary and physically active subjects the differences are slight. The COCM patients did not show a marked difference from the sedentary and physically active people. The slow twitch fibre proportion of the alcoholic patients differed more from the normals.

A similar tendency was found in the G muscle of the two groups of patients (Tables III, VII). The ST% in the G of the ACOCM patients was lower as compared to both the COCM patients and the healthy sedentary subjects sampled from the literature (Table VII). On the contrary, a higher

Table VII

ST percentage in m. gastrocnemius in heart muscle patients compared with sedentary subjects

Subjects	No	ST%
COCM	7	71.1 ± 5.6
ACOCM	9	51.6 ± 8.5
Sedentary (data in literature)	58	56.6

Table VIII

FT/ST area rations in athletes

VL		G	
Walkers	0.89	Karate	0.98
Marathon runners	0.92	Walkers	1.03
Middistance runners	0.98	Sprinters	1.14
Karate	1.01	Long jumpers	1.18
Sprinters	1.08	High jumpers	1.19
High jumpers	1.08	Middistance runners	1.27
Long jumpers	1.15		
Weight lifters	1.27		

ST% ratio was found in the G of (Table VII) the COCM patients differing from was found in their VL (Table III) and from the collected data in the literature. On this basis it may be assumed that the lower ST% in both VL and G of the ACOCM group might be a consequence of some kind of degenerative process.

Also, the cross-sectional area of the ST and FT fibres in the VL and G muscles was significantly lower in the ACOCM than in the COCM patients (Fig. 1). This observation might be explained by the suggestion of Langohr et al. [41] that chronic alcoholics with normal heart function perform a reduced amount of physical activity. It should, however, be pointed out that a limited amount of exercise is typical prior to the appearance of the disease so that alcoholics might have an inclination to be less active than normal subjects.

An additional finding was that both in the VL and G muscle of the two groups of patients, the fibres proved to be smaller than the ST fibres. This observation was revealed by calculating the FT area/ST area ratio, which was found to be almost identical FT/ST area ratios in patients

Table IX

	VL	G
COCM	0.82	0.83
ACOCM	0.84	0.83

The FT/ST ratio in the tibialis anterior muscle of control subjects and alcoholics with normal heart function was reported by Langohr et al. [41] to be 1.49 and 1.16, respectively. Their conclusion was that the FT fibres of alcoholic patients were atrophied. Kiessling et al. [38] published similar findings. Slavin et al. [54] and Brumback [8] reported on atrophy of the FT_b fibres in alcoholic patients without any malfunction in the heart. If one compares the FT/ST ratios listed above with those obtained for athletes (Table VIII), it seems evident that the lack of physical activity plays an important role in negatively affecting this ratio.

As for the occurrence of degenerative symptoms in the muscles of the ACOCM patients, it seems convincing that it is the alcohol rather than the heart disease which exerts a damaging influence on the skeletal muscles.

It may be concluded that comparison of the two kinds of congestive myopathy revealed a deteriorating effect on the skeletal muscle of the lack of physical exercise and the degenerative symptoms due to alcohol consumption. It was also pointed out that there was a decrease in the proportion of ST fibres in both the vastus lateralis and gastrocnemius muscles, especially in the ACOCM patients.

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CALCAEMIC RESPONSES IN THE YELLOW MONITOR, VARANUS FLAVESCENS TO VITAMIN D_3 ADMINISTRATION

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The effect of a daily intramuscular injection of vitamin D_3 (2000 IU/100 g b.wt) on serum calcium level was investigated in Varanus flavescens. This treatment evoked hypercalcaemia on day 3 which progressed up to day 7. At day 14 a decline was noticed in the serum calcium level which was followed by a rise from day 21 to day 28.

Keywords: calcium, vitamin D_3, Varanus flavescens

Vitamin D_3 is hydroxylated first by the liver at position 25 and then by the kidney at carbon —1, thus producing the biologically active form, 1.25 dihydroxycholecalciferol [3, 6, 7]. The enzyme responsible for the renal transformation is referred to as 25-hydroxycholecalciferol-1-hydroxylase (1-hydroxylase). Although Henry and Norman [4] have reported the presence of 1-hydroxylase in reptiles (lizards and turtles) there are no studies concerning the physiological effects of either the native vitamin or its metabolites in this class [2]. In the present report, we have described the effects of vitamin D_3 on the serum calcium level of a lizard, Varanus flavescens.

Material and methods

Eighty-four adult specimens of V. flavescens (b.wt 430—510 g) were acclimatized to laboratory conditions for 2 weeks prior to use. They were then divided into 2 numerically equal groups. Animals of Group A were given daily intramuscular injections of 0.02 ml/100 g b.wt of vehicle (arachis oil). Animals of Group B were injected daily with 2000 I. U./0.02 ml/100 g b.wt of vitamin D_3. Six animals of each group were anaesthetized and blood samples were collected by cardiac puncture (4 h after the last injection) on day 1, 3, 5, 7, 14, 21 or 28 following the start of the experiment. Serum calcium level was estimated by Trinder's [13] method.

To avoid the effects of circadian rhythm, the injections were given at the same time and the blood samples were collected at approximately the same time of the day throughout the experiment. Differences between vehicle-injected and vitamin D_3-injected specimens were evaluated using Student's t-test.

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Results and discussion

Table I shows the effect of vitamin D_3 administration on serum calcium concentrations of V. flavescens. Administration of vitamin D_3 had no significant effect on calcium level on day 1. After day 3 calcium level started to increase significantly which progressed up to day 7. Thereafter, a decline was noticed on day 14 which was again followed by a rise from day 21 onwards.

Table I

Effect of vitamin D_3 on serum calcium level (mg/100 ml) of Varanus flavescens

Days	Vehicle-injected	Vitamin D_3-injected
1	12.95 ± 0.26	13.05 ± 0.51
3	12.85 ± 0.16	13.69 ± 0.42a
5	12.90 ± 0.26	13.87 ± 0.25a
7	12.96 ± 0.24	14.47 ± 0.25b
14	12.86 ± 0.23	13.05 ± 0.24
21	12.80 ± 0.44	13.81 ± 0.24a
28	12.83 ± 0.17	13.26 ± 0.12a

The values are mean ± SD of 6 determinations.
a and b indicate significant differences $P < 0.01$ and $p < 0.001$, respectively.

The results obtained in the present study clearly indicate that vitamin D_3 is in fact inducing hypercalcaemia in V. flavescens. This is in agreement with similar effects of vitamin D in other vertebrates [5, 9, 10, 11, 12]. Henry and Norman [4] have reported the presence of 1-hydroxylase (the enzyme responsible for renal transformation of vitamin D_3) in lizards. They have stated that: "The presence of 1-hydroxylase in renal tissue of a species would be strong presumptive evidence that the species in question had a requirement for vitamin D metabolism for normal calcium homeostasis". The result of the present study is the first demonstration of the effect of vitamin D_3 in a lizard, V. flavescens.

The decrease in calcaemia on day 14 may be attributed to the possible increased secretion of some hypocalcaemic factor. The presence of calcitonin, which is a hypocalcaemic hormone in mammals, has been reported in the ultimobranchial gland of some lower vertebrates [1, 8].

The analysis of the present results may give a clue to the physiological function of vitamin D_3 in reptiles.

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EFFECT OF THIOUREA ON THE HYPOTHALAMIC NUCLEI OF THE MUD EEL *AMPHIPNOUS CUCHIA* (HAM.)

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Immersion of fish in thiourea solution (0.33×10^{-6} ppm) caused atrophic changes in the NPO neurones only but no change in the cell bodies of the NLT was recorded.

Keywords: Nucleus lateralis tuberis (NLT) Nucleus preopticus (NPO), Pars magnocellularis (PMC), Pars parvocellularis (PPC), Pars anterior (PA), Pars posterior (PP), Mud eel, Ovarian recrudescence.

Although NLT has been described as a second neurosecretory centre in the teleostean hypothalamus [10, 16] compared to the NPO it has drawn little attention. Peter and Crim [11] observed that in the goldfish nucleus preopticus, the lesions caused ovarian regression and blocked ovarian recrudescence. Dodd et al. [6] observed that in male Salmon parr. and Salmo saler lesions of the NLT blocked spermatogenesis, reduced testicular size and lowered pituitary gonadotropin content. Thus, the importance of the role of NPO and NLT in the regulation of gonadotropin secretion cannot be overruled. The hormonal control of the pituitary on the activity of gonads has been well established [1, 7].

The effect of drugs on the brain, hypophysis and reproductive cycle have been studied by several workers in a number of teleosts [2, 4, 9, 14, 15, 18, 20]. All these studies were inadequate to provide a clearcut conception of the effect of drugs on the hypothalamic nuclei which largely control the gonadotropic function of the pituitary. We have therefore studied the changes in different regions of the hypothalamic nuclei following thiourea (a chemosterilant) treatment in the mud eel.

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Materials and methods

Two-hundred acclimatized adult mud eels were used in this investigation. They were divided into two group. One group was used for thiourea immersion and other group served as control. All the fish were maintained under normal conditions of light (about L 12 h ± D 12 h) and temperature (25 ± 1 °C). Group I was maintained in 0.33% thiourea solution for a period of 30 days and sacrificed in intervals of 15 and 30 days. The controls were kept in dechlorinated tap water. Thiourea solution was renewed every fourth day (96 h). The brains together with the pituitary were collected and treated for routine histological study. AF and ATH stains were used to stain the hypothalamic nuclei. Nuclear diameter was measured by oculometer and P-values were calculated by utilising the procedure of Campbell [5].

Table I

Nuclear diameter of NPO neurones after thiourea immersion

Duration of treatment, days	Nuclear diameter of PPC (μ)		Level of significance*	Nuclear diameter of PMC (μ)		Level of significance*
	Control	Treated		Control	Treated	
15	5.48 ± 0.18	5.26 ± 0.18	$P < 0.01$	7.68 ± 0.22	7.32 ± 0.22	$P < 0.05$
30	5.41 ± 0.15	5.20 ± 0.19	$P < 0.01$	7.35 ± 0.20	7.07 ± 0.28	$P < 0.05$

Each value represents Mean ± SE of 30 observations.
* P-values calculated for nuclear diameter of thiourea immersed and untreated control group.
NS = Not significant.

Table II

Nuclear diameter of NLT neurones after thiourea immersion

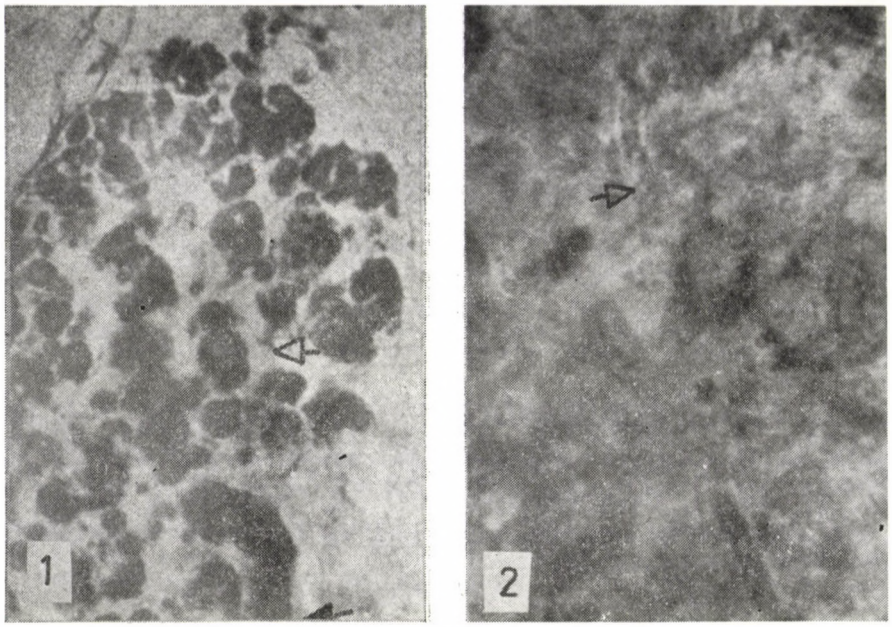
Duration of treatment, days	Nuclear diameter of PA (μ)		Level of significance*	Nuclear diameter of PP (μ)		Level of significance*
	Control	Treated		Control	Treated	
15	16.28 ± 0.56	15.78 ± 0.58	NS	9.40 ± 0.35	9.35 ± 0.35	NS
30	16.16 ± 0.66	15.76 ± 0.60	NS	9.38 ± 0.35	9.18 ± 0.27	NS

Each value represents Mean ± SE of 30 observations.
* P-values calculated for nuclear diameter of thiourea immersed and untreated control group.
NS = Not significant.

Results

Tables I and II summarize the changes observed in the nuclei of the NPO and NLT neurones after thiourea treatment. The nuclei of the PPC and PMC of thiourea treated fish were smaller than those of the controls (Fig. 1). The atrophic changes were significant statistically P 0.05 and P 0.01 in the PMC and PPC neurones, respectively, after 15 days and later after of immersion into thiourea. The NSM of NPO was also reduced (Figs 4 and 5)

There atrophic changes are reflected even in the perikarya which are feebly stained and the nuclei showed pycnosis, both in the PMC and in the



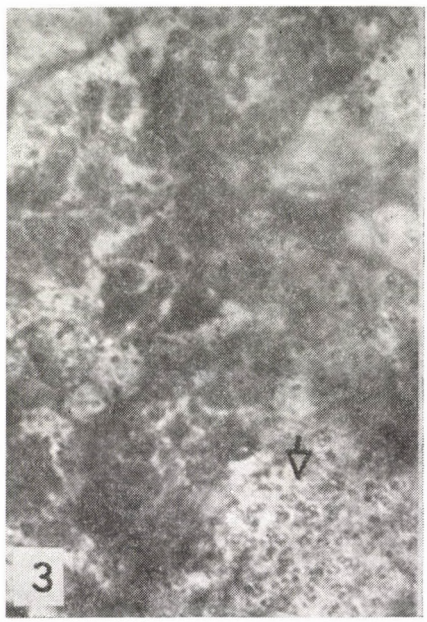
Figs 1—3. Photomicrograph of sagittal section of hypothalamo-neurohypophysial complex of *Amphipnous cuchia* in control and treated specimens. 1. PMC (→) and PPC (→) in control. ×1000. 2. NLT (→) in control. ×1000. 3. Greatly reduced PA nuclei (→) of NLT after 30 days of thiourea treatment. ×400

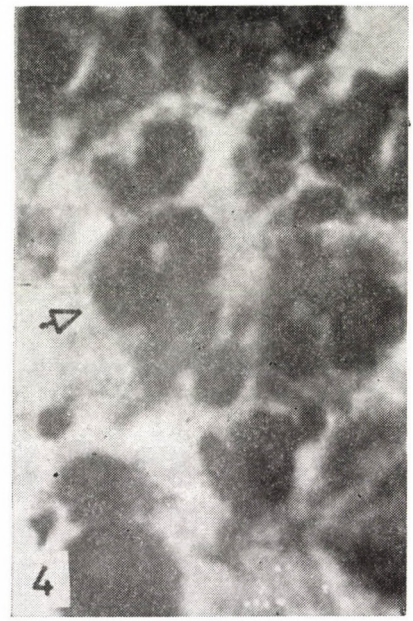
PPC. The nuclei of the PA neurones were also reduced in size as compared to those of the controls (Figs 2 and 3). The changes, however, were not significant and no remarkable variation was observed in the staining intensity of NLT neurones.

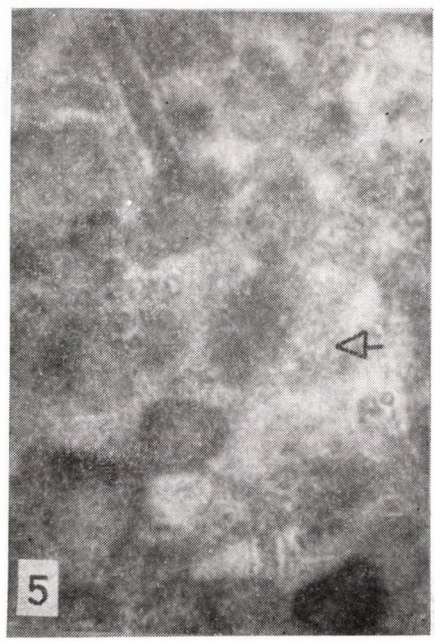
Discussion

It is well known that neurosecretory activity in the hypothalamus changes in response to cyanotrops [3, 7, 8, 12].

Hypophysectomy has been found to cause depletion of NSM in the NPO of fishes [13, 17, 19]. Immersion in thiourea solution caused atrophic changes in the PMC and PPC activity and the gonadotropin secreting cells as well, indicating that the hypothalamic neurones exercise an inhibitory action on the gonadotropin secreting cells. The atrophic changes observed in the hypothalamic nuclei after thiourea treatment may also be attributed to its pharmacological action which, however, requires further study. The present investigation revealed that after thiourea treatment the hypothalamus exerts an inhibitory influence on the gonadotropin secreting cells and these observations have shown that whatever the cause of the effect of thiourea, its action on the hypothalamic nuclei cannot be ruled out.







Figs 4—5. Photomicrographs of sagittal section of hypothalamo-neurohypophysial complex of *Amphipnous cuchia*. 4. Large amount of NSM (→) in NPO of control. ×1000. 5. Reduced amount of NSM (→) in NPO after 30 days of thiourea treatment. ×1000

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PRESENCE OF COVALENTLY BOUND ENERGY-RICH PHOSPHATES IN HUMAN TRACHEAL SMOOTH MUSCLE MYOSIN

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In a preliminary report, the tracheal NaCl-myosin prepared from an old and a young subject was discussed [8]. In the present paper, the total bound phosphate (P) content and its distribution is described in two parallel preparations of human muscle myosin. It was shown that a considerable amount of covalently bound P was present not only in NaCl, but in the fresh preparations of tracheal KCl-myosin. Analysing this phosphate fraction in the alkaline hydrolysate of RNA- and lipid-free preparations of myosin it was confirmed that phosphate was linked to the basic amino acid residues and their hydrolytic derivates.

As the phosphoryl binding sites are partly saturated, the phosphate concentration can be enhanced nearly three-fold compared to the fresh preparation. Phosphate incorporation is an autophosphorylation process depending on the ATP and Mg^{2+} concentraton.

Studying the actomyosin fraction in the presence of ATP it was found that its phosphate content can also be increased to a certain degree. It is supposed that the changes in phosphate content of myosin are associated with the formation of cross-bridges between the actin and myosin filaments in the processes of muscle contraction and relaxation. The process can be influenced directly and indirectly by some natural factors and drugs resulting in the concentration or relaxation of bronchial muscles.

Keywords: tracheal muscle myosin, covalently bound phosphate, autophosphorylation, distribution of bound P, amino acid phosphates

Human tracheal myosin preparations from an aged and a young subject were described earlier [8]. Several preparations have been presented from mammalian tracheal muscles (horse, bovine, dog) but these were mainly actomyosin preparations [21, 22] and few were related to myosin [4, 5, 6, 29]. The latest investigations studied the ATPase activity of the preparations and light chain phosphorylation as well [4, 5, 22, 29].

A rather low phosphate (P) concentration was found in the light chain (LC_2) that could be enhanced to about 2—3-fold P-Ser concentration [4, 5], and it was assumed that phosphorylation had a role in the regulation of muscle contraction.

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In our earlier experiments a significant amount of endogenous (preformed) P was found in the NaCl-myosin preparation of two human patients, namely 172 mol in the young and 60 mol P/mol myosin in the old patient's muscle. It was presumed that the high endogeneous P concentration in myosin alloved its participation in muscle contraction.

Therefore myosin was prepared from human tracheal muscle to study the total P content of myosin. This time the myosin was isolated with a KCl-containing solution in order to be compared with the NaCl-myosin preparations [7, 8]. The high amount of preformed P content in tracheal myosin was in contrast to the opinion of other authors, according to whom only a low molecular weight subunit of myosin (LC_2 which has a regulatory role) can be phosphorylated. This view is considered here as due to the considerable P content of these preparations, the formation of P-Ser might be a regulated or non-regulated side effect as a result of a nitrogen-to-oxygen migration [20, 30]. Moreover, the changes in P concentration of myosin and the phosphoryl group incorporation from ATP into myosin called our attention to the possibility of a direct participation of bound P in muscle contraction.

The aim of the present investigation was therefore to study the preformed P content covalenty linked to the peptide chains, and its distribution in tracheal preparations of lipid, RNA and actomyosin-free KCl-myosin. For this purpose it seemed valuable to obtain data on the distribution and role of bound P in human tracheal muscle.

Material and methods

Two freshly dissected human tracheas were obtained from subjects free of any airway and pulmonary disease. The tracheas were cleaned from connective tissue and minced in a pre-cooled electric mincer, used for the preparation of myosin processed as described before [8], except that instead of NaCl, a KCl and $KHCO_3$-containing solution was applied. Further, the significant amount of actomyosin sediment obtained by ultracentrifugation (105 000 g for 120 min) was also used for further purification prior to the investigations. The purification of ultracentrifuged myosin was completed by gel filtration [8, 10].

The actomyosin fraction was also gel filtered successively with myosin on the same Sepharose 4B column as detailed in Figures 1 and 2, and investigated similarly.

The molecular masses of myosin and actomyosin were calculated to be 500 and 650 kD, respectively, moreover an approximate ratio actin : myosin = 3 : 1 was assumed for the composition of the latter compound.

Protein and P concentration measurements, the removal of lipid and RNA traces and phosphorylation were carried out as already detailed [8]. The distribution of P was studied in the alkaline hydrolysates of fresh lipid-free myosin gained in teflon ampoules (3 M KOH, 105°, 10 h) [9]. The hydrolysates were diluted to 50 ml volume and its aliquots were used for total P determination before separation. The main portion of the hydrolysates were diluted further to 0.005 mol KOH concentration, percolated through a Dowex I×8 column (0.9×6 cm) and separated by chromatographic technique (see Figure 7). The total volume of each effluent tube was applied for P determination.

In the chromatograms the P concentration of each fraction was plotted against the increasing $KHCO_3$ concentration. The P containing peaks were identified by comparing the profiles to the profiles of synthetic amino acid phosphates and by their specific reactions [9, 10, 11].

Results

Myosin was prepared from 20 and 12 g samples of the tracheal mince in parallel experiments. Purification was terminated by gel filtration of the ultracentrifuged (uc-) myosin. The profiles of gel filtration shown in Figures 1 and 2 obtained 19 mg myosin from the 20 g and 12.5 mg myosin from 12 g mince.

It could be seen that both uc-myosins were accompanied with a significant amount of low molecular weight protein and non-protein materials.

The scanning electron microscopic picture of the filamentous system of tracheal myosin induced by 1 mM $K[Au(CN)_4]$ has been presented previ-

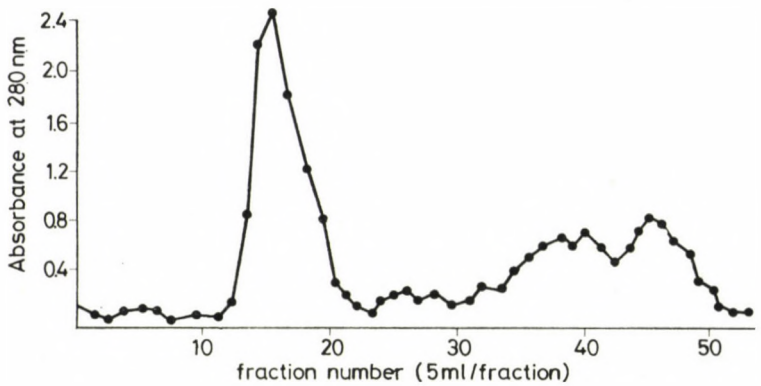

Fig. 1. Gel filtration profile of human tracheal smooth muscle myosin on Sepharose 4B column (1.9×84 cm), prepared from 20 g tracheal mince with KCl-containing solution. The column was equilibrated and eluated with 0.5 M KCl containing 5 mM $KHCO_3$. 19 mg gel — filtered myosin was recovered from 30 mg uc-myosin applied on the column

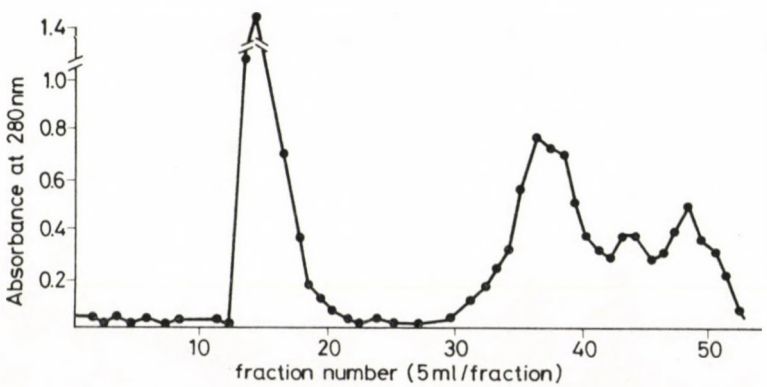

Fig. 2. Gel filtration profile of human tracheal smooth muscle myosin from 12 g mince, on the same column as in Figure 1. 13 mg myosin was recovered from 24 mg uc-myosin applied on the column

ously [8]; now the formation of a filamentous system in gel filtered tracheal myosin is shown in Figure 3.

For comparison, studies were performed with uc-actomyosin. After fractionation, actomyosin was redissolved and diluted to an appropriate volume, and then gel filtered. Gel filtration was carried out under similar circumstances as in the case of myosin, on the same column. Similarly to uc-myosin, the uc-actomyosin fraction also contained a considerable amount of accompanying material. Actomyosin, as shown by the elution profile, was eluted before myosin, starting at tube 9 and was gained and pooled into 45 ml effluent fraction (data not shown).

Both gel filtered myosin and actomyosin could be phosphorylated. Both fractions were able to incorporate a significant amount of phosphoryl group from ATP in spite of the short incubation time. As it is shown in Figure 4, the fresh preparation was saturated to nearly three-fold of the preformed P value. P incorporation into the stored preparation was less, while the P saturation time became somewhat longer; myosin phosphorylation was only about 10% of the preformed P content (data not shown).

The P saturation tracheal myosin depended on the ATP and Mg^{2+} concentration. It is seen in Figure 5 that P incorporation was already reduced at 4 mM ATP, and similarly at 40 mM Mg^{2+} concentration (Fig. 6).

P distribution in the myosin hydrolysate is represented in Figure 7. The alkaline hydrolysate of lipid-free myosin could be resolved by chromatographic separation into 8—9 P containing fractions. Their identification and chemical

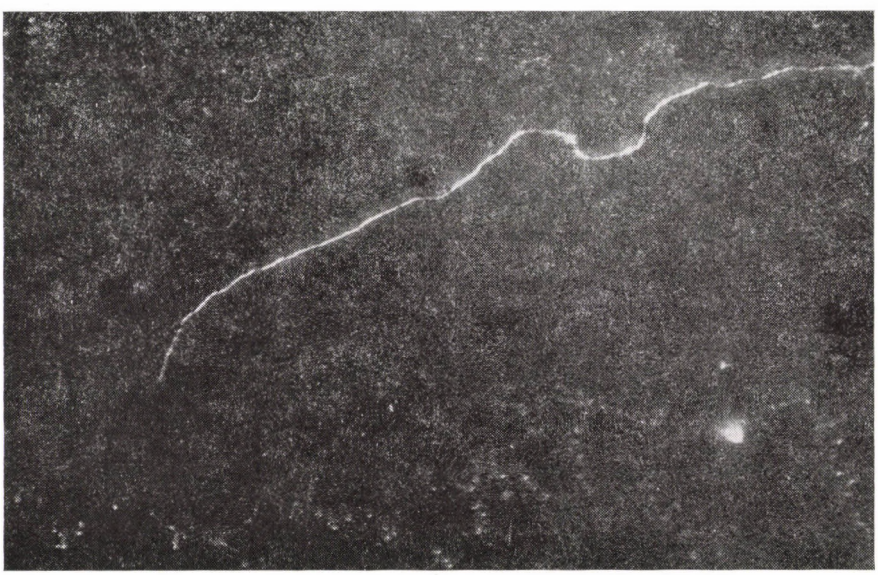

Fig. 3. Scanning electron microscopic picture of the formation of a solitary filament from fresh gel filtered tracheal myosin under the effect of 1 mM $K[Au(CN)_4]$. Magnification $\times 18\,500$

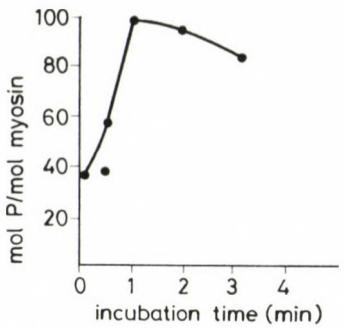

Fig. 4. Dependence of P saturation on incubation time in tracheal myosin in the presence of 1 mM Mg-ATP (1.1 mg fresh gel filtered myosin). Phosphate saturation was started with the addition of Mg-ATP mixture, and terminated by ice-cooled acetone at the marked incubation time. The precipitated protein samples were sedimented by centrifugation, purified from superfluous nucleotide and lipid before determination

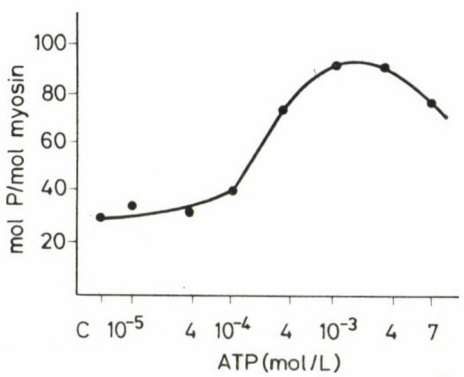

Fig. 5. Effect of ATP concentration on the saturation of tracheal myosin. Incubation was started with the addition of myosin to the ATP containing medium at the marked concentration and terminated after 1 min incubation. Other legends as in Figure 4

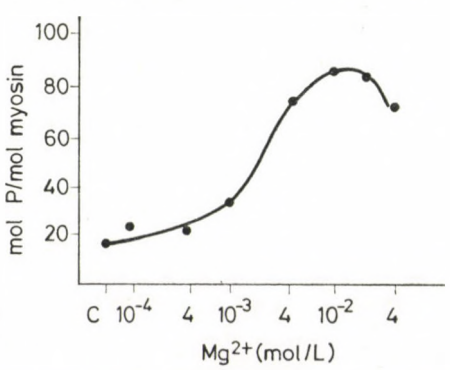

Fig. 6. Dependence of phosphorylation of tracheal myosin on Mg^{2+} concentration. The marked Mg^{2+} concentration was applied in 1 mM ATP containing medium. Phosphorylation was started with the addition of 1.1 mg myosin. Other legends as in Figure 4

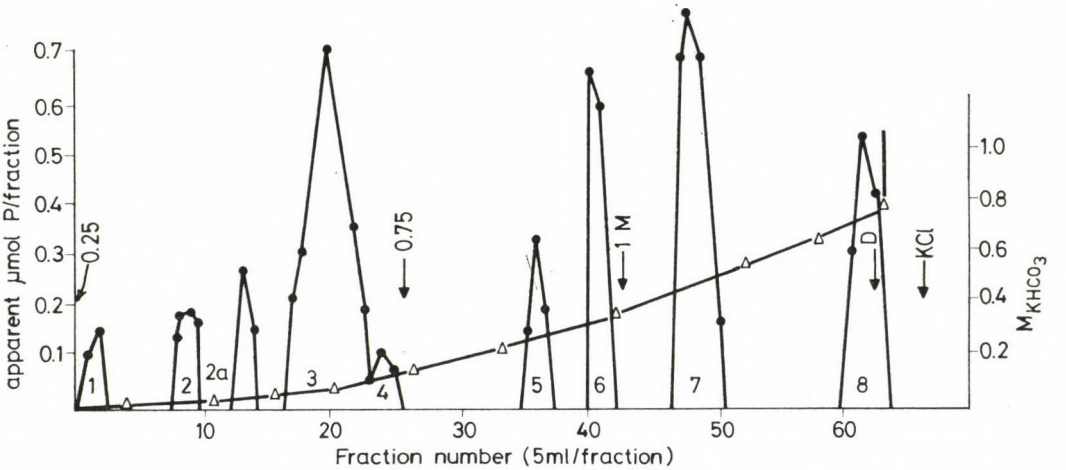

Fig. 7. Elution profile of P containing compounds in the hydrolysate of tracheal myosin. Hydrolysis of lipid-free myosin (5.51 mg containing 0.888 μmol P) was performed in a teflon ampoule (3 M KOH, 105 °C, 10 h). The hydrolysate was diluted to 0.01 M KOH concentration and applied onto a Dowex 1×8 column (0.9×6 cm). Separation was performed with a linear step gradient chromatographic technique using a mixing chamber with a capacity of 160 cm^3 containing 0.02 M $KHCO_3$ and 120 ml of 0.25 mM $KHCO_3$ in the reservoir equipped with an electromagnetic stirrer. The $KHCO_3$ concentration was increased and changed as indicated by the arrows. 1 M $KHCO_3$ was applied onto the column directly (D) without mixing, and 2 M KCl for regeneration of the column. The peaks are represented by P content of tubes in apparent μmol P per total effluent fraction. Peaks in turn: 1, 2, 6 . . . unidentified, 3 . . . P-Arg, 4 . . . Pi, 5 . . . P-Lys, 7 . . . N^{π}-P-His, 8 . . . N^{τ}-P-His. (See in text)

properties were determined partly by comparing their chromatographic profiles to profiles of synthetic amino acid phosphates, and by specific reactions, and thin-layer and paper chromatographic technique [9, 10]. The P containing effluent fractions of the chromatogram were numbered in the order of elution: P-Arg (3), Pi (4), P-Lys (5), N^{π}-P-His (7), N^{τ}-P-His (8). Some other peaks could not be identified. Higher concentrations were found for N^{π}-P-His (7), N^{τ}-P-His (8) and the unidentified component in peak 6 in the tracheal myosin compared to the other myosins. In the samples of mitochondrial, brain, cell and skeletal muscle myosin P-Arg occurred in higher concentration than in tracheal myosin [7, 9, 10, 11].

The chromatographic profile of a phosphorylated myosin hydrolysate is shown in Figure 8. Only a moderate increase was observed in P-Arg concentration as compared to other smooth and skeletal muscle myosins. Nevertheless, less myosin is sufficient for phosphorylation to obtain a proper amount of hydrolysate for the separation of P containing compounds.

The chromatographic profile of the actomyosin hydrolysate is shown in Figure 9. It can be seen that in the hydrolysate of actomyosin, the concentration of P-Arg and N^{π}-P-His (7) was increased. Inorganic P derived from P-Ser

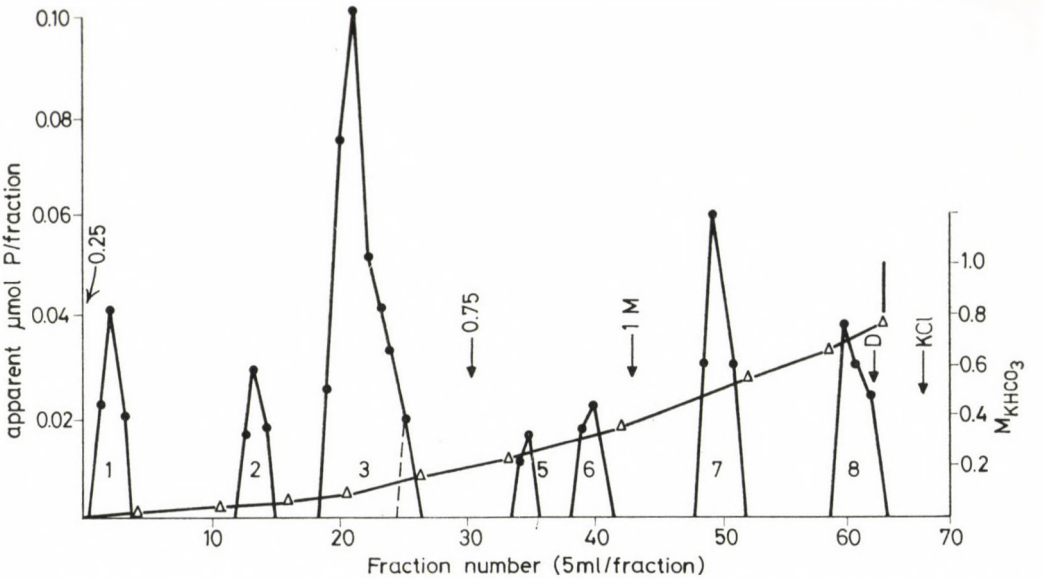

Fig. 8. Effect of phosphorylation on P containing individual fractions of tracheal myosin. The alkaline hydrolysate of lipid- and nucleotide-free myosin (2.25 mg with 0.80 μmol real P content) was separated on the same column, described in Figure 7

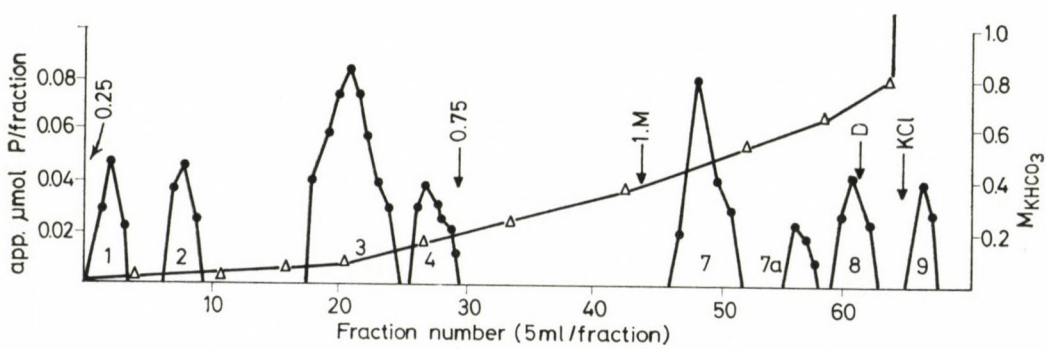

Fig. 9. Distribution of P containing fraction in alkaline hydrolysate of lipid-free tracheal actomyosin (6.5 mg protein with 0.7 μmol real P applied on column). The hydrolysate was separated on the same column as in Figure 7

by alkaline hydrolysis was found in a more significant concentration, than generally occurring in other myosins.

The phosphorylation of an actomyosin hydrolysate is presented in Figure 10. Comparing it to the control sample in Figure 9, it is seen that in the hydrolysate of the phosphorylated sample the peaks of N^{π}-P-His (7) and N^{τ}-P-His (8) as well as peak 6 are represented in a higher ratio than usual with other myosins.

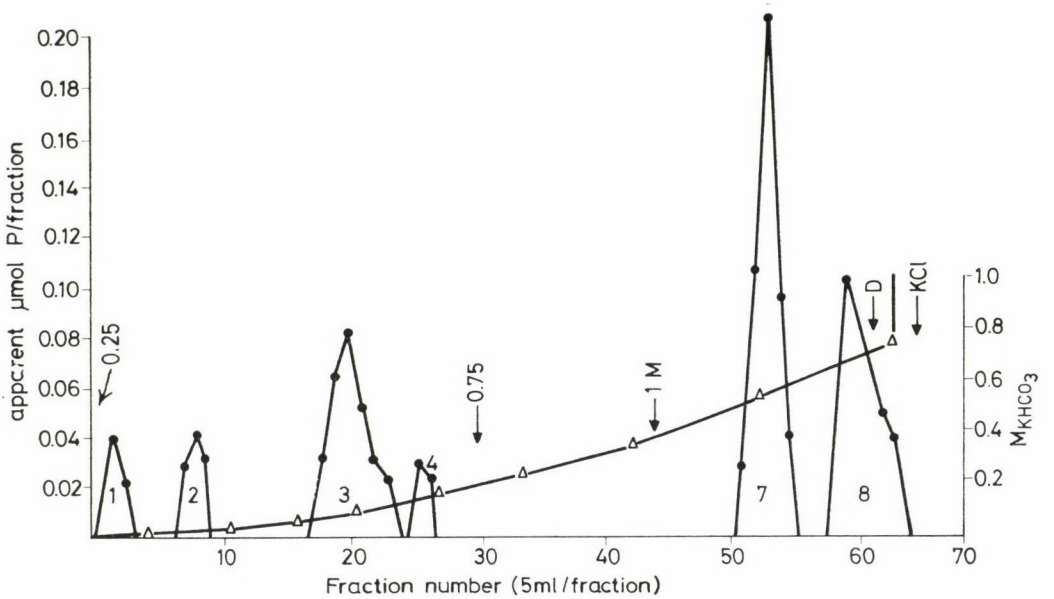

Fig. 10. Effect of phosphorylation on the P containing compounds of phosphorylated tracheal actomyosin (2.5 mg protein with 0.41 µmol real P content applied on column). Separation was performed as in Figure 7

As the N—P linkage in the P-guanidino group of P-Arg, in the P-ε-amino group of P-Lys, and in the P-imidazolyl group of P-His conformers resist basic hydrolysis, the total N—P content can be determined with systematic measurement in the hydrolysates. Still, the effect of alkaline hydrolysis is not known sufficiently in the case of various P peptide linkages and P-amino acid residues, therefore the exact distribution of individual acid labile N—P fractions is uncertain.

Discussion

It has been described that 170 mol P was found in the lipid-free NaCl-myosin preparation of a young patient's tracheal muscle [8]. The present experiments in KCl-myosin 102 mol P and in actomyosin fraction 195.7 mol P/mol were found. A lower P content, 60 mol P was in an old patient's NaCl-myosin, and much less, 38 mol P in KCl-myosin, and 42.5 mol P/mol in KCl-actomyosin. These findings confirmed that myosin with a higher P content was obtained by using a NaCl containing solution instead of a KCl containing one [7]. We analysed both kinds of myosin preparations and observed more lipid-P and RNA as accompanying materials in the NaCl preparation compared to the KCl preparations [7, 19].

The P content of KCl-myosin and KCl-actomyosin could be increased in a phosphorylating medium. Only a short incubation time (1 min, see Figures 4 and 5) was required to increase the 30 mol P content of fresh myosin nearly three-fold. The P content of tracheal myosin and actomyosin became lower at long storage, therefore P release occurs simultaneously, as detected by the P determination. The slow continuous P release is associated with an irreversible alteration of the myosin molecule. Therefore, after a long storage time, P incorporation becomes less by the 20th day; even if the incubation time is longer (3 min), the P content can only be increased from 19.5 mol P to 40 mol P.

P release could be facilitated by 0.42 mM Cu^{2+} treatment to a lower (6—10 mol P) but relatively stable P concentration.

The P content of the light chain fraction could not be increased in stored preparations during a 2—3 min incubation. Long incubation times (15—20 min) have in our opinion no biological significance. The phosphorylation of light chain in these cases may be the result of the formation of ester type phosphates as a side effect; P-Ser may be formed from N-phosphorylated amino acids [6, 20, 29, 30]. In the smooth muscle the formation of P-Ser is possibly an enzymatic process or a regulated side effect which participates in the regulation of the contraction process [4, 5, 6, 17, 18, 22, 28, 29], though Cole et al. [1] have not found a correlation between phosphorylation of the P-light chain and actin activation of the ATPase of chicken gizzard myosin.

It has been concluded from the present results that the filamentous tracheal myosin must exist in its phosphorylated form, thus the P content of fresh preparations must be the highest. As the purified myosins are not any more protected by their environment, P release and weakening of the phosphoryl group uptake and irreversible alterations will result in the structure of the myosin molecule.

Simultaneously a small decrease of ATPase activity of stored myosin was found but this was not associated with a weakening and final cease of the phosphorylating capacity. This was confirmed by the property of subfragment 1 that had been subjected to the intensive procedure and still had a considerable ability for ATP hydrolysis [12], but only traces of a P incorporation capacity could be registered.

The movements of phosphoryl groups have been discovered prior to P release in the course of [^{18}O] and γ-^{32}P ATP hydrolysis while the formation of various [^{18}O] Pi species in myosin and actomyosin was detected "which means that for a phosphoryl group at least 100 reversals of exchange reaction must have occurred" [23, 24, 25, 26]. P movement is induced at the Mg-ATP binding site by rephosphorylation, this results in relaxation and P release [2, 3, 17, 18, 28].

In experiments in vitro we found that some chemicals and bronchial drugs inducing relaxation (adenosine [27] caffeine and especially β_2 stimulators

such as terbutaline [19]) exerted their action not only at the membrane or receptor level, but were directly effective at the molecular level acting on the filamentous system, similarly to spasmogen [14, 15, 16, 22, 28], but dilatatory drugs and adrenergic β_2 stimulators prevent the rephosphorylation and cause a dissociation between the thin filament and the heads of myosin. These results, however require further examinations.

Finally, the results showed that endogeneous phosphate is less in myosin of old subjects compared to young one. Alterations in the diminished macroerg P content (with other changes) are responsible for shortcomings in the performance of bronchial muscle. Bronchospasm [2, 3, 12] becomes more frequent with age and an appropiate relaxation can be maintained only by drugs [13, 16, 22, 27, 28, 29].

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SEASONAL PECULIARITIES OF THYROXINE INFLUENCE ON THE WAY OF SEROTONIN METABOLISM IN THE PINEAL GLAND OF THE RAT

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In intact Wistar pubertal male rats [74] held on LD 8 : 16 (winter) and 16 : 8 (summer) pineal melatonin (M) production and other pineal indoles content serotonin (S), 5-methoxytriptamine (5-MT), N-acetylserotonin (N-aS), 5-hydroxyindoleacetic acid (5-HIAA) and 5-methoxyindoleacetic acid (5-MIAA) were investigated in basic conditions and after 10-days T_4 administration. The results suggest, that intact control in winter comparing with summer demonstrates high M, N-aS, 5-HIAA and 5-MIAA level, but low 5-MT concentration, S level was not changed. T_4 administration in winter produced a pronounced augmentation of all parameters (excluding serotonin), in summer M production not only increased, but appears to be dependent on the active metabolism of his precursors (S, 5-MT, N-aS). The experimental data favour more profound comprehension to the mechanisms of pineal activity regulation.

Keywords: pineal, methoxyindoles, serotonin, environment, seasonal variations.

In previous experiments [1] performed in the dark (in the winter), it has been shown that serotonin metabolism in the rat pineal gland was in a certain connection with the thyroid hormone level in the circulation, as surplus saturation with thyroxine directed the process of serotonin utilization towards its N-acetylation and melatonin, while thyroidectomy induced a contrary effect resulting in the predominance of hydroxydative deamination with the primary formation of 5-hydroxyindoleacetic acid with an acute inhibition of methoxyindole production.

Taking into consideration the role of the pineal gland in the regulation of daily and seasonal biological rhythms, in the present experiments we have studied the reaction of pinealocytes to thyroxine in summer and in winter, in the seasons of the lowest and the highest organ activity.

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Method

The study was performed on 74 pubertal male Wistar rats maintained under conditions of natural alteration of day and night. The experiments were conducted in winter with 8 hours light and 16 hours dark and in summer withs 16 hours light and 8 hours dark. They were injected daily intramuscularly with 100 μg/100 g body weight of thyroxine (Reanal, Hungary), then decapitated in the night between 1 and 3 a.m., that is during the period of the highest pineal gland activity. The indole content in the pineal gland taken from 2—3 animals was evaluated quantitatively with the fluorometric method of Miller and Maickel (1970). Fluorescence was determined by a fluorimeter BIAN-130 at wave length of 365 nm and 470 nm. The indole content was expressed per unit organ weight.

Results and discussion

The results have indicated that administration of T_4 for 10 days induced a decrease of body weight both in summer ($P < 0.001$) and in winter ($P < < 0.001$). As the same time, epiphyseal weight in animals given T_4 in comparison with the control ones increased in winter by 11.25% and in summer by 62.89%. The findings were even more demonstrative data for the relative weight of the pineal gland, as the increase in epiphyseal weight was calculated for body weight which decreased significantly both in summer and in winter.

The findings in Table I showed that in the pineal gland melatonin((M), N-acetyl-serotonin (N-aS), 5-hydroxyindoleacetic acid (5-HIAA) and 5-methoxyindoleacetic acid (5-MIAA) control levels were significantly higher in the winter while the pineal concentration of 5-methoxytryptamine (5-MT) was higher in summer. Total indole concentration averaged 32.77 ng/organ in summer and 72.20 ng/organ in winter. This pointed to seasonal peculiarities of epiphyseal function under physiological conditions. T_4 administration to experimental rats in different seasons elicited a concentration change of practically all serotonin derivatives in the pineal gland. An elevation of the pineal melatonin level under the effect of T_4 occurred in summer ($P < 0.001$) as well as in winter ($P < 0.02$), but in summer the reaction to T_4 was more pronounced. Similar changes were observed in the fraction with 5-HIAA and 5-MIAA: in winter under T_4 effect only a tendency to increase was note but in summer their production was highly significantly augmented ($P < 0.001$). Under the conditions of the present experiment 5-MT and N-aS fractions showed different alterations. Thus, in winter, T_4 administration resulted in a marked elevation of the pineal 5-MT level, in summer the opposite picture was observed as 5-MT the level decreased to traces, N-aS content showed no changes but in summer it was decreased significantly by T_4. Serotonin content in rats with experimental thyroxine toxicosis was stable in the winter but it decreased distinctly during the summer.

The findings indicated that surplus T_4 doses naturally caused on intensification of melatonin biosynthesis but the significance of the process appeared to have seasonal distinctions.

Table I

Effect of surplus doses of thyroxine on indoleamine concentration in the rat pineal gland in different seasons of the year

Season	Groups of animals	Significance	Indole content in the pineal gland (ng/organ)				
			5-MT	Melatonin	Serotonin	N-aS	5-HIAA + 5-MIAA
Winter	Intact control	n	5	5	5	5	5
		$\bar{x} \pm S_{\bar{x}}$	0.40 ± 0.06	12.60 ± 1.71	15.70 ± 3.53	12.80 ± 3.42	30.70 ± 2.99
	10-days thyroxine administration	n	7	7	6	5	5
		$\bar{x} \pm S_{\bar{x}}$	2.49 ± 0.52	21.47 ± 3.26	14.24 ± 3.30	20.70 ± 8.77	46.60 ± 10.27
		P	< 0.01	< 0.02	< 0.8	< 0.5	0.05 < P < 0.1
Summer	Intact control	n	8	7	9	9	8
		$\bar{x} \pm S_{\bar{x}}$	3.93 ± 0.79	1.85 ± 0.60	16.49 ± 1.34	2.06 ± 0.59	8.44 ± 1.01
	10-days thyroxine administration	n	8	8	8	8	8
		$\bar{x} \pm S_{\bar{x}}$	0.09 ± 0.04	14.87 ± 1.45	5.37 ± 1.05	0.61 ± 0.35	21.54 ± 1.98
		P	< 0.001	< 0.001	< 0.001	< 0.05	< 0.001

In winter the prease of melatonin biosynthesis occurred against a stable serotonin content and N-aS, it was indicative of a catalysis of tryptophan utilization as well as the maintenance of the normal serotonin level. In other words, though in pinealocytes the process tryptophan → 5-hydroxytryptophan → serotonin → N-acetylserotonin melatonin accelerated the yield of melatonin the final product, no exhaustion of the initial and intermediate products in melatonin biosynthesis could be observed.

In summer one could observe an other situation. The prolonged T_4 administration caused an acceleration of melatonin biosynthesis even more significant than in winter but this occurred at the expense of a maximum use of the initial products serotonin and N-aS.

We could also observe serotonin metabolism by oxidized deamination. If in winter under the influence of T_4 the process serotonin → 5-hydroxyindoleacetaldehyde → 5-hydroxyindoleacetic acid → 5-methoxyindoleacetic acid was insignificantly augmented, the concentration of 5-HIAA + 5-MIAA showing a tendency to increase at $0.05 < P < 0.1$, but in the summer these changes were more pronounced ($P < 0.001$). Taking into consideration that 5-MIAA is a metabolite of all pineal methoxyindoles, one may suppose that a high melatonin level observed in the pineal gland in winter has been the results of the catalysis of its biosynthesis and, to some extent of an inhibition of its metabolism what seemed to be justified biologically.

We have observed similar changes under the influence of T_4, observing serotonin metabolism in way of its direct orthomethylation with the formation of 5-MT depending on the season. Thus, if in winter the 5-MT level was elevated insignificantly, in summer it decreased to traces ($P < 0.001$). Despite the pronounced lowering of the 5-MT level in the pineal gland of rats treated with T_4 for long, there was no blockade of the direct way of serotonin orthomethylation, as we had previously shown in analogous conditions (Rom-Bugoslavskaya and Goncharko 1980) the catalysis of pineal hydroxyindole-ortho-methyltransferase activity, appearing to be a key enzyme in the biosynthesis of the whole epiphyseal methoxyindolyl group. In the present case the subsequent transformation of 5-MT into melatonin has probably, taken place.

The pineal gland under T_4 influence in light seems to be capable of rapid switches of serotonin metabolism towards a maximum melatonin production and the inhibition of thyroid activity while in the dark time of the year these processes are obviously inhibited. At the same time the indoleamine content (Table I) is indicative of the fact that in summer even the thyroxine stimulated maximum elevation of melatonin production (14.87 ± 1.45 ng/organ) was practically approaching the values characteristic for methoxyindole in winter in control animals (12.60 ± 1.71 ng/organ).

The results thus indicated that a surplus saturation of the organism with thyroid hormones irrespective of the season induced an intensification of

pineal melatonin production which is, in the opinion of a great number of investigators [4, 5, 6, 7] an inhibitor of thyroid activity. Still, the nature of the T_4 effect serotonin metabolism in the pineal gland is considerably conditioned by the season.

The experimental data favour more profound comprehension to the mechanisms of epiphyseal activity regulation with the simultaneous alteration of the environment (lighting) and internal life of the organism (hormonal background).

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CORRELATION OF THE FIBRINOGEN LEVEL WITH BLOOD PRESSURE AND PLASMA RENIN ACTIVITY IN RATS WITH EARLY GOLDBLATT HYPERTENSION

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Goldblatt hypertension was induced in rats by constricting the renal artery on one side. In one group of animals the contralateral kidney remained untouched (two-kidney hypertension), while in the other it was removed (one-kidney hypertension). In the two-kidney hypertension group, renin activity was higher than in the control animals, the fibrinogen was normal both in arterial and venous blood while in one-kidney hypertension the PRA was normal, but the fibrinogen was increased.

A close significant correlation could be demonstrated between blood pressure and fibrinogen.

Keywords: hypertension, fibrinogen, plasma renin activity.

It has been reported [14] that in hypertensive subjects a variation in the fibrinogen level displays a close correlation with systolic blood pressure, a somewhat weaker correlation with diastolic blood pressure, and an even weaker one with plasma renin activity (PRA). In connection with the correlation of hypertension and fibrinogen, assumptions have been put forward as concerns the pathogenesis [10, 11, 16], but we could find no data in the literature on the correlation of fibrinogen and PRA. In an attempt to shed light on the correlations of hypertension, fibrinogen and PRA, we have sought an experimental hypertension model in which these correlations could easily be followed. The choice fell on rats with early Goldblatt hypertension, as was shown [7, 12] that if one of the renal arteries of rats is constricted, in parallel with the development of hypertension (which is not very severe) the PRA too rises in the first 3 weeks (two-kidney hypertension). In contrast, if one of the renal arteries is constricted but the contralateral kidney is removed, blood pressure rose even higher, but the PRA will be normal (one-kidney hypertension). It appeared worth-while to examine how the fibrinogen level and the PRA behave as functions of the change in blood pressure in these two types of hypertension.

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Material and methods

Male R-Amsterdam rats weighing 180—200 g were used. The animals were divided into three groups: Groups I and II contained rats with Goldblatt hypertension, while Group III served as control.

In the animals of Group I (one-kidney hypertension: 10 rats), the left renal artery was constricted with a clip and the right kidney was removed. In the animals of Group II (two-kidney hypertension: 10 rats), the left renal artery was constricted similarly as in Group I, but the right kidney remained untouched. In Group III (8 rats), a sham operation was performed. Surgical interventions were carried out as described previously [13]. Blood pressure was measured by means of a microphonic method in the alert state, after the animals had been heated to 37 °C for 15 minutes [5]. This was done on three occasions before the operation, at 5-day intervals postsurgically, and finally immediately before the animals were killed. On the 18th day postsurgically, the rats were anaesthetized superficially with ether. Through an abdominal incision, the aorta was punctured above the bifurcation, and 3 ml blood was taken for PRA and fibrinogen determination. Simultaneously, from the vena cava inferior below the renal veins 1 ml blood was taken for birninogen determination: this blood was mixed with 3.8% Na-citrate solution in a ratio of 9 : 1. Pretreatment and determination of blood PRA were described earlier [12]. Fibrinogen was determined by the method of Lázár [9] as modified by Gallimore et al. [6]. The results were subjected to statistical evaluation with Student's *t*-test and correlation and regression analysis; data are reported as means ± SE.

Results

Results are presented in Table I.

Blood pressure. Blood pressure in the control group was 121.3 ± 2.3 mmHg, in the two-kidney group, 150.5 ± 4.6 mmHg ($p < 0.001$), and in the one-kidney group, 161.0 ± 0.5.4 mmHg ($p < 0.001$).

Table I

Blood fibrinogen level, PRA and blood pressure in rats with early Goldblatt hypertension. Correlations of these parameters

	Control	One-kidney	Two-kidney
BP mmHg	121.30 ± 2.30	161.00 ± 5.40***	150.51 ± 4.60***
PRA ng/ml	8.51 ± 0.43	8.96 ± 0.56	20.68 ± 2.00**
A. fibr. mg/100 ml	305.00 ± 7.30	483.00 ± 16.10***	307.00 ± 13.90
V. fibr. mg/100 ml	254.40 ± 12.20	328.00 ± 13.10***	246.00 ± 15.50

	Control	One-kidney group	Two-kidney group
Bp — PRA	r = +0.45 n.s.	r = +0.05 n.s.	r = −0.09 n.s.
Bp — A. fibr.	r = +0.05 n.s.	r = +0.34 n.s.	r = +0.77 $p < 0.01$
Bp — V. fibr.	r = +0.12 n.s.	r = +0.80 $p < 0.01$	r = +0.76 $p < 0.02$
PRA — A. fibr.	r = +0.48 n.s.	r = +0.46 n.s.	r = − 0.08 n.s.
PRA — V. fibr.	r = +0.30 n.s.	r = +0.05 n.s.	r = − 0.33 n.s.

A = arterial, V = venous
* $p < 0.05$
** $p < 0.01$
*** $p < 0.001$, compared to the control

PRA. PRA in the control group was 8.51 ± 0.43 ng/ml. In the two-kidney group it was significantly higher than in the controls, whereas in the one-kidney group it was essentially unchanged. No correlation was found between blood pressure and PRA.

Arterial plasma fibrinogen had a value of 305.0 ± 7.3 mg/100 ml in the control group, and 307.0 ± 13.9 mg/100 ml in the two-kidney group, i.e. there was no difference between the two groups. In the one-kidney group, however, the fibrinogen value was 483.0 ± 16.1 mg/100 ml, i.e. significantly higher than in the control group.

Venous fibrinogen had a value of 254.4 ± 12.2 mg/100 ml in the control group, and 246.0 ± 15.5 mg/100 ml in the two-kidney group. Thus, as with arterial fibrinogen, there was no significant difference between these two groups. In contrast, the fibrinogen value for the one-kidney group was 328.0 ± 13.1 mg/100 ml, i.e. significantly higher than in the control group. It is seen that the arterial fibrinogen level was higher than the venous fibrinogen level in all groups, the difference being significant ($p < 0.001$) for each group. Significant linear correlations could also be demonstrated between blood pressure and the arterial fibrinogen in the two-kidney group, between blood pressure and the venous fibrinogen level in both, the one-kidney group, and the two-kidney group.

Discussion

According to the latest observations the renin-angiotensin system, even in Goldblatt hypertension, is not the only factor featuring in the development of hypertension. It was observed that in hypertension the fibrinogen level is elevated both in humans [3, 10] and in experimental animals [11, 16]. Still the role of fibrinogen in the development of hypertension is not known: it is not even known whether it has a primary role in it. Earlier it was reported that the fibrinogen level in hypertensive patients is correlated not only with the elevated blood pressure, but also with the PRA [14]. In our present study, blood pressure and the PRA in the two hypertensive groups behaved similarly as described previously [12, 13]. It is noteworthy that in the individual groups no correlation could be demonstrated between blood pressure and the PRA.

Table I reveals that the fibrinogen level in the two-kidney hypertensive group did not differ from the corresponding value for the control group. However, in the one-kidney hypertensive group both the arterial and the venous fibrinogen levels were significantly higher than those in the control or the two-kidney hypertensive group. This indicates that the presence of an intact kidney may play an important role in regulation of the fibrinogen level. The kidney is known to produce an appreciable amount of fibrinolytic substance [1, 15] and it is possible that this has a role in the regulation of fibrino-

lytic activity of circulating blood [4], though this has not been confirmed by others [1].

It could again be demonstrated that the fibrinogen level is correlated with blood pressure also the two-kidney hypertension group, even though the arterial and venous fibrinogen levels in this group did not differ essentially from the control values. Remembering that such correlations were not observed in the control group, this result appears to confirm that the correlation between blood pressure and the fibrinogen level is a real one. While, however the correlation between blood pressure and venous fibrinogen level in the one-kidney hypertension group was marked, that between blood pressure and arterial fibrinogen level was not significant in the same group.

The absence of the intact kidney in one-kidney hypertension in all probability means a considerable loss of fibrinolytic activity, the kidney with the constricted artery presumably producing less fibrinolytic activity. It seems an acceptable explanation that the extent of the increase varies in accordance with the change caused in renal function by the renal artery constriction responsible for the hypertension. It is also conceivable that while the correlation is marked for the venous fibrinogen, such a correlation is not observed on the arterial side, because of the rapid utilization of fibrinolytic substances. These conclusions may be accepted if it is assumed that the fibrinolytic activity released from the kidneys plays a fundamental role in the change of the fibrinogen level. It should be noted, however, that substances with fibrinolytic activity are also found in other tissues and vascular walls. Nor should it be neglected that both specific and non-specific antifibrinolytic activity occurs in high titre in the blood of humans and experimental animals [8].

In each experimental group, the venous fibrinogen level was significantly lower than the arterial fibrinogen level: this may be explained by the high fibrinolytic activity found in the endothelial layer of the veins [2]. In the present study we have failed to find a correlation between PRA and fibrinogen. Further investigations are needed to provide data concerning the role of fibrinogen in the pathogenesis of hypertension.

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CHROMATOGRAPHY

These Chromatography conferences are part of the series of American—Eastern European Symposia on Liquid Chromatography initiated in 1981. The interest in these symposia has been increasing ever since due to the high scholarly standard. The conferences are regularly attended by chromatographers from up to twenty countries including the USA, the USSR, Sweden, Canada, the GDR, Italy and France.

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