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EXPERIMENTAL AND CLINICAL ASPECTS OF THE BIOSYNTHESIS OF ANDROGENIC STEROIDS

By

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Two important milestones in the history of endocrinology relate to the function of the testicles. BERTHOLD discovered the endocrine nature of testicular function more than one hundred years ago and the first use of androgens for therapeutic purposes is linked with the name of BROWN-SÉQUARD.

Sudden upswings in scientific research are always connected with the development of new methods. The new progress in biochemistry would have been inconceivable without the chromatographic procedures and isotope techniques. It is by these methods that we have gained insight into the successive phases of steroid metabolism and that isolation, identification of the individual products and their measurement in terms of nanograms have become possible.

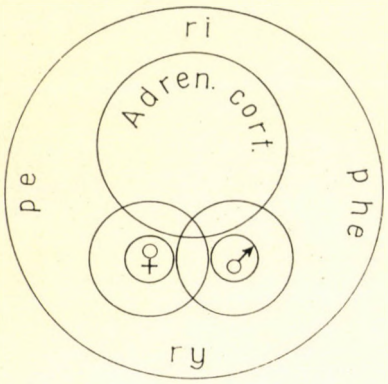

Fig. 1

The three-gonad theory formulated by the present author a few years ago expresses the fact that each of the three endocrine glands has all enzyme systems involved in steroid genesis at its command, in other words, that there is no sharp functional demarcation between these organs.

First of all it has to be made clear what we understand biologically and chemically by the term androgens. From the biological point of view, androgens are substances stimulating the growth of the male reproductive organs (BAR-

DIN and MAHOUDEAU, 1970). As regards their chemical structure, the androgens are C_{19}-steroids. The bisexual function of LH is reflected by the close relationship between the structures of the steroid hormones of the yellow body and those of the interstitial cells of the testicles. The structural formulas of the gonadal hormones, i. e., the two steroids of the female gonads, 17β-oestradiol and progesterone, and the testicular steroid testosterone, are presented in Fig. 2. It can be seen that progesterone is more closely related to testosterone than to the other female hormone. Both progesterone and testosterone are subject to LH-control, therefore it is not surprising that progesterone has a virilizing effect. Progesterone administered during pregnancy produces virilization of a female foetus.

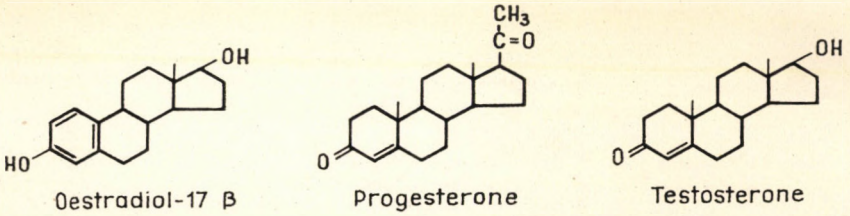

Fig. 2

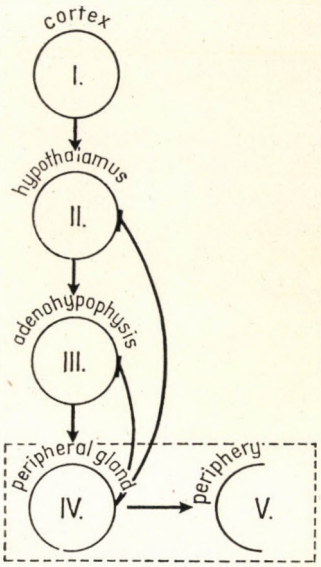

Fig. 3

It is no longer felt by the present author that the endocrine hierarchy is limited to the three central systems where his earlier theory has confined it. In his present view, it extends far beyond these systems, upward to the cortex of the psychoassociative sphere and downward to the totality of target organs, tissues and cells, in brief to the periphery (Fig. 3). A closer study of

the periphery is, therefore, not only of theoretical but also of clinical importance. The concept also implicates that all endocrine syndromes, irrespective of their origin, invariably manifest themselves at the periphery. This makes it our objective to follow up the various hormones formed in the individual endocrine glands all along their pathways down to the target organs, as far as the cellular and subcellular elements and to locate the individual cell structures—microsomes, mitochondria, cell membrane, nucleus—which may constitute the site of action of the given hormone. The question whether there exist any specific androgenophile receptors also belongs to this line of study.

The main sources of androgens are the testicles, the ovaries, the adrenal cortex and the placenta. There has been some uncertainty concerning the question whether the biosynthesis of androgens is confined to the last-named organs. On the evidence of our studies (JULESZ et al., 1971) the human skin is provided by all enzyme systems required for androgen synthesis. As regards the site of production, we have two sources of information. Estimation of androgens in the respective organs provide reliable evidence. Studies of the other type are based on the hormone concentration in the plasma of effluent venous blood of the organ. If it exceeds that of peripheral blood, the respective endocrine organ is presumably the site of production of the androgen in question.

The pathway of transport is the blood plasma. Transformation of androgens begins as soon as they enter the blood stream, though at this phase it is prevalently a binding to the plasma proteins rather than a radical transformation that takes place (WESTFAHL, 1970). The essential factor here is the reversibility of binding. Earlier, binding to proteins was regarded as part of the transport function. The present view is that the binding of steroids to proteins serves for the biological inactivation of the hormones. Release of the bonds makes the reactivated hormone available at the necessary site at the required time. The blood plasma is thus considered a reservoir of active steroids and of other hormones.

Binding to protein weight also provides a protective barrier to the hormone against chemical or enzymatic damage.

The question which now arises concerns the other end of the path. What happens in the target organs? Our knowledge concerning this point is still uncertain. We do not know whether it is the hormone which passes the nuclear membrane and occupies the place of the receptor after its release from the carrier-protein, or whether it is rather the carrier-protein which forms links with the receptor-structures. Certain new facts may, however, furnish some clues to the understanding of the problem. It has been found, for instance, that testosterone administered in vivo to normal rats significantly stimulates lipid biosynthesis in the mitochondria of the accessory sexual glands (DOEG, 1969). Other authors (TWETER and UNHJEM, 1969) noted a selective uptake of androgens in a macromolecular fraction of the supernatant of ultracentrifuged

homogenisates of seminal vesicles of rats treated with [1,2-^{3}H] testosterone. This fraction is regarded as an androgen-receptor. In other experiments in which labelled testosterone was administered to castrated rats, 55% of the total radioactivity of the prostate was demonstrable in the prostatic nuclei and only 30% in the supernatant. The finding that the 11β-hydroxylase-system takes effect at the inner mitochondrial membrane subfraction of the rat adrenal (DODGE et al., 1970) shows that localization has indeed reached a high degree of accuracy.

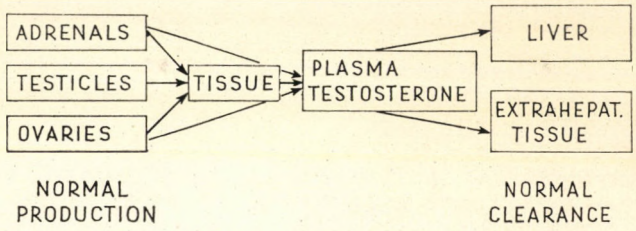

Fig. 4

Testosterone as well as precursors of minor androgenic activity pass from the adrenal cortex, the testicles and the ovaries into the blood stream. Conversion of the prehormones to active androgens may occur in addition to these endocrine glands also in the target-organs. A purely static interpretation would be inadequate to account for the height of the plasma testosterone level which is determined, apart from the extent of production, also by the amount of androgens extracted from the blood and utilized or inactivated thereafter, in other words, by the normality of clearance. This is illustrated by Fig. 4. From these considerations it follows that the production rate is measurable by metabolic clearance techniques on the grounds of the equation,

$$PR = MCR \times T,$$

where PR represents the production rate, T the plasma testosterone concentration and MCR the metabolic clearance rate, by which we understand that plasma volume which is irreversibly cleared of the given steroid in the unit of time (TAIT, 1963).

In view of these facts it is understandable why the plasma testosterone level remains normal in numerous virilized females. This can only occur at the cost of an increased rate of testosterone metabolism (SOUTHREN et al., 1969).

The part played by the binding of steroids to proteins in their metabolism and inactivation has been referred to earlier. Testosterone binding β-globulin is identical with the oestradiol binding β-globulin (STEENA et al., 1968). It is, moreover, known that testosterone can be displaced from the β-globulin by

oestradiol. In other words, there is a competitive antagonism between the two substances. Incidentally, androgens may attach to other plasma proteins including albumin.

Discovery of the conjugation mechanisms has helped to gain a closer insight into the metabolism of steroid hormones (SMITH and WILLIAMS, 1970).

The fact that steroid sulphates are synthetized in active endocrine tissue, is at variance with earlier theories and lends support to the hypothesis that the steroid sulphates play a specific metabolic role. In fact, the sulphate esters formed in the presence of sulphokinase need not split off the sulphate group before being metabolized with considerable intensity.

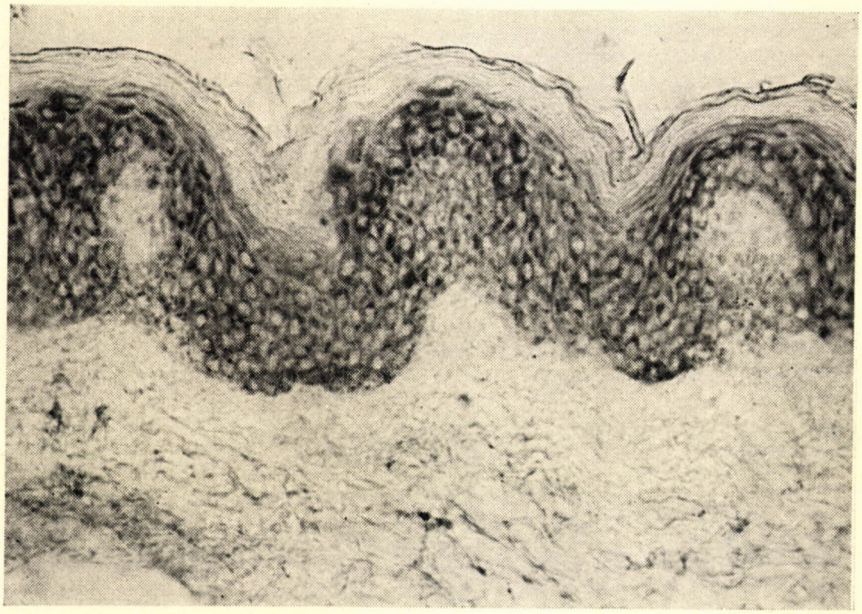

Fig. 5

The specific enzymes play a primary part in the biogenesis of steroids including androgens. Though the metabolites formed in the course of incubation permit their indirect identification, it must be sought to bring direct proof of their activity by histochemical methods. The importance of this is shown by the following observation. It has been possible to show the presence of dehydroepiandrosterone soon after embarking upon studies of the biosynthesis of the androgens of human skin, and at the same time we were the first to demonstrate Δ^5-3β-hydroxysteroid dehydrogenase activity in human skin by histochemical methods (JULESZ and HORVÁTH, 1963) (Fig. 5). If this finding was to prove true, then androst-4-ene-3,17-dione was among the steroids revealed by chromatography but not yet identified at that time. This proved to be the case, indeed, on the evidence of later studies.

The sequence of androgen biosynthesis is fairly well known. The question will not be discussed here in closer detail; Fig. 6 merely serves to recall the process.

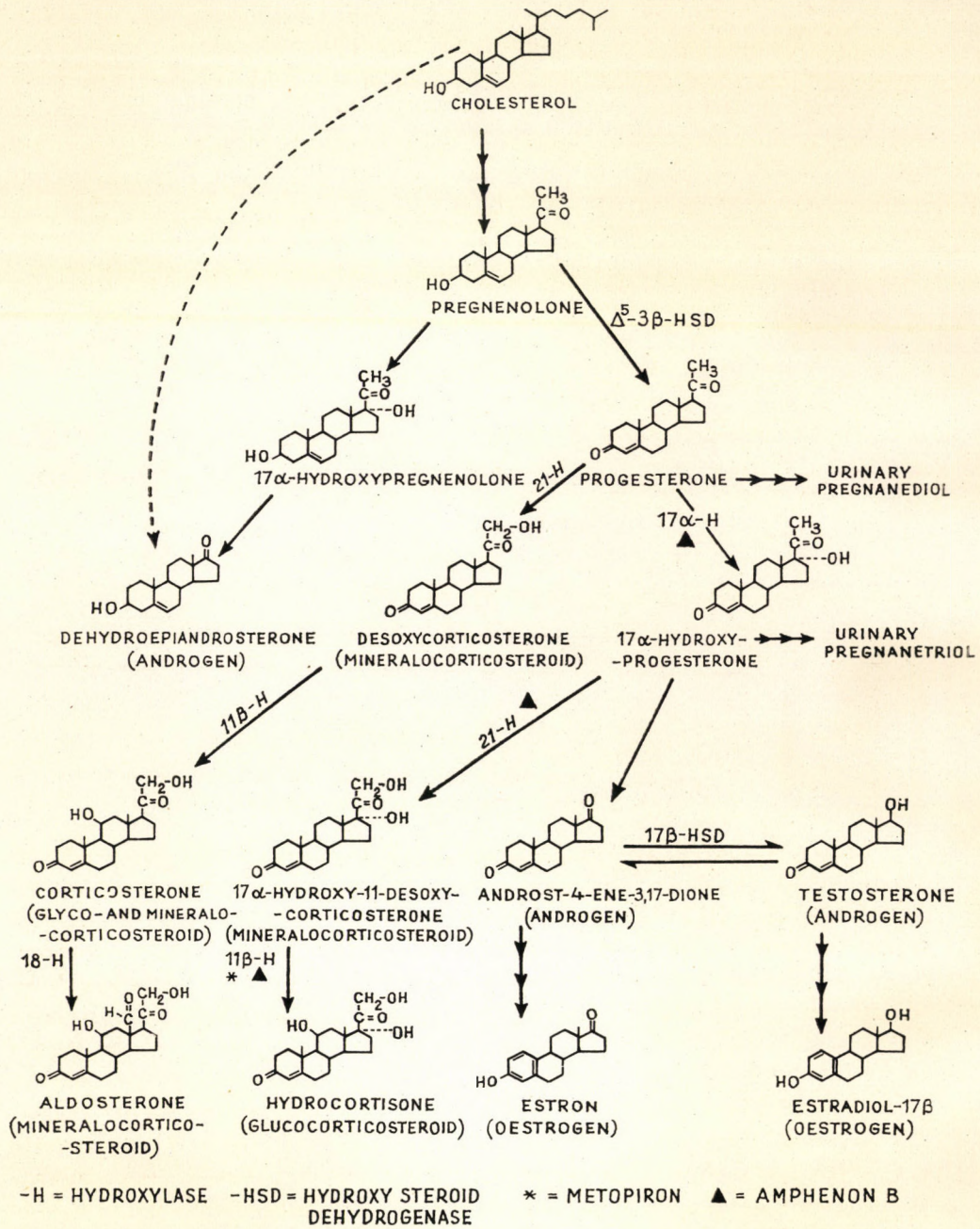

Fig. 6. Biosynthesis of adrenocortical steroids

It is on the basis of Fig. 7 that I have adapted the current haemodynamic terms backward and forward failure to the present subject. Certain enzymes are indispensable for a successive, uninterrupted sequence of steroid biosynthesis in the direction of glucocorticoid synthesis. Congenital errors of metabolism or the presence of certain steroid analogues are liable to block these enzymes. As a result, the production of cortisol is inhibited (forward failure), the control of ACTH is lacking, the secretion of adrenal steroids is enhanced up to the site of blockade, and the transformation of the steroid into the successive stage is inhibited. Consequently, blocked steroids and their metabolites accumulate in the urine: backward failure.

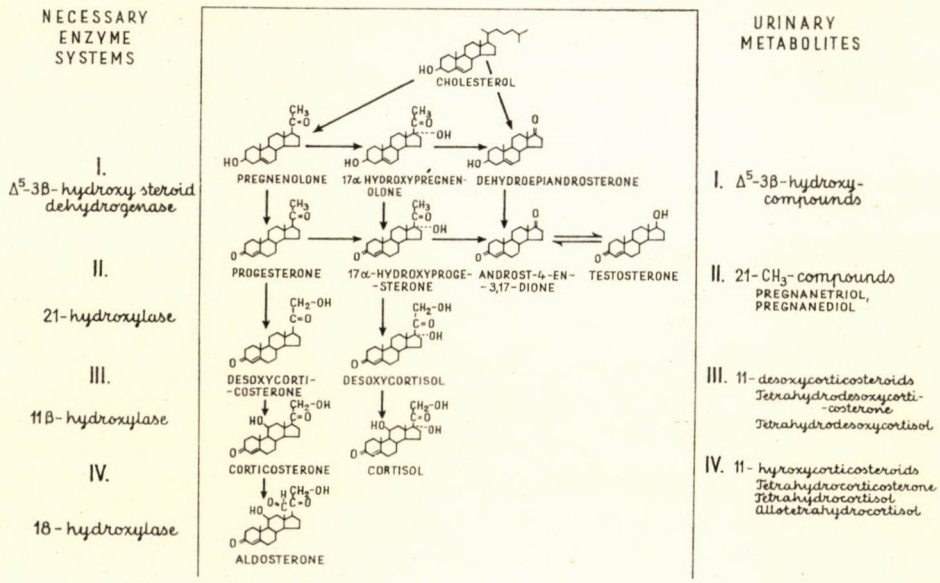

Fig. 7. Enzyme defects in congenital adrenocortical hyperplasia

Interesting facts have come to light in recent years (REDDY and STREETO, 1968). ACTH increases the intracellular amount of cyclic adenosine-3',5'-monophosphate (HAYNES, Jr. 1958). This has been found to be due to an intensification of adenylcyclase-activity. 3',5'-AMP penetrates the membranes of adrenal cells, stimulates steroid genesis and, in some way, also the synthesis of proteins. The results of other studies seem to support the hypothesis that cyclic adenosine-3',5'-monophosphate is actually the intracellular mediator of ACTH (FARESE et al., 1969).

Of the new substances of high biological activity it is in the first place the prostaglandins which offer promising avenues of study. Gynaecological research workers are well acquainted with the in-vitro effect of prostaglandin in progesterone synthesis and on steroid genesis in general (SPEROFF and RAMWELL, 1970). PGF_2 closely resembles LH in its effect, only LH acts on

the interstitial cells in addition to the yellow body. It might, therefore, be rewarding to extend the lines of research to the biosynthesis of androgens.

Since we had become acquainted with the androgens, testosterone was generally believed to possess the greatest androgenic activity of all these substances and that its reduction products formed in the course of its metabolism were of slighter biological activity. This, however, is not the case. In recent years, several androgen metabolites have been identified which proved superior to testosterone in their androgenic activity. Dihydrosterone (17β-hydroxy-5α-androstane-3-one) is regarded as the intracellular effector of testosterone (BRUCHOVSKY and WILSON, 1968; MAINWARING, 1969; BASHIRELAHI and VILLEE, 1970). Androstenediol (3β,17β-dihydroxyandrost-5-ene) is also an androgen more active than testosterone.

The clinical aspects of androgen metabolism will be dealt with here quite briefly. The adrenocortical enzymopathies are hereditary errors of metabolism due to particular enzyme defects accounting for the failure of the adrenal cortex to form steroids of normal pattern or in the necessary amounts. This constellation is prejudicial to the normal feed-back mechanism, in consequence, an excessive production of androgens and consequently a virilization of the female ensues. (Feminization of males is less common.)

The syndrome in its simple form, is confined to virilization, due to an incapacity of forming C_{21}-hydroxylated steroids. The complete failure of C_{21}-hydroxylation is associated with a salt-losing condition, production of 11-deoxysteroids being likewise absent. An atypical form is marked by a deficient Δ^5-3β-hydroxysteroid dehydrogenase activity and, as a result, by a failure of Δ^4-3-ketosteroid production.

One of the types of congenital adrenal hyperplasia is characterized by arterial hypertension, attributable to an excessive production of 11-deoxysteroids (DOC or an abnormal mineralocorticoid) as a result of a congenital 11β-hydroxylase defect.

A particular form of congenital adrenocortical hyperplasia is characterized by febrile periods (GONZALES and GARDNER, 1956; KAPPAS et al., 1957; SEGALOFF et al., 1957) which have been connected with an excessive production of etiocholanolone (COHN et al., 1961).

The identification of enzyme defects allowed to make proper use of glycocorticoids for their management (WILKINS, 1950) and helped to develop new diagnostic procedures including the estimation of the trophormone reserves.

The significance of enzyme inhibitors has increasingly been recognized. Numerous substances which would not have been expected to interfere with steroid genesis, possess an activity of this kind on the evidence of recent studies. For instance, the hypolipaemizing clofibrate (chlorophenoxyisobutyrate) has been found to block the 11β-hydroxylation of steroids (MCINTOSH et al., 1970).

In 1950, WILKINS reported the case of a person who, despite being in possession of testicles, had a feminine appearance with the absence of body hairs and proved completely resistant to the virilizing effect of long-term massive-dose testosterone and methyltestosterone treatment. From this the author concluded that the responsible factor was a genetically determined testosterone-unresponsiveness of the target organs rather than an inadequacy of testosterone production. It has then been confirmed by other authors that in testicular feminization the plasma testosterone level is normal. Still, the patient fails to respond to long-term administration of androgens. It has been demonstrated by BRUCHOVSKY and WILSON (1968) that testosterone remains inactive unless converted by the respective tissues to 5α-dihydrotestosterone. These findings would seem to indicate that the cause of the resistance to testosterone in testicular feminization lies in a genetically determined inadequacy of the cell system responsible for the conversion of testosterone to dihydrotestosterone (NORTHCUTT et al., 1969). According to other authors, in testicular feminization the 5α-reduction of testosterone by the skin is at fault (MAUVAIS-JARVIS et al., 1969a, 1969b).

As to our own experiments they have been centred on the issue of hirsutism. This is decided in the skin and in its accessory structures. The female develops hirsutism if the androgen-oestrogen proportion in the environment of the hair follicles shifts to the profit of androgens. In roughly 40% of our hirsute female patients, none of the potentially involved endocrine organs displayed any abnormality. The syndrome is termed, on these grounds, idiopathic hirsutism. This suggested the possibility that the skin itself might be a site of androgen formation. We indeed succeeded in demonstrating the presence of androgens in the skin and subsequently to find out a variety of steroids in large amounts, partly as sulphate ester conjugates, in the sweat of apocrine glands. We also found the water-soluble sulphate esters to imbibe the hair and to be extractable from there by simple soaking techniques. Via in vitro incubation studies, we have been able to show with the aid of the reverse radioisotope dilution method that in the presence of skin 11 to 15 metabolites and testosterone are formed from the labelled steroid substrates studied. It is a point of interest that steroids of poor androgenicity are transformed into testosterone of potent androgenic activity. From the incubation studies it also emerged that female or male skins have identical enzyme systems at their command. The difference seems to be therefore merely quantitative. In one of our cases, even the skin of an agonad completely hairless male of the karyotype XY, was capable of testosterone synthesis.

On the evidence of these studies we feel justified in attributing idiopathic hirsutism, in a proportion of cases, to an enzymopathy of the skin. Here, the use of antiandrogens (cyproterone acetate) seems to offer definite therapeutic possibilities in the future, the inhibitory effect of the drug having

been found to affect those tissues which contain androstenedione in substantial amounts (WHALEN et al., 1969). It has furthermore been demonstrated that antiandrogens inhibit the DHA, A and T induced growth of the prostate and the seminal vesicles in castrated rats (WALSH and GITTES, 1970).

In conclusion, it is seen that in the research team formed for the purpose of steroid studies, the clinician has to become familiar with steroid chemistry and the biochemist with the clinical aspects of endocrinology. However, further investigations into steroid chemistry can no longer dispense with protein and enzyme chemistry, thus exemplifying the old truth that there is no such thing as standstill in science.

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EXPERIMENTAL AND CLINICAL ASPECTS OF OESTROGEN METABOLISM

By

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1. History

Of all steroids the oestrogens have been known for the longest time and studied the most closely. The relevant literature is too extensive to be summed up in the framework of a brief report. Every account of this kind is, therefore, necessarily subjective, reflecting the individual sphere of interest of its author.

Allen Doisy	Demonstration in ovaries	1923
Aschheim Zondek	Demonstration in pregnancy urine	1927
Doisy Butenandt	Isolation	1929 1929
Marrian	Separation	1930
Butenandt	Structure	1931

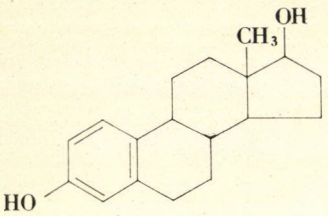
Fig. 1

In 1923, ALLEN and DOISY showed that ovarian extracts induce the appearance of anuclear cells, or rather, of clots ("Schollenzellen") in the vaginal smear of castrated rodents, a pattern identical with that of natural oestrus (Fig. 1). A few years later, ASCHHEIM and ZONDEK (1927) reproduced this effect with pregnants' urine from which DOISY et al. (1929) and, independently, BUTENANDT (1929) succeeded in isolating oestrogens. Shortly thereafter (1930) oestradiol and oestriol were isolated by MARRIAN, likewise from pregnants' urine and the structural formulas of the substances were clarified by BUTENANDT and HILDEBRAND in 1931.

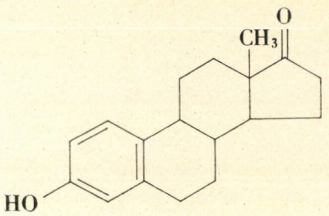
1.1. Oestrogens of the human body

More than 20 oestrogens are known which occur in body fluids and tissues and are extractable from them. The three most important substances of this kind are *17-beta-oestradiol*, the most potent oestrogen, ***oestrone*** and ***oestriol***. The last-named product is the least potent of all but occurs in the largest amounts in urine (Fig. 2).

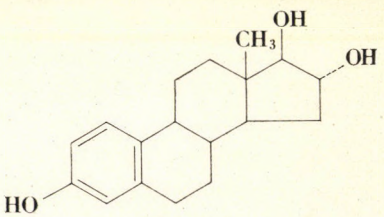
Two other derivatives, *17-epioestriol* and *15-alpha-oestetrol*, the structural formulas of which are also presented here will be dealt with in closer detail later (Fig. 3). The other oestrogens so far identified and assumed to be of lesser significance, will be merely listed here (Fig. 4).



17 β-Oestradiol

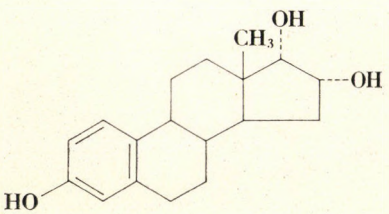


Oestrone

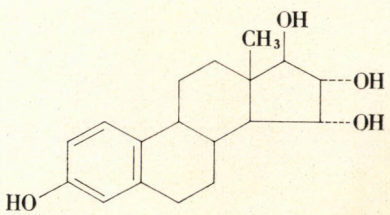


Oestriol

Fig. 2



17-Epioestriol



15 α-Oestetrol

Fig. 3

16-Epioestriol
16,17-Epioestriol
16 α-Hydroxyoestrone
16 β-Hydroxyoestrone
15 α-Hydroxyoestradiol
16-Ketooestrone
16-Keto-17 β-oestradiol
2-Methoxyoestrone
2-Methoxyoestradiol
2-Methoxyoestriol
18-Hydroxyoestrone
11 β-Hydroxy-17 β-oestradiol
Equilenin
Equilin
6-Dehydro-oestrone
Lumioestrone

Fig. 4

2. Chemical properties of the oestrogens

The oestrogens are compounds with a sterane nucleus of 18 carbon atoms. They dissolve readily in organic solvents and in fats and belong to the steroids which are particularly resistant to chemical and physical factors. This is due to the aromatic nature of ring A which with its three double bonds typifies the tendency to attain the lowest energy level which confers maximum stability to cyclic compounds of this kind. They owe their reactivity to the phenolic OH group at position 3.

The essential chemical characters of the three main oestrogens have long been established. They are essential for the isolation and estimation of these substances (Fig. 5).

	m.p. °C	(α) D	pks CH_3OH	max—mi
Oestradiol	178	+ 81° Di	—	—
Oestrone	260	+163° Di	9.36	280 (2300)
Oestriol	280	+ 68° Al	9.11	—

Di = dioxane
Al = alcohol

Fig. 5. Chemical properties of oestrogens

2.1. Nomenclature of the oestrogens

The oestrogens are called follicular hormones (folliculin), on the basis of their site of production, oestrogens on the basis of their activity and phenol steroids on the basis of the hydroxyl group at position 3 of the aromatic ring A. The cyclopentane-phenanthrene ring system with 18 C atoms is the *oestrane-nucleus*. The prefix "epi" indicates a β instead of the β-position, i. e., a reverse configuration with respect to the given position (Fig. 6).

According to	occurrence	follicular hormone
According to	effect	oestrogen
According to	aromatic (A/3 OH)	phenol steroid
Oestrane-nucleus: a cyclopentano-phenanthrene nucleus of 18 C-atoms with a methyl group at C-13		
EPI = β instead of β, i.e., a reversed configuration		

Fig. 6

3. Occurrence of oestrogens in the human body

The follicular production of oestrogens has been ascribed by many authors to both the theca cells and the granulosa epithelium. McKAY and ROBINSON (1947), DICZFALUSY and LAURITZEN (1961) and others, however, have been able to show that the sole site of oestrogen production within the follicle is the *theca*, even in the case of granulosa-cell tumours (McKAY et al., 1945), though the presence of granulosa-cells also seems to be involved in some manner (Fig. 7).

Theca folliculi (granulosa cells)
Sertoli cells (Leydig-cells)
Adrenal cortex
Chorion (syncytium)

Fig. 7

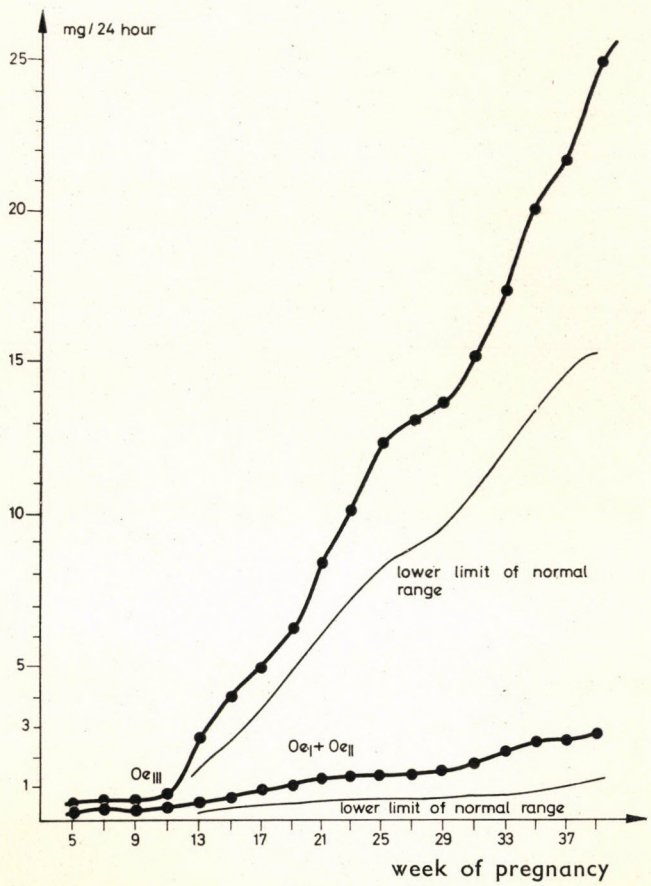


Fig. 8

Production of oestrogen in the testicles is confined, under normal conditions, to the Sertoli-cells (MADDOCK and NELSON, 1951; NELSON, 1951); in abnormal conditions, Leydig's cells may also participate in oestrogen-production (BERTHRONG et al., 1949).

Oestrogens are secreted by the adrenal cortex, too, but not to an extent of any significance, at least not under normal conditions, in the absence of enzymopathies.

The principal site of oestrogen production is the placenta, more precisely the superficial epithelial layer of the chorion, the syncytium.

The steroid-forming capacity of the placenta is excessive, since, by the end of pregnancy, secretion of oestrone and oestradiol attains 100 times, and that of oestriol 1000 times, the normal values with a daily progesterone secretion amounting to 100 mg (Fig. 8).

4. Biosynthesis and metabolism of oestrogens

The three main oestrogens are interconvertible, though only within certain limits (Fig. 9). As illustrated by the diagram, if biosynthesis has advanced to the stage of oestrone, this can be transformed into oestradiol, or conversely, and both can be converted into oestriol. On the other hand, reversal or oestriol into oestradiol or oestrone is not possible. It is probably for this very reason that oestriol accounts for the largest part of the oestrogens excreted with urine.

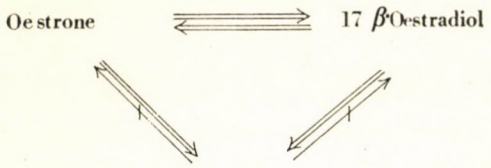


Fig. 9

According to present knowledge, oestrogens are built up from compounds of lower C-number by enzymatic activity, the source of energy being provided by ATP (Fig. 10).

The magnitude, direction and rate of local oestrogen production depends on the genetically determined pattern of the enzymes of the endocrine organs subject to neuro-endocrine control. However, the individual structures involved in oestrogen production have particular enzyme systems, for instance,

the gonads contain large amounts of 3-beta-hydroxy-dehydrogenase and 11-beta-hydroxylase.

The oestrogen constellation prevailing during pregnancy involves the participation of the foetus, although the processes taking place in the foetal

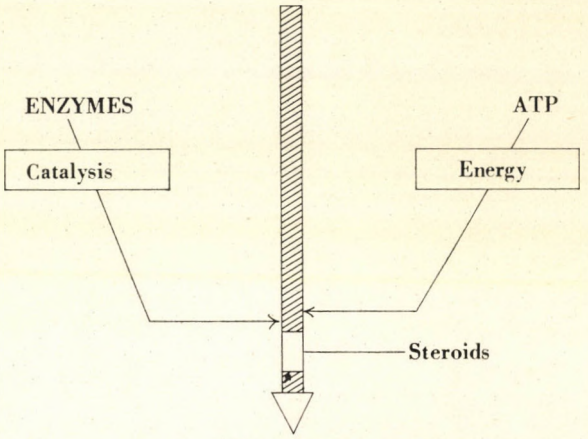


Fig. 10

Enzyme system	Placenta	Foetus
3β-ol-Dehydrogenase	high	low
Aromatization	high	low
Sulphatase	high	low
Sulphokinase	low	high
11β-Hydroxylase	?	high
16α-Hydroxylase	low	high
17α-Hydroxylase	low	high
21-Hydroxylase	?	high
17,20-Desmolase	low	high
Steroid synthesis from acetate	low	high

Fig. 11. Enzyme activities in placenta and foetus

organism are different from those of the placenta, the presence of enzymes being restricted to individual sites of the foetal body (Fig. 11).

The normal succession of enzyme-directed steroid production requires an organic integration of the enzyme systems within the mitochondria and an adequate induction of the reactions by the trophormones. If these prerequisites are satisfied, the biosynthesis of oestrogens proceeds as follows:

Acetate is converted through cholesterol to pregnenolone and progesterone (Fig. 12). The subsequent stages comprise the 17-hydroxy derivatives of these compounds and DHA or androstenedione, respectively, each formed by the agency of the appropriate enzymes (Fig. 13). Finally, 17-beta oestradiol and oestrone are formed as a result of aromatization involving the activity

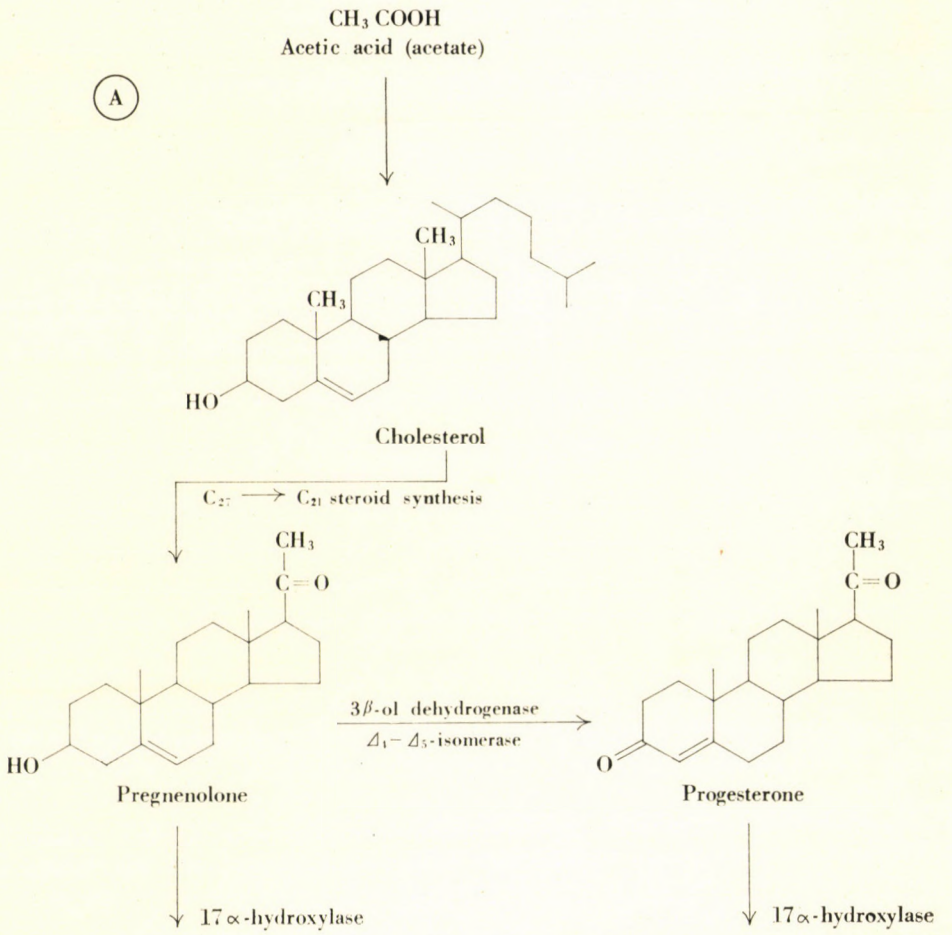


Fig. 12

of 17-beta-ol dehydrogenase (Fig. 14). Dimethylation is rapid, as confirmed by MEYER (1955) who obtained oestrone in good yield from androst-4-ene 3,17-dione of 19 C-atoms in the presence of placenta or of corpus luteum. Oestrone is still more readily formed from 19-hydroxy-androstenedione under the action of human placenta.

Placenta perfused with labelled acetate has been found to form oestrogens (LEVITZ et al., 1955). Conversion of testosterone to oestrogens was noted

under similar conditions by BAGGETT et al. (1956). The profound involvement of cholesterol in oestrol and oestradiol synthesis was confirmed by WERBIN et al. (1957). Having administered 38 mg 3-^{14}C-cholesterol (87.4 μC in 6 days) to a pregnant woman, they noted the excretion of labelled oestrone. Conversion of progesterone to oestrone has also been proved by experimental evidence.

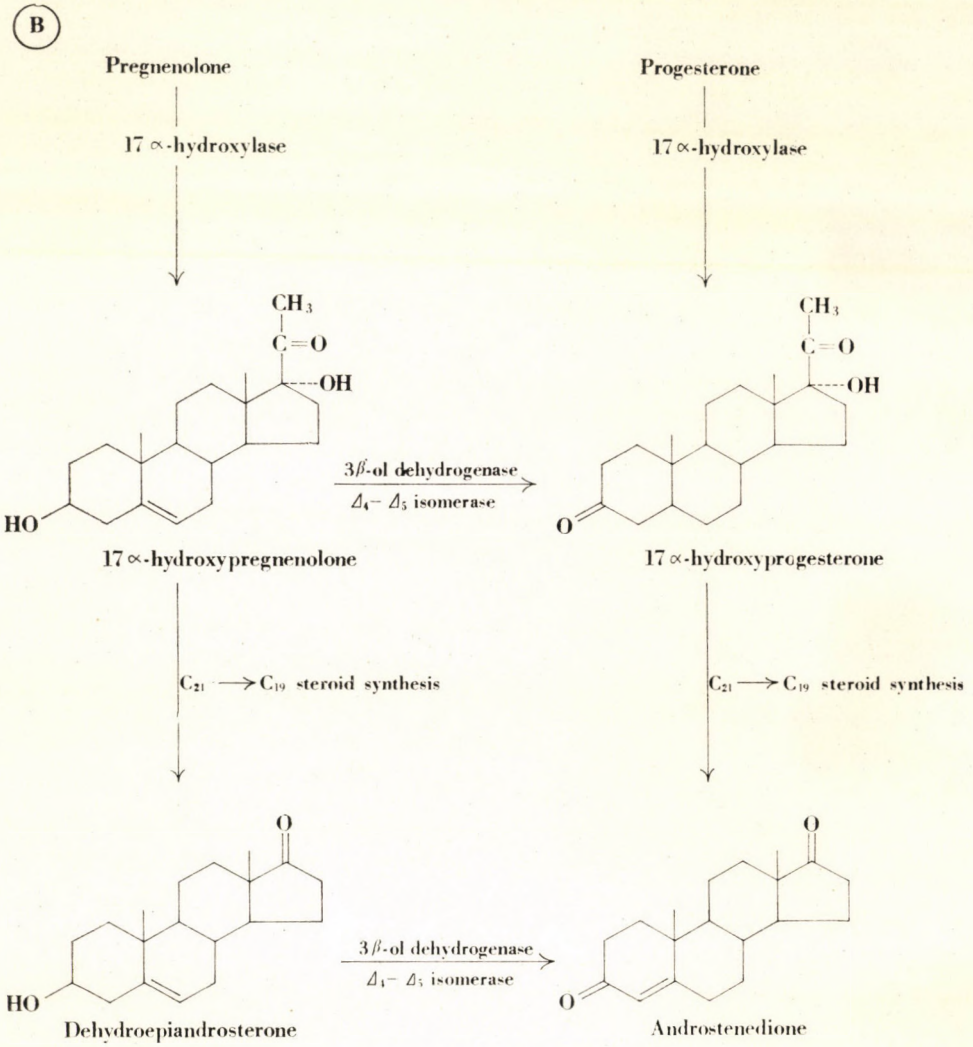


Fig. 13

In this manner, all essential steps of oestrol-oestradiol synthesis have been verified experimentally. It must be added that, as emerged from subsequent investigations, this pathway of synthesis applies to the foetoplacental system only with certain modifications.

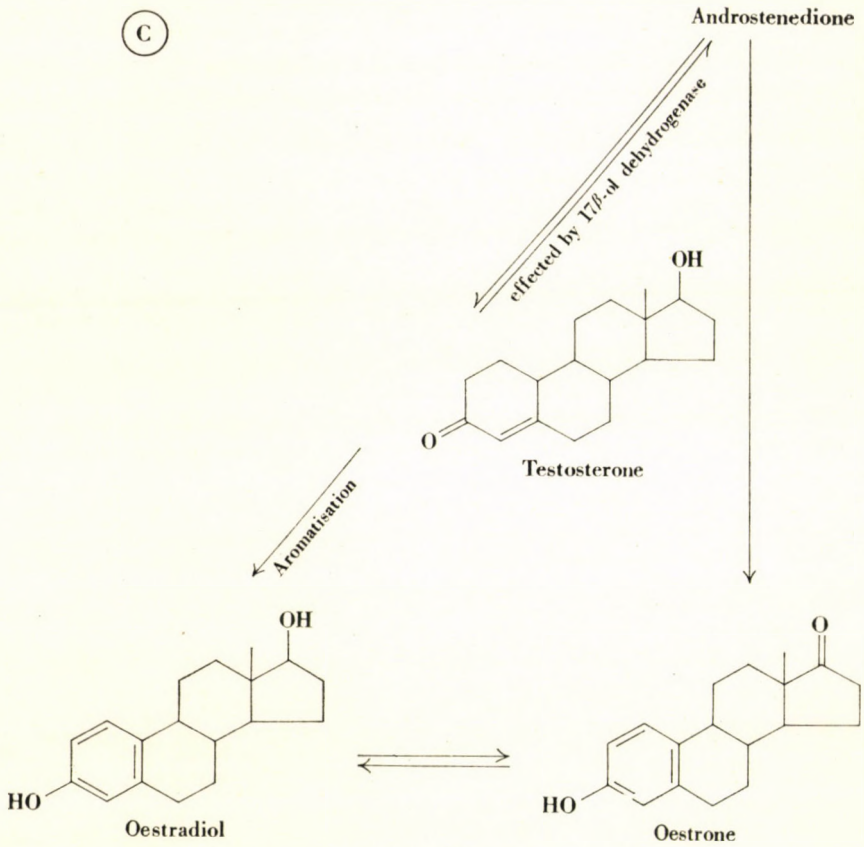


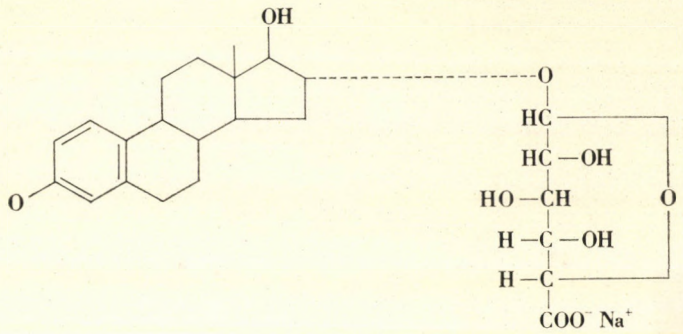
Fig. 14

4.1. Conjugation of oestrogens

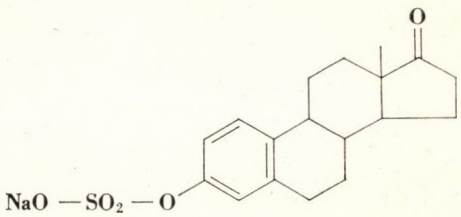
The oestrogens of poor water solubility are conjugated (SZEGŐ and WOLCOTT, 1954) after their binding to proteins so as to be suitable for excretion.

The water-soluble compounds are sulphate or glucuronate esters but double conjugations are also possible. The reaction occurs generally, though not necessarily, at position 3 or 16. This is exemplified by the structures of oestradiol glucuronide and of oestrone sulphate shown in Fig. 15. The studies of GOEBELSMANN and JAFFE (1971) have thrown new light on the conjugation of oestriol. Women in the second third of pregnancy were subjected to ^{3}H-oestriol and ^{14}C-oestriol-16 glucuronide infusion over 6 hours. The metabolites excreted in the urine were isolated, identified and measured (Fig. 16). Ninety per cent of the tritiated compound and 98 per cent of the ^{14}C-compound were recovered from the 48-hour urine. Only conjugated steroids were demonstrable

in the urine, prevalently bound to glucuronide at positions 16 and 3, whereas the sulphate and mixed conjugations were of minor significance in the case of urinary oestriol.



Oestriol glucuronide



Oestrone sulfate

Fig. 15

Derivative	Formed from ^{3}H-oestriol, per cent	Formed from 4-^{14}C-oestriol-16-glucuronide, per cent
Oestriol-16-glucuronide	63	~72
Oestriol-3-glucuronide	29	~25
Oestriol-3-sulphate-16-glucuronide	~5	~ 3
Oestriol-3-sulphate	~3	negligible
Oestriol-17-glucuronide	—	—

Fig. 16. Conjugation of oestriol after perfusion with ^{3}H-oestriol and 4-^{14}C-oestriol-16 glucuronide

4.2. Steroid synthesis "de novo" in the foetoplacental system

Hormone metabolism of the foetoplacental unit has been most closely studied by DICZFALUSY (1969).

The infusion apparatus constructed by LERNER and DICZFALUSY (1968) is suitable for the perfusion of the surgically delivered foetus together with the

placenta, allowing thus a separate collection of metabolites of the intervillous maternal and those of the foetal circulation. By means of this system TELEGDY et al. (1970a, 1970b, 1970c) have established the following facts concerning the de novo synthesis from labelled acetate.

In the second trimester of pregnancy, the placenta is unable to transform acetate into cholesterol but it does have the capacity of synthetizing placental squalene and lanosterol. This, however, is not converted to cholesterol. In contrast, the foetal liver, adrenals and testicles are capable of synthetizing cholesterol from labelled acetate.

pregnenolone(3 β-hydroxy-pregn-5-ene-20-one)

progesterone (pregn-4-ene-3,30-dione)

20 α- and β-dihydroprogesterone (20 α and 20 β-hydroxy-pregn-4-ene-3-one)

17 α-hydroxy-pregnenolone (3 β, 17 α-dihydroxy-pregn-5-ene-20-one)

dehydroepiandrosterone (3 β-hydroxy-androst-5-ene-17-one)

17 β-oestradiol (oestra-1,3,5(10)-triene-3,17 β-diol)

Fig. 17

Perfusion of the placenta with ^{3}H-7α-cholesterol and ^{14}C-acetate yielded the labelled steroids listed in Fig. 17. With the exception of pregnenolone and progesterone, all steroids were found in small amounts in the perfusion fluid. ^{14}C-steroids were absent, as a sign that no metabolite of this type had formed from acetate. Foetuses from the second trimester of pregnancy were found to synthetize prevalently pregnenolone and dehydroepiandrosterone (the two products accounting for 95% of the total production), ^{14}C and ^{3}H atoms being equally represented in the compounds. The foetal organism is thus capable of de novo synthesis from acetate, the products of which process contain oestrogen precursors. The placenta forms oestradiol which is readily converted to oestrone, while for the production of oestriol foetal factors too are necessary.

4.3. The part played by dehydroepiandrosterone sulphate in oestrogen biosynthesis

From the evidence outlined above and from the studies of RYAN (1959) it emerges that the placenta converts primarily the C-19 steroids to oestrogens with a substantial yield and at a high rate.

Of all C-19 derivatives it is dehydroepiandrosterone, a steroid prevalently of foetal origin, which constitutes the most important source. Since the rate of foetal dehydroepiandrosterone sulphate excretion is not known, reliable calculations concerning the yield cannot be made.

We (MIRHOM and SZONTÁGH, 1971) have succeeded in showing that early placental tissue forms 7-oxygenated derivatives from (4-^{14}C)-dehydroepiandrosterone in vitro in the following order: 7-oxo-DHA $>$ 7α-hydroxy-DHA $>$ 7β-hydroxy-DHA. Fig. 18 illustrates the process of one of the assays and Fig. 19 its evaluation.

Interesting facts have emerged from our experiments in which early trophoblast tissue was incubated with (4-^{14}C) DHA for various periods. At the end of the experiment we were able to isolate DHA, androstenedione, testosterone and androsterone in a radiochemically pure state. At 5 minutes

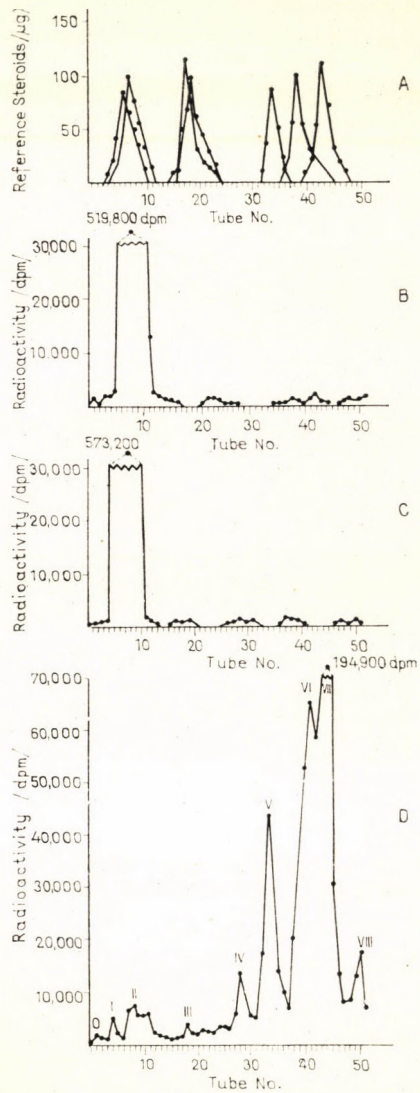


Fig. 18

Steroid identified	Carrier μg	Chemical reaction	Derivative	TLC system	R_f value	S. A. dpm/μM	Total dpm incorp./g	% dpm incorp./g
7-keto-DHA	108.3	—	7-keto-DHA	Lisboa "E"	0.52	30.640		
		—	7-keto-DHA	"E"	0.60	25.783		
		Acetylation	7-ket-DHA-acetate	"1 a"	0.41	15.668	5.497	0.35
		Hydrolysis	7-keto-DHA	Lisboa "E"	0.52	15.033		
7β-OH-DHA	67.2	—	7β-OH-DHA	Lisboa "E"	0.48	—		
		—	7β-OH-DHA	"E"	0.46	15.425	3.072	0.20
		Acetylation	7β-OH-DHA-diacetate	"1 a"	0.49	14.052		
		Hydrolysis	7β-OH-DHA	Lisboa "E"	0.50	13.779		
7α-OH-DHA	84.0	—	7α-OH-DHA	Lisboa "E"	0.44	—		
		—	7α-OH-DHA	"E"	0.33	14.491	2.776	0.18
		Acetylation	7α-OH-DHA-diacetate	"1 a"	0.49	9.940		
		Hydrolysis	7α-OH-DHA	Lisboa "E"	0.43	10.180		

Fig. 19. Isolation of 7-keto-DHA, 7 β-OH-DHA and 7 α-OH-DHA after incubation of placental tissue homogenate obtained from 15-year-old woman with 1,559,000 DPM [4-^{14}C]-DHA for 5 minutes

45%, at 10 minutes 71%, and at 30 minutes 99% of the DHA was completely metabolized. Androstenedione was formed at a higher rate (peak at 5 minutes) and in 6.5 times larger amounts than testosterone (peak at 10 minutes), but was also utilized more rapidly. This indicates that androstenedione is a more prevalent intermediary product in the biosynthesis of oestrone and oestradiol than is testosterone (Fig. 20).

We were the first to demonstrate the presence of 3α-reducing enzymes in the placenta and were able to isolate small amounts of androsterone which basically follows the pathway of testosterone. Its formation may be ascribed to the inactivation of those small androgen quantities which have escaped aromatization.

On the other hand, from the study by LEHMANN et al. (1971) it also emerges that placental biosynthesis substantially changes its enzymatic pattern in the course of pregnancy. Incubation with 4-^{14}C DHA of placental microsomal fractions obtained in the mid-term and terminal stages of pregnancy showed that the mature placenta was capable of metabolizing the precursors into oestrogens four times as rapidly as did the mid-term placenta. It was furthermore demonstrated that conversion of DHA into oestrogens by the placenta of diabetic or toxaemic women, was far less intensive than by the placenta of healthy women. There is a definite relationship between

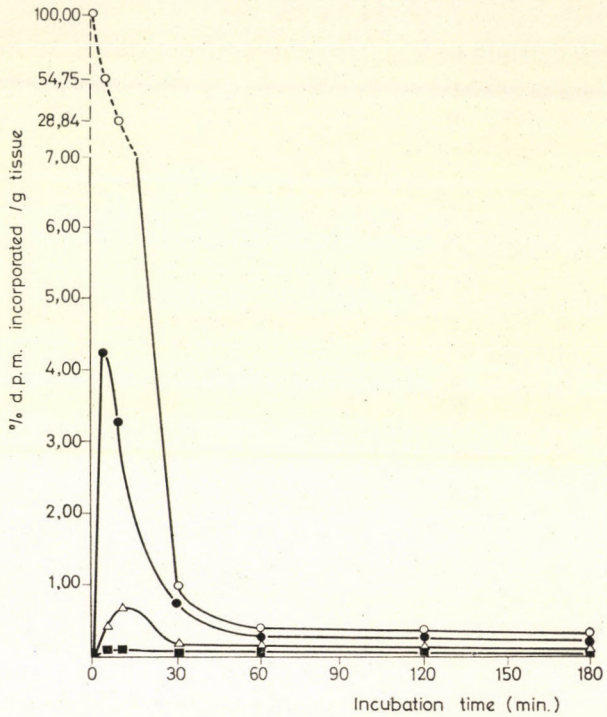


Fig. 20 ○—○ DHA, ●—● Androstenedione, △—△ Testosterone, ■—■ Androsterone

placental incompetence of this kind and the inadequate activity of A^4—A^5-isomerase, 3-beta-hydroxysteroid dehydrogenase, as well as of the aromatizing enzyme system.

4.4. Oestetrol

In 1965, HAGEN et al. isolated a new oestrogen metabolite from the urine of newborns. The substance, named oestetrol was synthetized by FISHMAN and GUZIK in 1967. GURPIDE et al. (1966) also identified the substance by means of intravenous or intra-amnionic injections of labelled oestradiol (1966).

Studies of the metabolism of 16α-hydroxy-C-19 steroids revealed in the urine a labelled steroid which was of greater polarity than oestriol and on the grounds of its chemical character could be identified likewise as oestetrol. YOUNGLAI and SOLOMON (1968) studied the *in vivo* metabolites of neutral steroids and found that these substances are metabolized into 15α-hydroxy-oestrogens, a process in which the foetal liver is closely involved.

The intricate oestrogen metabolism of the foetoplacental unit requires highly elaborate methods of investigation (Fig. 21). The possibilities are illustrated by the model, where V_{MF} represents the rate of passage of the substance from mother to foetus; V_{FM}, the passage from foetus to mother; Q,

the uptake of the substance by the maternal and foetal circulation; and V_F and V_M, the final output of the substance. It must be noted that e.g., V_{FM} represents all the pathways through which a given substance may pass from the foetus to its mother, and Q_F, the figure of de novo passage of a given substance into the foetal circulation, thus from other sources than the maternal circulation. For instance, in the case of oestriol, Q_F obviously represents the amount of hormone passing from the placenta into the foetal circulation.

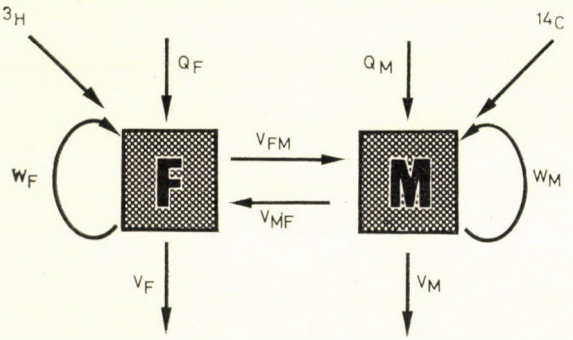


Fig. 21

GURPIDE and van de WIELE (1968) infused the ^3H and ^{14}C-labelled forms of the same substance simultaneously into the maternal and the foetal circulation. In (Fig. 22). P represents the rate of infusion and *a* the specific activity, The results obtained after injection of the two types of labelled 17β-oestradiol showed that the production of oestetrol (E_4) was confined to the foetal organism (Fig. 23).

	Foetal circulation	Maternal circulation
^3H	$P_F^{3H} + V_{MF}\, a_M^{3H} = (V_{FM} + V_F)a_F^{3H}$	$V_{FM}\, a_F^{3H} = (V_{MF} + V_M)a_M^{3H}$
^{14}C	$V_{MF}\, a_M^{14C} = (V_{FM} + V_F)a_F^{14C}$	$P_M^{14C} + V_{FM}\, a_F^{14C} = (V_{MF} + V_M)a_M^{14C}$
Endogenous	$Q_F + V_{MF} = V_{FM} + V_F$	$Q_M + V_{FM} = V_{MF} + V_M$

Fig. 22

Specific activity (cpm/μg)			Rates of secretion and metabolism of E_2 (mg/day)	Precursors of urinary E_3
Metabolite	^3H	^{14}C		
E_2	123	30	$Q_F < 1.8$	E_2 in foetal circulation < 5%
E_4	660	negligible	$Q_M > 3.5$	E_2 in maternal circulation = 10%
E_3	36	2.8	$V_{FM} < 0.7$	Other sources > 85%
			$V_{MF} = 0$	

Fig. 23

5. Mode of action of the oestrogens

The metabolic aspects of the production and excretion of oestrogens are still far from completely understood. This is primarily due to methodological difficulties.

Investigation of the various metabolic processes through which the specific activity of oestrogens takes effect, poses still greater difficulties. Within the limits of this report only a brief reference can be made to the present lines of investigation concerned with this problem which are directed at the target cell, down to its subcellular structures, and rely on various elaborate methods such as tissue culture, ultracentrifugal analysis, radiocytography, cytochemistry, cytophotometry, microfluorometry, etc.

It is known that while the peptides act on the cellular surface, the site of action of the steroids is intracellular.

The first essential question concerns the receptor, the most appropriate definition of which has been given by BAULIEU et al. (1971). In a general sense, this is a molecular unit which comprizes a receptor and an effector site, the two being linked by a coupling mechanism. The first phase of steroid activity takes effect at the receptor site through which the hormone imparts its information to the cell. However, attachment of the hormone to the receptor need not result in a hormonal effect. The criteria of the receptor are hormone-specificity, tissue-specificity, high affinity and saturability.

What happens to the oestrogen molecule while imparting its information to the receptor? This is the other essential question. In radioisotope studies with ^{3}H-oestradiol, the molecule remained chemically unaffected, while the ten times less potent oestrone molecule was converted to oestradiol within the receptor cell during the production of its effect.

Radioautographic studies and cell fractionation have made it possible to locate the ^{3}H-oestradiol retained in endometrial receptor cells to the nucleus. It has been demonstrated by Sephadex-chromatography that the hormone attaches to a cytosole macromolecule (STUMPF and ROTH, 1966; JENSEN et al., 1967; TALWAR et al., 1964), the sedimentation coefficient of which has been established by TOFT and GORSKI (1966). Moreover, BAULIEU et al. have confirmed its high affinity to the oestrogen-molecule and its saturability with oestradiol.

These have been merely a few illustrative data chosen from the great wealth of experimental facts which still leave us with a great many unanswered questions. It is not known for instance, what happens at the effector site of the receptor apparatus during the production of the hormonal effect. The advances in endocrinology since the discoveries of ALLEN and DOISY to the present day are non the less immense, and further progress will doubtlessly add increasing evidence to our knowledge of oestrogens. However, the clinician's ultimate aim is action. To him, knowledge is only a means to this

end. What he seeks by studying the various processes and the ways and means of influencing them is the benefit of the patient.

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RECENT ADVANCES IN MINERALOCORTICOID RESEARCH

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Among the adrenocortical hormones containing 21 carbon atoms, aldosterone which owes its name to the presence of an aldehyde group at position 18, is regarded today as the most important mineralocorticoid hormone. In fact, aldosterone is essential in the maintenance of fluid and electrolyte balance, i. e., for homeostasis and it is closely involved in the aetiology of various clinical syndromes.

The site of aldosterone production is the zona glomerulosa of the adrenal cortex, whereas the other two zones, the zona fasciculata and zona reticularis, form no aldosterone under normal conditions. Steroid production of the individual zones is determined by their different enzyme contents. Since of the three zones it is only the zona glomerulosa which possesses 18-hydroxylase, an enzyme essential to the biosynthesis of aldosterone, the production of this mineralocorticoid is obviously confined to this area.

The schematic representation of a steroid secreting cell reproduced from the monograph of CHRISTENSEN and GILLIM (1969) is seen in Fig. 1. The 18-hydroxylase enzyme-system is localized to the mitochondria, at the site of aldosterone production. The significance and specificity of the 18-hydroxylase enzyme in aldosterone biosynthesis is convincingly illustrated by observation to its deficiency, as in the case of selective hypoaldosteronism (ULICK et al., 1964), where aldosterone production is practically absent. In other cases, e. g., in Conn's syndrome, the surgically removed adenoma shows an increased 18-hydroxylase activity. In these cases the microscopic appearance of the aldosteronoma was reminiscent of the zona fasciculata, indicating that under abnormal conditions not only the zona glomerulosa but also the other zones of the adrenal cortex are forming aldosterone, probably as a result of enzymatic alterations of the tumour tissue.

Daily aldosterone secretion ranges from 50 to 250 μg in normal individuals. In comparison with the daily secretion of other corticosteroids which attains several mg, this quantity, however, is not negligible in consequence of high biological activity of aldosterone, which is emphasized by the fact that only 50 to 60 per cent of its total amount is bound to plasma proteins (SIEGEN-

THALER et al. 1964), mainly to albumin as opposed to the 90 per cent for the glycocorticoids. Actually, it is the free hormone concentration which determines the biologically active hormone level.

Though aldosterone has well-defined glycocorticoid properties, its main activity concerns the metabolism of minerals, particularly of sodium and potassium, this activity forms the subject of the present study in view of its prime importance in human pathology.

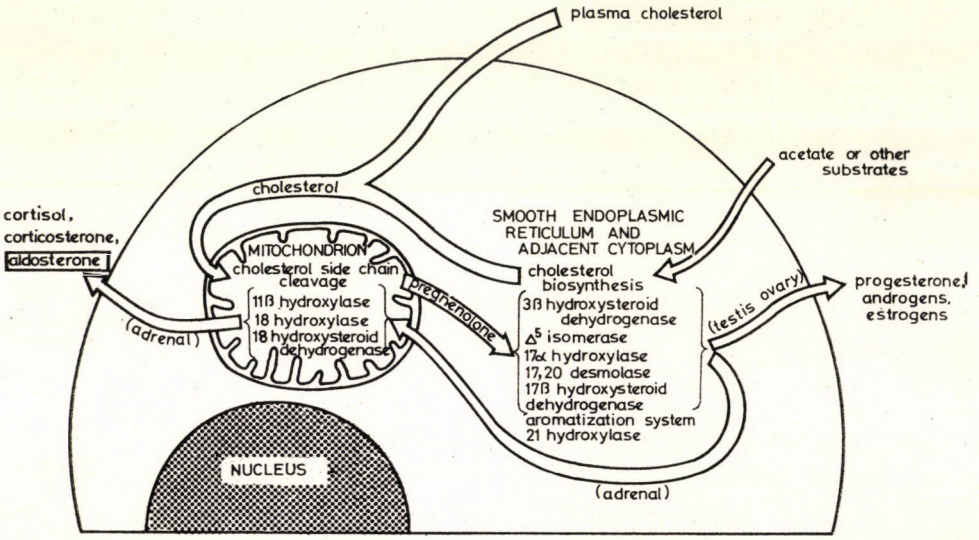

Fig. 1. Schematic representation of a steroid-secreting cell. From CHRISTENSEN and GILLIM (1969)

Fig. 2 demonstrates the mineralocorticoid effects of aldosterone and of the other corticosteroids as compared with DOC. Although the various methods yield different indexes expressing the ratio of activity of the given hormone to that of DOC, it stands beyond doubt that the sodium retaining capacity of aldosterone is approximately 25 to 50 times that of DOC. Research concerned with mineralocorticoid hormones has revealed interesting facts in recent years. It has been demonstrated that DOC, regarded earlier essentially as an intermediary product of corticosterone and aldosterone biosynthesis, is secreted in excessive amounts in pathological conditions of the adrenal cortex associated with certain enzyme defects, as well as in the case of ACTH-secreting tumours and that, similarly to aldosterone, its hypersecretion may give rise to arterial hypertension and to grave hypopotassaemia (BIGLIERI et al., 1966, 1968; NOLTEN et al., 1968; BROWN and STROTT, 1971). Recently, BROWN and STROTT (1971) have developed a highly sensitive procedure based on competitive protein binding, suitable for the estimation of DOC concentrations in the blood of normal individuals. The figures thus

	Survival of adrenalectomized dog	Sodium retention in adrenalectomized		Potassium excretion in adrenalectomized		$^{24}Na/^{42}K$ ratio in adrenalectomized rat	Threshold of sensitivity in Ussing's toad bladder preparation
		rat	dog	rat	dog		
DOC	100	100	100	100	100	100	100
Aldosterone ...	2500	2500	3900	500	2900	10 000	2000
11-dehydro-corticosterone	14	10.3		10		7	
Corticosterone .	14	0.5		15		14	weak
Compound S ..		3.0		1.5		8	
Cortisol	7	0.03		24	4	10	weak
Cortisone	2.5	0.01		14	2.1	2.5	no effect
18-OH-DOC ..		6—8**		6—8			weak
18-OH-corticosterone							substantially milder effect than with aldosterone

* Data are referred to the effect of DOC (= 100).

** BIRMINGHAM et al. (1965, 1968) have found the sodium retaining activity of 18-OH-DOCA (!) considerably greater, i.e., 25 to 50% of that of DOC.

Fig. 2. Comparison of the mineralocorticoid effects of some corticosteroids (after WOLFF, 1959; KAGAWA and PAPPO, 1962; GAUNT and CHART, 1962; SHARP and LEAF, 1964).* From GLÁZ and VECSEI (1971)

obtained (5 to 10 ng/100 ml) largely correspond to the normal plasma concentration of aldosterone. It has moreover been demonstrated that the mineralocorticoid activity of 18-hydroxy-DOC, regarded earlier as negligible, amounts to 25 to 60% of that of DOC (BIRMINGHAM et al., 1968; PORTER and KIMSEY, 1971). MELBY et al. (1967) showed 18-OH-DOC to be a product of the human adrenal cortex by identifying the compound in the blood of the adrenal vein obtained by percutaneous catheterization from a patient with mineralocorticoid hypertension. The findings of MELBY et al. (1971) have revealed that the secretion of 18-OH-DOC in healthy individuals is not far below that of aldosterone and of DOC, its normal range being between 50 and 120 μg/24 hrs, though KÜCHEL (1971) gives a value of approximately 200 μg/24 hrs. The biosynthesis of 18-OH-DOC is assumed by MELBY et al. (1971) to take place in the zona fasciculata of the adrenal cortex according to the scheme presented in Fig. 3, where the pathway of aldosterone biosynthesis can also be seen. The zona fasciculata may well be the site also of a process involving no 17-alpha-hydroxylation which would account for the biosynthesis of this steroid. As illustrated by Fig. 4, it is assumed that, similarly to the relationship of 18-OH-B to aldosterone, 18-OH-DOC may well represent a precursor of a potential mineralocorticoid which is more potent than aldosterone. The precursor was termed 11-deoxy-aldosterone (Fig. 4).

Zona glomerulosa

Cholesterol —(Angiotensin, ACTH, K^+)→ Progesterone → DOC → Corticosterone → 18-OH-B → Aldosterone

Zona fasciculata

17α-OH-Preg → 17α-OH-Prog → 11-Deoxycortisol → Cortisol

Cholesterol —(ACTH)→ Pregnenolone → Progesterone → DOC → 18-OH-DOC

Fig. 3. Pathways of aldosterone and 18-OH-DOC biosynthesis. From MELBY et al. (1971)

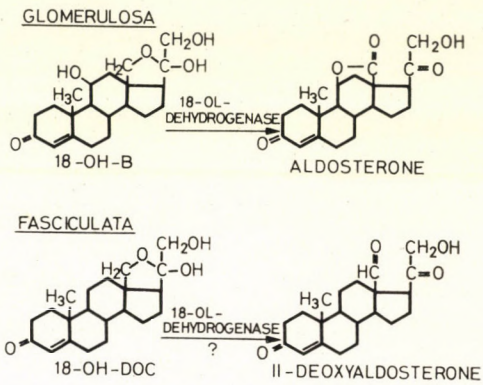

Fig. 4. Chemical structure of aldosterone, 18-OH-B and 18-OH-DOC and of 11-deoxyaldosterone. From MELBY et al. (1971)

On these grounds, as far as the mineralocorticoids of the human organism are concerned, we have to take into account, in addition to aldosterone, the production and mineral effects of DOC and of 18-OH-DOC.

The site of action of the mineralocorticoid hormones is best understood on the basis of its effect on one of its effector organs, the renal tubule. By its action on the distal tubules, aldosterone increases the reabsorption of Na^+ and Cl^- and stimulates the excretion of K^+, H^+ and NH_4 ions. However, we are still ignorant of how aldosterone acts; in other words, how its mineralocorticoid mechanism works. The existence of a special mineralocorticoid receptor or receptors, in the effector organ is an old problem. Some light has been thrown on the question by the studies of FANESTIL and EDELMAN (1966) who identified ^3H-d-aldosterone in the subcellular fraction of the tubular cells after injection of the labelled substance into adrenalectomized rats. SHARP and LEAF (1965) and later EDELMAN (1968) demonstrated that aldosterone enhanced Na-uptake by increasing protein synthesis through the DNA-dependent RNA, as seen in Fig. 5 reproduced from the study of EDELMAN et al. (1964). It has also been shown that actinomycin D and puromycin, both RNA-blocking substances, thus also inhibitory to protein synthesis, block the effect of aldosterone on Na-transport at the level of the cell

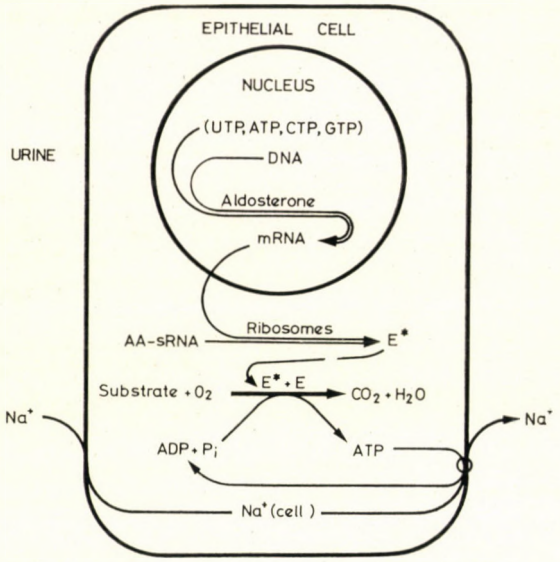

Fig. 5. Theoretical pathway of the mechanism of action of aldosterone. From EDELMAN et al. (1964)

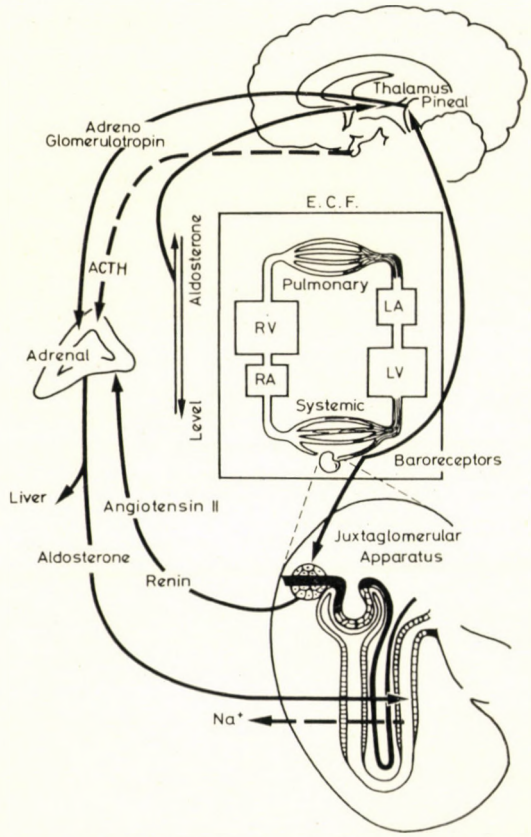

Fig. 6. Schematic representation of the factors involved in the regulation of aldosterone secretion. From VENNING and SOLOMON (1964)

membrane. Though the involvement of aldosterone in certain oxidative processes has been confirmed by LOSERT et al. (1967), the successive steps of this process are not yet known. The theory represented by EDELMAN's diagram (Fig. 5) also rests on incomplete evidence.

The extrarenal mineralocorticoid activity of aldosterone, though likewise of great significance, is outside the scope of the present paper.

The organism adjusts the aldosterone level to its continuously changing demands through a highly intricate regulatory mechanism even under physiological conditions, and still more so in abnormal states (GLÁZ, 1970). As it emerges from the diagram in Fig. 6, reproduced from the monograph of VENNING and SOLOMON (1964), though this mechanism involves numerous factors such as the changes in the circulating blood volume, shifts in potassium and sodium balance, ACTH, the renin–angiotensin system, the central nervous system, none of them in itself is responsible for the regulation of mineralo-

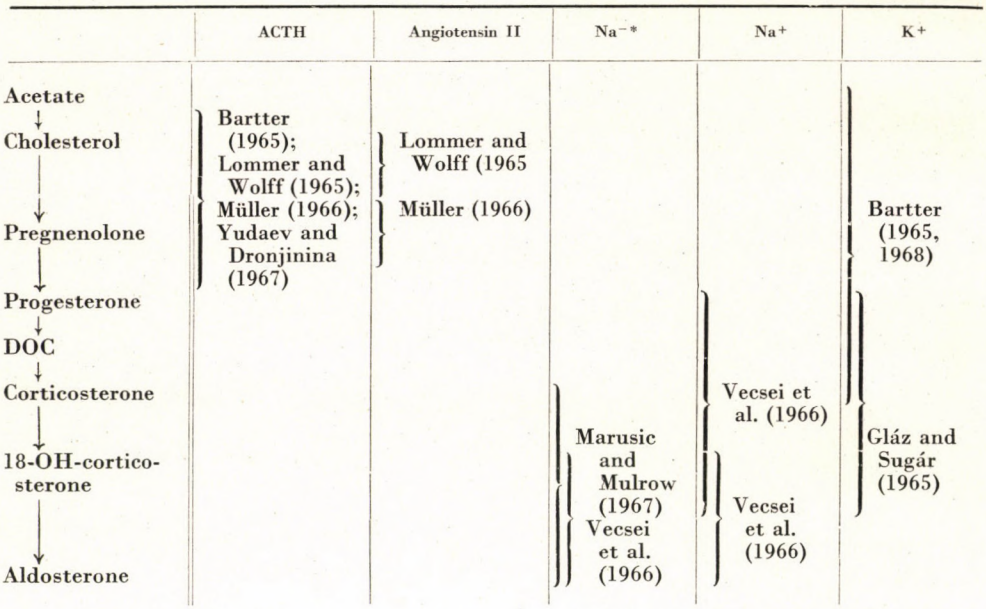

Fig. 7. Schematic pathway of aldosterone biosynthesis and the sites of action of the essential factors involved in this process. From GLÁZ and VECSEI (1971)

corticoid hormones (Fig. 6). The scheme in Fig. 7 represents the pathway of aldosterone biosynthesis (GLÁZ and VECSEI, 1971). The sites of action of some of the regulatory factors for the biosynthesis of corticosteroids are also presented in Fig. 7.

It is not possible to deal with all these factors within the scope of the present report, therefore we have to confine ourselves here to a few examples of the renin–angiotensin system and ACTH-effects for the illustration of the highly

complicated mechanism of mineralocorticoid hormone regulation. The diagram in Fig. 8, reproduced from the study of STOLLERMAN (1969), represents the feedback mechanism asserting itself in the renin–angiotensin–aldosterone system under physiological conditions. However, it is not only the effective blood volume, and the pressure prevailing in the renal artery, which regulates the secretion of renin by the JGA, but also the intrarenal, i.e., intratubular, concentration of sodium at the level of the macula densa (according to the theory of THURAU), furthermore the sympathetic nervous system and the potassium concentration in the blood of the renal artery, to name only some of the most essential factors. The part played by the renin–angiotensin system — has been substantiated by ample clinical observations and experimental evidence. There are none the less certain points which require some comments, first of all the mechanism and specificity of the angiotensin effect as regards the stimulation of aldosterone production. Angiotensin II, just as ACTH, affects the early phase of steroid biosynthesis by stimulating the conversion of cholesterol into pregnenolone, as illustrated in the diagram.

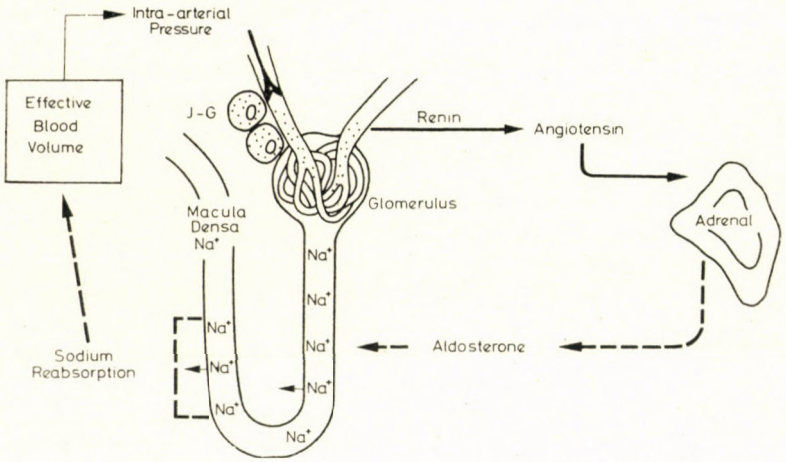

Fig. 8. Schematic representation of the feedback mechanism of the renin–angiotensin–aldosterone–system. From STOLLERMAN (1969)

Despite this similarity, there are some differences in the mechanism of action of the two hormones, as, on the evidence of the studies of KAPLAN (1965), angiotensin, in contrast with ACTH, does not augment the phosphorylase activity of the adrenal cortex. Furthermore, the effects of ACTH and of cyclic-3',5'-AMP on corticosteroid biosynthesis are not additive, whereas ACTH and angiote nsinII as well angiotensin II and cyclic-3',5'-AMP do potentiate each other's effects. Similarly to ACTH, angiotensin II administered in pressor doses stimulates, in addition to the biosynthesis of aldosterone, that of cortisol and corticosterone, whereas the activity of its subpressor doses

is confined to aldosterone biosynthesis in man (RAYYIS and HORTON, 1971). However, in certain animal species the compound has no stimulating effect on aldosterone biosynthesis unless administered in pressor doses, as it has been confirmed by our earlier experiments in the rat (GLÁZ and SUGÁR, 1962) (Fig. 9). It is a characteristic feature of angiotensin activity that its aldosterone-stimulating effect is potentiated by sodium depletion.On the other hand, ACTH primarily stimulates the secretion of cortisol and of corticosterone, though there is evidence that in opposition to the earlier view it considerably enhances the production of aldosterone. Dissimilar as the two hormones are in their mechanisms of action, there is none the less a close relationship between them, at least as far as the human organism is concerned. On the evidence of the recent studies of RAYYIS and HORTON (1971), blocking of endogenous ACTH by dexamethasone results in a significant suppression of the angiotensin II-induced elevation of the plasma aldosterone level even if pressor doses of angiotensin II have been used, as a sign that production of maximum angiotensin II effect requires a normal production of corticotrophin by the pituitary gland.

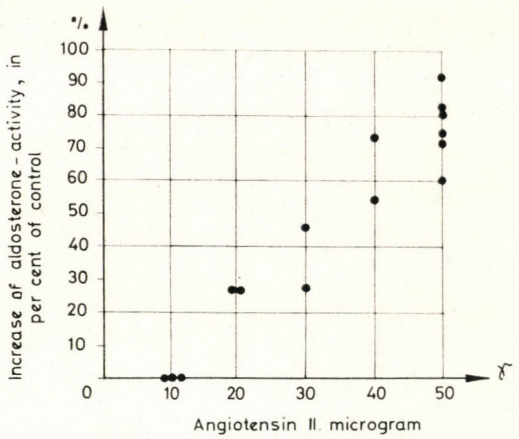

Fig. 9. Stimulating activity of increasing doses of angiotensin II on aldosterone biosynthesis in the rat. From GLÁZ and SUGÁR (1962)

The long-term use of angiotensin II confronts us with particular problems in respect of aldosterone production. While in man and in the dog its stimulating activity remains unaffected even for long periods (LARAGH et al., 1964;, URQUHART et al., 1963), in sheep (BLAIR-WEST et al., 1962) and in rats (CADE and PERENICH, 1965) it disappears within a few hours. Conversely, in humans it is ACTH which loses its stimulating activity on aldosterone production when used over longer periods, probably because, as suggested by NEWTON and LARAGH (1968), the ACTH-induced hypersecretion of cortisol and cortisone produces a distinct mineralocorticoid effect, i.e., sodium retention,

which by a feedback mechanism leads to a suppression of aldosterone secretion. The reduction in the secretion of aldosterone attained by the use of exogenous glycocorticoids in clinical cases (NEWTON and LARAGH, 1968) seems to fit in with this interpretation. The results of rat experiments—by examining the corticosteroid biosynthesis in the presence of H^3-pregnenolone (GLÁZ et al., 1971)—are in conformity with these findings: instead of the ACTH-induced characteristic reduction in aldosterone specific activity indicative of an enhanced production of non-labelled aldosterone (Fig. 10), an increase in specific activity of aldosterone, as a sign of reduction in its absolute amount, was noted in case of long-term use of dexamethasone in rats aimed at the suppression of endogenous ACTH secretion (Fig. 11).

	Specific activity			
	DPM/mg		DPM/mg in per cent of the controls	
	Aldosterone	Corticosterone	Aldosterone	Corticosterone
Control	364.83	374.65	100	100
$ACTH^{1-24}$ (Synacthen)	126.72	98.57	—60.88	—71.66
$ACTH^{1-28}$ (Humacthid)	104.05	101.72	—68.97	—69.46

Fig. 10. Effect of synthetic ACTH on aldosterone biosynthesis by the rat adrenal, in the presence of ^{3}H-pregnenolone. From GLÁZ et al. (1971)

The correlations between angiotensin II and aldosterone level also pose interesting problems. In the studies of FRASER et al. (1965), during the intravenous infusion of angiotensin II the aldosterone level was increased. After the infusion the level of aldosterone returned to its initial value in 30 min, in accordance with the biological half-life of aldosterone. This observation would seem to be suggestive of a close correlation between the two factors, the recent studies by BLAIR-WEST et al. (1971), however, indicate that the said effect too is closely connected with the sodium balance prevailing in the body, these authors having been able to reduce the increased aldosterone level in sodium-depleted sheep by the administration of sodium in a stage when the plasma angiotensin II level was still high. A dissociation between the levels of aldosterone and renin and of aldosterone and angiotensin II, has been confirmed by clinical studies of other authors as well (SLATER et al., 1969; CHINN et al., 1969; VEYRAT et al., 1967). Despite this evidence, with the exception of particular situations, there are intimate correlations between the activity of the renin–angiotensin system and the production of aldosterone.

Attention must be directed to the changes effected by the above discussed regulatory factors in the secretion of DOC which also has an important

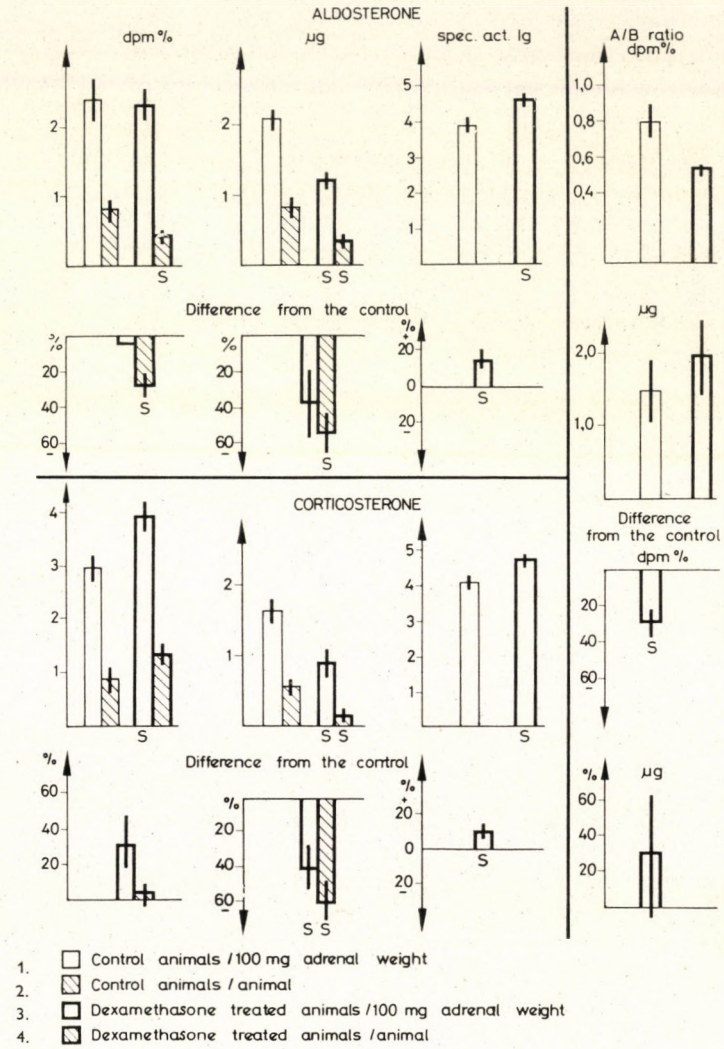

Fig. 11. Effect of dexamethasone treatment on aldosterone and corticosterone biosynthesis by the rat adrenal in the presence of ^{3}H-pregnenolone (GLÁZ et al.)

mineralocorticoid effect. On the evidence of the studies of BROWN and STROTT (1971), a low-sodium diet, in the same manner as angiotensin II infused over one hour in pressor doses, fails to increase the plasma DOC level in man. In opposition to this, the infusion of ACTH as well as the enhanced ACTH secretion in the ectopic ACTH syndrome induced a significant rise in the plasma DOC level. These data fit in with earlier observations of the increased production of DOC in congenital adrenocortical hyperplasia in 11-beta, or in 17-hydroxylase enzyme defects (GOLDSMITH et al., 1967; BIGLIERI et al., 1968), or in adrenocortical carcinoma inducing Cushing's syndrome (CRANE and HARRIS,

1966). From these facts it clearly emerges that, while ACTH plays a definite part in the production of DOC hypersecretion, angiotensin II lacks any stimulating activity of this kind. This is consistent with clinical observations relative to syndromes associated with secondary hyperaldosteronism where in spite of the activity of the renin–angiotensin–aldosterone system is significantly enhanced, the DOC level is unchanged (BROWN and STROTT, 1971).

The release of 18-OH-DOC, the third steroid of mineralocorticoid activity, has closely been studied in human pathology by MELBY et al. (1971) and KÜCHEL (1971). In the studies of MELBY et al., the 18-OH-DOC level, while remaining unaffected by angiotensin II as well as by a high sodium intake, definitely increased in response to ACTH administration, and the potentiating effect of sodium depletion on the secretion of 18-OH-DOC was shown by KÜCHEL.

These facts clearly indicate that, together with the renin-angiotensin system, ACTH secretion plays a decisive part in the regulation of aldosterone and of other mineralocorticoids.

Hypertensive conditions
- Essential hypertension
- Malignant hypertension
- Renovascular hypertension
- Acute renal disease
- Robertson's syndrome (renal haemangiopericytoma)
- Pheochromocytoma
- Coarctation of the aorta

Conditions associated with oedema
- Nephrotic syndrome
- Congestive heart failure
- Portal cirrhosis accompanied by ascites
- Idiopathic oedema

Miscellaneous conditions
- Pregnancy
- Diabetes insipidus
- Bartter's syndrome (JGA-hyperplasia)
- Pseudo-Bartter's syndrome (excessive use of cathartics, diuretics)
- Anorexia nervosa
- K, Na losing renal disease
- Pyloric stenosis
- Acute blood loss

Fig. 12. Conditions associated with increased renin–angiotensin activity and with high aldosterone level

We shall now attempt to discuss the practical significance of the renin–angiotensin–aldosterone system in some clinical syndromes. Actually, the increased activity of the renin–angiotensin system shows a parallelism with the elevation of the aldosterone level in various pathological conditions and clinical syndromes associated with hyponatraemia, hypovolaemia, hypertension or oedema (Fig. 12).

Hypotensive conditions
- Selective hypoaldosteronism
  - idiopathic
  - congenital (18-hydroxylase and 18-dehydroxylase deficiency)
  - iatrogenic (therapeutic use of heparin and heparinoids: block of 18-hydroxylase)
- Diffuse adrenocortical failure
- Addison's disease
- Adrenalectomy (bilateral)

Fig. 13. Conditions associated with increased renin–angiotensin activity and reduced aldosterone level

Fig. 13 lists the syndromes or conditions, where the fall in the level of aldosterone or of other mineralocorticoids is accompanied by an excessive activity of the renin–angiotensin system (absence of feedback effect). These include bilateral adrenalectomy, Addison's disease, hypoaldosteronism or hypomineralocorticism. Recent observations have made us more familiar with the states of hypoaldosteronism. A new type of hypoaldosteronism has been described by BIGLIERI et al. (1966), by STOCKIGT et al. (1971) and by PEREZ et al. (1971). In the presence of the typical signs of hypoaldosteronism, they found a surprisingly low plasma renin activity instead of the high values to be expected on the basis of earlier experience. Even the level of angiotensin I, as determined by radio-immunoassay, was extremely low and failed to respond to the conventional renin-stimulating factors, i. e., salt-deprivation, diuretics, erect posture, etc. The normalization of hyperpotassaemia by means of substitution therapy of hypoaldosteronism also left the renin–angiotensin values unaffected. It was assumed on these grounds that the hypoaldosteronism consequent upon an atrophy of the zona glomerulosa was due to an impaired production of renin.

However, in syndromes accompanied by excessive production of aldosterone or of other mineralocorticoid hormones (Fig. 14) the activity of the renin–angiotensin system is gravely affected and its response to the usual stimulation is poor or absent (feedback effect as e. g., in Conn's syndrome). Recent studies have offered closer insight into many aspects of Conn's syndrome, but the present discussion will be confined to its problems relative to the morphology of the adrenals. As it is seen in Fig. 15 (classification of

Hypertensive conditions
- Primary aldosteronism (adrenocortical adenoma)
- Pseudo-primary aldosteronism
  - congenital aldosteronism (adrenocortical hyperplasia)
  - aldosteronism of adult-age onset (micro- and macronodular hyperplasia of adrenal cortex)
  - dexamethasone-responsive type
- Tertiary aldosteronism associated with renal disease
- Cushing's syndrome
- Adrenogenital syndrome (11-, 17-hydroxylase deficiency)*

* Increased aldosterone levels are uncommon in adrenogenital syndrome of this type.

Fig. 14. Conditions associated with a reduced renin-angiotensin activity and increased aldosterone level

DHOM and STÄDTLER, 1968), an "aldosteronoma" of the adrenal cortex is not the only possible cause of primary hyperaldosteronism; micronodular hyperplasia, even diffuse hyperplasia of the adrenal cortex may also be responsible for it (Fig. 15). From our material (Fig. 16, 17, 18) a case of solitary adenoma and one of micronodular hyperplasia as the primary cause of hyperaldosteronism is presented (Dr. É. Konyár, and Dr. J. Mohácsi, of the Second Institute of Pathology Univ., Budapest). It must be noted that in these cases, in addition to the production of aldosterone also that of cortisol was excessively high. This was, however, only to be expected in view of the microscopic structure of adenoma, reminiscent of the zona fasciculata rather than of the zona glomerulosa.

(1) Adenoma of the cortex:
  (*a*) solitary
  (*b*) multiple, unilateral or bilateral
  (*c*) solitary, associated with micronodular hyperplasia of the remaining cortex
  (*d*) multiple with micronodular hyperplasia
(2) Bilateral micronodular hyperplasia of the cortex:
  (*a*) congenital hyperaldosteronism
  (*b*) hyperaldosteronism of the aged
(3) Adrenal carcinoma
(4) Adrenocortical hypoplasia (aberrant aldosteronoma)
(5) Adrenal cortex without morphological abnormality

Fig. 15. Adrenal morphology in primary aldosteronism. From DHOM and STÄDTLER (1968)

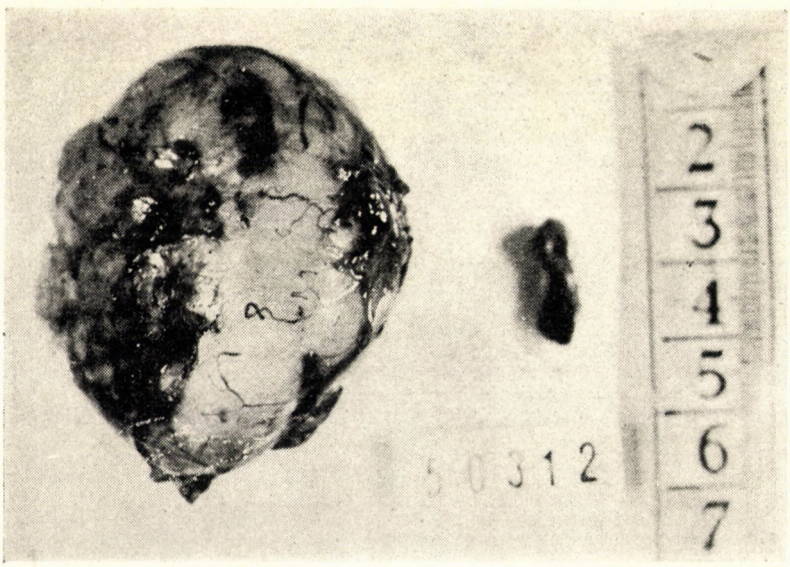

Fig. 16. Gross appearance of an adrenocortical adenoma causing Conn's syndrome (GLÁZ et al.)

The poor, unresponsive, renin activity in the presence of high or even normal aldosterone levels not only confirms the diagnosis of the classical

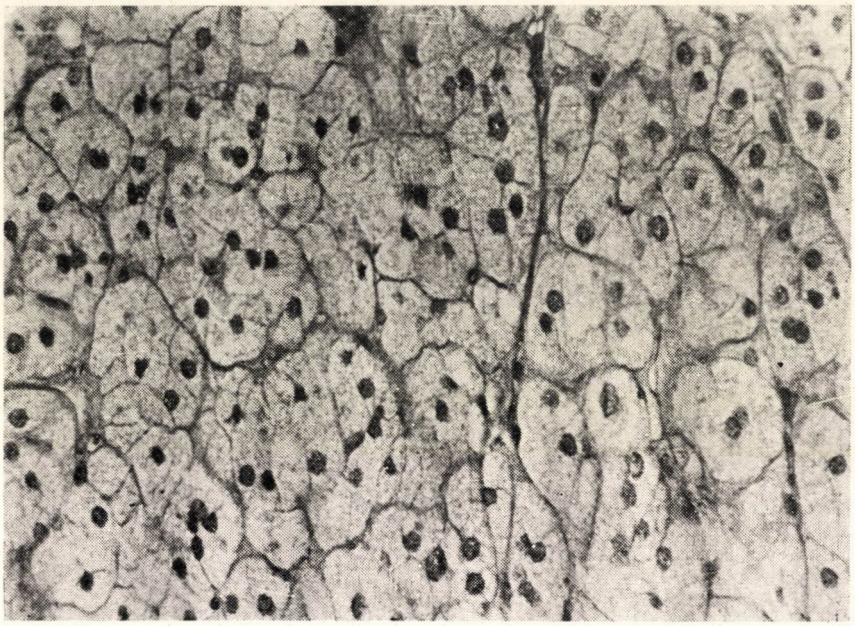

Fig. 17. Microscopic appearance of the adrenocortical adenoma shown in Fig. 16

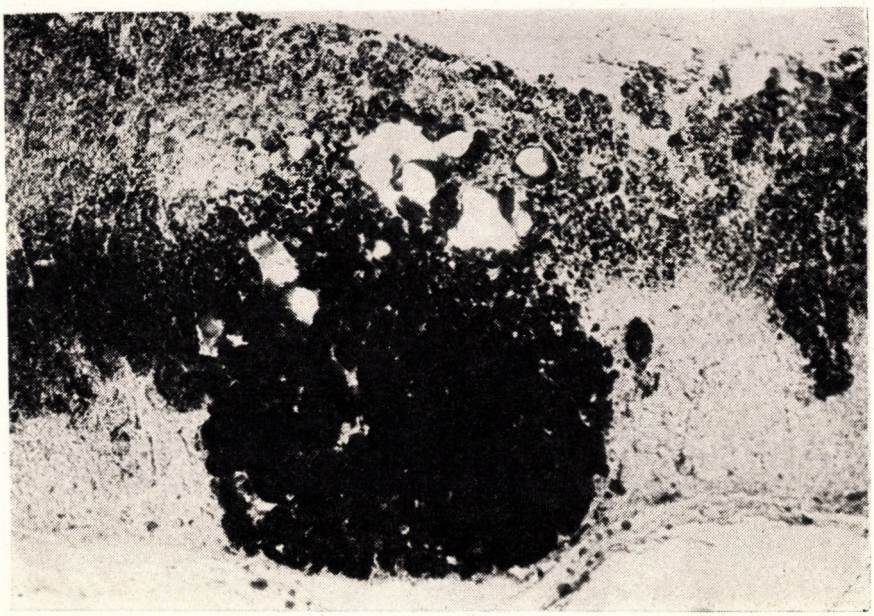

Fig. 18. Microscopic appearance of nodular adrenocortical hyperplasia causing Conn's syndrome (GLÁZ et al.)

Conn's syndrome, but also allows to recognize definite cases of oligosymptomatic endocrine hypertension due to adrenocortical hyperplasia thus offering a procedure for the screening of essential hypertension. On the evidence of our observations (GLÁZ et al., 1970, 1971) (Fig. 19) the hypertension as a sign of oligosymptomatic adrenocortical disease was proved in a considerable number of cases. Correct diagnosis makes it possible to introduce an aetiologic therapy.

Estimation of plasma aldosterone and plasma renin activity under basal conditions and after salt withdrawal and administration of diuretics allows to diagnose the endocrine type of hypertension at a stage where other

Number of patients examined:	169	%
Essential hypertension	9	5.3
Progressive hypertension	6	3.5
Forme fruste Cushing's disease	76	45.0
Cushing's syndrome	8	4.7
Oligosymptomatic adrenocortical hypertension	61	36.1
Conn's syndrome	9	5.3
(surgical evidence)	(4)	

Fig. 19. Patients with endocrine hypertension due to oligosymptomatic adrenocortical hyperfunction in a clinical series investigated for the aetiology of hypertension. Final diagnosis in hypertension patients of suspected adrenocortical origin (GLÁZ et al., 1970, 1971).

signs of adrenocortical disease are still absent. As Fig. 20 shows, in patients with essential hypertension the plasma aldosterone level increased nearly by 170 per cent and the plasma renin level by 64 per cent in response to the usual stimuli. The difference is remarkable since the elevation in the group of endocrine hypertension was of the order of 20 to 25 per cent only. The response to stimulation of renin and aldosterone in endocrine hypertension is of the same pattern as in Conn's syndrome (Fig. 20).

In the literature of recent years we find references to cases of hypertension regarded as "essential" hypertension despite the unresponsiveness of renin activity, on the grounds that surgical exploration of the adrenals for suspected Conn's syndrome had revealed no adenoma (KAPLAN, 1967; LEDINGHAM et al., 1967; FISHMAN et al., 1968; LARAGH et al., 1970). This would seem to cast doubt on the diagnostic value of the unresponsiveness of the renin system, at least under certain conditions. It should, however, be borne in mind that, while on the one hand, apart from a solitary adenoma, a micronodular hyperplasia of the adrenal cortex, accessible only to microscopical diagnosis, may also be involved in the aetiology of Conn's syndrome, on the other hand an excessive uptake of sodium — very common in hypertensive subjects — as well as of potassium may suppress renin activity in true hypertensive disease (VEYRAT et al., 1967). New light has been thrown on the problem by WOODS et al. (1969), who were able to reduce the blood pressure in subjects with "essential" hypertension with suppressed renin activity by the therapeutic use of aminoglutethimide, a substance inhibiting the biosynthesis of steroids, thus also of aldosterone. Spironolactone, a drug of antialdosterone-activity, was also used

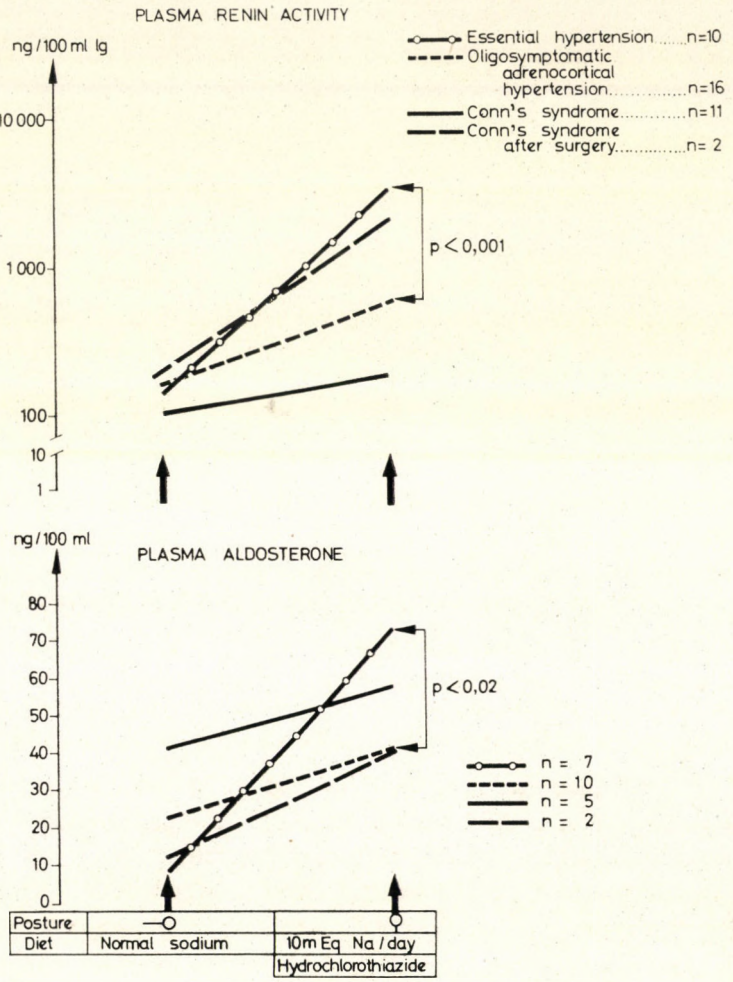

Fig. 20. Plasma renin activity and plasma aldosterone concentration in hypertension of various causes (GLÁZ et al.)

with benefit by CRANE and HARRIS (1970) in hypertensive patients of a similar type. These observations also emphasize the diagnostic and aetiologic significance of suppressed renin activity in hypertensive patients.

The therapeutic results obtained in our hypertensive material are consistent with the observations of the above mentioned authors. Fig. 21 presents the values for five patients with oligosymptomatic endocrine hypertension where the plasma aldosterone and renin level proved less responsive than in the cases of essential hypertension. After unsuccessful rauwolfia and methyldopa treatment, spironolactone induced a considerable fall of blood pressure. Combination of this treatment with aminoglutethimide resulted in a further

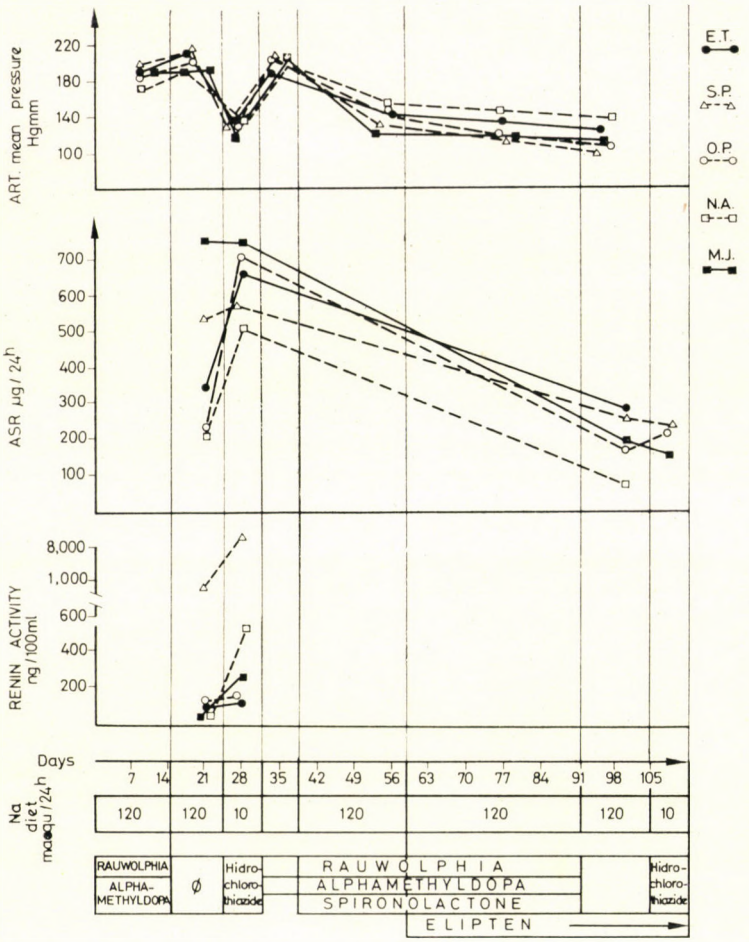

Fig. 21. Effect of aminoglutethimide in patients of oligosymptomatic adrenocortical hypertension (GLÁZ et al.)

decline of arterial pressure parallel with a significant reduction in aldosterone secretion. It is known from various studies including those of FISHMAN et al. (1967) and of our observations that aminoglutethimide is one of the most potent inhibitors of aldosterone biosynthesis. Fig. 22 demonstrates the steroid biosynthesis by the rat adrenal under chronic aminoglutethimide treatment. It can be seen that the conversion of labelled pregnenolone, a precursor of aldosterone, as well as the absolute aldosterone production, were significantly reduced as compared to the controls. This reduction did not affect corticosterone production.

Fig. 23 sums up the pathological conditions and clinical syndromes associated with a suppression of renin–angiotensin activity and also of aldo-

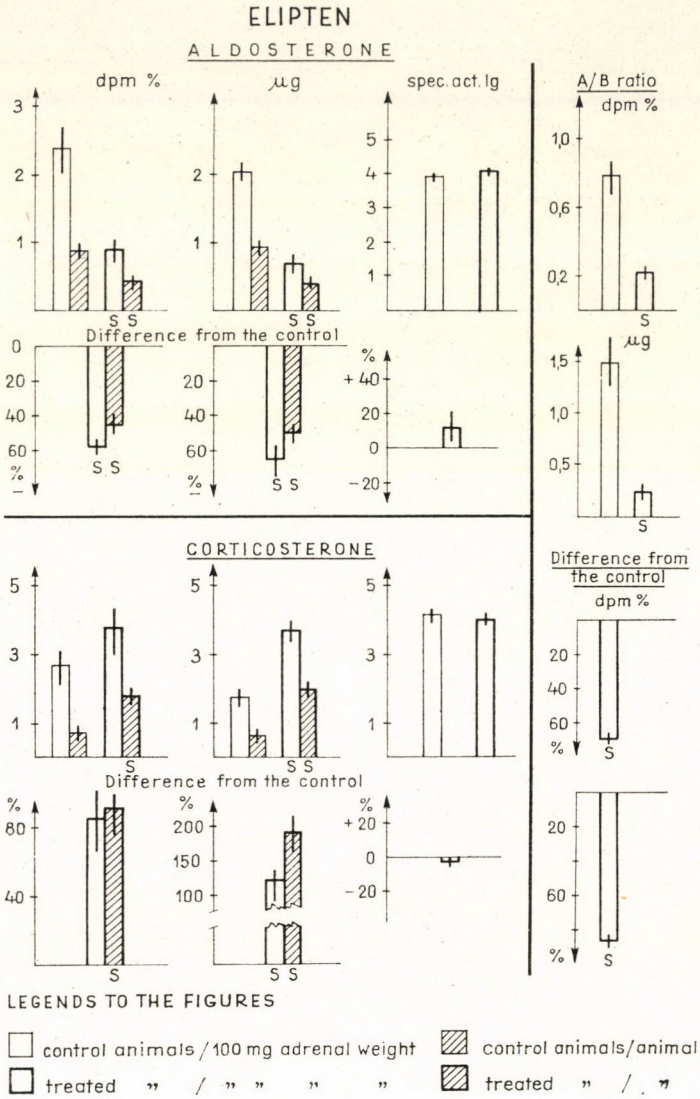

Fig. 22. Effect of aminoglutethimide on the aldosterone and corticosterone biosynthesis in rat adrenal cortex (GLÁZ et al.)

sterone secretion in consequence of an overproduction of mineralocorticoids, but not of aldosterone. As a result, the levels of renin–angiotensin II and also of aldosterone are low.

The evidence furnished by the recent studies of MELBY et al. (1971) is highly relevant to this issue, these authors having found an abnormally high urinary excretion of 18-OH-tetrahydro-DOC in the presence of low or normal

Hypertensive conditions
Biglieri's syndrome
(17-hydroxylase defect: hypersecretion of 11-desoxycorticosterone and corticosterone)
Congenital adrenocortical hyperplasia
(11-beta-hydroxylase defect: hypersecretion of 11-desoxycortisol and 11-desoxycorticosterone)
Pseudo-Conn-syndrome (excessive use of glycyrrhizic acid)
Renal tubular cell defect
Liddle's syndrome
Other conditions
Nephrectomy (bilateral)
Parkinson's disease

Fig. 23. Conditions associated with a reduced renin-angiotensin activity and low aldosterone level

aldosterone and DOC-plasma levels in patients with suppressed renin activity. A high secretion rate of 18-OH-DOC was demonstrated by KÜCHEL (1971) in patients of the same type. In view of the moderate mineralocorticoid activity of the substance in question further observations are needed for bringing these findings in harmony with the aetiology of hypertensive disease accompanied by reduced renin and aldosterone levels.

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ROLE OF GLUCOCORTICOIDS IN CONTROLLING PITUITARY-ADRENAL FUNCTION

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The control of pituitary-adrenal function by plasma corticosteroid concentration has been recognized years ago and widely discussed in the light of the recent investigations (see reviews by HARRIS, 1955; GUILLEMIN, et al., 1958; LISSÁK and ENDRŐCZI, 1965; YATES, 1967, etc.). An importance of the free as opposed to the bound corticosteroid in the negative feedback signalization has been suggested by numerous authors (see review by FORTIER, 1966). Thus, the corticosteroids are distributed in three compartments of the plasma; the free, the albumin-bound and the transcortin-bound fractions. An equilibrium of the three compartments as the function of dissociation constants was estimated by SLAUNWHITE and SANDBERG (1959), and the protein-bound fraction was found to be inactive in testing corticosteroid activity. On the other hand, it was found that the thyroid and ovarian hormones exert a significant influence on the plasma transcortin level and induce a shift of the free fraction which is followed by an excess activation of pituitary ACTH secretion (see review by FORTIER et al., 1970). Obviously, if someone studies the role of plasma corticosteroid concentration as a controller of pituitary-adrenal function, he must be aware of the dynamics of the equilibrium between free and bound compartments of the plasma corticosteroids. Moreover, the factors involved in the control of the transcortin level show a marked species difference, and observations from animal experimentations are not necessarily valid for humans.

Time patterns of pituitary ACTH and adrenal corticosterone production in the rat

An increase in the plasma corticosterone concentration on ACTH administration or on an increase of endogenous ACTH production in response to various stressors has been observed in many laboratories. The time pattern of the two variables has been also assumed although a minute-by-minute comparison is lacking in the relevant literature. By using the conditioned fear as a constant stimulus we have studied the changes of plasma ACTH

and corticosterone concentration in the rat. Conditioned fear was established in a box and the animals were given, through an electrified grid serving as the floor of the box, 10 electrical shocks in 10 consecutive days. No escape was allowed and the situation itself meant the conditional fear stimulus. On the 11th day the rats were placed in the box and killed in different intervals for determination of the plasma ACTH and corticosterone levels. Plasma ACTH estimation was performed according to the ultramicro technique of SAYERS et al. (1970) and corticosterone was determined fluorometrically by the method of GUILLEMIN et al. (1958).

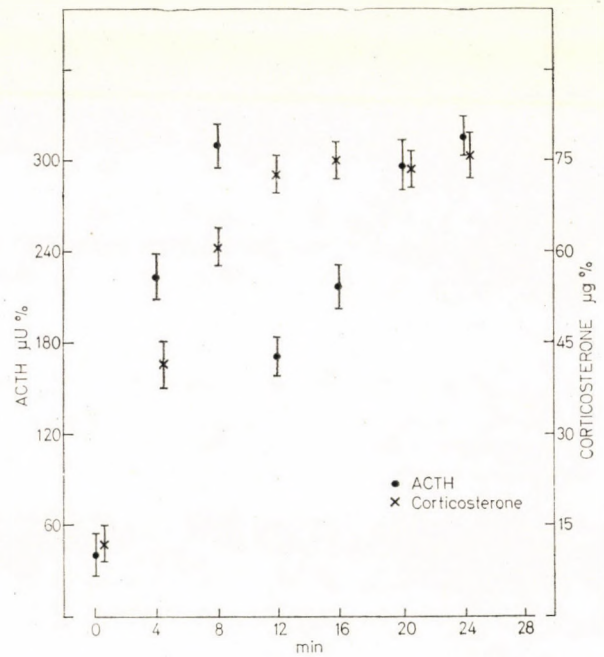

Fig. 1. Effect of conditioned fear on plasma ACTH and corticosterone concentration in the rat. Each bar corresponds to the mean and standard error of six animals killed in different intervals after exposure to the test situation

The plasma ACTH concentration showed a rise within 4 to 5 minutes following exposure of the animals to the conditioned fear situation but this increase was followed by a transient decrease in a minimum of 12 to 14 minutes and a subsequent increase in the further observation period. In contrast, plasma corticosterone showed an increase somewhat later and reached the maximum level after 12 to 15 minutes (Fig. 1). These observations led us to assume that an intermittent increase of pituitary ACTH secretion under the influence of a constant stimulus pattern cannot be attributed to the change of the plasma corticosterone level as a supposed controller and a fluctuation

of the ACTH release reflects some changes of the central nervous excitatory states involved in the activation of corticotropin producing elements. A repetition of the investigation in rats with chronically implanted electrodes revealed that a release of pituitary ACTH occurs in 10 to 12-minute periods and is usually preceded by an EEG arousal reaction (Fig. 2). A fluctuation of the arousal state of rats in a situation with constant stimulus pattern or in the absence of motivation stimuli has been described in electrophysiological studies (see review by LISSÁK and ENDRŐCZI, 1965). The present data clearly demonstrated a correlation between the onset of EEG arousal and pituitary

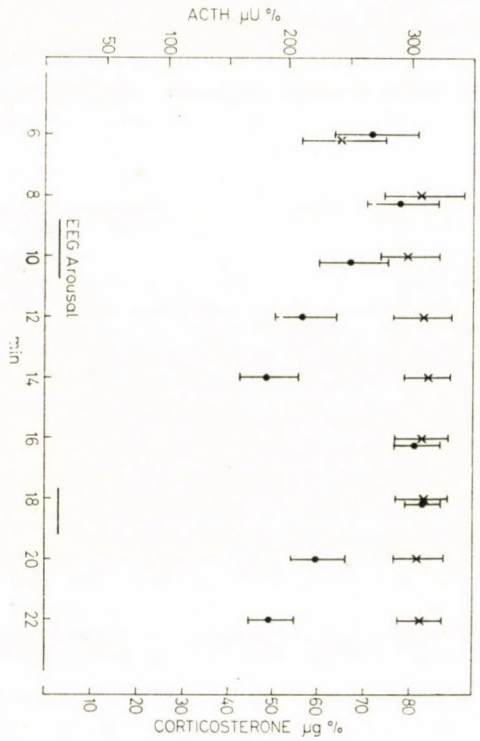

Fig. 2. Correlation between EEG arousal and the fluctuation of plasma ACTH level by rats in a conditioned fear situation. Each bar shows the mean and standard error of 5 to 8 rats killed in different intervals after beginning the EEG recording

ACTH release in the conditioned fear situation. However, there were no data about the fluctuation of ACTH secretion in a non-motivated state when a periodical shift of the alert and non-alert states is still observable in the electrocorticographic records.

A different time pattern in the secretion of pituitary ACTH and adrenal corticosterone has been observed when rats were exposed to 10 electrical shocks within 1 minute and the animals were killed in the 8th or 20th minute

for determination of the plasma ACTH and corticosterone levels. It was found that the daily repetition of shocks resulted in a decrease of the ACTH level, although the rise of plasma corticosterone concentration did not change during the observation period (Fig. 3).

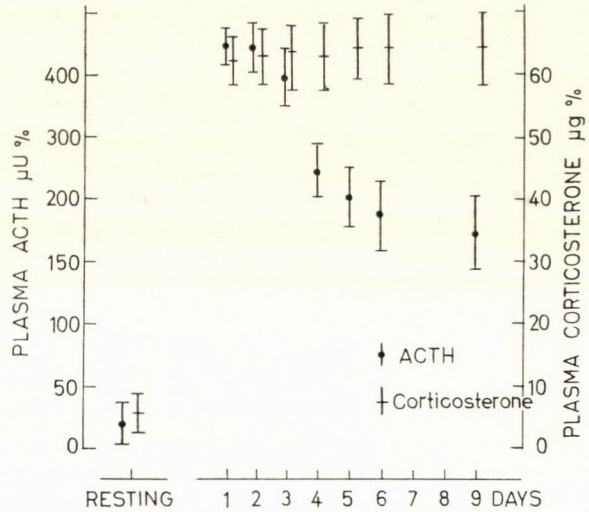

Fig. 3. Effect on pituitary-adrenal function of repetition of 10 electrical shocks on consecutive days. Ten shocks were given daily and the animals were killed in the 8th and 20th minutes for determination of the plasma ACTH and corticosterone concentration

Corticosterone binding property of the central nervous structures, corticosterone adjusted behaviour

Chemosensitivity of the central nervous structures for corticosteroids in term of the control of pituitary ACTH secretion has been recognized by implantation (ENDRŐCZI, 1969), deafferentation (HALÁSZ et al., 1962) and electrophysiological studies (FELDMAN et al., 1961; ENDRŐCZI, 1969). The use of iontophoretic dexamethasone phosphate application into the cells of the hypothalamus and thalamic nuclei revealed that the majority of cells responded by a decreased firing rate (RUF and STEINER, 1967). Moreover, both electrophysiological and behavioural experiments revealed that the brainstem and hypothalamic connections are sensitive to hydrocortisone and both facilitatory and inhibitory influences have been reported (see review by ENDRŐCZI, 1969; DEWIED and WEIJNEN, 1970).

The use of labelled corticosterone clearly demonstrated that the hippocampal cells can accumulate much greater amounts of this steroid than do other parts of the brain (MCEWEN et al., 1969). It was also found that the corticosterone uptake of an adrenalectomized rat cannot be suppressed by

the previous administration of hydrocortisone or dexamethasone which steroids are not species-specific for the rat. A selective accumulation of the septal-hippocampal unit merits considerations in the light of the findings that both rostral septal lesions destroying the fornix and hippocampectomy led to failure of the organization of corticosterone-adjusted behaviour. Moreover, it seems that the hippocampus is a specific receptor structure not only for corticosterone but also for triiodothyronine and in a lesser extent for oestradiol (DUPONT et al., 1970, 1971).

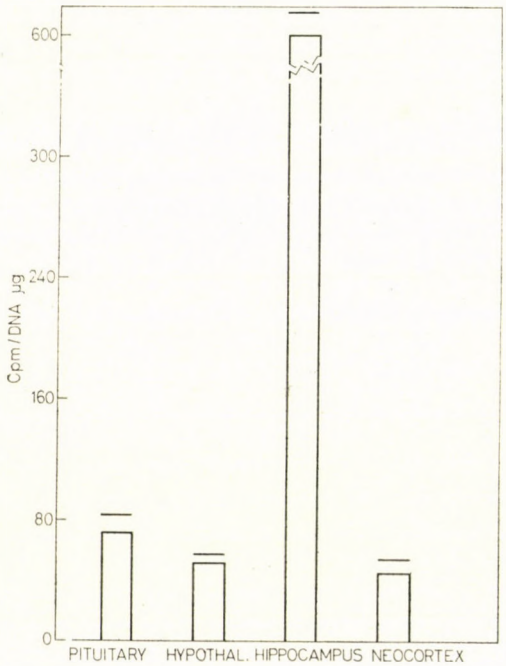

Fig. 4. ^{3}H-corticosterone uptake by the different parts of rat brain 24 hours after adrenalectomy and 60 minutes following intravenous injection of the steroid. All data are expressed on the basis of the DNA content

Rats with rostral septal lesions or hippocampectomy were kept under 23-hour water deprivation for 5 days and water was provided only in the experimental box. On the 6th day drinking attempts were associated with electrical shocks for 20 minutes. This passive avoidance learning showed an exponential character and the learning rate could be expressed by a plot of the logs of consecutive intertrial intervals on the sequence of trials (Figs 4 and 5). Intravenous injection of 200 μg/100 g corticosterone one hour prior to the experimental session produced a significant increase of the learning rate in the adrenalectomized rat. This facilitatory influence could be prevented by hippocampal ablation or rostral septal lesion (Fig. 6). On the other hand, dexamethasone remained effective after hippocampectomy.

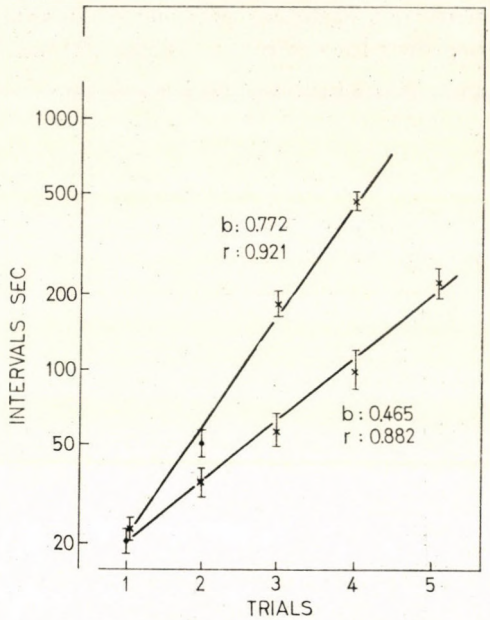

Fig. 5. **Effect on the learning rate in adrenalectomized rats of 200 μg/100 g corticosterone given intravenously one hour prior to the experimental session. x = physiological saline, ● corticosterone administration**

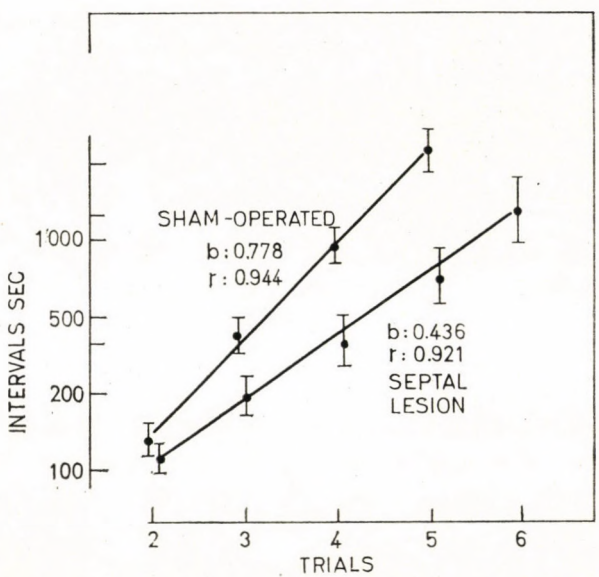

Fig. 6. **Effect of 400 μg/100 g corticosterone on learning rate of sham-operated and septal lesioned animals. The figure indicates a lack of the response following septal lesion**

The exploratory activity of both hippocampectomized and rostral septal lesioned rats was higher than that of the sham-operated controls. Corticosterone resulted in a suppression of the exploration, when injected in an amount of 400 μg/100 g subcutaneously 3 hours prior to the exploratory test in sham-operated but adrenalectomized rats. In contrast, the rats with septal lesion showed a marked resistance to the suppressive effect of corticosterone on the exploration, although dexamethasone resulted in a decrease of the exploratory scores of both groups (Fig. 7). In view of the increased resistance to corticosterone of septal-hippocampal lesioned rats, which could be observed

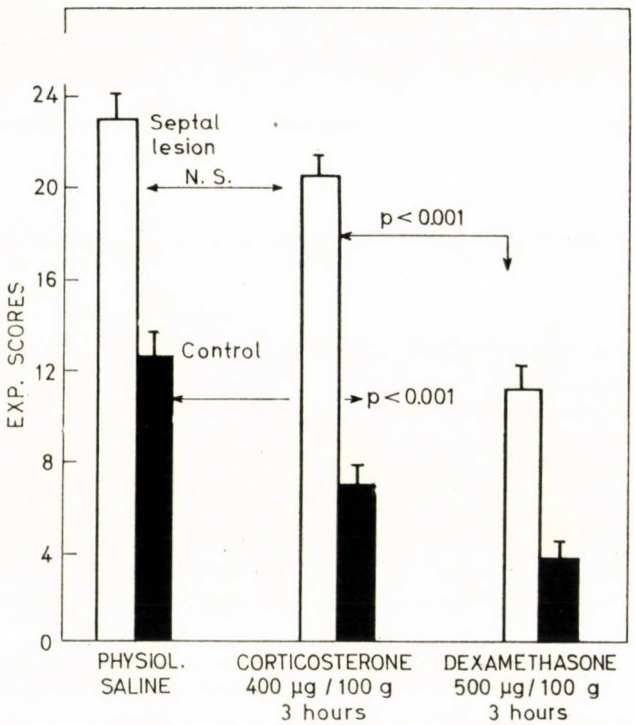

Fig. 7. Effect of corticosterone and dexamethasone on the exploratory activity of sham-operated and septal lesioned rats (black columns). Exploratory activity was tested in a 12-cell maze; scoring was made on the basis of the crossings of gates during a 10-minute observation period

in both the exploration and the passive avoidance conditioning, we may assume that the natural glucocorticosteroid as corticosterone exerts an influence on the brain and behaviour relationship through the septal-hippocampal unit.

A primary involvement of the septal-hippocampal unit in the corticosterone-adjusted behaviour is supported by the finding that corticosterone administration produced a suppression of the hippocampal theta rhythm,

which is in accordance with the suppression of exploratory activity (ENDRŐCZI and NYAKAS, 1971).

The above findings led us to assume that corticosterone exerts an effect on the brain through the specific binding sites of the hippocampus and other glucocorticoids like hydrocortisone or dexamethasone produce changes through non-specific receptors of the hypothalamus and the brain stem. This view is supported by the electrophysiological finding in that hexamethasone iontophoresis did not induce changes in the firing rate of hippocampal cells but markedly changed the unit activity in broad areas of the hypothalamus (RUF and STEINER, 1967; see review by ENDRŐCZI, 1971). On the other hand, hydrocortisone or dexamethasone administration results in behavioural changes very similar and frequently more powerful than corticosterone. This, however, does not mean that species-specific and non-specific steroids are acting at the same neuroanatomical substrate.

Interactions between the pituitary-adrenal and the pituitary-thyroid and gonadal functions

Endocrine control of the pituitary ACTH release in the sense of a closed loop feedback regulation (YATES, 1967) depends primarily on the plasma corticosterone concentration. Other endocrine factors exert an effect through the changes of the corticosteroid-binding capacity of the plasma compartment or by changes of the corticotropin releasing factor acting on the hypothalamus or the ACTH release directly at the pituitary level.

Recently, we have been interested in studying the involvement of pituitary-thyroid function in the oestradiol-induced activation of the pituitary-adrenal axis. The compensatory hypertrophy of the adrenals following thyroidectomy is markedly suppressed, and the role of thyroid hormones in the maintenance of normal pituitary-adrenal function is supported by numerous observations (see review by FORTIER, 1966). Moreover, it was also assumed that the oestradiol-induced adrenal hypertrophy is mediated through an increased TSH secretion (FORTIER et al., 1970). Such a regulatory process seems, however, operating only under limited physiological conditions.

Implantation of 4.5 μg oestradiol into the median eminence produced a significant compensatory adrenal hypertrophy in thyroidectomized rats (Fig. 8). Thyroidectomy was performed 3 to 4 weeks prior to steroid implantation; one group received cholesterol implants and other groups oestradiol plus 1 μg triiodothyronine daily for 12 days. The compensatory hypertrophy was expressed as per cent of the initial wet weight. These observations clearly demonstrated that oestradiol increases ACTH secretion in the absence of a concomitant increase of pituitary-thyroid secretion.

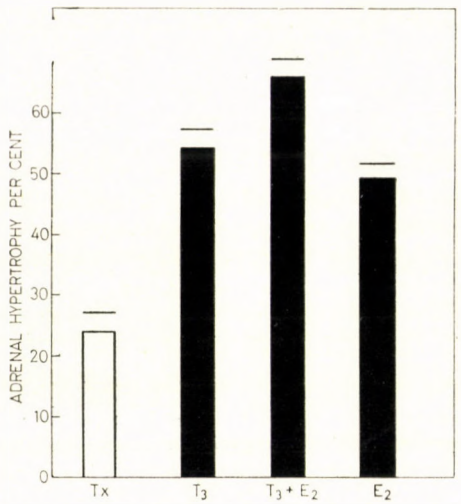

Fig. 8. Effect of oestradiol implantation on the compensatory hypertrophy of adrenals in thyroidectomized rats. Cholesterol implantation was performed in the Tx (thyroidectomized group), and daily 1 μg triiodothyronine was given in the second and third groups. The fourth group received oestradiol implants without substitution therapy. Each column shows the mean and standard error of 6 animals

Discussion

Recent data on the control of pituitary-adrenal function have revealed that a short-term control by the changes of plasma corticosterone concentration as postulated by the "closed loop feedback theory" of YATES (1967) or its modification by several authors is not consistent with numerous experimental findings. Thus, a rise of the plasma corticosterone concentration failed to induce a suppression of pituitary ACTH release in response to either nervous or humoral stressors, and an inhibition has been observed after 3 to 16 hours when corticosterone output had returned to the resting level (SMELIK, 1963). This time lag of the inhibition may be attributed to those changes which occur at the central nervous level during the course of a feedback action of corticosterone and the inhibition seems to be of nervous origin. The participation of the septal-hippocampal unit in controlling both the sensory input of the hypothalamus and pituitary ACTH secretion is known from earlier studies (see reviews by LISSÁK and ENDRŐCZI, 1965; ENDRŐCZI, 1969, 1970; and ENDRŐCZI and NYAKAS, 1971). A selective accumulation of corticosterone by the hippocampal cells is also suggesting an important role of the septal-hippocampal unit in the organization of the corticosterone-adjusted behavioural reactions and in the control of daily variations of the pituitary-adrenal activity under the synchronizing influence of light and dark periods. By all means, we must be aware that these conclusions derived from studies with species-

specific glucocorticosteroid such as corticosterone, and the use of other glucocorticosteroids does not exclude their effect on the endocrine and behavioural processes through other receptor structures of the hypothalamus and brainstem. Moreover, there is no doubt that the hippocampus is one of the structures which stores the greatest amount of corticosterone but other parts of the brain also contain cells with a similar property.

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MORPHOLOGICAL BASIS OF THE HYPOTHALAMIC CONTROL OF STEROID PRODUCTION

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Synthesis and release of hormonal steroids are controlled by adrenocorticotrophic and gonadotrophic hormones. One of the most important factors in the regulation of secretion of these pituitary hormones is the feedback action of hormonal steroids. Experimental data accumulated in the last two decades support the assumption that the feedback action of gonadal and adrenocortical steroids is mediated, at least partly, through multiple neuronal elements situated in various parts of the brain, especially in the hypothalamus. This is termed now as indirect or neuro-hormonal feedback.

Data concerning the control of the production of adrenocortical steroids will be presented during this meeting in the Symposium on the Regulation of ACTH secretion. Therefore, I limit my presentation to the role of hypothalamic structures in the control of pituitary-gonadal function by feedback of gonadal steroids.

It was as early as 1932 that HOHLWEG and JUNKMANN (1932) suggested that the ovarian hormones might influence gonadotrophic functions through a hypothetical "sexual centre" located somewhere in the brain. It was demonstrated later by SAWYER et al. (1949) and by EVERETT and SAWYER (1949) that the effects of oestrogen and progesterone in enhancing the spontaneous release of luteinizing hormone (LH) could be eliminated by neural blocking agents. This clearly showed the neural character of the "positive" (stimulating) feedback action of ovarian steroids on the secretion of gonadotrophic hormones. The first experimental evidence of the existence of a "negative" (inhibiting) neurohormonal feedback in the control of gonadotrophin secretion has come from our laboratory.

In 1954, our attention was especially attracted by the effect of an anterior hypothalamic lesion resulting in persistent vaginal cornification and polyfollicular ovaries in the rat (FLERKÓ, 1954). HILLARP (1949) and GREER (1953) who at that time confirmed the effect of the anterior hypothalamic lesion resulting in polyfollicular ovaries first described by DEY (1941), were of the same opinion as DEY (1941) in that this effect might primarily reflect a deficiency in LH secretion, while the follicle-stimulating hormone (FSH)-

oestrogen component of the pituitary-ovarian interplay was presumably spared. It was suggested that a discharge of LH insufficient for ovulation should be ascribed to the destruction of an LH-mobilizing centre localized in the anterior hypothalamus. Especially on the basis of the pioneer experiments of MARKEE, EVERETT and SAWYER (1952), which have clearly shown the existence of a neural LH-releasing mechanism somewhere in the hypothalamus, we could agree with this explanation. Keeping in mind, however, the principal interplay of gonadotrophic and ovarian hormones, we realized the difficulty in accepting this explanation based merely on the destruction of a hypothalamic LH-releasing mechanism. The principal inconsistency of the explanation arises from a neglect of the well-known fact that the secretion of FSH was also inhibited by the continuous action of oestrogens on the pituitary. Simultaneously with the presence of the clear-cut signs of continuous oestrogen secretion (enlarged pituitary, cystically dilated endometrial glands, persistent vaginal cornification, etc.), the ovaries of rats with anterior hypothalamic lesion contain normally growing follicles indicating a normal release of FSH. DEY's (1941) and HILLARP's (1949) explanation for the anterior hypothalamic lesion effect failed to answer the question why the continuous oestrogen action, present in these lesioned animals, should not inhibit the secretion of FSH? In order to eliminate this contradiction we assumed a nervous mechanism in the preoptic-anterior hypothalamic area indispensable for the inhibitory action of physiological amounts of oestrogens upon FSH secretion. We and others were able to substantiate this assumption in a series of experiments in the late fifties.

The inhibitory effect of oestrogen (1 μg/day) on the castration-induced rise in FSH output could considerably be diminished by electrolytic lesions placed in the anterior hypothalamus of juvenile rats, as revealed in parabiosis experiments (FLERKÓ, 1956, 1957a). — Ovaries of juvenile rats given 1 μg of oestrogen daily and bearing anterior hypothalamic lesions contained a larger number of corpora lutea than did ovaries of oestrogen-treated rats without anterior hypothalamic lesion. The poor luteinization or its complete absence in ovaries of oestrogen-treated intact juvenile rats have been explained by lack of FSH. These ovaries contained a smaller number (if any) of follicles capable of being luteinized than did ovaries of rats with anterior hypothalamic lesion (FLERKÓ, 1957b). — Oestrogen released from small fragments of ovarian tissue autotransplanted into the anterior hypothalamus inhibited FSH secretion. Similar ovarian implants in the adenohypophysis and in the posterior hypothalamus failed to produce this effect (FLERKÓ and SZENTÁGOTHAI, 1957). — Testosterone (500 μg per day) failed to cause intrasplenic ovaries to involute in spayed adult rats with anterior hypothalamic lesions, although it did in control animals with intrasplenic grafts but without hypothalamic lesions (FLERKÓ and ILLEI, 1957). — In HOHLWEG and DAUME's (1959) ex-

periment, oestrogen when injected into the anterior hypothalamus of rats, had an antigonadotrophic effect 125 times more intensive than if administered subcutaneously. — Uninhibited FSH-oestrogen secretion following anterior hypothalamic lesions brought about an increase in pituitary weight resembling to cytological changes similar to those observed after oestradiol treatment (FLERKÓ and BÁRDOS, 1960). — The nuclei of nerve cells in the preoptic-anterior hypothalamic area showed a consistent decrease in size when the blood sexual steroid level was increased or decreased (FLERKÓ, 1962).

On the basis of the above experimental results we (FLERKÓ and SZENTÁGOTHAI, 1957) assumed that there are oestrogen-sensitive neurons in the preoptic anterior-hypothalamic area, through which FSH release is inhibited by a slight (physiologic) elevation of the oestrogen level in the blood. On the other hand, the absence of compensatory ovarian hypertrophy following hemispaying of animals with anterior hypothalamic lesion (D'ANGELO and KRAVATZ, 1960; FLERKÓ and BÁRDOS, 1961a) supported the idea that anterior hypothalamic oestrogen-sensitive neurons play a role also in the mechanism by which the decrease in the blood oestrogen level enhances FSH release.

The postulate that oestrogen-sensitive neurons are present in the preoptic-anterior hypothalamic area was later supported by the experimental results of LITTLEJOHN and DE GROOT (1963), FENDLER and ENDRŐCZI (1965—1966), KÖVES and HALÁSZ (1969) as well as by the findings that individual neurons in this part of the brain accumulate oestrogen (MICHAEL, 1962; ATTRAMADAL, 1964; STUMPF, 1968), and the anterior hypothalamus, including the preoptic area, takes up and retains oestradiol in the same way as do the peripheral oestrogen-reactive tissues (EISENFELD and AXELROD, 1965; KATO and VILLEE, 1967; FLERKÓ et al., 1969). Concerning progesterone, RALPH and FRAPS (1959, 1960) and BARRACLOUGH and YRARRAZAVAL (1961) were the first to show that the anterior and ventral hypothalamus in the hen, and the medial preoptic area in the rat, are specific sites at which this hormonal steroid acts in the sense of a "positive" feedback facilitating ovulation.

In a brilliant series of experiments (see HALÁSZ, 1968, 1969), HALÁSZ et al. have shown that the medial-basal part of the rat hypothalamus, termed "hypophysiotrophic area" (HTA) by HALÁSZ et al. (1962), contains the neurons which produce the hypothalamic releasing and inhibiting factors, among those the follicle-stimulating and luteinizing hormone-releasing factors (FRF and LRF). According to SZENTÁGOTHAI's (1962, 1964) investigations, these neurons build up, at least partly, the tractus tuberoinfundibularis or hypothalamic parvicellular neurosecretory system. The fibres of this system originate mainly from the small cells of the arcuate nucleus and of the suprachiasmatic region of the hypothalamus. They enter the pituitary stalk and terminate on or in the immediate neighbourhood of the capillary loops emerging

from the hypothalamo-hypophyseal portal system and penetrating the median eminence.

Depression of the activity of the LRF-producing neurons of the HTA by continuous oestrogen action appeared to be an important factor in evoking the anovulatory condition following anterior hypothalamic lesion. When the continuous negative oestrogen feedback on the LRF-producing neurons was greatly reduced or eliminated, these neurons stimulated again the anterior pituitary to release LH sufficient for luteinization. In this way, we have succeeded in inducing formation of corpora lutea in the formerly polyfollicular ovaries of rats made anovulatory by anterior hypothalamic lesions (FLERKÓ and BÁRDOS, 1961b). It seemed therefore reasonable to assume that the FRF- and LRF-producing neurons of the HTA are also influenced directly by the gonadal hormones in the sense of a negative neuro-hormonal feedback. This assumption was supported by the experimental results of LISK (1960, 1962, 1963), DAVIDSON and SAWYER (1961a, 1961b), ARAI (1962; 1963), IFFT (1962), MCCANN (1962), KANEMATSU and SAWYER (1963a, 1963b; 1964), KOBAYASHI et al. (1963), HILLIARD et al. (1964), RAMIREZ et al. (1964), DAVID et al. (1965), DAVIDSON and SMITH (1966), KOBAYASHI et al. (1966), KOBAYASHI and FARNER (1966), PIACSEK and MEITES (1966) and PASTEELS (1970). All these experimental results indicated the presence of oestrogen and testosterone sensitive neurons in the medial-basal part of the hypothalamus. Further support for this assumption came from the findings that a considerable number of neurons in the medial-basal part of the hypothalamus, especially in the arcuate nucleus, accumulate oestradiol (STUMPF, 1968), and this part of the brain shows a pattern of uptake and retention of tritiated oestradiol which is similar to the pattern found in the uterus and anterior pituitary, i.e., in the peripheral oestrogen-reactive tissues (FLERKÓ et al., 1969).

It may be assumed with considerable certainty that the sensitivity or responsiveness to oestrogen of the neurons in the preoptic-anterior hypothalamic area is not identical with that of the neurons in the HTA, i.e., in the medial-basal part of the hypothalamus. Ovarian compensatory hypertrophy was blocked in animals with a frontal cut behind the anterior hypothalamic area, but this deafferentation did not interfere with the pituitary response to castration (KÖVES and HALÁSZ, 1969). Similar observations have been reported in animals with complete deafferentation of the HTA; ovarian compensatory hypertrophy did not occur in these rats, but at the same time pituitary LH content increased and castration cells developed in the anterior pituitary after bilateral ovariectomy (HALÁSZ and GORSKI, 1967). These findings suggest that the neurons in the preoptic-anterior hypothalamic area are more sensitive to changes in the blood oestrogen level than are the elements of the HTA-pituitary complex. This may account for the fact that HTA by itself is not able to maintain the cyclic secretion of gonadotrophic

hormones (tonic mechanism of trophic hormone secretion). To do this, the tonic mechanism, i.e., the FRF and LRF-producing neurons in the HTA, needs afferent impulses from the more sensitive steroid-receptor neurons of the preoptic-anterior hypothalamic area (cycle mechanism of gonadotrophin secretion). According to the varying oestrogen level in the blood the steroid receptor neurons modulate (i.e., enhance or inhibit) the tonic activity of the FRF- and LRF-producing neurons and, in this way, secure the cyclic release of FSH and LH. If the sensitivity or responsiveness of the neurons of the cycle mechanism is greatly reduced — like in the male (PETRUSZ and FLERKÓ, 1965) or in the androgen-sterilized female rats (PETRUSZ and NAGY, 1967), — the tonic mechanism by itself can maintain only the non-cyclic, tonic release of FSH and LH, characteristic of the male pattern of gonadotrophin secretion.

In an attempt to clarify the mechanism by which perinatal androgen action results in reduced responsiveness, and hence in diminished functional capacity of the hypothalamic oestrogen-sensitive neurons, a preliminary experiment (FLERKÓ and MESS, 1968) has revealed that the oestradiol-binding capacity of the pituitary and the uterus of androgen-sterilized rats was significantly reduced as compared to controls without early postnatal androgen treatment. This raised the possibility that perinatal androgen action might disturb the synthesis of the oestrogen-receptor proteins and in this way interferes with the oestradiol uptake and/or retention by the pituitary and the uterus. JENSEN et al. (1967), KING (1967) and others have suggested that uptake and retention of oestradiol occurs at receptor sites that are specific for oestrogens. The specificity of action of oestradiol on the oestrogen-responsive tissues has been explained by the ability of these tissues to take up and retain oestradiol in an unconverted form for a few hours. Since the experimental results, mentioned before, indicated that the anterior and middle hypothalamus contain oestrogen-sensitive neurons, one might postulate that the perinatal androgen action might damage the specific trapping mechanism in these neurons. If so, the oestradiol-binding capacity of the hypothalamic areas containing oestrogen-sensitive neurons should be reduced in the androgen-sterilized rat. We have succeeded in showing this in 1968 (FLERKÓ et al., 1969; FLERKÓ, 1970) and similar observations were reported by other authors (MCGUIRE and LISK, 1969; TUOHIMAA et al., 1969; VÉRTES and KING, 1969; MCEWEN and PFAFF, 1970). GREEN et al. (1969) also found that 30 minutes after an injection of 6,7-^{3}H-oestradiol-17β, some hypothalamic parts of the brain and pituitaries of spayed rats retained more radioactivity than did similar tissues in castrated males or spayed, neonatally androgenized females.

In the first experiment of GREEN et al. (1969), however, the males and neonatally androgenized females were heavier than the control female rats. To estimate the role of body weight in the uptake of oestradiol, a second experiment was performed, using male and female rats of equal group body

weight but of different age. Both sets of animals were sexually mature. In this second experiment, GREEN et al. (1969) did not find any difference between the sexes in the binding of oestradiol by any tissue, and concluded that the effect observed by us might reflect weight differences rather than a blockage of oestrogen receptor sites by neonatal androgenization as we have suggested.

We have therefore repeated our original experiment, using control and androgen-sterilized females of approximately equal group body weight since the control and androgenized rats used in our earlier experiments were of equal age but different in body weight. Similarly to our previous findings on control and androgenized rats of equal age but different body weight, the oestradiol-binding capacity of the neural (anterior and middle hypothalamus) and of the non-neural (uterus) target tissues was significantly reduced in androgen-sterilized rats as compared to controls of approximately equal body weight (FLERKÓ et al., 1971).

The finding that androgen-sterilized rats had significantly lower anterior and middle hypothalamic radioactivity levels than the controls without neonatal androgen action, supports our hypothesis that perinatal androgen action might interfere with the normal synthetic process of oestrogen-receptor proteins and, hence, with normal uptake and/or retention of oestradiol by the hypothalamic oestrogen-sensitive neurons. In this way, the neurons of the hypothalamic cycle mechanism become desensitized and functionally inactive in mediating positive and negative oestrogen feedback on the anterior pituitary cells producing gonadotrophic hormones. Thus, the reduction of oestradiol binding and the consequential loss of neurohormonal oestrogen feedback, induced by perinatal androgen action, might account for the non-cyclic pattern of gonadotrophin secretion and for the presence of anovulatory sterility in the androgen-sterilized rat.

Conclusion

The male or female pattern of gonadotrophin and gonadal steroid secretion is determined by different hypothalamic mechanisms.

The continuous, male type hormone secretion is maintained by the "tonic mechanism" composed of the tubero-infundibular neurons in the hypophysiotrophic area of the medial-basal hypothalamus, which produce and release the follicle-stimulating- and luteinizing hormone-releasing factors (FRF and LRF) into the hypothalamo-hypophysial portal circulation.

To secure the female type cyclic release of gonadotrophic and ovarian steroid hormones, the tonic mechanism needs afferent impulses originating from the steroid receptor neurons in the preoptic-anterior hypothalamic area ("cycle mechanism"). A number of experimental data indicate that the steroid sensitive neurons of the cycle mechanism are instrumental in the neurohor-

monal ovarian steroid feedback which modulates (i.e., enhances or inhibits) the activity of the FRF and LRF producing neurons and, in this way, maintains the cyclic gonadotrophin release.

YAZAKI (1959, 1960), HARRIS and LEVINE (1962, 1965), GORSKI and WAGNER (1965) have shown that the non-cyclicity of gonadotrophic function in the adult male rat is established only around the third day after birth, and the presence of testes in the first few days of postnatal life determines the male specificity of the hypothalamus. These experimental results suggest that the hypothalamus of the newborn rat of either sex has the inherent ability to maintain a cyclic release of gonadotrophins. It is only in the first few postnatal days that normal male rats under the influence of testicular androgens, or female rats that are given testosterone, lose the ability to release gonadotrophins in a cyclic manner and, thereby, to cause ovulation.

The finding that androgen-sterilized rats given tritiated oestradiol had significantly lower hypothalamic radioactivity levels than controls without perinatal testosterone-treatment, supports our hypothesis that a perinatal androgen action might interfere with a normal development of oestrogen-receptor proteins and, hence, with normal uptake and/or retention of oestradiol by the hypothalamic oestrogen-sensitive neurons of the cycle mechanism. In this way, these neurons might become desensitized and functionally inactive in mediating positive and negative oestrogen feedback on the anterior pituitary cells producing gonadotrophic hormones. If so, the reduction of oestradiol binding and the consequential loss of neurohormonal steroid feedback induced by perinatal androgen action, might account for the non-cyclic pattern of gonadotrophic and gonadal steroid hormone secretion in the male and androgenized female rat.

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NEURAL CONTROL OF PITUITARY ACTH SECRETION UNDER RESTING CONDITIONS

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It is generally accepted that anterior pituitary function is controlled by the central nervous system, first of all by the hypothalamus, and that the mechanism of this action is neurohumoral, i.e., some substances (called releasing and inhibiting factors) are released by the nerve endings in the superficial zone of the median eminence and pituitary stalk, which substances are then carried by the portal circulation to the anterior lobe cells.

This view contains two main assumptions, viz. (1) the hypothalamus produces substances which act on the anterior lobe, and (2) these are conveyed by the portal blood to the adenohypophysis. The data available support both assumptions.

Ad 1. With regard to pituitary ACTH secretion it has been demonstrated by SAFFRAN and SCHALLY [27] and by GUILLEMIN and ROSENBERG [9] that hypothalamic extracts cause a release of pituitary ACTH. The active substance has been called Corticotrophic Releasing Factor (CRF). Further studies confirmed the mentioned observation and, in addition, they suggested that hypothalamic extracts not only influence the release of ACTH, but probably act on its synthesis, too. This latter view is supported by UEMURA's [33] finding showing that pituitary ACTH content increases significantly if hypothalamic extract is given to the medium *in vitro*. The functional significance of CRF is underlined by the data that there is a similar diurnal variation in hypothalamic CRF activity as in pituitary ACTH secretion and that the daily peak of CRF activity occurs three hours earlier than the plasma corticosterone peak [2, 15]. — The chemical structure of CRF and the mechanism of its action on the anterior lobe are not known.

Ad 2. It has been reported by PORTER [23] that the blood collected from the proximal part of the transected pituitary stalk exhibits CRF activity. CRF activity has not yet been demonstrated in the peripheral plasma of intact animals. It was found only under special experimental conditions such as after hypophysectomy, when presumably the releasing factors are released in a larger amount [1, 3, 29]. It is very likely that CRF is present in the peripheral blood of intact animals, but in such a small amount that it cannot be detected by the current methods.

There is no direct information concerning the site of CRF production. The data available suggest that this function is restricted to the medial basal hypothalamus. The following observations support this idea.

(i) By implanting anterior pituitary tissue into various brain regions or under the kidney capsule of hypophysectomized rats we [12] found that ACTH secretion of the grafted anterior lobe is significantly better maintained if the pituitary is located in the medial basal hypothalamus than if it is outside this region, either in other parts of the hypothalamus, in extrahypothalamic regions or under the kidney capsule. We called the medial basal hypothalamus the hypophysiotrophic area (HTA) [11]. This area includes the ventral part of the periventricular nucleus, the medial part of the retrochiasmatic region and the arcuate nucleus.

(ii) CRF activity was found only in extracts from the median eminence region, i.e., from HTA [34].

(iii) The neurons in the HTA send their axons to the median eminence, forming the tubero-infundibular tract [32].

(iv) Nerve cells outside the medial basal hypothalamus have no axon terminals in the superficial zone of the median eminence [25].

HTA appears to be able to synthesize and release CRF in the absence of neural afferents. This is indicated by the observation that following neural isolation of the area pituitary basal ACTH secretion is unchanged [7, 17, 35] or, according to other authors [10, 13, 14], even enhanced (Table I). (An extremely small amount of ACTH is secreted if the anterior lobe is disconnected from the hypothalamus.) This suggests that presumably the HTA is primarily responsible for the maintenance of pituitary ACTH function.

However, the diurnal ACTH rhythm, existing in intact animals, fails after the interruption of the neural connections of the medial basal hypothalamus [13, 14, 22]. We [13, 14] found that under such circumstances pituitary ACTH content and plasma corticosterone levels are high in the morning as well as in the afternoon (Table I). This finding shows clearly that the HTA by itself is not capable of controlling normal ACTH activity, but requires afferent impulses. According to our findings [13, 14] the afferents critical for the diurnal ACTH rhythm reach the HTA from the anterior direction; interruption of the anterior connections of the area, leaving intact all the other pathways (frontal cut) blocks this rhythm, whereas the severance of the bilateral, superior and posterior connections (incomplete deafferentation) does not interfere with this function (Table I). Concerning the neural structures being involved in the diurnal ACTH rhythm, interesting observations were published by MOBERG et al. [20]. These authors found that transection of the fornix above the 3rd ventricle inhibits the diurnal changes in rat pituitary ACTH activity. Similar findings were obtained earlier by MASON [18] in monkeys. Further studies are required to clarify the origin of the critical afferents.

Table I

ACTH content of pituitary and corticosterone content of peripheral plasma and of adrenal gland of rats decapitated four weeks after sham operation or partial or total deafferentation of the hypophysiotrophic area [13, 14]

Group		No. of animals	Pituitary ACTH content	
			mU/ant. pit.	mU/mg ant. pit.
Sham operation	a.m.	5	131	13.3 (10.8—15.1)[a]
	p.m.	5	74	7.1 (5.8—10.1)
Complete	a.m.	2	191	26.8 (19.0—30.9)
deafferentation	p.m.	2	165	24.2 (20.1—28.7)
Incomplete	a.m.	4	125	13.3 (8.1—15.0)
deafferentation	p.m.	5	54	7.0 (3.2—10.2)
Frontal cut	a.m.	4	174	20.4 (15.6—24.1)
	p.m.	4	170	21.1 (15.3—26.7)

Group		No. of animals	Corticosterone content		
			adrenal gland		periph. plasma μg/100 ml
			μg/adr.	μg/g adr.	
Sham operation	a.m.	15	0.13 ± 0.01[b]	5.1 ± 0.4	7.5 ± 0.6
	p.m.	14	0.65 ± 0.01	26.6 ± 2.7[c]	25.6 ± 2.6[c]
Complete	a.m.	7	0.55 ± 0.02	21.0 ± 5.1	36.0 ± 5.1
deafferentiation	p.m.	5	0.69 ± 0.17	31.2 ± 5.0[d]	38.6 ± 5.4[d]
Incomplete	a.m.	6	0.18 ± 0.06	11.2 ± 4.7	6.7 ± 0.9
deafferentation	p.m.	8	0.42 ± 0.08	24.4 ± 5.1[c]	14.2 ± 1.3[c]
Frontal cut	a.m.	8	0.19 ± 0.08	10.1 ± 4.0	15.6 ± 3.4
	p.m.	7	0.14 ± 0.05	9.5 ± 3.6[d]	11.0 ± 2.4[d]

a: 95% confidence limits
b: mean and standard error of mean
c: $p < 0.01$ vs a.m. value
d: difference between a.m. and p.m. value is not significant

Corticosterone content of adrenal gland and peripheral plasma and ACTH content of pituitary were measured in the same animals but pituitary ACTH levels were not determined in all rats

Regarding the neural structures involved in the regulation of ACTH secretion but located outside the HTA, in the first place the limbic system and mesencephalon have to be taken into account. Some of these structures probably exert mainly a stimulatory, while others an inhibitory influence. The amygdala complex and some ill-defined mesencephalic regions (ventral tegmentum) appear to have a stimulatory effect; electrical stimulation of these regions induces ACTH release [4, 19, 24, 30, 31]. In contrast, stimulation of

the hippocampus, septum and mesencephalic dorsal tegmentum results in a decrease of the blood corticosteroid level in dogs, cats and monkeys [5, 19, 21, 26, 30] and destruction of these structures causes an opposite effect [4, 16] suggesting that these structures are mainly inhibitory. According to ENDRŐCZI and LISSÁK [5] the anterior and lateral hypothalamus also inhibit ACTH secretion.

Taking into account that neural structures outside the HTA have no nerve endings in the median eminence, it is close at hand to assume that the mentioned elements do not act directly on the anterior lobe, but might exert their influence via the HTA, by modulating the synthesis and/or release of CRF. This means that two levels exist in the neural control of pituitary ACTH secretion.

The first level is the HTA. This region produces CRF and appears to be responsible by itself for the maintenance of basal ACTH secretion, except the diurnal ACTH rhythm.

The second control level is represented by the neural structures outside the HTA. This level controls the diurnal rhythm of pituitary ACTH activity, among others.

It is not clear what sort of synaptic transmitters mediate the impulses within the various structures inside the second control level and from the second level to the first one, but it seems likely that acetylcholine, noradrenaline, serotonin and presumably other substances are also involved in these events. ENDRŐCZI et al. [6] have shown that injection of a cholinergic substance (eserine, carbaminoylcholine) into the septum, preoptic region, ventral lateral hypothalamic area or into the mesencephalic dorsal tegmentum inhibits ACTH secretion, whereas the same substances injected into the ventral tegmentum of the mesencephalon have an opposite effect. Serotonin given into the preoptic area, the anterior, mid- or posterior hypothalamus causes an increase in pituitary ACTH secretion. Interesting observations were made by SCAPAGNINI et al. [28]. They found that the diurnal variations in 5-hydroxytriptamine content of the amygdala and the hippocampus and the plasma corticosterone levels go parallel in time, both values are the lowest at 8 a.m. and the highest at 8 p.m. If 5-hydroxytriptamine synthesis was blocked by p-chlorophenylalanine, the morning level of plasma corticosterone was higher and the afternoon value lower, thus the diurnal variations disappeared. p-Chlorophenylalanine caused at the same time a 50 to 63% decrease in the 5-hydroxytryptamine content of the amygdala and the hippocampus. Based on these findings SCAPAGNINI et al. assumed that serotoninergic neurons play an important role in the control of the diurnal ACTH rhythm.

In summary, there are two levels in the neural control of pituitary ACTH secretion. One level is in the medial basal hypothalamus (the hypophysiotrophic area, HTA). This region produces the corticotrophic releasing

factor (CRF) and is probably responsible by itself for the maintenance of basal ACTH secretion. The other level is in the neural structures located outside the HTA, such as the limbic system, mesencephalon, etc. Some of these regions presumably stimulate, others inhibit ACTH activity. This level controls the diurnal ACTH rhythm among others. It probably does not affect directly ACTH secretion, but might act through the HTA by modulating the synthesis and release of CRF.

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REGULATION OF ACTH SECRETION IN STRESSFUL CONDITIONS

THE NATURE OF AFFERENT PATHWAYS INVOLVED IN STRESS-INDUCED ACTH SECRETION

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Ever since it has been generally recognized that a wide variety of stressful stimuli increase ACTH-glucocorticoid secretion, much attention has been devoted to clarifying the nature of the mechanism(s) by which these stimuli bring about rapid ACTH secretion. Over the past two decades, two theories have been advanced to account for the secretion of ACTH induced by acute stress: one emphasizes corticoid feedback or autoregulation, the other regulation by the central nervous system. DE GROOT and HARRIS (1950) were the first to produce direct evidence of the important role of the hypothalamus in increasing stress-induced ACTH secretion. Today the view is favoured that ACTH production is regulated by the corticotrophin releasing factor (CRF) and that this substance is released in the hypophyseotrophic area of the medial basal hypothalamus (MBH). Little is, however, known of the afferent pathways through which the various stressor agents are stereotypically leading to enhanced ACTH secretion.

In this report, the term "afferent pathways of the stress reaction" is meant to denote all the information-transmitting mechanisms which by activating the hypothalamo-hypophyseal unit lead to ACTH release. Such mechanisms are: nerve impulses, one or more humoral mediators that have entered the systemic circulation, and any circulatory, metabolic, and other alterations which activate the hypothalamo-hypophyseal unit directly or indirectly, one inducing the other.

FORTIER (1951) classified the stressful stimuli into two groups. Those that enhance ACTH secretion, even in animals with their hypophysis grafted into the anterior chamber of an eye, he designated as "systemic" because he thought that under such conditions the stress acts directly on the hypophysis by way of the systemic circulation. The stressful stimuli effective only if the hypophysis is not severed from the hypothalamus, i.e., that exert their effect by way of the CNS, he denominated "neurotrophic".

MIALHE-VOLOSS (1958), SMELIK (1959, 1960), DE WIED (1968) and other authors found that after mutilative interferences with the hypothalamus or the hypophysis — such as electrolytic lesioning of the hypothalamus or

neurohypophysectomy — some stressors are ineffective whereas others are effective.

The tentative division of stresses into "neural" and "systemic" groups by FORTIER (1951) was not accepted unanimously (see FORTIER 1966); partly, because in earlier experiments inadequate criteria (ascorbic acid depletion, lymphopenia) had been used to demonstrate ACTH release, and partly, because no allowance had been made for the possibility that in stress CRF might enter the transplanted hypophysis from the median eminence *via* the systemic circulation, as ANDERSON (1966), BRODISH (1964a), and, recently, SIRETT and KENDALL (1969) have justly hypotethized.

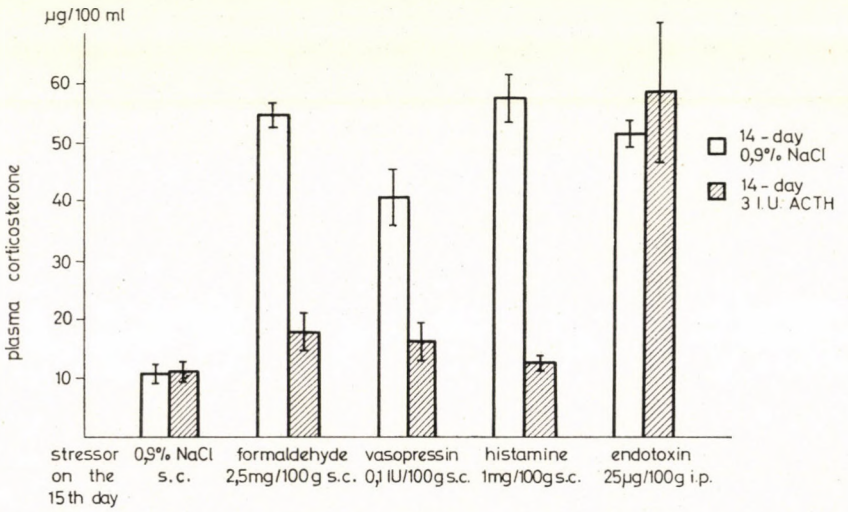

Fig. 1. Hypophyseal-adrenocortical response to different stressors 24 hours after the last of daily ACTH injections

Investigations of our own, in which the plasma corticosterone level was used as the indicator of ACTH secretion, suggested that different stressor agents increase the secretion *via* different afferent pathways.

If rats were exposed to the same stress daily for two weeks and the effect of it on the plasma corticosterone level was examined after the last exposure, some stressors failed to raise the corticosterone level, or raised it significantly less than after the first stress, whereas other stressors increased the plasma corticosterone level on the last like on the first day. A possible interpretation of these observations seemed to be that whereas the afferent pathways of certain stressors adapt themselves, those of others do not; this means, that the stressors exert their effects *via* different pathways (STARK et al., 1968a).

Results which may be interpreted similarly have been obtained in experiments in which the effect of a few stressors had been studied following treatment with ACTH for two weeks (STARK et al., 1968b). Fig. 1 shows that

on the day following the last ACTH injection the resting plasma corticosterone was at the control level, and an injection of endotoxin increased it to the same level as in the controls. On the other hand, the effect of histamine, or of a small dose of formaldehyde, etc., injected on that day caused no ACTH secretion. An explanation of the phenomenon might be that the last dose of a prolonged ACTH treatment raises the plasma corticosterone level more than does a single dose (STARK et al., 1963). It is this higher level which then may inhibit ACTH secretion 24 hs later (feedback action), when the corticosterone concentration is again normal.

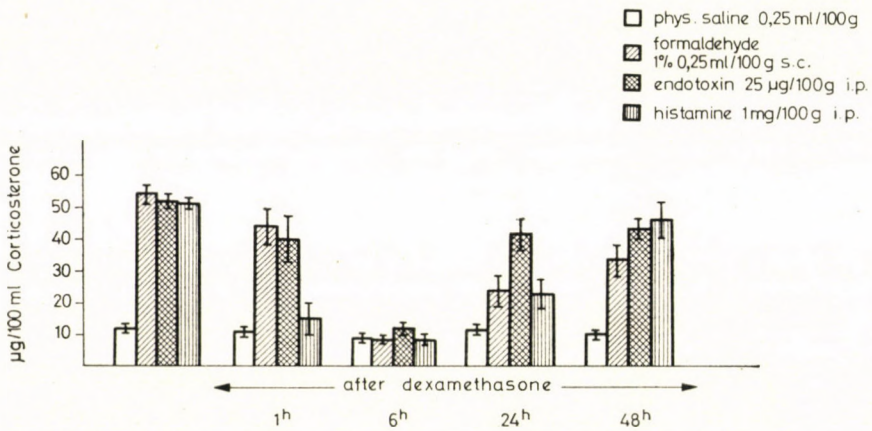

Fig. 2. Inhibitory effect of dexamethasone on ACTH release induced by different stimul- blood withdrawn 1 hour after formaldehyde, 3 hours after endotoxin, 30 min after histamin

Studies reported by YATES (1967) gave rise to the question whether endotoxin may exceptionally exert its effect *via* corticoid-insensitive pathways. Fig. 2 shows that the effect of endotoxin following dexamethasone is inhibited, which means that the endotoxin acts *via* corticoid-sensitive pathways; further, that in dexamethasone-treated animals stressor inhibition neither begins nor ends at identical points of time.

If it is assumed that the feedback action of corticosterone is exerted exclusively at the hypothalamo-hypophyseal level, what is the explanation of the observation that certain stressors release ACTH at a time when others do not?

As we see it, the feedback action of the high plasma cortisterone level is exerted not only at the hypothalamo-hypophyseal level but operates on afferent pathways through which stressful stimuli reach the hypothalamo-hypophyseal system. It is therefore conceivable that afferent pathways for endotoxin are available to stressful stimuli at a time when those for other stressors are not. On this basis it is safe to claim that some stressors reach the hypothalamo-hypophyseal unit by pathways different from those of other stressors.

We have assumed that stress-induced acute ACTH release results from increased CRF release and that the CRF-producing cells are in the MBH. Theoretically, the pathways which mediate the stressor effects to this area may be divided into a neural and non-neural group.

In the following, we shall designate as "neural" all the stressors which reach the MBH by neural pathways, and as "humoral" all those after whose application the information that enhances ACTH secretion reaches the CRF-producing cells by non-neural pathways such as the blood, CSF, and/or lymph circulation.

On the basis of such considerations, we have studied the ACTH-increasing effect of a number of stressors after both partial and total deafferentation of the MBH.

Surgery was performed by the method of HALÁSZ and PUPP (1965), and the increase in stress-induced ACTH secretion was observed 7 or 8 days after it, and successful surgery was checked on serial sections (MAKARA et al., 1969a).

In animals with anterolateral deafferentation of the MBH, noise and vibration, surgical stress, and a small dose of formaldehyde failed to influence the plasma corticosterone level, but histamine and capsaicin produced a significantly lower one than in the control, whereas endotoxin enhanced ACTH secretion like in the controls (Fig. 3). Adrenal sensitivity to ACTH remained unchanged.

These experimental findings were interpreted as meaning that certain stressors increase CRF production by travelling to the MBH along anterior, lateral, and dorsal pathways, i. e. by neural ones. No rise in ACTH secretion ensued because after intersection of the pathways the stimulus could not reach the MBH. One or the other of these pathways is involved in the action of such stressors as histamine and capsaicin, as is shown by the fact that with the pathways intersected the efficacy of these stressors is considerably weakened. The residual efficacy of capsaicin and histamine is mediated by the posterior pathways left intact, since they are ineffective in the same doses after total deafferentation. Endotoxin proved to be as efficaceous in the deafferentated as in the sham-operated animals: it exerted its effect *via* the posterior or a humoral pathway.

In a separate experimental series we studied the ACTH-releasing effect of different stressors after total deafferentation of the MBH (MAKARA et al., 1970a).

Both after partial and after total deafferentation (Figs 3 and 4) the resting plasma corticosterone level was similar to that in the controls. This agrees with data reported by VOLOSCHIN et al. (1968), FELDMAN et al. (1970), but conflicts with those published by HALÁSZ et al. (1967), PALKA et al. (1969) and DUNN and CRITCHLOW (1969a, 1969b), who observed significantly in-

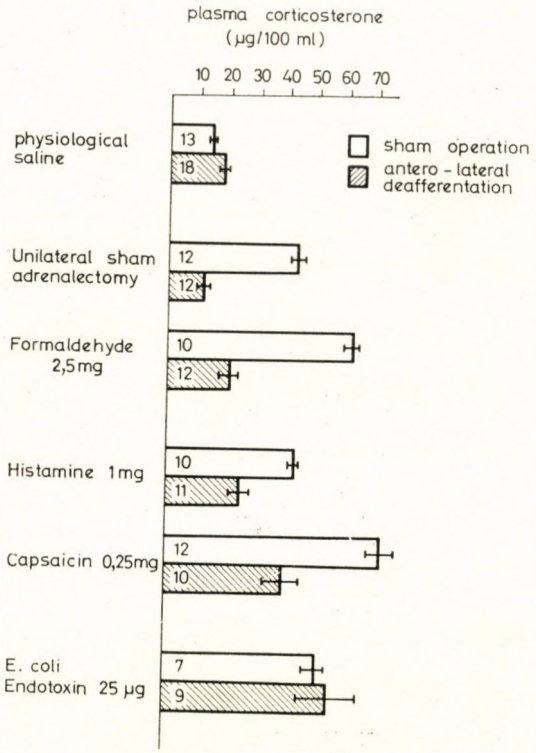

Fig. 3. Effect of stressful stimuli in rats with anterolateral deafferentation of the MBH The doses indicated were injected for 100 g weight

creased corticosterone levels after total deafferentation of the medial basal hypothalamus. As can be seen in Fig. 4, stressors ineffective after anterolateral deafferentation were ineffective also in totally deafferentated animals. E. coli endotoxin, a large dose of formaldehyde, and insulin raised the plasma corticosterone to the same level as in sham-operated animals (MAKARA et al., 1970a). It cannot be claimed that neural afferents would have no part at all in the action of these stressors, for it may be that in the intact animal ACTH secretion is increased by the combined action of humoral and neural afferents. It is, however, safe to state that to the neural afferents no decisive role can be assigned in this reaction.

Our results and data in the literature make it obvious that the stressful stimuli which equally increase ACTH secretion in the normal animal, are not equally effective after division of the nerves entering the MBH. Some are wholly ineffective, these are the neural stressors, while others are wholly effective, these are the humoral stressors. These data appear to assist in characterizing the afferent pathways through which the stressors release CRF.

In connection with the studies described so far, various questions present themselves, some of which I will attempt here to answer in the light of our experiments.

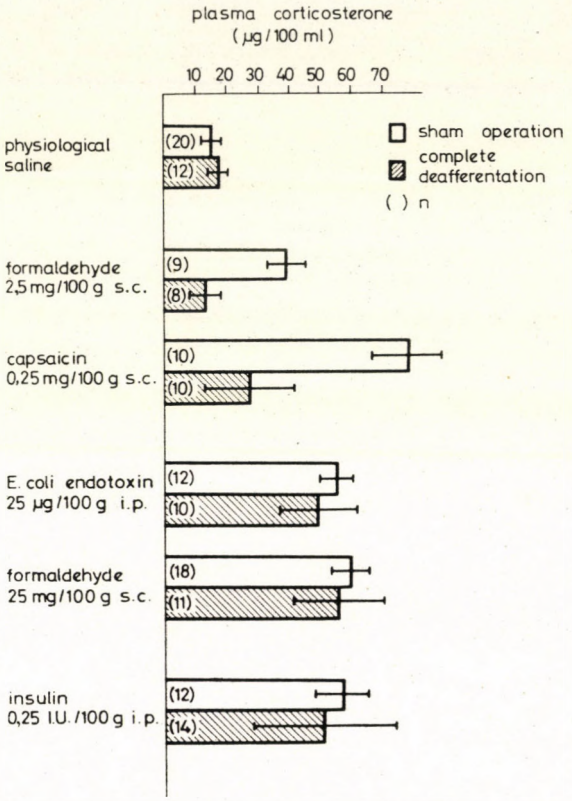

Fig. 4. Effect of stressful stimuli in rats with complete deafferentation of the MBH

(i) Do the stressors, which must have intact neural pathways to the MBH, to exert their effect, — travel along neural pathways at the periphery?

You will remember that a small (2.5 mg/100 g) dose of formaldehyde failed to increase ACTH secretion after both partial and total deafferentation of the MBH. When injected into a denervated hind limb, this small dose of formaldehyde produced no rise in ACTH secretion. Nor did it raise it in animals with their spinal cord severed (MAKARA et al., 1969b), which indicates that the afferent pathway of a small dose of formaldehyde is neural, not only centrally but also peripherally. The peripheral afferent pathway of surgical stress likewise proved to be neural (MAKARA et al., 1970b).

(ii) Since BRODISH (1964b) performed his investigations of surgical stress, the view has frequently been expressed that the stress reaction runs a two-phase course. Therefore, we have studied the question whether a stressor which has proved ineffective a certain time after deafferentation of the MBH, may not increase ACTH secretion at some later point of time.

Surgical stress failed to raise the corticosterone level in anterolaterally deafferentated animals in the first hour, but raised it significantly two hours

later (STARK et al., 1970a) (Fig. 5). This is in keeping with observations made by BRODISH (1964b) in animals with a lesioned hypothalamus. On repeating the experiment using formaldehyde we found that a small dose of it failed to enhance ACTH secretion 30, 60, or 120 minutes after its administration (Fig. 6). This shows that both after surgical stress and after a small dose of formaldehyde the information which stimulates rapid CRF release is mediated to the MBH by nervous pathways, but in the case of surgical stress the delayed

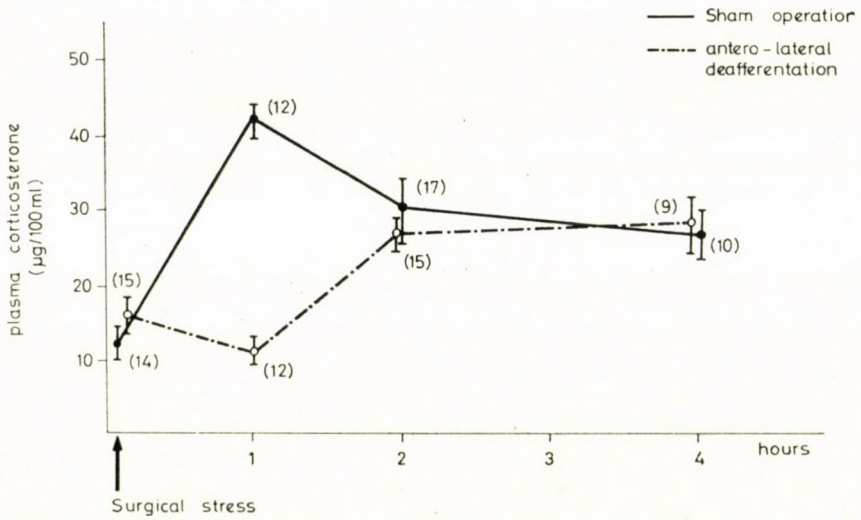

Fig. 5. Effect of surgical stress at various points of time in rats with antero-lateral deafferentation of the MBH

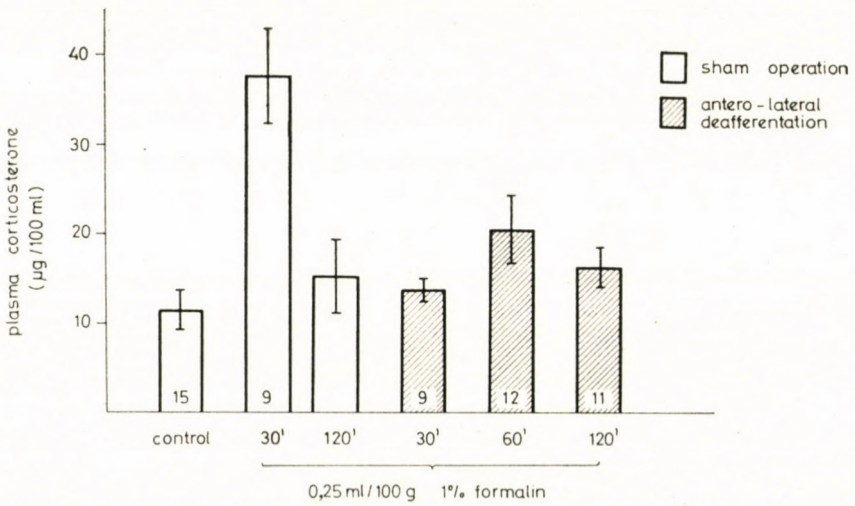

Fig. 6. Effect of formaldehyde at various points of time after injection in rats with antero-lateral deafferentation of the MBH

phase comes about *via* another pathway than in the rapid phase. Accordingly, the two-phase course is not a characteristic feature of every stressor agent.

(iii) According to some authors, stressors applied after certain interferences — such as hypothalamic lesioning, corticosteroid blockade, etc., — depend for their effects not on differences between their afferent pathways but on their intensity. The question is as to whether or not a change in intensity brings about a change of the afferent pathway through which these stressors exert their effect. We determined the plasma corticosterone level after increasing the amount of the stressor agent applied (formaldehyde or histamine), a procedure which can be regarded as equivalent to increasing the intensity (MANGILI et al., 1966).

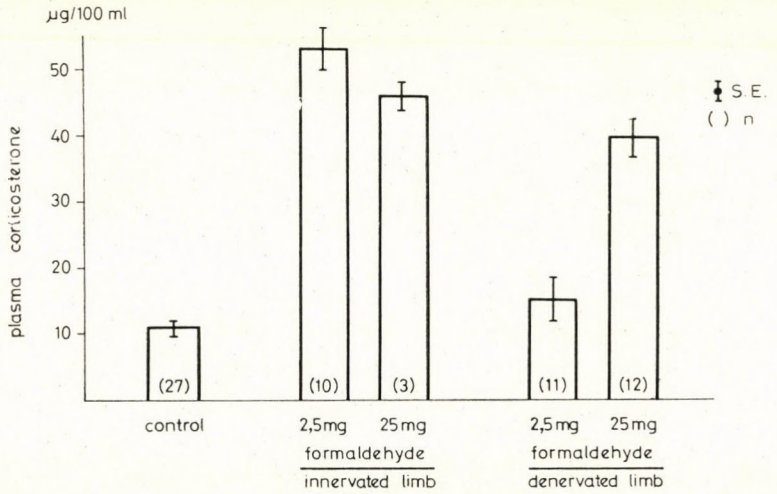

Fig. 7. Effect of different doses of formaldehyde injected into innervated or denervated hind limb

In sham-operated rats, different doses of formaldehyde produced identical corticosterone levels. Yet, when different doses were injected into rats with a denervated limb or a totally denervated MBH, only large doses raised the corticosterone level, and the increases differed insignificantly from those in the control animals (Fig. 7). This permits the conclusion that if we increase the intensity of a stressor, the afferent pathway(s) through which it increases ACTH secretion, undergoes a change.

(iv) A large dose of formaldehyde, endotoxin and histamine each were effective after total deafferentation of the MBH. The question is, did these stressors increase CRF secretion only, or are they capable of increasing ACTH secretion by bypassing the MBH?

The answer to this question was expected from experiments on animals deprived of the MBH.

A 4 mm wide strip of the part of the hypothalamus between the optic chiasma and the mammillary body, together with the brain tissue overlying that part, was removed through a glass tube by suction (Fig. 8).

Neither a small dose of formaldehyde nor surgical stress raised the plasma level of corticosterone. A large dose of formaldehyde, as well as endotoxin raised it significantly, though in a lesser degree than in the normal animal, probably for the lack of hypothalamic mediation, or because of the infarct in the adenohypophysis (STARK et al., 1970b). Dexamethasone even in a dose of 50 μg/100 g prevented these two stressors from increasing the secretion of ACTH (Figs 9, 10).

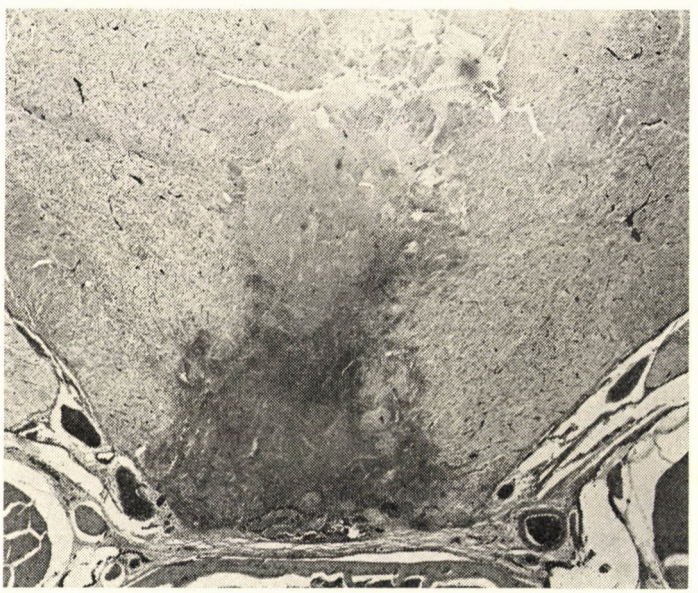

Fig. 8. Coronal section through the brain of a rat 24 hours after removal of the MBH (haemalum-eosin stain, 20×)

Against the suggestion that the endotoxin might further deteriorate the circulation of the isolated hypophysis and allow the ACTH to flow out from the ischaemic tissue, argues the finding that dexamethasone prevents the endotoxin from stimulating ACTH secretion, the same as it does in the normal animal.

As measured by fluorometry, dexamethasone prevented the endotoxin from inducing ACTH secretion, but failed to reduce the resting level. As measured by the more sensitive method of MURPHY (1967), based on competitive protein binding, the resting level, too, was found to be reduced. These experiments appeared to show that some stressors are capable of enhancing

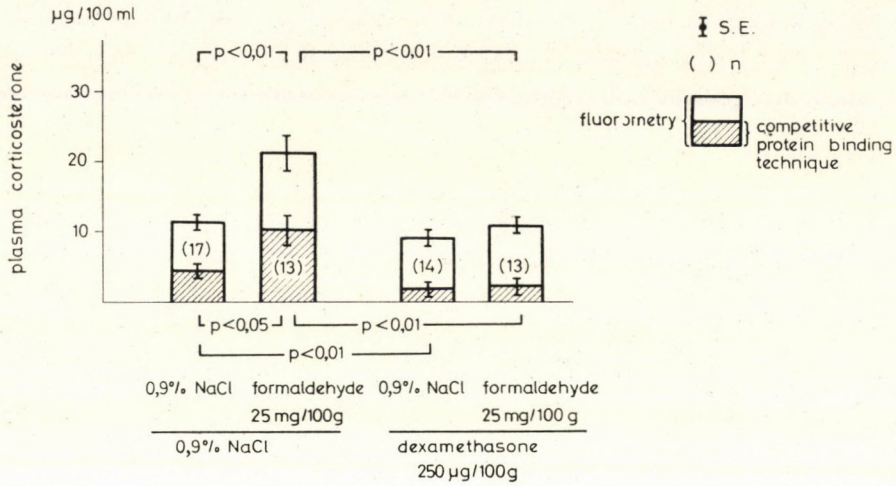

Fig. 9. Effects of formaldehyde and dexamethasone (250 µg/100 g) in rats with pituitary island

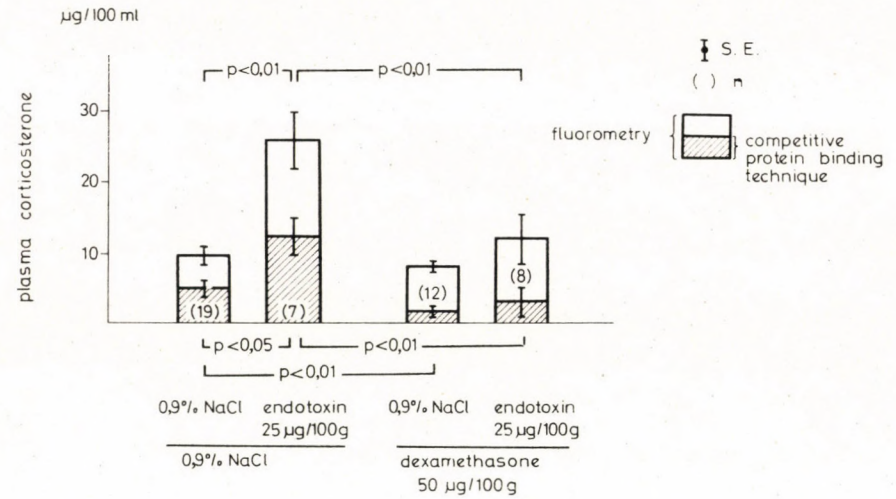

Fig. 10. Effects of endotoxin and dexamethasone (50 µg/100 g) in rats with pituitary island

ACTH secretion without mediation through the medial area of the hypothalamus or the median eminence, and that this is inhibited by dexamethasone.

Two points arise in relation to endotoxin and a large dose of formaldehyde: (1) they release stored CRF or ACTH from the neurohypophysis; (2) they possess the ability to stimulate ACTH secretion without CRF mediation.

Four weeks after transection of the hypophyseal stalk we removed the MBH; 24 hours later we found that endotoxin considerably enhanced the secretion of ACTH (MARTON et al., in press). This tends to show that, as a

source of CRF or ACTH, the neurohypophysis is unlikely to have a share in the increased ACTH secretion observed after removal of the MBH (Fig. 11).

These data raise the possibility that the humoral stressors have the ability to stimulate ACTH secretion without mediation through the CRF, perhaps by acting directly on the adenohypophysis. ANDO et al. (1964) failed in increasing ACTH secretion with endotoxin in vitro. Thus, the humoral stressors may increase ACTH secretion through some mediator(s) that reach the hypophysis by way of the systemic circulation.

The above results have allowed to distinguish the stressors acting *via* neural from those acting *via* humoral pathways.

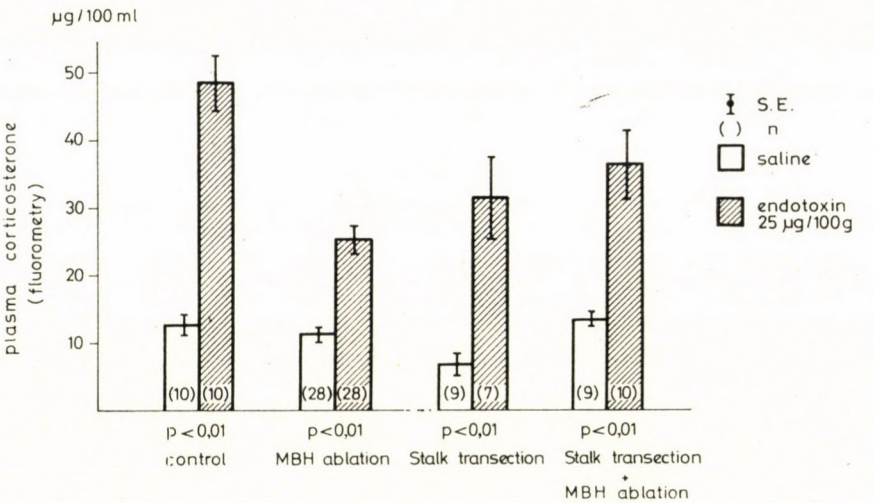

Fig. 11. Effects of endotoxin in rats after MBH ablation, stalk transection, and stalk transection followed by MBH ablation

Conclusions

1) Direct evidence has been presented to show that certain types of stressor agents stimulate CRF-ACTH release *via* neurons to the MBH (neural), whereas others are effective even after transection of these neurons (humoral).

2) Stressors which for their effect depend on intact neurons to the MBH also travel along neural pathways at the periphery.

3) Some stressors increase ACTH release in two phases: a rapid phase *via* a neural pathway and a delayed phase by a humoral one. The two phases are not characteristic of every stress reaction.

4) If we alter the intensity of a stressor agent, the afferent pathway by which it leads to increased activity of the hypothalamo-hypophyseal system, may alter with it.

5) Certain stressor agents increase ACTH release in the absence of the hypothalamus. Dexamethasone inhibits this increase.

6) Enhanced ACTH release induced by endotoxin can be observed in animals deprived of the hypothalamus, even if the hypophyseal stalk has been transected weeks earlier. In this case the neurohypophysis does not come into consideration as a source of CRF or ACTH.

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FEEDBACK ACTIONS OF ADRENOCORTICAL HORMONES

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It is generally accepted that adrenocortical hormones exert a negative feedback action on the secretion of ACTH from the anterior pituitary. However, the role of such action in the regulation of the pituitary-adrenal system is not clear at all. In fact, at present it appears to be more difficult to conceive a model of the system than some time ago. There have been several theories concerning the control of the system which proposed a dominant role for the corticoid feedback, but it appeared that they were not entirely correct. When we would try to separate facts from fiction, what is the evidence for the existence of a feedback action? A true negative feedback would exist if two conditions are fulfilled:

1. a decrease in corticosteroid blood levels should provoke an increase in ACTH secretion;

2. an increase in corticosteroid levels should inhibit ACTH release.

In the early days it seemed that both suppositions were valid, for it had been demonstrated both experimentally and clinically that removal of the adrenal glands resulted in an increased production of ACTH, and conversely, administration of corticoid hormones blocks ACTH secretion.

Consequently, a feedback theory on the control of ACTH secretion was proposed (SAYERS and SAYERS, 1947). It implied a balance system between the pituitary and the adrenal cortex: a stressful situation would increase the need for corticoids; due to the increased utilization in the peripheral tissues the blood levels would fall; this would present a stimulus for the pituitary to secrete more ACTH; consequently, corticoids would be produced in greater amounts and blood levels would increase, thereby gradually inhibiting ACTH release. This inverse relationship between ACTH and corticoid levels was supposed to be the only controlling system; the only decisive factor was the feedback by the peripheral corticoid levels on the ACTH-producing cells in the pituitary. This simple theory failed, however, at one incorrect assumption, i.e., that stress would cause an initial fall in corticoid levels. As soon as direct measurements of plasma corticoid titers could be performed, it appeared that such an initial fall does not take place.

During the fifties it became increasingly clear that the central nervous system dominated the function of the pituitary-target organ systems and that the controlling center was to be sought in the hypothalamus. In 1961 YATES introduced a modern version of Sayers' feedback control theory (YATES et al., 1961). He argued that a hypothalamic controlling device would have a certain set-point, and that the corticoid levels in the blood would feed back information to this controller as to the degree of activity of the system. In terms of a central heating system: the variations in room temperature are recorded by a thermostat which compares the actual temperature with the desired temperature (the set-point), and which switches on and off the heater system. In such a system the heater can be activated by two means: first, a decrease in room temperature, and second, a higher set-point of the thermostat. This theory proposed that stressful stimulation of the system would in fact cause a reset of the set-point at a higher level, thus inducing not an actual but a virtual decrease in corticoid levels.

This attractive theory again failed on experimental data. It implied that the system would not be activated if the corticoid levels would be raised artificially as to meet the new required set-point. However, we could show that an infusion of corticosterone in the rat, raising the blood levels to the height of that reached by a stress stimulus, does not block the ACTH release due to that stimulus. Only if much higher levels were induced, a gradual inhibition of the stress response was observed (SMELIK, 1963a).

Moreover, such an inhibition by pharmacological doses of corticoids is only established at the time when the induced blood levels have already returned to normal (SMELIK, 1963b). This means that inhibition does not follow closely the blood levels, but that a considerable delay occurs. This period of delay is not constant, but dependent on parameters which are not known precisely. If one compares inhibition after subcutaneous injection with corticosterone with that after intraperitoneal injection, it appears that the time lag is more reduced than could be accounted for by the difference in rate of appearance in the blood. It also seems that the degree of inhibition may not be proportional to the absolute level of corticoids in the blood, but perhaps to the time integral of concentration.

Quantitative data are too scarce to draw any conclusion, but these examples show that certain relationship between dose, route of administration, and the characteristics of inhibition exist.

So far we may conclude that for *inhibition* of the system the adrenal feedback has a slow action and a low capacity. It does not necessarily follow that the same is true for *activation* of the system. Very little is known about the effect of experimental decrease of the corticoid blood levels. BRODISH and LONG (1956) have shown long ago with a cross-circulation technique that unilateral or bilateral adrenalectomy causes ACTH hypersecretion, but only

after several days. Surprisingly, they observed a fall in ACTH secretion after 6 or 12 hours, and possibly an initial rise, which with the indirect measurements of that time is difficult to evaluate. More recently, however, BOHUS and ENDRŐCZI (1964) deviced a technique in dogs by which they were able to make a temporary shunt between the adrenal vein and the liver. They found that short periods of shunting did cause a rise in corticoid production within about half an hour. Although these experiments were done in severely stressed dogs, they may indicate that the reaction time is in the same order as is the case with inhibition.

As a preliminary conclusion one may say that the feedback system probably has a rather *low capacity*, which means that only extreme variations in blood levels may modify pituitary-adrenal activity, and a *sluggish action*, which implies that it cannot account for the rapid activation during stress. It seems likely that in resting conditions the adjustment to a certain basal level of activity is mainly performed by the peripheral corticoid levels. There are indications that the system in steady state is much more sensitive for feedback control. For instance the diurnal rhythm in activity is easily suppressed with small amounts of corticoids. During stress, however, the controlling center is activated by inputs, which are independent of the existing corticoid levels. One might say that a stressful stimulus overrides the stabilizing influence of corticoid feedback, or even that stress as a pathological condition may disturb the neuro-endocrine regulations.

When *pharmacological* doses of corticoids are administered, the system can be blocked, even under stress conditions.

From several studies it can be concluded that there must be a quantitative relationship between the intensity of the noxious stimulus and the level of cortical hormones. The more severe the stress, the more corticoids must be given to prevent the stress response. Whether or not certain stress stimuli are suppressible, or "corticoid-sensitive", can only be answered if dose-response curves have been made. Much of the confusion in the literature is due to conclusions drawn from experiments in which only one dose, or one stimulus strength was given, or measurements were done at only one time interval, etc.

These aspects also play an important role in the discussions around the question of the *site of action* of corticoid inhibition. There has been much controversy in the literature whether the feedback action is on the pituitary or on the hypothalamus. The relevance of this problem can be illustrated by the following dilemma: if one wants to assay hypothalamic extracts for their CRF-activity, one should do this in animals in which the endogenous production of CRF is blocked. A number of people have been using corticoid-blocked animals, happily convinced that they had produced a hypothalamic blockade. However, if the extract was not active, they were faced with the

embarrassing choice between two possibilities: either the block was at the pituitary level (contrary to the current view), or (even worse) the extract did not contain CRF-activity.

Theoretically, since the available evidence indicates that the controlling center of the pituitary-adrenal axis is situated in the hypothalamus, the most obvious idea is that the information is conveyed by corticoids to this structure. When the technique of local implantation of crystalline steroids in the central nervous system came into use, the results were in complete agreement with this view. The general experience was that the basal hypothalamus was the most effective implantation site, and that implants in the anterior lobe of the pituitary were ineffective.

The same was true when micro-infusions of corticoid solutions were employed: again blockade of the stress response could only be obtained by infusions within the central nervous system, and pituitary infusions were reported to be ineffective.

This general agreement at that time was only disturbed by a few reports which did show that corticoids can act on the pituitary itself (De Wied, 1964; Kendall and Allen, 1968; Arimura et al., 1969). Although in these cases the powerful synthetic steroid dexamethasone had been employed, it was clear that at least in certain conditions adrenocortical steroids are capable of blocking the pituitary directly. Moreover, it had been pointed out by Bogdanove (1963) that implantation in the hypothalamus might be a more effective mean of reaching the anterior lobe cells than local implantation in the anterior lobe itself, because the portal vessel system very effectively transports material from the median eminence region to the pituitary.

The problem has been re-investigated recently by several groups. The group of Yates, who had earlier reported that micro-injections of 1 μg of dexamethasone into one side of the anterior lobe did not cause a blockade, now found that the injection of 1 μg into either side of the pituitary did prevent the stress response and also the response to a CRF-preparation. Moreover, the same dose of 2 μg of dexamethasone, when injected into the septal region, also prevented the response to CRF, indicating that spread of the dexamethasone to the pituitary had occurred (Russell et al., 1969).

A similar approach in dogs was followed by Stark et al. (1968), who could not find an inhibition of the adrenal corticoid production up to three hours after the infusion of 200 μg of dexamethasone directly into the pituitary.

In contrast, the group of Yates quite recently reported that in dogs. the systemic injection of 4 mg of dexamethasone prevented the adrenal response to a CRF-preparation injected into the pituitary.

The blockade was apparent 2—6 hours after dexamethasone administration, but had disappeared after 15—24 hours. At that time, however, the response to histamine was still completely inhibited, so that the conclusions

would have been entirely different if only the 24-hour observations were made. They concluded that apparently the effectiveness of the inhibition depends on the strength of the ACTH-release stimulus (GONZALEZ-LUQUE et al., 1970).

It must be clear from these studies that massive doses of dexamethasone are capable of rendering the pituitary refractory against the stimulation by CRF. Whether natural steroids like cortisol and corticosterone would have a similar action, remains to be seen. It is to be expected that the dosage of these much weaker inhibitors must be enormous. In view of the possible spread of implanted solid material, we have studied the effect of dexamethasone implants in the hypothalamus on the response to CRF. It appeared that 10 hours after implantation strong ACTH-releasing agents like vasopressin are completely blocked, whereas the effect of a CRF-preparation is not im-

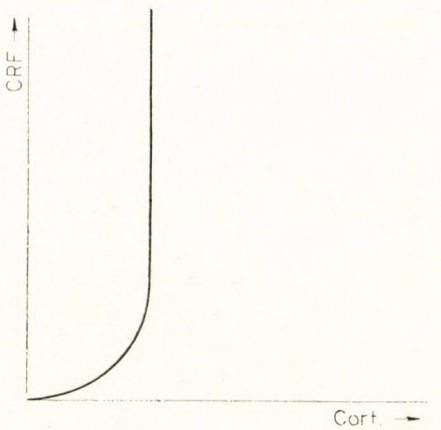

Fig. 1

paired. During the following days, however, there is a gradual decrease in responsiveness of both the pituitary and the adrenal cortex, presumably because of the non-activity of the system, in the absence of the hypothalamic drive (SMELIK, 1969).

The question of a hypothalamic or a hypophyseal site of feedback action can also be considered from the viewpoint of systems analysis. If we would accept the idea that the system becomes less sensitive proportional to the magnitude of the stress signal, it can be argued that this can only be explained if both hypothalamic centers and the anterior pituitary are sensitive for feedback signals, but at a different level of sensitivity.

Theoretical considerations would show that a dual relationship must exist ultimately between the production of CRF and the production of corticoids. On the one hand, a correlation will be present between the amount of CRF released and the output of the adrenal cortex. This relation will have the character of a dose-response curve (Fig. 1). On the other hand, since the

output of the system is fed back to the input, an inverse relationship exists between the level of corticoids and the amount of CRF released, in this sense that an increase in corticoid production will inhibit CRF release (Fig. 2). This feedback can occur at different levels, dependent of the magnitude of "stress" signals arriving at the system. Since both characteristics of the system are operating simultaneously, they should be combined, which results in a stable operating point for each level of activity of the system (Fig. 3). It can

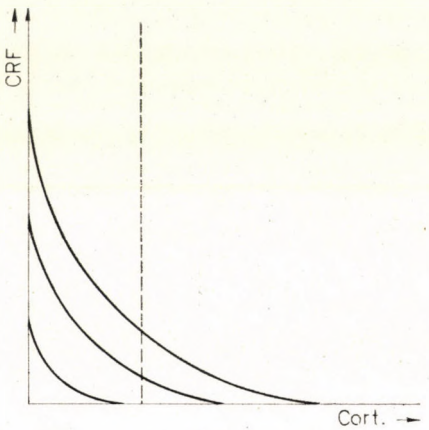

Fig. 2

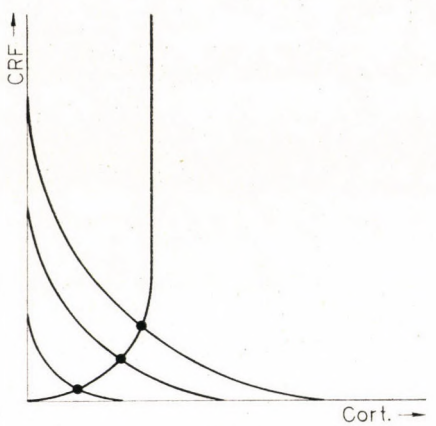

Fig. 3

easily be seen that at any activity level of the system (induced by "stress" signals which are independent of the level of circulating corticoids), this activity is maintained at that level automatically, since every deviation from this operating point will be corrected for.

Such a system would be equally sensitive at all operational levels for the corticoid feedback, irrespective of the site of feedback action, be it either

pituitary or hypothalamic. This is not the case, however, as argued before; at high activity levels (during stress) the sensitivity is much lower.

This can be explained, if we propose that not only the hypothalamus, but also the anterior pituitary is capable of monitoring corticoid levels. This would mean that the sensitivity of the ACTH-producing cells for CRF can be modulated by corticoids (Fig. 4). Incorporation of such characteristics into the output-input relationship of the system results in a stable operating region

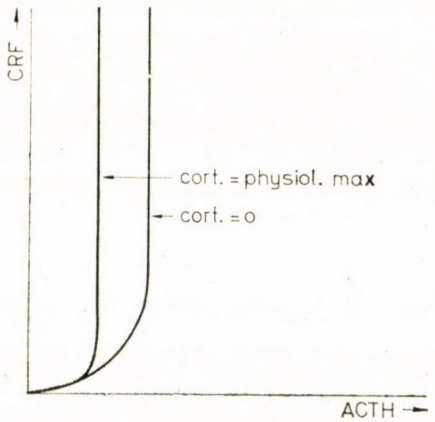

Fig. 4

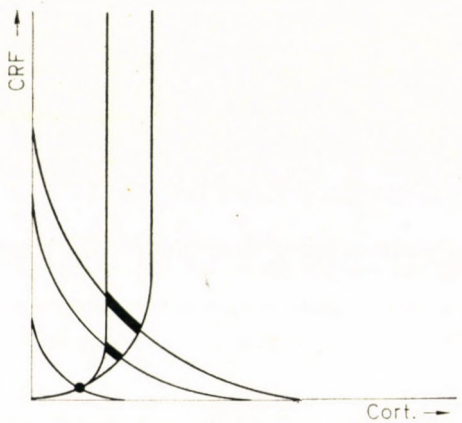

Fig. 5

(rather than a point), which means that within this region the activity of the system is insensitive for minor deviations (Fig. 5). Such an arrangement, in which the corticoids primarily act on a hypothalamic center, but are capable of modifying pituitary sensitivity as well, would then account for the decreased sensitivity of the system for corticoid feedback during stress.

In an attempt to summarize it can be said that many conflicting results

still exist, but that these often can be reduced to differences in timing, dosage or technique. During the last years more attention has been paid to such variables, and perhaps the following picture may emerge from the now available data.

In basal conditions, the pituitary-adrenocortical system is under permanent control of a hypothalamic center, which exerts a tonic influence on the pituitary. Diurnal variations are due to rhythmic variations in the activity of the controlling centre.

In this state the system is rather sensitive for fluctuations in the level of adrenocortical hormones, which act as a stabilizer through their feedback action on this center.

Minor disturbances of the system can be suppressed rather easily by corticoids, but the amount of corticoids needed for inhibition increases with the strength of the stimulus. The blocking dose of corticoids exceeds by far the maximal physiological levels, indicating that the feedback capacity of the system is rather small. Moreover, the feedback action has a slow onset: there is a delay period of at least 30 minutes.The site of action of the corticoid feedback is the controlling structure in the hypothalamus, presumably located in the cell bodies of the CRF neurons. These neurons are capable of summating excitatory and inhibitory stimuli, which can be either neural or humoral, and after summation they will send a releasing signal of an exactly determined intensity to the anterior pituitary. The pituitary itself is sensitive for a modulating action of corticoids, but the local concentration must reach very high values in order to block the release of ACTH completely. It is unlikely that such conditions can ever be obtained physiologically. However, in cases of treatment with high doses of corticoids such as dexamethasone the possibility of a blockade on the pituitary level should be taken into consideration.

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REGULATION OF ACTH SECRETION AND ITS CLINICAL ASPECTS

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Regulation of ACTH secretion is being extensively studied and a considerable wealth of evidence has been accumulating on the subject over the years. On the other hand, the question is still far from being clarified in all its aspects. It is doubtlessly the clinical study of the subject which poses the greatest difficulties. The most conventional line of approach to the understanding of functional disorders of the ACTH-adrenocortical system in man has been the experimental study of the problems since the results of animal experiments are not applicable, at least not directly, to clinical endocrinology. One of the essential tasks of clinical observations, therefore, is to make use of the experimental facts with regard to homeostasis in man. Unfortunately, however, the hypothalamus, which plays the central role, is not accessible to direct study, owing to the location of the midbrain (Reichlin, 1967). Information to be drawn from post-mortem material is confined to the terminal stage of the disease. As regards studies in vivo, here the compensatory mechanisms tend to mask the primary process, and moreover the complexity of neural organization makes proper interpretation difficult.

Before the sixties, it was only in an indirect manner, i.e., by the study of adrenocortical function, that an assessment of ACTH-secretion was possible. Direct estimation of ACTH was expected to offer a closer insight into the problems involved. Liddle et al. (1962) elaborated a sensitive bioassay. This is in fact a modified procedure of Lipscomb and Nelson's method (1959), consisting in the measurement of the corticosterone concentration in adrenal venous blood 7 to 10 min after administration of standard ACTH and of the blood-extract to rats which had been hypophysectomized 48 hours before. The method, therefore, is basically a "three point assay." The results thus obtained were published in 1962 by the authors. The graph representing their findings is shown in Fig. 1, from which it can be seen that the lowest ACTH concentrations have been found in hypopituitarism. The values for healthy subjects comprise four different patterns: the lowest concentrations are found after dexamethasone-suppression, the concentrations in the morning and in the afternoon are different, according to the diurnal rhythm, and

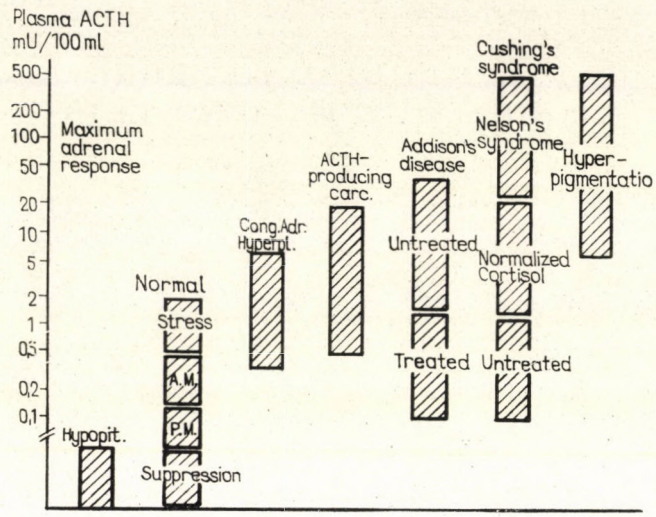

Fig. 1. Plasma ACTH level in various disorders of the adrenal cortex (after LIDDLE et al., 1962)

there is finally a mobilization of hormone in response to stress. In congenital adrenocortical hyperplasia which results from an inadequacy of cortisol production in consequence of an enzyme defect, ACTH secretion is obviously increased. Normal ACTH levels have been found in Addison's disease subjected to adequate substitution therapy and in untreated Cushing's syndrome. Abnormally high ACTH concentrations have been noted in ACTH-producing non-endocrine tumours, in untreated Addison's disease, in Cushing's syndrome after bilateral adrenalectomy and subsequent substitution therapy. Extremely high values occur in Nelson's syndrome caused by a pituitary adenoma, as well as in the case of a functional impairment of the adrenals associated with extensive hyperpigmentation.

The bioassays for ACTH, though having proved highly informative, are too time- and labour-consuming for general use. In 1959, FISHMAN, MCGARRY and BECK published an immunoassay and in 1964 YALOW et al. described a procedure for the radioimmunoassay of ACTH which has since been adopted all over the world. This procedure helped to confirm the results obtained by the current bioassays (the normal range of the blood levels of circulating ACTH being 0.3—0.6 mU/100 ml plasma) and contributed to the accuracy of the measurements even in conditions associated with low ACTH levels. Gradually, however, it has been realized that there is often a discrepancy between the results of bioassays and those of immunoassays. In this context we have to refer to the study by IMURA et al. (1965) in which it is pointed out that the C-terminal portion of the ACTH molecule is responsible for its immune activity and its N-terminal portion for its biological activity. On these

grounds, the results of radioimmunoassay are only considered fully reliable if confirmed by those of the bioassay.

Adoption of direct assays for ACTH, while having helped to gain a closer insight into the mechanism of ACTH regulation, has brought to light many interesting and sometimes conflicting facts. It is not possible to deal here with all the relevant questions of major interest, and therefore discussion will be confined to certain pathogenetic aspects of pituitary-adrenocortical hyperfunction, i.e., of Cushing's syndrome, which we intend to illustrate by a few clinical observations.

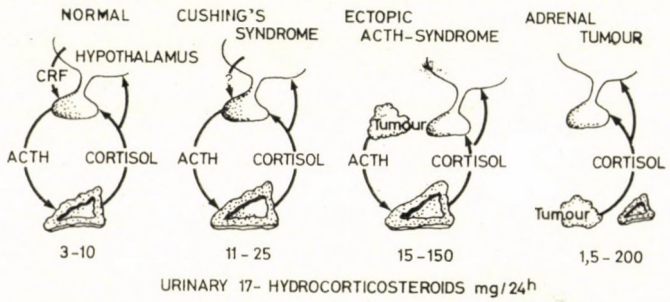

Fig. 2. Pituitary-adrenocortical function in various types of hyperadrenocorticism (after LIPSETT et al., 1964)

Anglo-American literature refers to all abnormalities characterized by a hypersecretion of cortisol as Cushing's syndrome. LIDDLE (1960) divides the syndrome into two major groups, according to whether or not the administration of dexamethasone in daily doses of 8 mg results in the suppression of corticoid secretion. LIPSETT et al. (1964) found it convenient to divide the non-suppressible Cushing-syndrome into two types (Fig. 2), the first being due to non-endocrine ACTH-producing tumours ("ectopic ACTH-syndrome") and the second, to adrenal tumours characterized by a hypersecretion of cortisol.

The first point to be discussed here concerns the pathogenesis of the hypothalamo-pituitary type of Cushing's syndrome. For more than thirty years Cushing's syndrome has been attributed to the excessive amounts of ACTH secreted by the pituitary gland, a claim based on the observation that while the syndrome could be induced experimentally by exogenous ACTH, hypophysectomy was followed by a regression of the symptoms (RAY, 1960). It was all the more surprising, therefore, to find normal ACTH-levels in Cushing's syndrome (LIDDLE et al., 1962), though increased ACTH-levels were also noted (LINN et al., 1967). Notwithstanding this, there is general agreement that in Cushing's syndrome due to pituitary adenoma, ACTH secretion is increased. LIDDLE (1962) even regards this type as a particular syndrome, referring to it as Nelson's syndrome (NELSON et al., 1960). Again,

NELSON pointed out in another paper (1966) that the course of Cushing's syndrome comprizes three phases, i.e.:

(1) normal ACTH-levels lacking any diurnal rhythm, in contrast to the increased serum cortisol level;

(2) increased ACTH and cortisol levels (particularly after partial adrenalectomy);

(3) extreme hypersecretion of ACTH (after bilateral adrenalectomy; pigmentation; poor suppressibility of ACTH secretion, the primary process often being a pituitary tumour).

It needs a proper explanation to reconcile Cushing's syndrome with normal ACTH levels, in other words, to account for the factors keeping them in the normal range. Of the possible interpretations, a feedback mechanism would seem to be the most probable explanation, assuming that the increased cortisol level exerted a suppressive effect on ACTH production. On the other hand, the adrenal cortex has been assumed to be of increased sensitivity in this condition, though JAMES et al. (1968) were unable to confirm this, except for a minority of their cases. The observation of LIDDLE et al. (1962) deserves interest, the authors having noted increased ACTH levels in Cushing's syndrome after bilateral adrenalectomy in the case of cortisol substitution with daily doses of 10 mg, in contrast to Addison's disease where the ACTH level remained normal despite a similar substitution. Cushing's syndrome would thus seem to be associated with an abnormally active driving mechanism and therefore the normalization of the ACTH level would require a considerably enhanced secretion of cortisol which would induce a readjustment to a new equilibrium. As reported by JAMES et al. (1968) Cushing's syndrome is marked by a poor responsiveness of the adrenal cortex to pyrogens and to dexamethasone and by an absence of the normal diurnal rhythm of the plasma cortisol level, while ACTH, ADH and metyrapone elicit normal or enhanced responses. On these grounds, Cushing's syndrome is believed to be due to a disturbed hypothalamic or cerebral regulation, rather than to a primary failure of the pituitary gland as had been assumed by CUSHING (1932). RAYYIS and BETHUNE (1969) have offered a closer definition of the aetiologic factors. Having found the administration of dexamethasone to result in a poor, and that of hydrocortisone in a normal suppression, they attribute Cushing's syndrome to some dysfunction of the feedback receptors.

Hypercorticism caused by non-endocrine tumours constitutes a special type of abnormality associated with ACTH hypersecretion. The syndrome has long been known. The first case of this kind, where a pulmonary tumour was responsible for Cushing's syndrome, was described by BROWN in 1928. Since that time approximately 240 cases have been reported, chiefly in connection with tumours of the bronchi, less often with those of the thymus or the pancreas. However, the manner of production of the endocrine disturbance

remained obscure until it was demonstrated by modern procedures that certain tumours are capable of secreting substances of hormonal activity including ACTH. It is on these grounds that LIDDLE (1963) applied the term "ectopic ACTH-syndrome" to hypercorticism of this origin, which constitutes one of the paraneoplastic endocrine syndromes. The fact that the tumours are ACTH-producing, has been confirmed by various procedures. A significant ACTH activity has been demonstrated in the primary tumour as well as in its deposits by means of immunofluorescent as well as by chemical procedures (ENGEL and KAHANA, 1963; GOLDBERG and MCNEIL, 1967; YARETT et al., 1964; LIDDLE et al., 1963; MARKS et al., 1963; MEADOR et al., 1962; PFOHL and DOE, 1963; PFOTENHAUER and KRACHT, 1967). Fig. 2 gives an illustration of the changed pituitary-adrenocortical relationships: the blood ACTH level attains extreme values, the adrenal cortex becomes hypertrophic, the serum cortisol level is abnormally high, and the amount of ACTH demonstrable in the pituitary is diminished. A case of extreme rarity, one of thyroid carcinoma accompanied by hypercorticism (SZIJJ et al., 1969) will be recalled here in order to illustrate the typical course of events in syndromes of this kind.

The patient was a 39 years old male. In February, 1967, he reported to have gained in weight suddenly in the last five months. At the same time his face had become round, reddish and he had noted a brownish coloration of the skin. He was put on the waiting list for admission which was due four weeks later. While waiting for admission, he started losing weight (and surprisingly lost 6 to 8 kg), and developed shortness of breath, cyanosis and diarrhoea. On admission, obesity confined to the trunk, signs of cardiac failure and pigmentation of the skin were found. The thyroid was moderately enlarged, the liver exceeded its normal size by 6 to 7 cm and was of uneven surface. There were no striae on the skin. Blood pressure was 175/120 mmHg.

Table I

N. A., 39, male

Diagnosis: Ectopic ACTH-syndrome

Laboratory findings:

Urine: specific gravity 1004, concentration test 1011, glycosuria, proteinuria

Sedimentation rate: 29 to 35 mm/hr

				mg/100 ml
Blood sugar:	126 mg/100 ml.	Oral glucose tolerance test:	fasting level	166
Serum Na:	155.5 mEq/l		30′	230
Serum K:	3.1 mEq/l		60′	286
			120′	280
			180′	218

Hormone excretion	Untreated	After ACTH administration
total 17-ketosteroids (mg/24 hrs)	18.1— 32.5	13.4— 17.6
17α-hydroxycorticosteroids (mg/24 hrs)	9.1— 21.3	10.6— 10.8
serum hydrocortisone (μg/100 ml)	80 —120	86 —114

The essential laboratory findings are summarized in Table I. There was a high ESR, a blood sugar tolerance curve of the diabetic type and a low serum potassium level. The conspicuously high ketosteroid and corticoid excretion was not raised further by ACTH administration. The serum hydrocortisone level was extremely high and also remained unaffected by ACTH.

Closer investigations (metyrapone, dexamethasone suppression) were made impossible by the rapid downhill course of the process. The patient died of circulatory failure three weeks after admission. The assumed diagnosis of ectopic ACTH syndrome was confirmed at post-mortem which revealed a primary tumour of the thyroid with the microscopical features of a medullary carcinoma (Fig. 3), containing sparse intercellular eosinophilic amyloid deposits (Fig. 4). There were metastases in the liver, lungs, regional lymph nodes and dorsal vertebrae. The adrenals were enlarged, showing hyperplasia particularly of the fasciculate zone (Fig. 5). The hypophysis contained large Crooke-cells with hyalinized, vacuolated cytoplasms typical of Cushing's syndrome (Fig. 6).

This case makes it timely to refer briefly to the question of what enables the malignant cell to form ACTH. In the view of WILLIAMS (1966a, b) the solid or medullary carcinoma with signs of amyloid degeneration, as in the present case, arises in the parafollicular or C-cells of the thyroid, regarded by PEARSE (1968) as part of the APUD (amine and precursor uptake and decarboxylation) cell system. The cells of this system are capable of uptake and decarboxylation of various amines and other precursors, and of the production of numerous biologically active substances including bradykinin, serotonin and ACTH. On the grounds of the hypothesis put forward by LIPSETT et al. (1964) and by KRACHT (1968) the cells of tumours originating in the APUD system might be assumed to contain an "ACTH-gene" in the dormant state, prevented from activity under normal conditions by various intracellular repressor systems. Cellular dedifferentiation in the course of malignant transformation suspends the inhibitory effects and brings a new messenger RNA into existence. The information originally contained in the DNA molecule is carried into effect, the cell starts to form a peptide of ACTH activity which it does not produce under normal conditions and which stimulates the secretion of adrenocortical steroids, thus leading to hypercorticism.

The next type of hyperadrenocorticism is that of hormone-producing adrenal tumours. This group also deserves some comments, it being particularly suited for the study of the clinical involvements of the negative feedback mechanism. Here, the pattern of regulation is basically as follows. Adrenocortical hypersecretion results in a diminished secretion of the corticotrophin releasing hormone, and therefore in a reduced ACTH content of the pituitary and in a low blood ACTH level. Atrophy and hypofunction of the

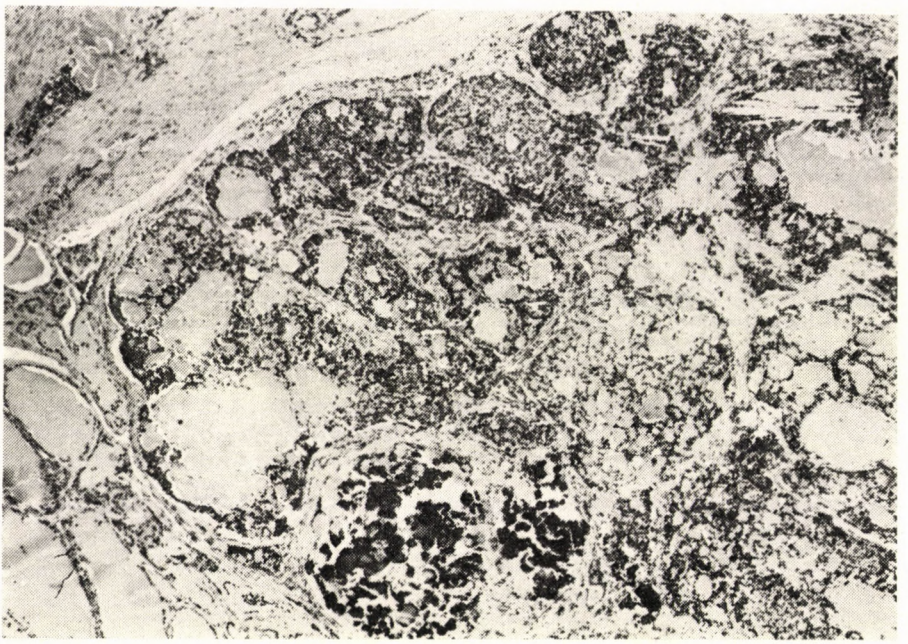

Fig. 3. Histological appearance of thyroid carcinoma with multiple scattered foci of calcification. H + E staining. ×45

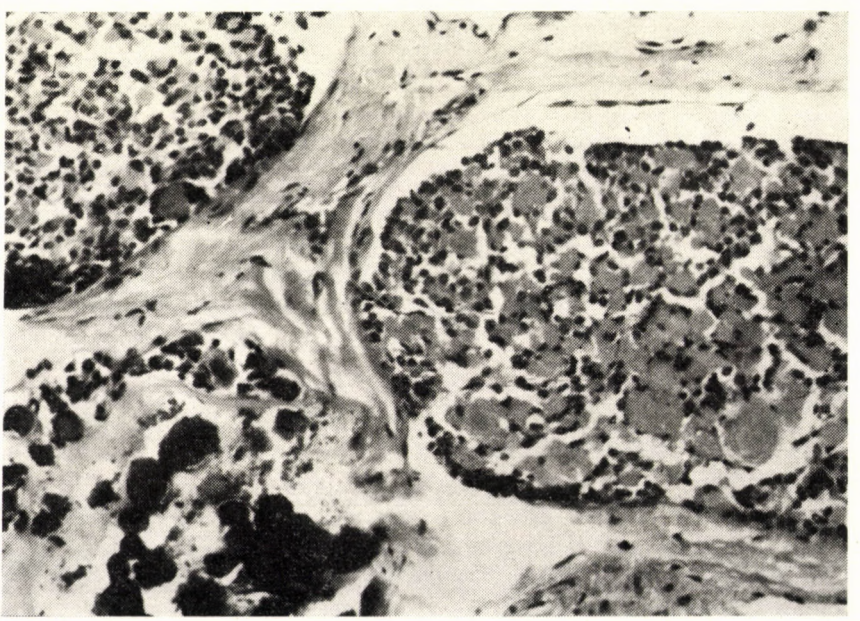

Fig. 4. Thyroid carcinoma with granules of amyloid and calcium deposits. H + E staining. ×170

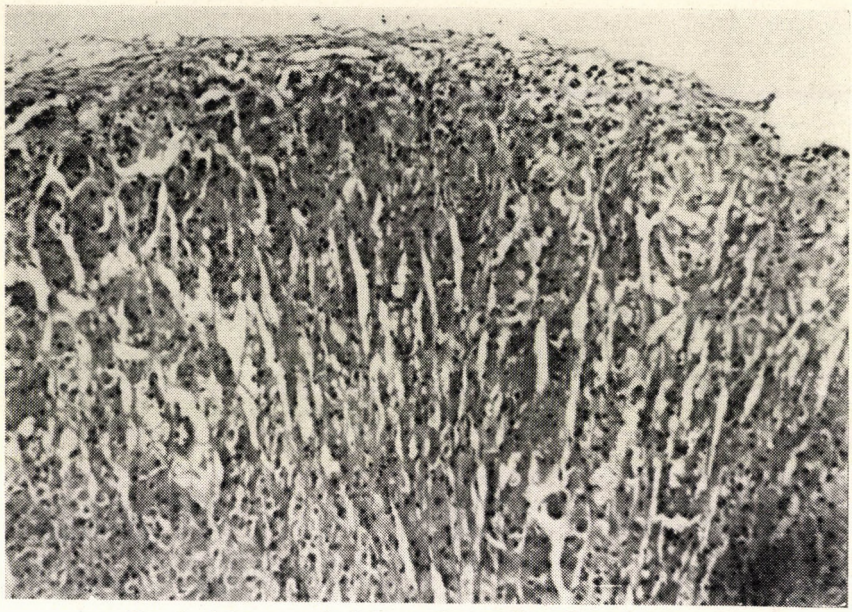

Fig. 5. Adrenocortical hyperplasia. H + E staining. ×72

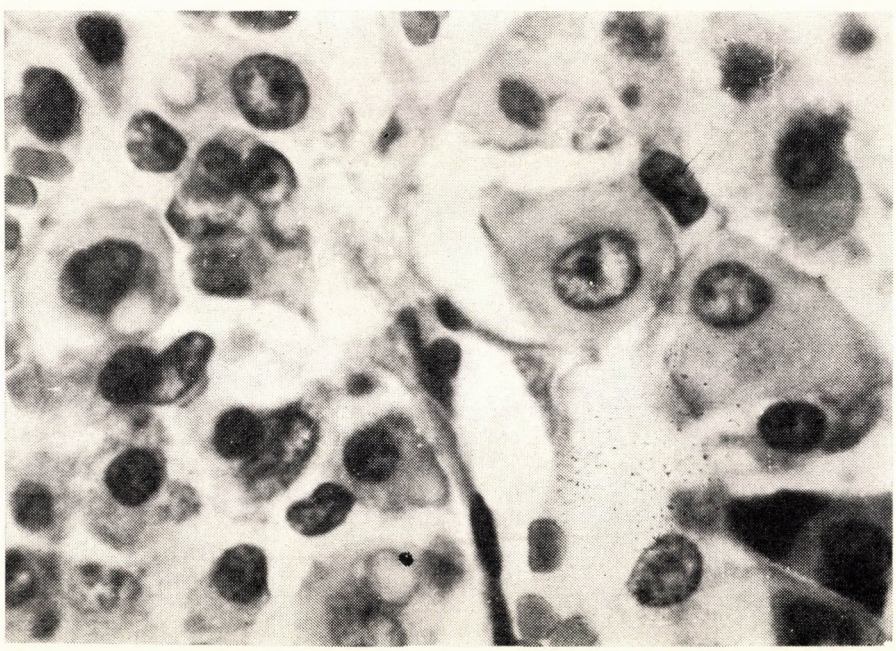

Fig. 6. Crooke-cells in the adenohypophysis. H + E staining. ×1120

contralateral adrenal gland ensue. The sequels of suppression do not, however, become manifest before removal of the tumour. Realization of the significance of this fact dates 30 or 40 years back, since, until the fifties, the majority of patients with Cushing's syndrome died soon after extirpation of the adrenal tumour of one side. These observations raise various issues. The question of prime importance is to know which types of adrenal tumour are most liable to hazards of this kind. This might be answered by presenting three case records, each being illustrative of a particular abnormal pattern produced by the individual type of adrenal tumour. Only the first case will be reported in detail.

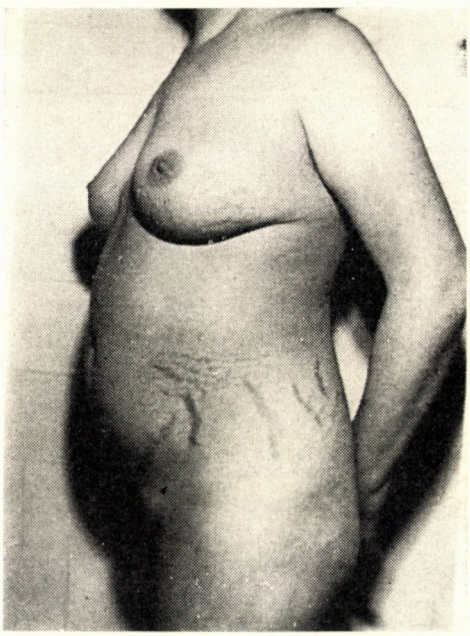

Fig. 7. Cushing's syndrome. Active striae on the skin of abdomen, buttocks and chest

The patient was a 27 years old female. The presenting symptoms were a rapid gain in weight, hirsutism, amenorrhoea for five years, and hypertension. Physical examination revealed active striae involving the skin of the abdomen and of the buttocks (Fig. 7). The most important laboratory findings included a blood sugar tolerance curve of the diabetoid type and a serum potassium level as low as 3.3 mEq/l. The hormonal constellation is shown in Table II. Excretion of 17-ketosteroids and corticoids was moderately increased and unaffected by dexamethasone regardless of the doses, while being enhanced to a certain degree by ACTH (in all probability as a result of stimulation of the contralateral adrenal cortex). The plasma hydrocortisone level was high, lacked any diurnal rhythm, remained unaffected by dexamethasone and rose

Table II

K. I., 27, female

Diagnosis: Adrenal tumour on left side. Cushing's syndrome

Hormone excretion, mg/24 hrs		After administration of		
		2 mg dexamethasone	8 mg dexamethasone	ACTH
Total 17-ketosteroids	14.8—21.7	15.3—18.0	22.7—26.9	24.9—31.6
17α-hydrocorticosteroids	10.0—10.8	11.6—12.3	15.9—20.3	21.7—27.8

Plasma hydrocortisone, μg/100 ml

Rhythm

hr				
8	26.2	After administration	After administration of	
14	27.0	of 1 mg dexamethasone	ACTH (2 μg/kg)	
20	27.6	28.2	fasting value:	29.6
2	26.2		hours	
8	28.8		1	36.8
			2	31.4
			4	27.2

but slightly in response to ACTH. X-rays combined with perirenal insufflation revealed an adrenal adenoma on the left side. This was removed at the 1st Department of Surgery. Its gross appearance was that of a yellow tumour measuring 35×40 mm (Fig. 8). Microscopically it was identified as an adrenal adenoma with structure similar to that of the reticular zone (Fig. 9). Cortisone was administered during the first ten postoperative days, and was then discontinued and the function of the ACTH-adrenocortical system was studied. The relevant data have been summarized in Table III which clearly shows

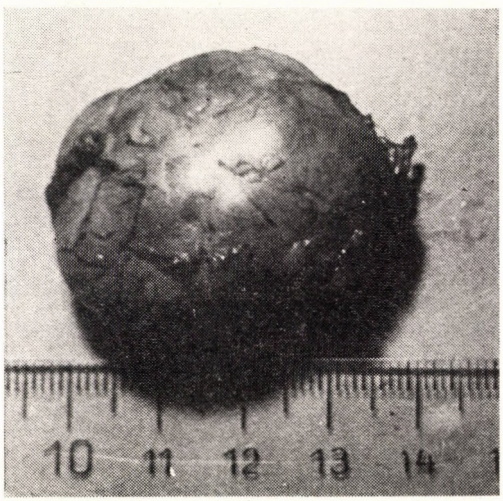

Fig. 8. Adrenal adenoma giving rise to Cushing's syndrome

Fig. 9. Adrenocortical adenoma reminiscent in its structure to the reticular zone. H + E staining. ×35

Table III

K. I., 27, female

Diagnosis: State after left adrenalectomy for adrenal tumour

Hormone excretion, mg/24 hrs		After administration of	
		Metyrapone	ACTH
Total 17-ketosteroids	6.2—7.8	6.6—8.2	7.8—8.8
17α-hydrocorticosteroids	2.0—3.1	1.3—1.5	5.8—8.5

Serum hydrocortisone, μg/100 ml

Rhythm hour		After administration of	ADH	ACTH
8	5.6	fasting:	5.4	4.8
14	8.8	1 hours:	4.6	7.8
20	5.6	2 hours:	5.0	7.8
2	4.0			
8	5.6			

that corticoid and ketosteroid excretion was at the lower limit of the normal range, unresponsive to metyrapone and responding but moderately even to ACTH. The plasma hydrocortisone level was reduced, lacked a diurnal rhythm and failed to respond to intramuscular ADH. Intravenous administration of 2 μg/kg ACTH resulted in a minimal rise of the cortisol level. The responsive-

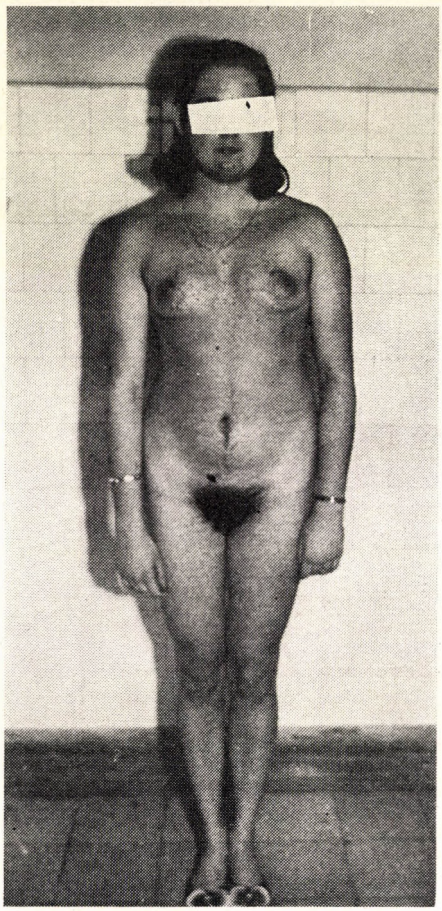

Fig. 10. Adrenogenital syndrome. Hirsutism, seborrhoeic manifestations affecting the skin of the chest

ness of the residual adrenal cortex was thus impaired and, moreover, a proper assessment of ACTH capacity would have required direct measurements of ACTH. The tests repeated three months later gave similar results.

The second patient was a 17 years old female. The presenting symptoms were hirsutism, amenorrhoea (Fig. 10), a bearded face, and seborrhoea (Fig. 11). The essential data of hormone excretion are shown in Table IV. There was an uncommonly high ketosteroid excretion which failed to respond to dexa-

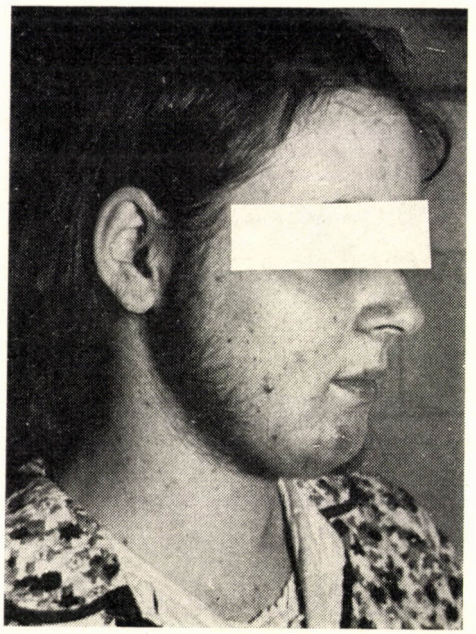

Fig. 11. Face of a female patient with adrenogenital syndrome. Distinct beard, folliculitis

Table IV

B. M., 17, female
Diagnosis: Adrenogenital syndrome

Hormone, excretion, mg/24 hrs		After administration of		
		2 mg dexamethasone	8 mg dexamethasone	ACTH
Total 17-ketosteroids	197.8—310.2	350.0—413.1	324.5—415.8	293.0—350.0
17α-hydrocorticosteroids	5.0— 6.0	0.8— 2.7	1.7— 1.9	23.7— 47.8
Pregnanediol	3.7— 5.9	7.0— 7.8	12.0— 13.0	9.2— 10.5
Pregnanetriol	0.7— 1.1	0.3— 0.7	1.6— 1.9	3.2— 3.3

Plasma-hydrocortisone, μg/100 ml

Rhythm		After administration of		
hour				
8	23.6			
14	16.8	1 mg dexamethasone	ACTH (2 μg/kg)	
20	14.4	10.2	fasting:	14.0
2	7.4		hours	
8	19.5		1	22.8
			2	19.6
			4	12.0

methasone and to ACTH. On the other hand, corticoid excretion remained normal; it was suppressed by dexamethasone and enhanced by ACTH. The plasma hydrocortisone level was normal, in respect to both diurnal rhythm and responsiveness to dexamethasone and ACTH. The tumour weighing 206 g occupied the upper pole of the right kidney (Fig. 12). It was identified as an adrenal adenoma composed of cells similar to those of the reticular zone at sites with atypical cells and structure, interspersed with haemorrhages (Fig. 13). After removal of the tumour basal hormone secretion, ACTH reserves and adrenocortical responsiveness to ACTH were completely normal.

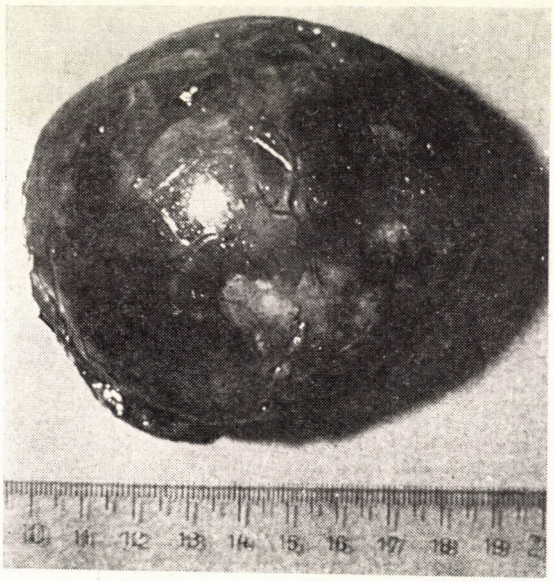

Fig. 12. Adrenocortical tumour responsible for adrenogenital syndrome

In the third patient with typical Conn's syndrome, ACTH and glycocorticoid functions were normal preoperatively as well as after removal of the tumour, which proved to be an adrenal adenoma, yellow in colour, 15 mm in diameter (Fig. 14), composed of cells corresponding to those of the fasciculate zone (Fig. 15). The findings in the second and third cases obviously dispensed with the need for the administration of cortisone or ACTH.

Though the cases presented are small in number, they will have sufficiently illustrated the fact that in the regulation of ACTH secretion in man, it is only cortisol or cortisone, that is the 17-hydroxycorticosteroids, which play a significant part. A fall in the plasma free cortisol level elicits an increase in ACTH secretion, whereas its elevation has an inhibitory effect on the mobilization of ACTH. It is this so-called servo-mechanism which accounts for the stability of circulating plasma cortisol within certain limits in stress-

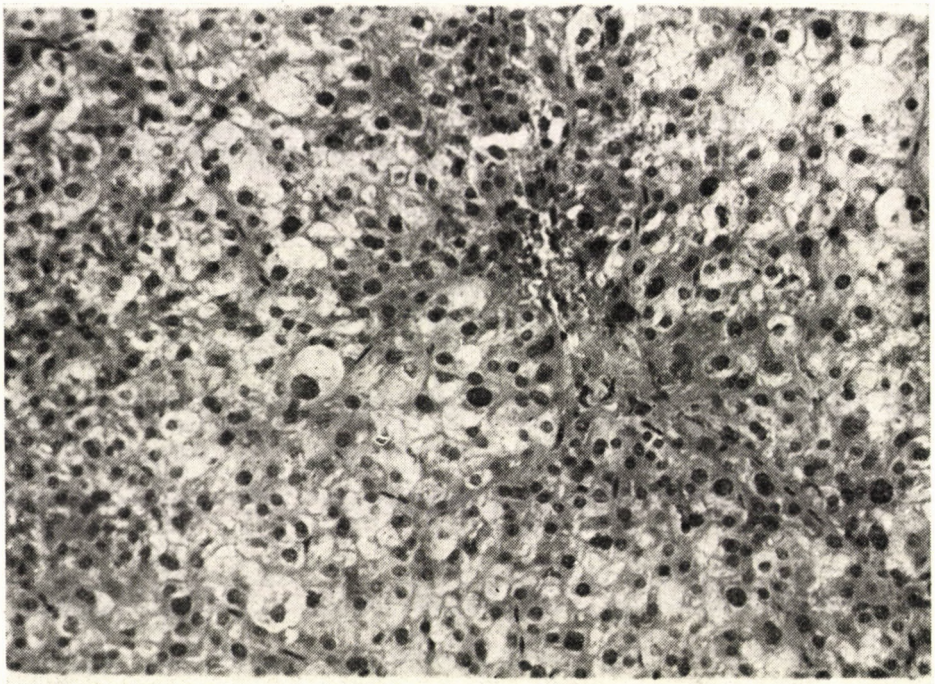

Fig. 13. Adrenocortical adenoma constituted by cells reminiscent of those of the reticular zone. In places, atypical cells and structure. H + E staining, ×287

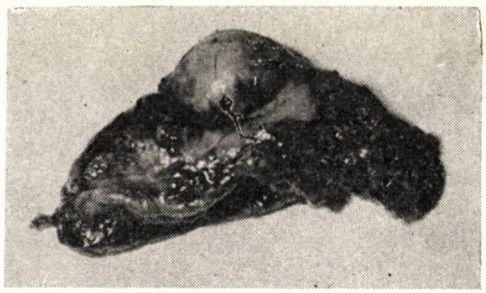

Fig. 14. Adrenal adenoma 15 mm in diameter, giving rise to Conn's syndrome

free conditions. The diagram representing these relationships (Fig. 16) shows that ACTH, by stimulating adrenocortical function, enhances the production of cortisol, 17-ketosteroids, oestrogens and of progesterone, and even of aldosterone. On the other hand, of all five components it is only cortisol which has any significant retrograde effect on ACTH production via the hypothalamus. It is assumed that impulses originating in the cerebral cortex and in other areas of the brain act on ACTH output through these pathways (WILLIAMS, 1968).

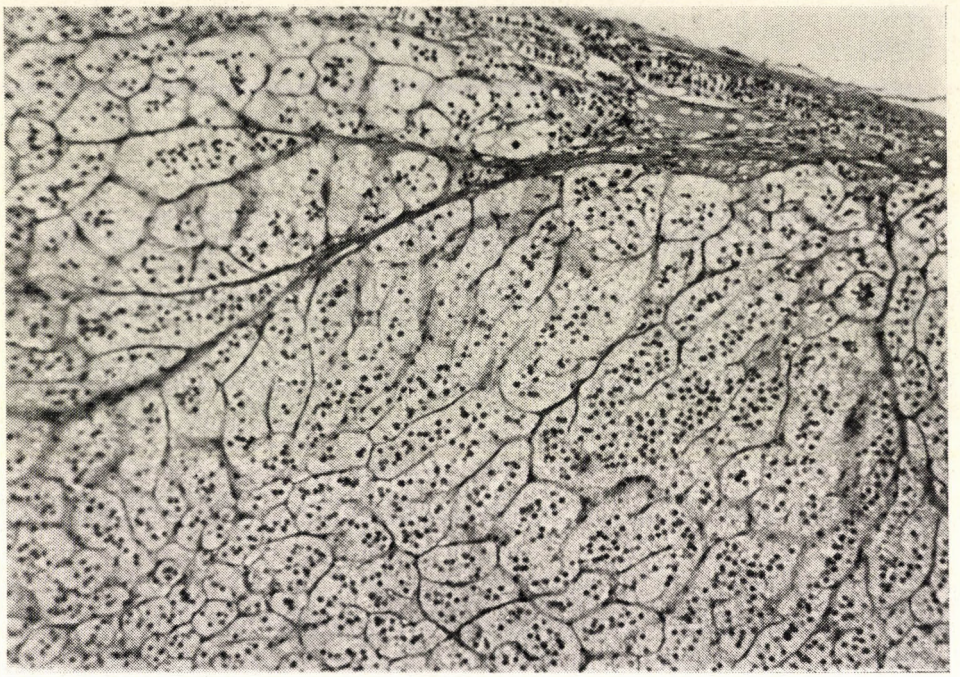

Fig. 15. Adrenocortical adenoma reminiscent in its structure to that of the fasciculate zone. H + E staining, ×70

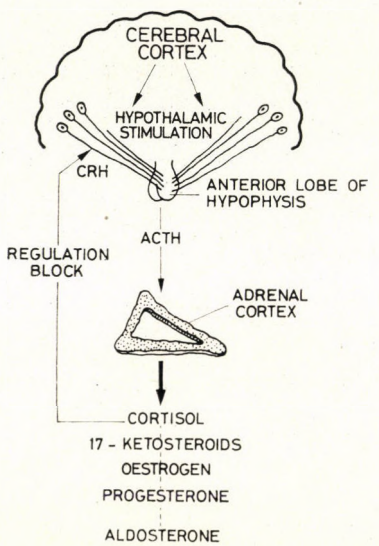

Fig. 16. Feedback regulation of ACTH-activity (after WILLIAMS, 1968)

A problem of importance is the restoration of ACTH-adrenocortical function. We have to refer here to the noteworthy observations of GRABER et al. (1965) who studied the plasma cortisol and ACTH levels over long periods simultaneously in subjects with Cushing's syndrome and with adrenal tumours having been subjected to long-term, massive cortisone treatment (Fig. 17). The dots on the diagram represent normal values, the crosses those of the subjects having been under long-term suppression. It is seen that the preoperative ACTH level was reduced, and that of cortisol increased. One month after withdrawal both parameters were subnormal; subsequently there

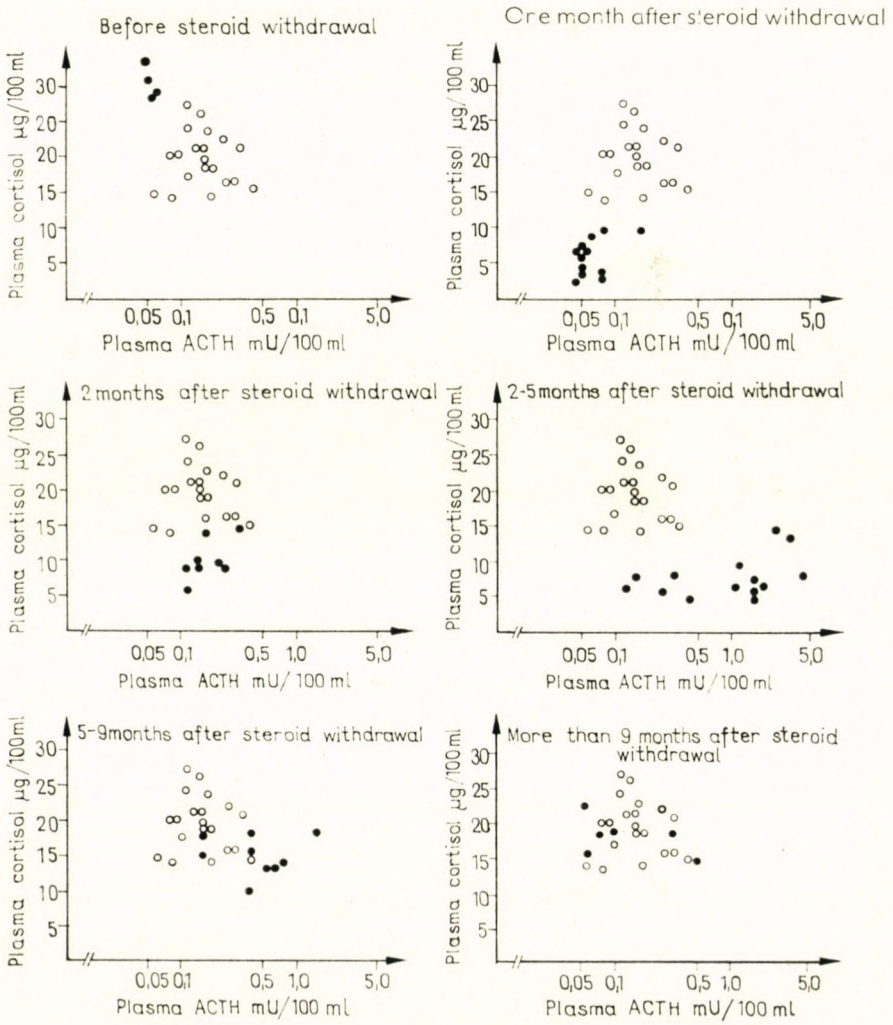

Fig. 17. **Relationship between plasma cortisol and ACTH levels in normal subjects and after long-term adrenocortical suppression (GRABER et al., 1965)**

was a gradual rise in the ACTH level, followed by that of the cortisol after 5 to 9 months, and full normalization required 9 months.

The results of this fundamental observation confirm the essential fact that not only does the adrenocortical responsiveness suffer during a long-term suppression but the pituitary also requires a certain (by no means short) period to be able to compensate the reduced cortisol level by an increased ACTH output. As regards the level at which the inhibition of ACTH secretion operates, no certain answer can be given. The secretion of CRH may be blocked, or the ACTH-producing cells which have remained inactive for long may have become unresponsive to the releasing hormone. Further studies should provide a definite answer. The clinical consequences to be drawn are that it would be a mistake to leave the patient to his fate after a few days' course of ACTH in the belief that the adrenals have been driven into action and that normal conditions have been restored. Nothing could be more erroneous. Major stress-effects and intercurrent diseases may be fatal even many months later. The current practice today is to keep the patients on minimum substitution doses of cortisol (10 to 15 mg daily) which have been reduced to these levels gradually (in the course of four weeks). Care must be taken to administer the whole dose in the morning so as to let its effect pass off before the nocturnal period of regeneration. It is also important to know that intercurrent diseases call for multiplied maintenance doses. An 8 to 10 days' course of ACTH can only help to restore the function of the pituitary-adrenocortical system.

In conclusion, we have to refer to the possible perspectives of the study of ACTH regulation. There seem to be two main lines of approach to the subject. Much may be expected from the progress in human neuro-endocrinology, as regards the development of technical facilities, a greater accuracy in the localization of hypothalamic tumours or of other lesions, and a closer insight into the afferentation of the hypothalamic as well as of other cerebral regions. What seems to be decisive, however, is the progress of endocrine biochemical methods, first of all as regards the clarification of the structure of CRH and, similarly to TRH, the synthesis of the corticotrophin-releasing hormone and the elaboration of assays for its estimation. A routine use of direct ACTH estimation would also be highly desirable. In addition, many problems involved in ACTH regulation remain to be clarified by future research.

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MODERN TRENDS IN STEROID ANALYSIS

INTRODUCTION

By

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The Hungarian Society of Endocrinology and Metabolism has answered a long-felt need of Hungarian steroid scientists by including into the program of the Vth Hungarian Endocrine Congress a symposium on "Modern trends in steroid analysis."

We have all been witness to the rapid progress in steroid research, most of which is due to procedures developed during the last decade.

The now classical procedures of column-chromatography and paper-chromatography have made a significant contribution to the possibilities of the specific determination of any steroid in biological substances containing other steroids and impurities. These separation methods have recently been succeeded by thin-layer chromatographic methods, which owe their popularity to the rapidity with which they can be carried out.

Efficient fluorometric methods now allow the quantitative determination of very small amounts of any steroid.

The gas-chromatographic methods lend themselves well not only to the separation of small amounts of steroids but also to micro-quantitative determinations and to steroid identification.

The introduction of ^{3}H and ^{14}C labelled steroids has provided almost limitless possibilities in steroid analysis and in the *in vitro* and *in vivo* study of steroid metabolism. The up-to-date radioactive methods are the results of a long and considerable development. The tracer techniques, such as isotope-dilution procedures, protein-binding and radio-immunoassays, marked significant stages in this development. Their application makes it possible to determine steroid hormones at submicrogram levels. The use of radioactive steroids permits not only the determination of hormone levels in biological substances, but also the following of hormone production and the dynamics of hormone or steroid metabolism in the organism.

Although steroid research in Hungary faces some difficulties, in many Hungarian laboratories advanced work is in progress, and the results of these new methods will be reported at this symposium.

Acta Medica Academiae Scientiarum Hungaricae, Tomus 29 (1—2), pp. 119—123 (1972)

PAPER CHROMATOGRAPHIC ANALYSIS OF CORTICOSTEROIDS

By

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Paper chromatography including that of steroids is regarded today as a classical separation procedure, though in certain respects it is doubtlessly less advantageous than either thin-layer or gas chromatography. If, however, the group reactions of the steroids extracted from blood or urine have to be completed by further purification or isolation of the individual fractions, for the purpose of prepurification of labelled steroids or for isotope dilution procedures, paper chromatography may be indispensable. Though the procedure is time-consuming and requires experience, it is of high efficiency, as shown by the wide range of corticosteroids mentioned in recent literature. Of the various possibilities of application of the method, we only refer to functional-group analysis and to some double isotope dilution techniques combined with paper chromatography.

Discussion of the general methods or of the pertinent theoretical questions are outside the scope of this paper, the only aim of which is to point out some practical issues.

The procedures currently used at our laboratory are as follows.

I. *Separation of corticoids by paper chromatography*

a) separation from interfering substances

b) separation from each other.

II. *Identification of corticoids*

a) by combination of chromatographic systems

b) after transformation of the steroid molecule and combination of repeated identifying runs.

Extraction from plasma, urine or incubated tissues is not steroid-specific, regardless of the solvent used, owing to the presence of large amounts of lipids and of chromogens in the extracts. Their elimination in the case of previous purification almost invariably requires additional paper chromatography. Some of these procedures used are as follows. There is, first of all the crude-extract purification of plasma and of incubated tissues. In the

benzene-containing systems in which the substances are run, particularly in the Bush A-system, the impurities migrate with the moving phase whereas the corticoids, with the exception of corticosterone, remain at the starting point (WEISZ and GLÁZ, 1960).

The situation is different when urinary contaminations have to be eliminated. In the case of aldosterone estimation or of extraction of metabolites of other corticoids requiring large urine samples, chromogens are present in such large amounts as to interfere with the further steps of the procedure, regardless of the type of purification prior to paper charomatography. In this case the extracts are run in water, as described by LOMMER and WOLFF (1964) which enables the interfering substances to remain at the starting

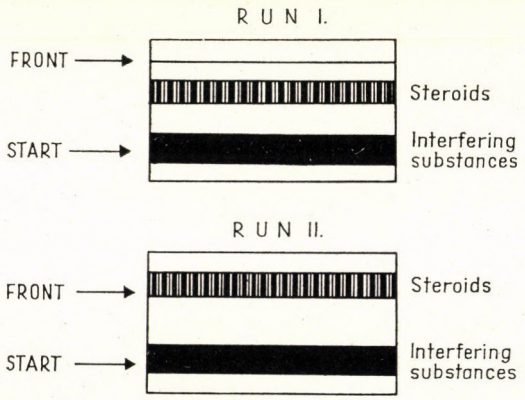

Fig. 1. Scheme of one of the purification procedures of the crude extract for the estimation of urinary aldosterone. Both ascending chromatographies have been performed in distilled water

point and the corticoids to move from the area of the contaminants. For quantitative separation of the corticoid fractions it is important to apply the extract to as large an area as possible. Ascending chromatography in water, repeated twice, allows the steroids to move with the front wherefrom they are eluted and subsequently separated by further chromatography (Fig. 1).

For the isolation from the urine of derivatives more polar than tetrahydrocortisol and tetrahydrocortisone (e.g., 6β-OH-cortisol, 20-dihydrocortisol), removal of the contaminants is possible by running the extracts e.g., in a Bush B-5 system for 24 hours. This eliminates the chromogens while the fractions of strong polarity move but slightly from the starting point wherefrom they are eluted.

As to the procedures suitable for the separation of corticoids and their metabolites, in the following the essential procedures employed by us will be presented together with some typical chromatograms.

The final chromatogram of urinary aldosterone in a system of butyl acetate–water–ethylacetate–formamide after impregnation with 30% acetone is presented in Fig. 2. The condition of a chromatography of this kind is a previous removal from the urinary extract by means of combined solvents of the fractions of a polarity stronger or weaker than that of aldosterone. The aim of each successive separation is to find a system by which the interfering fraction which may have been left back after previous separations can be removed, leaving exclusively the derivative to be isolated on the paper. It is often necessary to isolate steroid analogues such as prednisolone. Separation from cortisol poses up difficulty. As we know, prednisolone is of similar polarity as the tetrahydroderivatives of cortisol and cortisone. Their complete

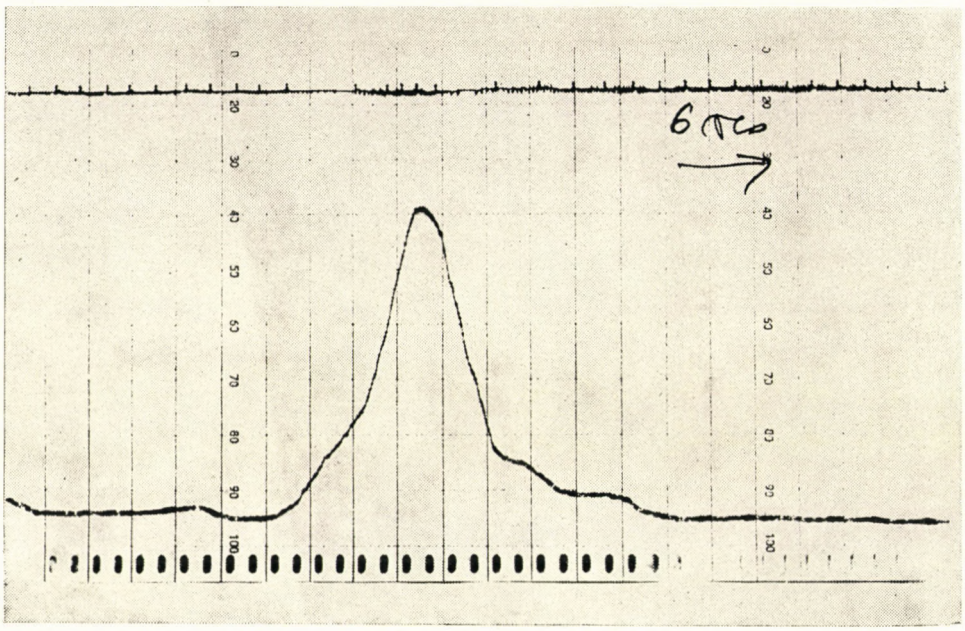

Fig. 2. Detection in situ of the final chromatogram of urinary aldosterone determination after development with TB. Registration was made with a "Vericord" electronic densitometer

separation is of importance for instance if the secretion rate of cortisol has to be estimated in patients on steroid therapy. In most of the current chromatographic systems the tetrahydroderivatives which form the target-compounds in secretion rate determinations, are not completely separable from prednisolone and form, as an inactive fraction, a source of error in the course of successive measurements. For their elimination, first the contaminants are run in a strongly polar system and eluted together with THF, THE, allo-THF. Subsequently the fraction is acetylated and the acetates thus formed are run again. The monoacetate of prednisolone thus separates from the

diacetates and can now be separated and after TB-development be eluted or scanned on paper (Bush, 1961).

Recent and most reliable procedures for the evaluation of chromatograms are based on the detection by densitometry or scanning in situ without requiring an elution of the target compounds.

Fig. 3 shows the evaluation of a chromatogram with an electronic densitometer of the "Vericord" type, equipped with an integrator for the quantification of the peaks which can be read directly from the record.

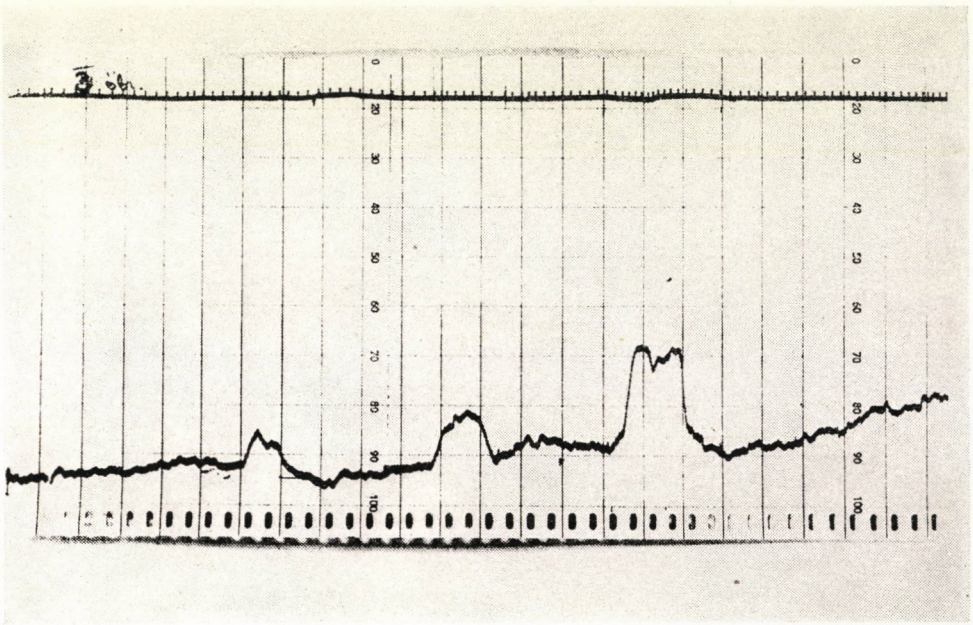

Fig. 3. Registration of a standard cortisol curve (5—15—21 μg) after development with TB, with a "Vericord" electronic densitometer

Fig. 4 shows a radiochromatogram made with a Packard chromatogram scanner. The sample was a tissue extract incubated with tritiated cortisol. Catabolized 20-dihydrocortisol can be located at the sites corresponding to the standard.

From the various physical and chemical identification procedures there are two chemical chromatographic techniques which are particularly reliable for the measurement of one or two Rf-values if run against reference substances, and they can be integrated with other, routinely used procedures. Of the microchemical group reactions for identification purposes it is the acetylation of the primary or secondary OH-groups which we are routinely applying in view of its simplicity. Though the reaction is not quantitative,

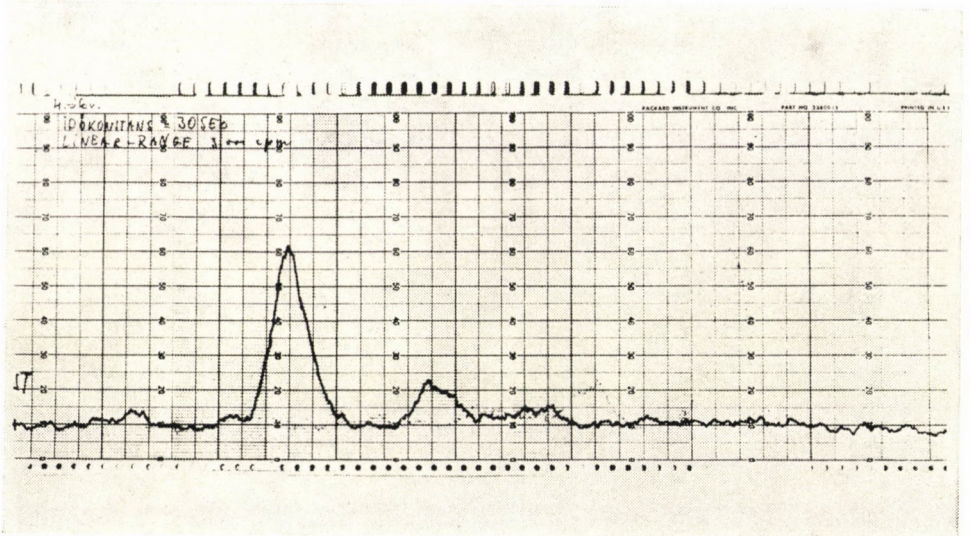

Fig. 4. Synovial membrane extract incubated with 1—2 ^{3}H cortisol and run in a benzene-methanol : water : acetate-system (1 : 1 : 1 : 0.1). The radioactive peaks are at the sites corresponding to 20β-OH-F, cortisol and cortisone standards. Evaluation with a Packard Tricarb chromatogram scanner

it is commonly used for identification purposes. If for instance of the urinary steroids excreted in a free state, 6β-OH-cortisol, 20β-OH-cortisol and cortisol are to be separated, acetylation and subsequent running in the Bush 3 system permits their separation without any difficulty.

One of the usual procedures for the identification of C-21-steroids is based on the oxidation of the dihydroxy-acetone or of the glycerol side chain with subsequent repeated chromatography of the 17-ketosteroids thus formed. For the identification of free 20-dihydro-derivatives, the periodic acid reaction is used. Their oxidation products are similar to those obtained with $NaBiO_3$, 20β-OH-cortisone giving adrenosterone and 20β-OH cortisol 11β-hydroxyandrosterone which readily separate in a Romanoff system and can be detected by the Zimmermann-reaction.

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FLUOROMETRIC ESTIMATION OF STEROIDS

By

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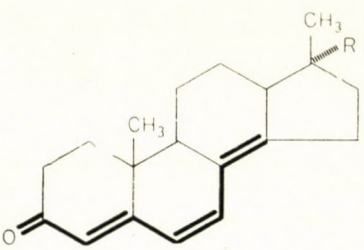
The steroids do not give any useful fluorescence unless the pH is manipulated: then they can be made to fluoresce by treatment with strong acids. This phenomenon, known since 1929, has been adapted for the quantitative fluorophotometric estimation of various hormonal steroids extracted from biological samples. The fluorogenic reactions of steroids in strong acid media are widely used for quantitative analysis and structure elucidation. The acid reagent contains sulphuric acid, phosphoric acid, perchloric acid or hydrochloric acid together with ethanol, methanol, acetic acid, phthalic acid anhydride.

Each steroid gives rise to several fluorescing species, the relative intensities varying with the type and concentration of acid, temperature and other factors.



The development of the fluorescent structure is probably an at least three-stage-reaction pattern. Reversible protonation at the Δ^4-C-3-ketone or of the C-3-hydroxyl at the aromatic A-ring is the first step. In the second one the protonated steroid is assumed to form intermolecular association complexes at reactive substituent sites of other steroid molecules. Finally, the postulated complexes are thought to break down irreversibly to yield several fluorogens. The fluorescent products are presumably trienones [1]. Little is, however, known about the mechanism of formation and the chemical structure of fluorescent products and it is difficult to find correlations between the chemical structure and fluorogenic features (absorption and emission

maxima, intensity of fluorescence) of steroids. For example, in sulphuric acid/ethanol reagent the Δ^4-3-keto- and 11-hydroxy structure is necessary to obtain the characteristic fast fluorescence of corticosteroids. A second double bond in ring A or halogenation in position 9 abolishes this fast reaction. The negative results obtained with 11β-hydroxyandrost-4-ene-3,17-dione suggest the presence of a side-chain. In this side-chain the 17-hydroxy group and the 20-keto group are not essential but the absence of the 21-hydroxy group causes an important decrease of the fast fluorescence. Fluorescence is reduced by the introduction of an 11-keto or 16-hydroxy group.

By treatment with strong sulphuric acid, corticosteroids possessing 11-hydroxy group yield fluorescent derivatives. Their characteristics are 470 to 475 nm excitation and 520 to 530 nm fluorescence. The basic procedure for the determination of corticosteroids in plasma is an extraction of the plasma with dichloromethane, chloroform, ethyl acetate, carbon tetrachloride or a mixture of these solvents, and re-extraction of the steroids from the organic solvent into the sulphuric acid/ethanol mixture, followed by estimation of the fluorescence of the acid phase. Since the methods of assay have been worked out empirically, many slight variations are found in the literature; several authors omit some steps for the sake of simplicity or add steps to improve specificity and sensitivity: e.g., washing of plasma with iso-octane or petroleum ether to remove neutral lipids, sterols; washing of the plasma with alkali to fix acid materials, notably oestrogens, which have a phenolic hydroxy group in the A-ring.

An important problem concerning the fluorometric assay of steroids is that of non-specific fluorescence and quenching. Several substances interfere with the rapid development of the sulphuric acid/ethanol fluorescence, e.g., surface active agents such as Triton. Traces of chromic acid are strong quenchers. Certain substances aspecifically increase the early fluorescence characteristic of the Δ^4-3-keto-11-hydroxysteroids. Such is the case for tobacco smoke, phenolphthalein, and still unknown products which develop in stored urine. Fortunately this aspecific fluorescence can often be detected by the yellow coloration of the final sulphuric acid/ethanol mixture. Cholesterol and cholesterol esters show a slight fluorescence but owing to their concentration in blood, they represent the major interfering fluorogen in the determination of plasma corticosteroids [2]. FLACK and STOCKHAM [3] presented a method to eliminate the interfering effect of cholesterol mathematically; it consists of reading the fluorescence over the 520—540 nm range. Specificity was greatly improved by the introduction of a correction based on the different development of fluorescence with time of specific (corticosteroid) and non-specific fluorogens in the sulphuric acid/ethanol reagent [4]. Rodent ovaries secrete 20α-hydroxypregn-4-en-3-one. This steroid interferes with the fluorometric assay of corticosterone; this interference may be eliminated by washing with

petroleum ether [5]. WERK et al. [6] and KENDALL et al. [7] showed that the benzyl alcohol preservative commonly found in commercial heparin solutions also interferes with the fluorometric technique. In partially clotted blood the degree or interference with benzyl alcohol is greater. The nature of the interfering substance in "impure" dichloromethane which reacts with benzyl alcohol is unknown. The emission spectrum of the interfering fluorogen is identical to that of cortisol.

Replacement of ethanol for acid diluents results in an increase of fluorescence emission; an improved reagent consists of sulphuric acid, acetic acid and formic acid in the volume ratio 3 : 1 : 1 in which the development of fluorescence takes 1 hr. 11-Deoxycortisol, corticosterone, cortisol and 21-deoxycortisol produce fluorescence in the same intensity range, therefore this reagent is applicable after chromatographic separation only [8].

Simultaneous determination of cortisol and corticosterone becomes possible after separation by chromatography [9, 10] or by solvent partitioning [11]. The separated fractions are then measured fluorometrically. Other methods make use of the different fluorescent properties of the two steroids to determine cortisol and corticosterone without actually separating them. Since the fluorescence of cortisol develops more quickly than that of corticosterone, they can be resolved by reading at two different times, first when only cortisol is giving the fluorescence and later when corticosterone is also contributing to it. MATSUMARA et. al. [12] developed a method which differentiates cortisol and corticosterone on the basis of the different relative fluorescence of these steroids in different strengths of acid [13]. CLARK and RUBIN [14] described a new fluorometric method for cortisol assay; it has the specificity and reliability of lengthier chromatographic procedures. It uses a metaperiodate oxidation of corticosterone and cortisol to their corresponding 17α-carboxylic acids (4-androstene-17α-carboxylic acid 11β-ol-3-one and 11β17α-diol-3-one). Only the cortisol derivative is fluorescent (475 → 530 nm) in sulphuric acid.

A number of steroids show a red (max. 540 nm) fluorescence after heating (85 °C) for 12 min in 70% H_2SO_4/ethanol and then diluting with absolute ethanol. The ratio of green/red fluorescence is constant and characteristic of each steroid [15]. EECHAUTE et al. [16, 17] reported a sensitive (from 0.005 μg), well reproducible and relatively specific reaction inducing red fluorescence for testosterone (17-α and 17-β epimer) and androstenedione. The steroids are heated in 65% H_2SO_4/ethanol reagent at 85 °C for 10 min. After cooling, ethanol is added; the fluorescence at 615 nm, with activation light of 598 nm is measured. The method allows determination of testosterone concentrations as found normally in male human plasma.

The presence of an oxygen function on C-11 is usually considered necessary for the fluorescence of corticosteroids in acid/alcohol media. By the

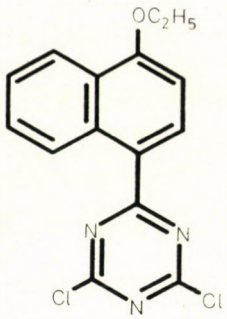
method of MUEHLBAECHER, Δ^4-3-keto-17-hydroxysteroids lacking an oxygen function on C-11 can be measured fluorometrically at 525 nm, with excitation at 465 nm, after heating in 15 M sulphuric acid or 9 M perchloric acid at 60 °C for 2 hours and adding methanol [18].

TOUCHSTONE and MURAWEC [19] described a fluorometric procedure for the measurement of progesterone in plasma. The sample is treated with 2 N methanolic potassium hydroxide and dissolved in 88% sulphuric acid. The fluorescence of the H_2SO_4 solution in the green region is then determined. Progesterone exhibits some fluorescence when dissolved in sulphuric acid; previous treatment with alkali causes a hundred-fold increase in the intensity of fluorescence. A sensitive assay for the quantitative estimation of progesterone was developed by HEAP [20]. The method consists of extraction of progesterone by ether from alkalinized plasma, paper chromatographic isolation, conversion on 20β-hydroxysteroid dehydrogenase, chromatographic purification and fluorometric estimation of the 20β-hydroxypregn-4-en-3-one in H_2SO_4/ethanol mixture. WOOD [21] adds the sulphuric acid to the progesterone in methanolic potassium hydroxide medium after oxygen and heating. The quenching effect of high concentrations of inorganic alkali on the fluorescence of progesterone disappears when the solution is diluted with methanol. Oxygen must take part in the reaction or catalyse it; it also increases the fluorescence intensity.

The aldosterone molecule possesses an 11-hydroxy group and a Δ^4-3-keto group but in solution aldosterone is in equilibrium with its hemi-acetal form. In sulphuric acid the equilibrium will shift to the aldehyde form and exhibit fluorescence. The excitation and emission wavelength of aldosterone is 460 and 505 nm, respectively, in 36 N H_2SO_4 after heating [22]. The recently described method of GÖDICKE and GERIKE [23] depends on the oxidation of aldosterone using periodic acid and condensation of the resulting formaldehyde with acetyl acetone and ammonia to the strongly fluorescent 3,5-diacetyl-1,4-dihydrolutidine at 60 °C. The oxidation of aldosterone takes one hour. Destruction of the oxidation medium is necessary to obtain a low blank value: it is equal to a fluorescence intensity of less than 0.2 μg aldosterone. The maximum of excitation is at 412 nm, that of emission, 498 nm. The sensitivity is roughly equal to that of cortisol or corticosterone determination by the sulphuric acid/ethanol fluorescence method.

CHAYEN et al. [24] described a fluorometric procedure for corticosteroid and oestrogen assay. Its principle differs from that of the above mentioned methods. EDTN (1-ethoxy-4-(dichloro-s-triazinyl)naphthalene) is a strongly fluorescent compound; it produces fluorescent complexes with the phenolic hydroxy groups of oestrogens and with the aliphatic hydroxyls of other steroids in alkaline medium at 45 °C. These fluorescent steroid-EDTN complexes are extracted with dichloromethane, separated on Kieselgel-G layer

and indicated in UV-light. After elution with ethanol, the fluorescence is measured at 352 nm excitation and 419 nm emission wavelength. The concentration-fluorescence intensity curve is linear in the 0.1—25 μg range. The method is very sensitive for oestrogens.



EDTN

For the further progress of the fluorometric analysis of steroids, a deeper knowledge on the formation and nature of fluorogens will be necessary.

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ESTIMATION OF ^{3}H AND ^{14}C LABELLED STEROIDS BY THE LIQUID SCINTILLATION TECHNIQUE

By

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The use of ^{3}H and ^{14}C labelling marks a considerable progress in the qualitative and quantitative studies of steroids. Both tracers emit low-energy, i.e., "soft" beta radiation, therefore their measurement requires excessive sensitivity. The liquid scintillation technique is superior to the procedures hitherto employed in respect of practically every parameter (efficiency, background, etc.), preparation of the sample is easy and the equipment lends itself to serial estimations. The difficulties involved by the measurement of soft radiation have thus been practically overcome. The liquid scintillation techniques can be traced back to the observations of RAYNOLD and KALLMAN (1950) who demonstrated that certain organic compounds dissolved in organic solvents scintillate on exposure to ionizing radiation (Fig. 1).

The mechanism of fluid scintillation comprizes three phases.

1. The radioactive particles excite the molecules of the solvent.

2. The excited molecules of the solvent transmit their energy successively from one molecule to the other and to the scintillator molecules. The excited scintillator molecules produce fluorescence and emit light.

3. The fluorescent light either attains the photosensitive cathode of the detector or is absorbed in it in the presence of "secondary scintillator" molecules and is reemitted at some other wavelength before attaining the measuring detector.

The properties of the solvent are essential. Toluol is considered the optimum solvent in every respect, in particular, as regards its counting efficiency, its favourable solvent properties, its high transparence in the range of the scintillator spectrum, its low freezing point, and the easiness of its purification.

The most extensively used primary scintillator is 2,5-diphenyloxazol, PPO. Since the spectrum of luminescent radiation of the primary scintillators does not correspond to the maximum range of sensitivity of the photosensitive photocathode, we have to use a wavelength shifter, called secondary scintillator, so as to shift the spectrum to the desired range. The substance commonly used for this purpose is dimethyl POPOP.

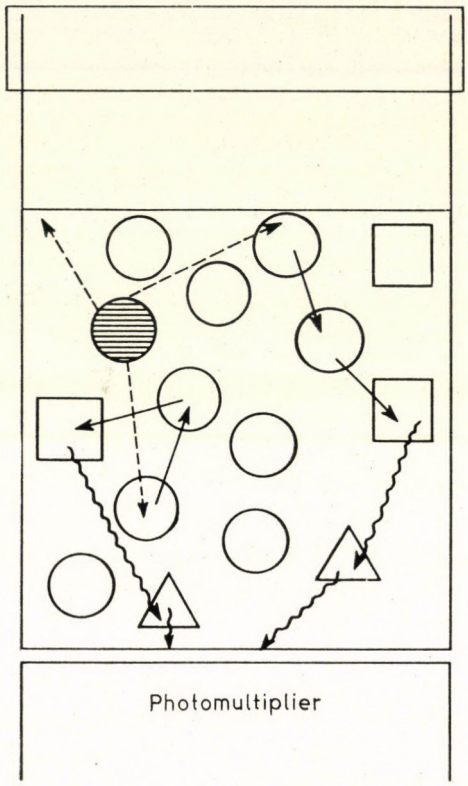

Fig. 1

The steroids after adequate extraction generally dissolve well in toluol. Aqueous media such as plasma, serum, urine or exclusively water-soluble steroid sulphates, pose some difficulty. Toluol forms no aqueous solution, therefore simple mixing is not practicable. Solvent-scintillator mixtures suitable for the measurement of the aqueous phase are too numerous to be kept in evidence. Bray's solution made up of dioxane and naphthalene, or a mixture of toluol and Triton-X-100 detergent (2 : 1) are generally used for this purpose. The efficiency of the individual mixture is expressed by the product of its hygroscopicity and of its counting efficiency. The greater this value, the more suited the mixture for the measurement of samples of aqueous media. In the last two years we have been using for measurement in aqueous phase a mixture of toluol and Triton X-100 detergent.

One of the greatest problems of liquid scintillation counting is the "quenching action" which term is applied to all factors that affect the efficiency of fluid scintillation. We have to deal with three types of quenching.

1. Concentration quenching or self-quenching in which the phenomenon

is brought about by the scintillator molecule itself. This effect increases with the concentration of the scintillator and the temperature of the solution.

2. External quenching. Many substances inhibit the transfer of energy to the scintillator. The energy of the excited solvent molecule is transformed into heat and is lost, by this fact, to measurement.

Some compounds involved in liquid scintillation:

Diluters	Quenchers
Alcohols	Ketones
Olefins	Aromatic Cl and Br compounds
Ethers	Amines
Saturated hydrocarbons	Thiophene derivatives
F and Cl alkanes	

The diluters reduce the counting efficiency to a slight degree, the quenchers have a strong effect of this kind.

3. Colour extinction. Luminescent radiation is reduced by certain substances which absorb within the range of the radiation wavelength. Substances of this kind, chromogens, produce a quenching effect as a result of absorption.

The quenching effect makes a checking of the counting efficiency obviously indispensable. Since practically every sample is quenched to some degree, the counting efficiency never attains 100 per cent. There are various methods for the ascertainment and continuous checking of counting efficiency.

Standardization procedures:

Internal standard method
Extrapolation method
Colorimetry
Integral counting method
Channel ratio method
External standard method.

One of the most reliable procedures is based on the principle of internal standard. To the measured sample an activity of known magnitude is added and measurement is repeated. The channel-ratio method has also gained general acceptance. The quenching effect not only reduces the count rate but also shifts the spectra to a lower energy level. If the sample is measured on two suitable channels, the counting efficiency can be inferred from the changes in the proportion of the counting rates recorded from the two channels. The external standard procedure is regarded today as the simplest and most modern technique. Here too it is the ratio of the count rates recorded from the two channels which defines the efficiency with the aid of a calibration curve. For this purpose one or two external gamma-emitting isotopes, usually

radium or americium, are employed. The gamma-radiation penetrates into the sample and generates compton electrons which produce a scintillation of the same kind as beta-radiation. The modern devices are all equipped with external standards.

The arrangement of the measuring cell and of the photomultipliers provide for a "volume-independent" measurement which is due to the fact that between 5 and 20 ml the changes in the volume of the sample leave the counting efficiency practically unaffected. This makes it convenient to use samples less than 10 ml in volume.

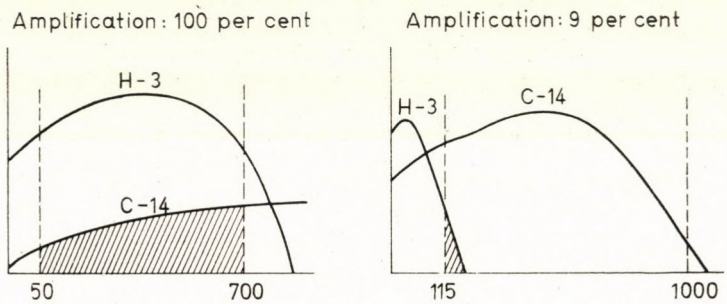

Fig. 2

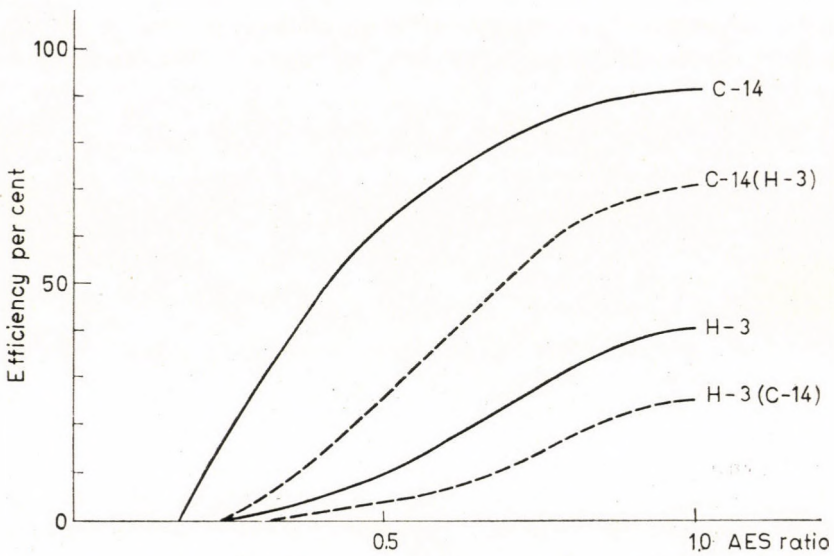

Fig. 3

Double labelled samples are increasingly used in recent years for the estimation of steroids. For instance the ^{3}H and the ^{14}C forms of the same substance simultaneously indicate the extraction losses and the substance itself. The modern multichannel equipments provide for the simultaneous

measurement of ^3H and ^{14}C in the same sample (Fig. 2). A selection of the degree of amplification and of the upper and lower level of the differential discriminator allows the ^3H to produce but a minimum increase in the counting rate of the ^{14}C-channel and, the ^{14}C, to produce an increase of a definite ratio in the counting rate of the channel selected for the measurement of ^3H. The net ^3H and ^{14}C counts are computed by substituting the overlap quotients into an equation of two unknown quantities. As the third factor, we have to know the quenching effect because of the influence of its variations on the magnitude of the overlap-coefficients (Fig. 3). In the case of double labelling the counting efficiency is obviously inferior to that attained by the use of a single label, but this short-coming is offset by numerous advantages. The tedious calculations involved by double labelling is eliminated today by the use of data-processing systems. For the calculation of net counts of double labelled samples we also use computer programs.

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COMPETITIVE PROTEIN BINDING ASSAY OF CORTICOSTERONE

By

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The method published by MURPHY in 1967 in which the principle of competitive protein-binding assay was applied for various steroids has opened new aspects in the analysis of steroid hormones. The basic steps of the procedure are as follows. Tritiated steroid is added to a specific steroid-binding protein, e.g., to transcortin. The protein binds the majority of the tritiated steroid. If cold steroid is now added to the system, it displaces the tritiated hormone from the protein receptor, the amount of displaced hormone being related to that of the cold hormone. The amount of cold hormone added to the system can now be determined by estimating the residual protein-bound fraction with the aid of a calibration curve. This simple procedure may be regarded as equivalent in sensitivity to the double isotope dilution techniques.

The method has recently been applied by SCHULSTER et al. (1970) for the assay of corticosterone (B), and by MAYES et al. (1970) and BAYARD et al. (1970) for that of aldosterone. Our aim has been to adapt these two procedures to the assay of both hormones in rat plasma or in the incubation medium of adrenals. This requires preliminary separation of the corticosteroids. Adequate separation has the additional advantage of increasing the specificity of the assay.

The procedure is basically as follows: 1000—1500 cpm of ^{3}H—B is added to the sample prior to extraction in order to check analytical losses. The plasma samples to be examined are of a volume containing 10 to 100 ng B. Extraction is carried out with 3×5 vol methylene chloride. At the first extraction the sample is alkalinized, and the alkali is removed from the collected organic phase by washing first with acetic acid, subsequently with distilled water. Na_2SO_4 is used for drying. In the case of larger plasma volumes the nonpolar lipids are removed by partitioning between 70% methanol and toluene-hexane 1 : 9. The steroids are separated by thin-layer chromatography, using a 0.3 mm thick Kieselgel G layer to which 0.5% Leuchtpigment has been added. The plates are activated at 110 °C for 40 minutes. The dry residue is applied with methylene chloride–methanol 1 : 1, and run by the sandwich technique. The solvent is a mixture of chloroform—acetone—acetic acid

100 : 40 : 2.5. This system reliably separates natural and synthetic steroids which may possibly interfere with one another. In order to determine the running distances, standards are run parallelly which are detected in UV-light at 254 μm. The spot corresponding to corticosterone is eluted, evaporated and redissolved in 1 ml methylene chloride—methanol. A 0.3 aliquot of the eluate is used for recovery-studies, and two other similar aliquots serve for the assay.

For the assay, a binding solution is used which contains 2 to 4 μCi ^{3}H—B and 2 ml mouse serum per 100 ml. Parallel assays are performed with the samples as well as with the standards. The extracts of the samples and the standards are transferred to stoppered centrifuge tubes, and evaporated to dryness. One ml of the binding solution is added to each tube, incubated at 38 °C for 5 minutes permitting equilibration between protein, labelled and cold B. For the separation of protein-bound B from the free one, 43 mg of pretreated Florisil, capable of adsorbing free B, is added to the mixture. Since, however, the protein would rapidly release the bound steroid, the system is previously cooled to 4 °C at which temperature the protein-steroid complex dissociates far more slowly. After vigorous shaking for 120 sec the tubes are left in the ice bath for 5 minutes during which time the Florisil settles. Now the samples are decanted and between the 10th and 20th minute, 0.5 ml of the decanted samples is pipetted over to the scintillation cocktail, the radioactivity of which is measured by a liquid scintillation spectrometer. The calibration curve is constructed by plotting the percentage of protein-bound ^{3}H—B against the standard B added. Calculation of protein-bound B involves correction for the inefficiency of Florisil to adsorb B, as estimated by the use of "protein-free" solution. This has the same composition as the binding solution with the exception of serum. Two protein-free tubes are subjected to the entire procedure, finally the degree of Florisil-adsorption is determined by measuring the activity of the tritiated supernatant. In addition to this correction, consideration is given to the fact that ^{3}H—B has been added to the samples at the start of the assay in order to check the recovery.

Fig. 1 shows the non-corrected and the corrected calibration curves. The values read from the calibration curve are corrected for recovery of the sample and the blank. Average recovery after chromatography was 52% in 48 cases.

To test the accuracy of the method, a known amount of B was added to 0.5 ml physiological saline and the analysis was carried out (Fig. 2). Between 10 and 100 ng there was a linear correlation between the added and the measured amounts, the correlation coefficient being 0.990 and mean recovery $92.6 \pm 13.6\%$.

The precision of the procedure was computed on the basis of parallel measurements of known amounts of B. Between 10 and 100 ng the variation

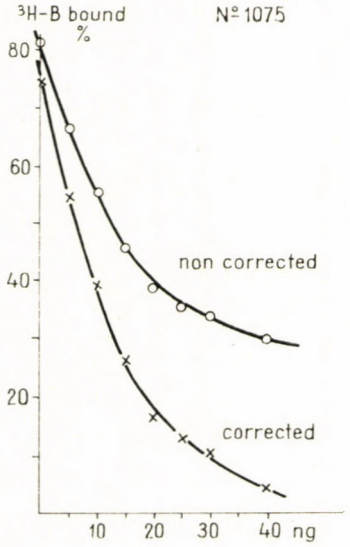

Fig. 1

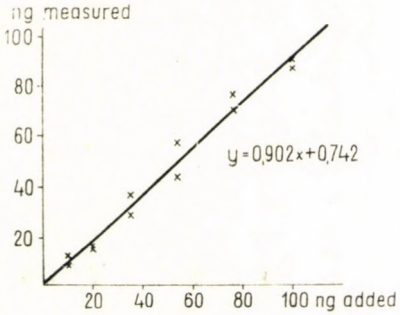

Fig. 2

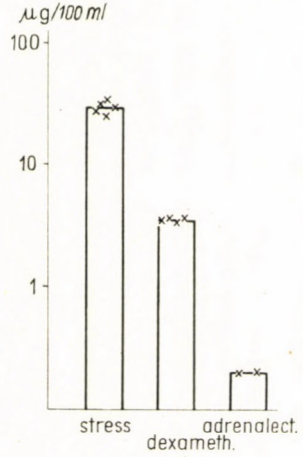

Fig. 3

coefficient of the duplicates was 10.7%. An alternative method for checking the precision was to perform repeated measurements from plasma pools of different B-contents (Fig. 3). The variation coefficient was 12.9% for the first, 3.8% for the second pool, whereas for the third pool two measurements yielded identical values. The concentration of B in the three pools varied in a range of more than two orders of magnitude.

Water-blank was 1.92 ± 2.08 ng in 12 cases and was unrelated to the volume of the sample in the 0.5 to 5 ml range.

The sensitivity of the method, i.e., the smallest amount significantly different from zero (after correction for the blank), was calculated to be about 2.4 ng.

In order to increase specificity mouse serum was used as a source of transcortin, since it binds corticosterone with great specificity. The rat samples contain no cortisol and cortisone. Dexamethasone, frequently used for the pretreatment of laboratory animals as well as progesterone being present in small amounts, are separated by thin-layer chromatography.

The normal values yielded by the present method correspond to those reported in the literature.

The results obtained by the procedure are largely due, in addition to the purification of the isotope, to the intense purification of the organic solvents of analytical grade. The introduction of the radioimmunoassay of aldosterone is in progress.

We are indebted to Dr. T. Garzó (Institute of Medical Chemistry, University Medical School, Budapest) for critical comments and valuable aid in the liquid scintillation measurements, to Mr. B. Kanyár and Miss Judith Szutrély (Section of Computer Technique, University Medical School, Budapest), for preparation of the computer program and their aid in the calculations; to Drs Sturcz and T. Harza (Institute of Physiology, University Medical School, Budapest) and to Dr. Zs. Ács (Institute of Experimental Medicine, Hungarian Academy of Sciences), for their valuable advices.

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SEPARATION AND IDENTIFICATION OF FREE AND ESTERIFIED 17-KETOSTEROIDS

By

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It has long been known that in human urine the 17-ketosteroids occur predominantly in the form of 17-ketosteroid sulphates (17-Ks-S) and 17-ketosteroid glucuronides (17-Ks-G). Dehydroepiandrosterone (DHA) and androsterone (A) in peripheral blood are also found in the main in the form of sulphate esters. Moreover, the testicles, ovaries and adrenal cortex have also been shown in recent years to be capable of synthesizing 17-Ks-S in addition to free 17-ketosteroids. This has directed attention to the biochemical and physiological role of 17-Ks esters. The present report deals with the procedures developed in our laboratories for the study of 17-Ks esters in human blood and sweat.

In earlier reports (Faredin and Tóth, 1964a, 1964b; 1965; Faredin, 1965) we pointed out the advantages of the separation of steroid groups of different polarities, in particular pregnanediols, pregnanetriols and 17-hydrocorticosteroids, on a "Nymco" florisil column. Since the free 17-ketosteroids and their sulphate and glucuronide esters have significantly different polarities, we have attempted their separation on a "Nymco" florisil column.

The scheme of the procedure is presented in Fig. 1.

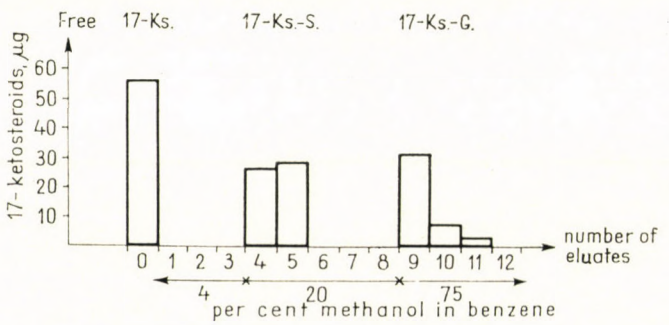

Fig. 1. Separation of free and esterified 17-ketosteroids on a 1.5 g "Nymco"-florisil column. Free 17-Ks = dehydroepiandrosterone, androsterone. 17-Ks-S = dehydroepiandrosterone sulphate, androsterone sulphate. 17-Ks-G = dehydroepiandrosterone glucuronide, androsterone glucuronide, etiocholanolone glucuronide

"Nymco" florisil (1.5 g) was suspended in 24 ml benzene in a column. The 17-ketosteroid mixture was dissolved in 1 ml methanol and transferred to the column. The solution was eluted dropwise from the column, and the entire procedure was repeated with a further 24 ml benzene and 1 ml methanol. The two 25 ml eluates were combined: this was fraction 0. The column was next washed with 3×20 ml 4% methanol in benzene, to give fractions 1—3. Fraction 0 contained all the free 17-ketosteroids.

The column was subsequently eluted with 5×20 ml 20% methanol in benzene; these fractions contained the 17-Ks-S. In order to obtain the 17-Ks-G, the column was eluted with 4×20 ml 75% methanol in benzene.

As can be seen from Fig. 1, this chromatographic procedure is suitable for the separation of 17-ketosteroids into free, 17-Ks-S and 17-Ks-G groups. However, the 17-ketosteroids occurring in biological fluids (blood, urine, sweat) are accompanied by large amounts of foreign chromogens, and without additional purification they are still not suitable for quantitative determination.

The separation of 17-ketosteroids into free, 17-Ks-S and 17-Ks-G fractions by thin-layer chromatography is based on the procedure described by SARFATY and LIPSETT (1966). As Table I shows, when chromatography was performed on a 0.25 mm thin-layer of silica-gel-G or Al_2O_3-G as adsorbents in solvent system I (absolute ethanol — ethylacetate — NH_4OH, 5 : 5 : 1, v/v/v), both adsorbents proved suitable for the separation of the fractions referred to above. The 17-Ks-G remained at the starting point, the 17-Ks-S were at the middle and the free 17-Ks ran near the front. Since the 17-Ks-G

Table I

Separation of free and esterified 17-ketosteroids by thin-layer chromatography

Steroids	Al_2O_3-G Solvent system I		Silica-gel-G			
			Solvent system I		Solvent system II	
	Running distance in cm	R_f	Running distance in cm	R_f	Running distance in cm	R_f
Dehydroepiandrosterone	16.8	0.96	14.8	0.85	—	—
Androsterone	16.8	0.96	15.2	0.89	—	—
Etiocholanolone	16.8	0.96	14.9	0.86	—	—
Dehydroepiandrosterone sulphate	9.8	0.56	9.7	0.56	15.0	0.86
Androsterone sulphate	9.7	0.56	9.8	0.56	15.5	0.89
Dehydroepiandrosterone glucuronide ..	0.0	0.00	0.7	0.04	7.5	0.43
Androsterone glucuronide	0.0	0.00	0.9	0.05	8.6	0.49
Etiocholanolone glucuronide	0.0	0.00	0.7	0.04	8.1	0.46

System I (SARFATY and LIPSETT, 1966): abs. ethanol : ethyl acetate : NH_4OH, 5 : 5 : 1 (v/v/v)
System II: methanol : ethyl acetate : NH_4OH, 50 : 50 : 18 (v/v/v)

did not move, this solvent system was unsuitable for their analytical study. The use of solvent system II (methanol — ethyl acetate — NH_4OH, 50 : 50 : 18, v/v/v) on a silica-gel-G thin-layer caused the 17-Ks-G fraction too to run and hence to be readily separable from the 17-Ks-S.

With these thin-layer chromatographic procedures, therefore, it is possible to isolate the 17-ketosteroids as the 17-Ks-S and 17-Ks-G ester fractions and also in the free form.

The applicability of these procedures for quantitative purposes depends on the extent of recovery of the free steroids and their strongly polar 17-Ks esters from the adsorbent. The results of our studies on this question are shown in Tables II and III. It can be seen that 95 to 96% of the authentic free 17-Ks, 89% of the DHA-S an 60% of the DHA-G were recovered from the "Nymco" florisil column.

Table II

Recovery of free and esterified 17-ketosteroids from a 1.5 g "Nymco" florisil column

Steroids	Number of tests	Recovery	
		Limits, per cent	Mean, per cent
Dehydroepiandrosterone	4	92—99	96
Androsterone	6	91—99	96
Etiocholanolone	6	92—99	95
[7α—^{3}H]-dehydroepiandrosterone sulphate	10	82—98	89
Androsterone sulphate	8	68—84	78
Dehydroepiandrosterone glucuronide	4	54—68	60

Table III

Recovery of free and esterified 17-ketosteroids from silica-gel-G and Al_2O_3-G thin-layers

Steroids	Silica-gel-G			Al_2O_3-G		
	Number of tests	Limits, per cent	Mean, per cent	Number of tests	Limits, per cent	Mean, per cent
Dehydroepiandrosterone	4	77—82	80.0	7	71—81	76.0
Androsterone	4	71—78	74.0	4	71—77	72.0
Etiocholanolone	4	80—83	81.0	4	59—65	62.0
Dehydroepiandrosterone sulphate	4	70—78	72.5	4	65—76	68.5
[7α—^{3}H]-dehydroepiandrosterone sulphate		—	—	7	65—77	70.0
Androsterone sulphate	4	77—80	79.0	4	71—81	77.0
Androsterone glucuronide	4	62—75	69.7	—	—	—

Recovery of 17-Ks-S extractable with methanol from silica-gel-G and from Al_2O_3-G thin-layers varied between 68.5 and 79% while that of A—G from silica-gel-G was on average 69.7%. Thus, our chromatographic procedures are suitable not only for qualitative but also for quantitative studies.

Only a few methods can be found in the literature for the separation and quantitative study of free and esterified 17-ketosteroids. BUSH (1961) described paper-chromatographic, and BAULIEU and EMILIOZZI (1961) column-chromatographic techniques. These methods are either time-consuming or require large solvent quantities. The procedure of SARFATY and LIPSETT (1966) adapted to thin-layer chromatography meant substantial progress, but it has the shortcoming of being unsuitable for the study of steroid sulphate unless this is present in large amounts. The new procedures reported in the present paper allow the simultaneous study of only a few μg of free and esterified ketosteroids and with their relative simplicity, may be of use in the analysis of steroids.

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GAS CHROMATOGRAPHY OF OESTROGENS IN BIOLOGICAL FLUIDS

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The first practical procedure for gas chromatographic measurement of oestrogens in urine was reported by WOTIZ and MARTIN [48] in 1961. This marks the origin of gas chromatographic analysis of hormones in body fluids. Since that time there have been numerous modifications of the original procedure.

Clinical or biochemical research is primarily concerned with the hormone content of biological fluids, and accordingly the gas chromatographic procedures will be discussed here from this aspect.

Oestrogens are compounds with a sterane nucleus of 18 carbon atoms, particularly resistant to chemical and physical factors. They are formed readily and in fairly large amounts from their precursors, probably owing to the fact that the three unsaturated bonds in ring A ensure the attainment of the minimum energy level. The oestrogens owe their reactivity to the phenolic OH-group at position 3. Their hydroxy-groups confer a polar character on the oestrogens which has to be taken into account in gas chromatography procedures.

Oestrone, oestradiol, oestriol and oestetrol have the greatest practical importance among the numerous oestrogens which are known to occur in the organism, and therefore most reports on the gas chromatography of oestrones concern these four hormones.

Fig. 1 shows some of the most important oestrogens (Fig. 1). According to GLEISPACH [14], any gas chromatograph is suitable for their estimation, if it is easy to handle, can be equipped with a glass column, can operate at high temperatures and if it is provided with a flame ionization or an electron-capture detector. For an exact quantitative evaluation, an integrator is indispensable.

Though the use of glass columns has been generally adopted for the estimation of oestrogens, certain workers, e.g., ROSENTHAL and YASEN [40], and WOTIZ and CHATTORAJ [49], make use of steel columns. These allow a good separation in our own experience too. Glass columns have other advantages: owing to their polyfunctional groups, the oestrogens are liable

to irreversible adsorption on the column surface. At high temperatures this may attain a far greater intensity with steel columns than with glass columns. On the other hand, connection of the glass column is far more cumbersome than that of a steel column since use has to be made of heat-proof silicone-rubber sealing rings or of teflon sealing material, which are subject to deformation and can be used only once.

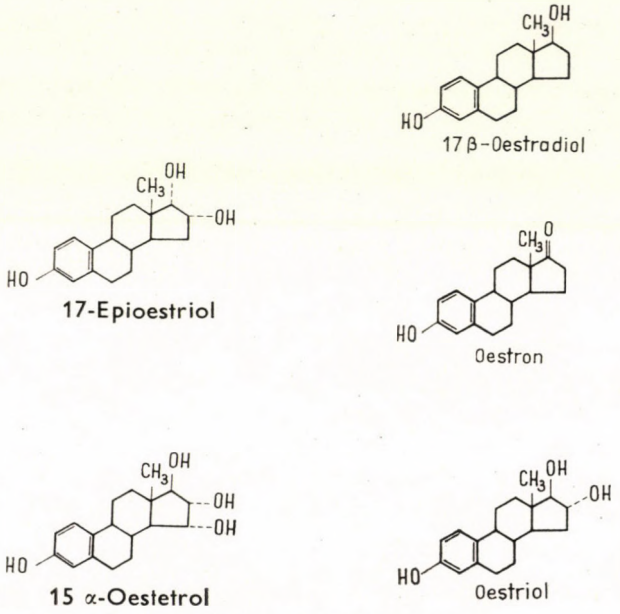

Fig. 1. Some essential oestrogens

Support medium: The currently used media for gas chromatography of oestrogens include Diatoport S, Diatomit CQ and Supelcoport 80 to 100, possibly 120 mesh.

Solvents: Polar or non-polar solvents are equally suitable. TOUCHSTONE [46] used a mixed solvent with satisfactory results.

The substances currently applied in gas chromatography of oestrogens include:

ketone-selective	QF 1 trifluoro-propyl-methyl-silicone-polymer;
non-polar	SE 30 dimethyl-silicone polymer;
polar	NGS polymer;
	XE 60 cyanomethyl-silicone;
	L 45 methyl-silicone polymer;
	Z co-polymer consisting of ethylene glycol, succinic acid methyl siloxane monomer;
	F 60 methyl p-chlorophenyl siloxane monomer;
	OV 17-phenyl-methyl-silicone polymer.

As regards capillary columns the only report known to us is that of GROENENDIJK [16].

Detector. Flame ionization (H_2) and LOVELOCK's [31] electron-capture detectors are used. Those of the latter type operate with ^{3}H, ^{63}Ni, Ra or ^{90}Sr isotope sources. The flame ionization detector has a sensitivity of 0.2 μg/100 ml plasma, the electron-capture detector one of 0.00005 to 0.05 μg/100 ml plasma. The latter device allows oestrogens to be estimated even in the absence of pregnancy when their concentration is very low.

Conversion to derivatives. The fact that the polyfunctional groups of the oestrogens may cause a firm and irreversible adsorption of these substances on the active surface of the column, requires the preparation of relatively non-polar products which not only inhibit this effect but also promote separation. Derivatives of this nature are readily formed and have considerably lower melting and boiling points than those of the oestrogens, and thus higher volatilities. This is a great advantage, since the thermolability of these compounds has been one of the major problems of gas chromatography. Their conversion to derivatives is therefore a step of the utmost importance, though according to SANGHVI et al. [41] free oestriol can be estimated quite as well as its acetate, and TOUCHSTONE [46] and KROMAN and BENDER [28] performed gas chromatography in the presence of QF 1 and of F 50, without forming derivatives.

The derivatives formed for the purpose of gas chromatography fall into two main groups.

For flame ionization detection, the acetates [48] or trimethylsilyl ethers, or trifluoracetates [32, 34] of the oestrogens are generally used. In routine work, however, preference is given to derivatives of the first type, acetates being less sensitive to humidity and to acids than are trimethylsilyl ethers, but the latter are easier to separate.

For electron-capture detection the oestrogens have to be converted to derivatives which are capable of increasing the electron-adsorptive capacity of these products. The halogenated oestrogen derivatives such as

monochloracetates [11]
hexadecafluoronanonates [23]
trifluoracetates [17, 18]
heptafluorobutyrates [19, 50]
pentafluorophenylhydrazones [3] are best suited for this purpose. Some of these derivatives are presented in Fig. 2.

For either type of detection mixed derivatives may be used, provided the compound has been converted previously to 3 methyl ether. This is advantageous for the stability of the product as noted during preliminary purification and phase-substitution (Fig. 3).

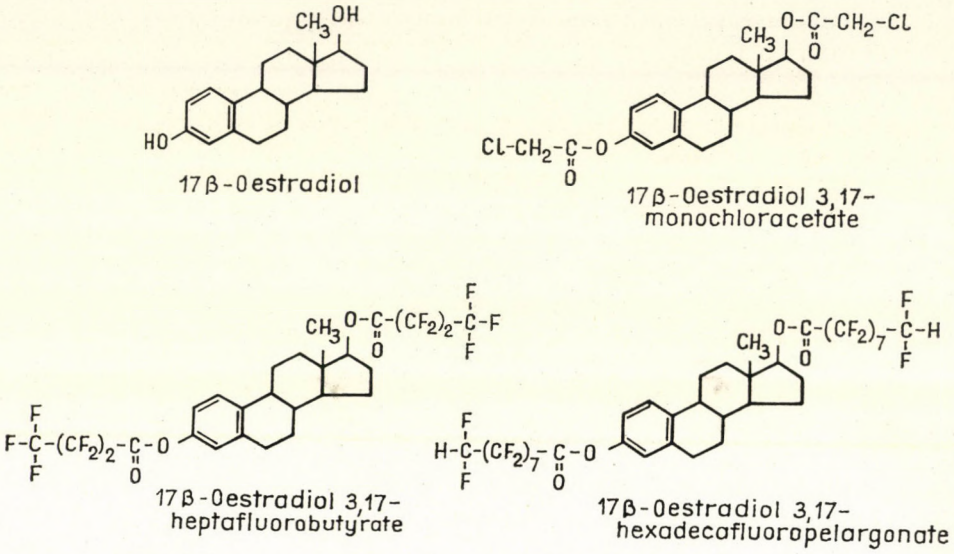

Fig. 2. Halogenated derivatives of oestradiol

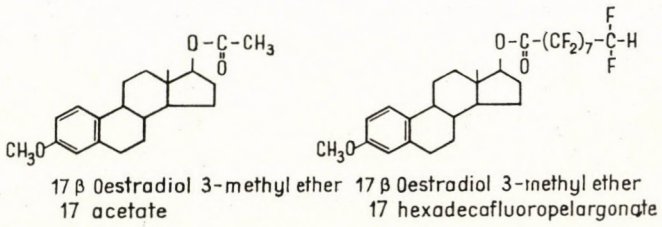

Fig. 3. 3-methoxy-oestradiol derivatives

Oestriol and oestrogens have been estimated in urine of pregnants or during menstruation by WOTIZ and CHATTORAJ [49], EBERLEIN [10], ADLERCREUTZ and LUUKKAINEN [1, 2], TOUCHSTONE [44], MENINI [36], SCHINDLER et al. [42], TOUCHSTONE et al. [47], KNORR et al. [23], ROSENTHAL and YASEEN [40], SANGHVI et al. [41], FOSTER and HOCHHOLZER [13], CAGNAZZO et al. [7], CHUN et al. [9];

in blood serum or plasma by TOUCHSTONE and MURAVEC [45], PANICUCCI and TAPONECO [39], ATTAL and EIK-NES [4], KROMAN et al. [26, 27], WOTIZ and CHATTORAJ et al. [49], MEAD et al. [35], KUSHINSKY et al. [29];

in amniotic fluid by SCHINDLER et al. [42], BIGGS et al. [6], HONDA et al. [20];

in human or animal tissues by EIK-NES et al. [11], MCKERNS and NORSTRAND [22], TAKÁCS et al. [43];

in bile by LUUKKAINEN and ADLERCREUTZ [33];

in solutions in oil by MORETTI et al. [38], together with pregnanediol and pregnanolone by BAYNTON and CAMPBELL [5].

Procedures for oestrol estimation have been described by LEE and WOOD [30], and for other derivatives by numerous workers including WOTIZ and CHATTORAJ [49], KNUPPEN et al. [24], MONTALUO and WHEELER [37], McGREGOR et al. [15].

A direct gas chromatographic oestrogen assay in biological fluids is not possible because of the contaminations. For this reason before gas chromatography hydrolysis and extraction of the oestrogen conjugates must be followed by column, gel or thin-layer chromatographic separation prior to conversion to derivatives; it has been attempted to simplify these elaborate procedures as far as possible [8, 13] so as to make them suitable for routine use.

The samples are usually applied in the form of solutions in organic solvents. CAGNAZZO et al. [7], KROMAN et al. [25], HONDA and KUSHINSKY [21] described a procedure in which the samples are used in solid form; we found this method too cumbersome, particularly as regards the equipment.

The essential features of the procedure are presented in Tables I to III.

Table I

Gas chromatographic estimation of oestradiol in pregnancy urine by the procedure of ADLERCREUTZ *and* LUUKKAINEN [2]

1 Gel filtration of the urine on Sephadex G 25;
2 Hydrolysis of the conjugated oestrogens with β-glucuronidase and phenol sulphatase;
3 Ether extraction after addition of Brown's carbonate buffer pH = 10.5;
4 NaOH extraction of ether solution;
5 Boric acid treatment and methylation of the alkaline extract;
6 Extraction of the methylated oestrogens with benzene, and washing of the benzene fraction with water;
7 Conversion of the methylated oestrogens to trimethylsilyl ethers and evaporation to dryness;
8 Gas chromatography on Gas-Chrom-P (100 to 140 mesh) with SE 30 as solvent, at 227 °C in a 3 m glass column, with a flame ionization detector, in argon as carrier gas (Fig. 4).

Table II

Gas chromatographic estimation of oestriol and oestetrol according to LEE *and* WOOD [30]

1 Hydrochloric acid hydrolysis of the urine;
2 Addition of Brown's carbonate buffer pH = 10.5, then extraction with ether;
3 NaOH extraction of ether solution;
4 Adjustment of the alkaline aqueous extract to pH = 9.5 and re-extraction with ether;
5 Evaporation of ether extract to dryness and production of trimethylsilyl ether of the dry residue;
6 Evaporation of the silylating mixture, dissolution, addition of cholesterol for use as internal standard;
7 Gas chromatography on Diatomite CQ (100 to 120 mesh) with a solvent of 3% SE 30 and 3% QF 1, in a 2.5 m glass column, with a flame ionization detector, with N_2 as carrier gas, at 208°C (Fig. 5).

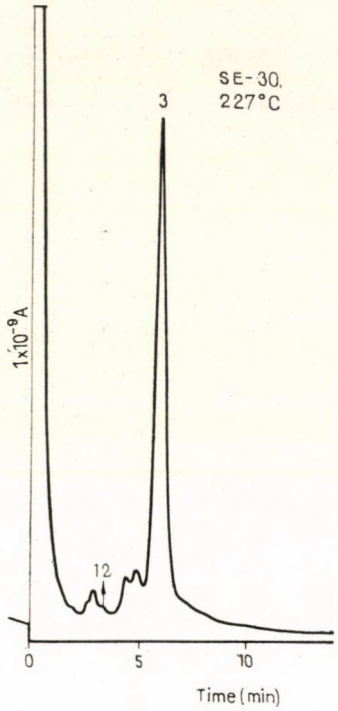

Fig. 4. 1) Oestrone, 2) 17β-oestradiol, 3) Oestriol

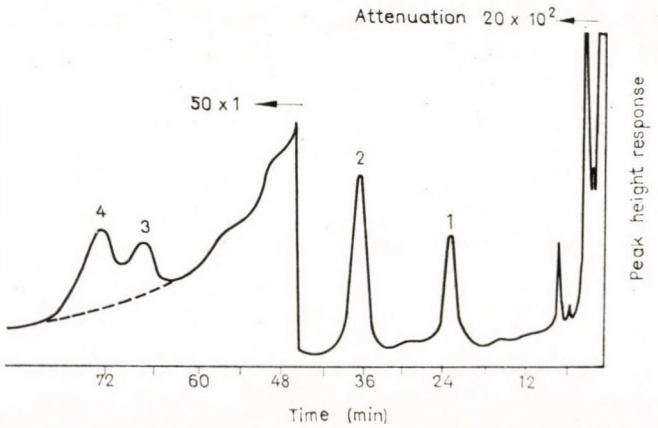

Fig. 5. 1) Cholestane, 2) Oestriol, 3) Cholesterol, 4) Oestetrol

Table III

Gas chromatographic estimation of oestrone and oestradiol with an electron-capture detector, according to KNORR *et al.* [23]

1 Enzymatic hydrolysis and introduction of labelled steroid in order to check procedural losses;
2 Adjustment of pH and extraction with ether;
3 Evaporation of ether;
4 Partition of the dry residue between NaOH and toluene;
5 Re-extraction of the alkaline aqueous fraction at pH = 9.5 with ether; washing, drying;
6 Thin-layer chromatographic separation of oestrone and oestradiol;
7 Methylation of the compounds;
8 Borohydride reduction of oestrone-3-methyl ether to oestradiol;
9 Thin-layer chromatography for purification of the methyl ethers;
10 Conversion to 17-hexadecafluoronanonate, purification by thin-layer chromatography;
11 Gas chromatography on Diatoport S (60 to 80 mesh); QF 1 as solvent, in a 3 m glass column at 215°C, with a ^{63}Ni electron-capture detector in N_2 carrier gas (Fig. 6)

Fig. 7 shows the chromatogram of acetylated standard oestrone, oestradiol and oestriol, obtained with a JEOL 810 gas chromatograph, on Gas-Chrom P 100 to 120 mesh as support medium, 2% SE 30 as solvent at a column temperature of 260°C, with a flame ionization detector in N_2 as carrier gas

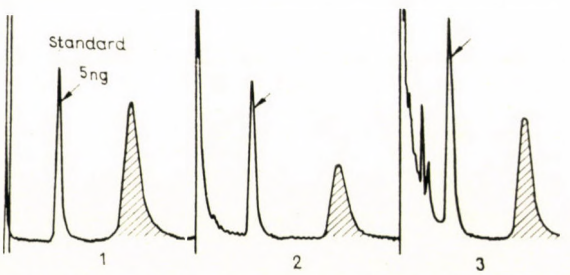

Fig. 6. 1) 3-methoxy-17-hexadecafluoronanonate (the hatched peak is that of episterone used as internal standard), 2) Oestrone, 3) Oestradiol

Evaluation was performed using the peak height of the curve, of the area under it, and the internal standard.

The advantages of gas chromatography in the estimation of oestrogens include its high resolution capacity, the rapidity of separation, and the possibility of direct quantation; its high sensitivity allows the estimation of hormone levels with adequate accuracy and selectiveness even within ranges where the colour reactions or fluorescent techniques no longer give reliable results.

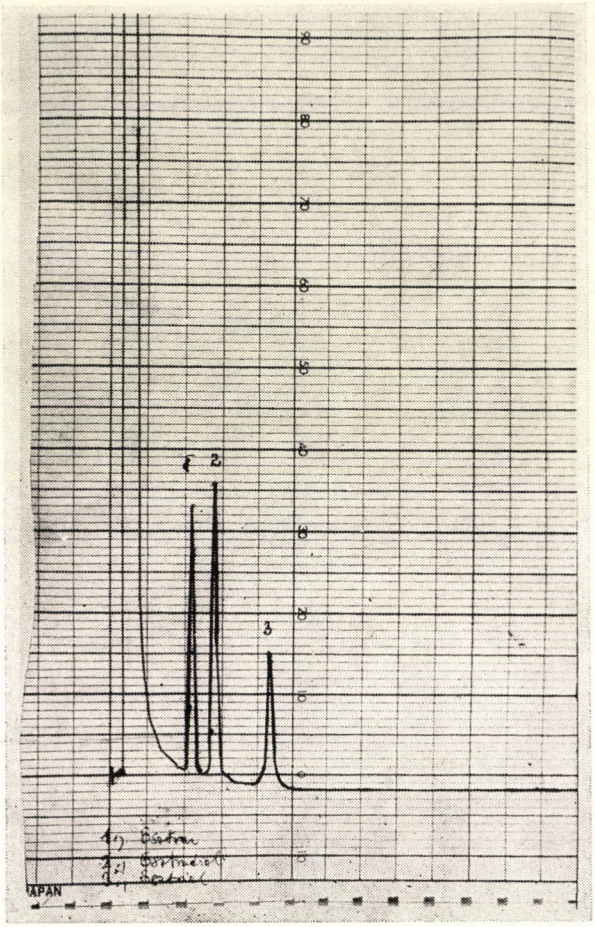

Fig. 7. 1) Oestrone, 2) Oestradiol, 3) Oestriol

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GAS CHROMATOGRAPHIC METHODS FOR THE SEPARATION, STRUCTURE ANALYSIS AND QUANTITATIVE DETERMINATION OF C_{19}-STEROIDS

By

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Gas chromatography is one of the most sensitive and effective methods for the separation and quantitative determination of steroid hormones, their metabolites and of synthetic hormone preparations. In the present paper the gas chromatographic methods applied for the separation and quantitative estimation of C_{19}-steroids (androgens, their metabolites and synthetic analogues) in biological materials and some aspects of structure analysis by gas chromatography will be reported.

A PYE-UNICAM Series 104 analytical gas chromatograph with flame ionisation detector and glass columns with SE-30 (non-selective) and QF-1 (selective) stationary phase were used. Column temperature was 222 °C, detector temperature 260 °C, the carrier gas was nitrogen.

Determination of testosterone in urine

Estimation of 17-ketosteroids has little value for the evaluation of normal and pathologic androgen metabolism. Determination of testosterone in urine has become one of the most reliable methods in laboratory diagnostics. The flowsheet of our method of urinary testosterone estimation is shown in Fig. 1. For the simultaneous determination of testosterone and individual 17-ketosteroids, paper, thin-layer and gas chromatography were employed. The urine specimen was hydrolized enzymatically or with HCl under mild conditions extracted with benzene, and the extract was purified and chromatographed on paper. One half of the paper strip was detected with m-dinitrobenzene/KOH reagent, the testosterone-zone of the undeveloped half strip eluted, acetylated, the testosterone-acetate was subjected to thin-layer chromatography, and the eluate to gas chromatography on a SE-30 column.

The following criteria ensured the reliability and precision of method:

i) The loss during the procedure was corrected by using [4-^{14}C] testosterone as a tracer.

ii) Mobility of testosterone and its separation from epitestosterone were determined by nortestosterone and methyltestosterone as marker steroids.

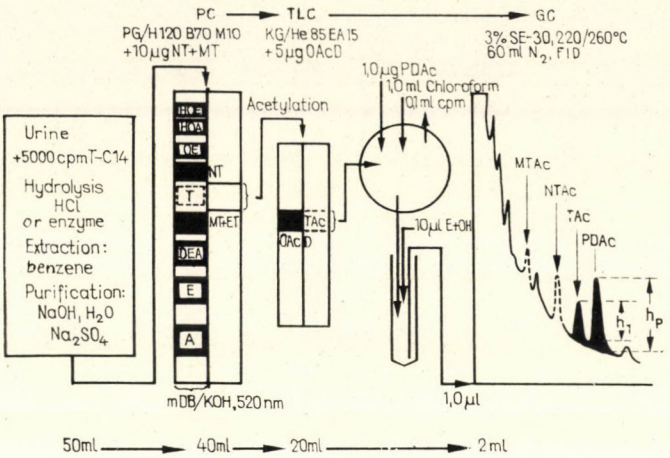

Fig. 1. Flowsheet of urinary testosterone and individual 17-ketosteroid determination (HOE 11-OH-etiocholanolone, HOA 11-OH-androsterone, OE 11-keto-etiocholanolone, DEA dehydroepiandrosterone, E etiocholanolone, A androsterone, NT nortestosterone, MT methyltestosterone, ET epitestosterone, T testosterone, OAcD oestrone-acetate-dinitrophenylhydrazone, TAc testosterone-acetate, PDAc pregnadienolone-acetate, mDB m-dinitrobenzene)

iii) Mobility of testosterone-acetate was checked by the steroid-marker, oestrone acetate-dinitrophenylhydrazone.

iv) The amount of testosterone was determined by using pregnandienolone-acetate as internal standard based on peak-height measurements. The recorder response for both steroids was identical. The great sensitivity of the method allowed to estimate testosterone in an extract corresponding to 2 ml male or 4 to 8 ml female urine (initial volume, 50 ml) [1].

Determination of dehydroepiandrosterone sulphate and androsterone sulphate in plasma

A gas chromatographic modification of our procedure [2] for the determination of the sulphate ester of dehydroepiandrosterone and androsterone in human plasma by twofold thin-layer chromatographic separation and subsequent gas chromatography is shown in Fig. 2. Following the addition of [7α^3H] dehydroepiandrosterone sulphate (or the parent free steroid tracer) to 3—5 ml plasma, it was deproteinized and solvolyzed at pH 1.0, then the ethyl acetate extract purified, evaporated and the residue partitioned between n-hexane and ethanol. The evaporated extract containing the free dehydroepiandrosterone and androsterone was chromatographed on silicagel G layer by benzene–ethanol (96 : 4), and the zone containing both steroids acetylated and rechromatographed in a system of n-hexane-ethyl acetate (85 : 15). Mobility of the steroids was checked by marker steroid-pairs. The zone containing the acetates was eluted and to the eluate 1.0 µg dihydrotestosterone

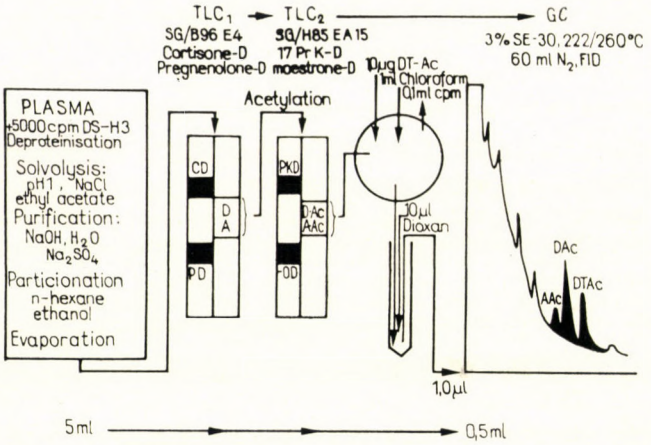

Fig. 2. Flowsheet of dehydroepiandrosterone sulphate and androsterone sulphate determination in plasma (CD cortisone-dinitrophenylhydrazone, PD pregnenolone-dinitrophenylhydrazone, PKD 17-OH-progesterone-capronate-dinitrophenylhydrazone, mOD methoxy-oestrone-dinitrophenylhydrazone, AAc androsterone-acetate, DAc dehydroepiandrosterone-acetate, DTAc dihydrotestosterone-acetate)

acetate as internal standard was added. Radioactivity was measured in a 1/10 volume of the extract and another 1/10 aliquot in dioxan subjected to gas chromatography.

The method, similarly to the preceding one, was controlled with a marker system and by using double internal standards in each sample. The procedure appears to be suitable for estimating steroid values in 5 ml plasma of children and in less of adults.

Determination of methandienone in human urine

The metabolism of certain hormone analogues is slow, and considerable amounts of some synthetic hormones are excreted with the urine without any change in their structure. These characteristics allow a direct gas chromatography of urinary extracts without preliminary purification by paper or thin-layer chromatography, if the compound can be chromatographed directly without derivative formation. An example of this is the determination of methandienone (17α-methyl-17β-hydroxy-androsta-1,4-dien-3-one) (Fig. 3). The method is suitable for studying the metabolism of steroid drugs and for the control of sports stimulants.

To a 5 to 10 ml aliquot of urine, 1.0 μg ethynyl-nortestosterone as internal standard for the correction of losses and determining retention properties of the drug as also for quantitation is added. After ether extraction, the extract was purified first with alkali then with water, dried over Na_2SO_4 and evaporated. A dioxan aliquot of the residue was gas chromatographed as indicated in Fig. 3.

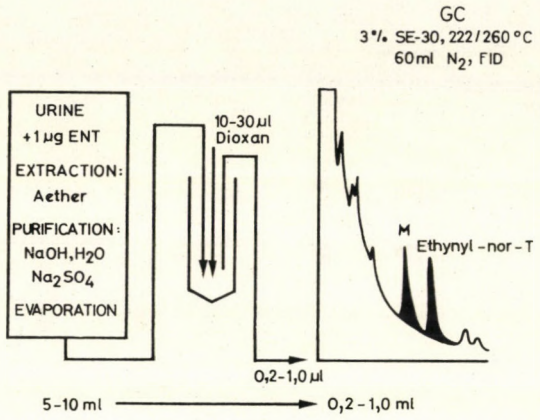

Fig. 3. Flowsheet of the urinary identification and quantitative determination of methandienone in urine (M methandienone)

Correlation between chemical structure and retention behaviour

Although gas-liquid chromatography is a separation method, it may be applied on structure analysis or for a rapid identification of steroids. Characteristic changes in retention properties observed following an introduction into or a chemical alteration of a functional group on, the molecule have substantiated this type of analysis. For the characterization of retention properties

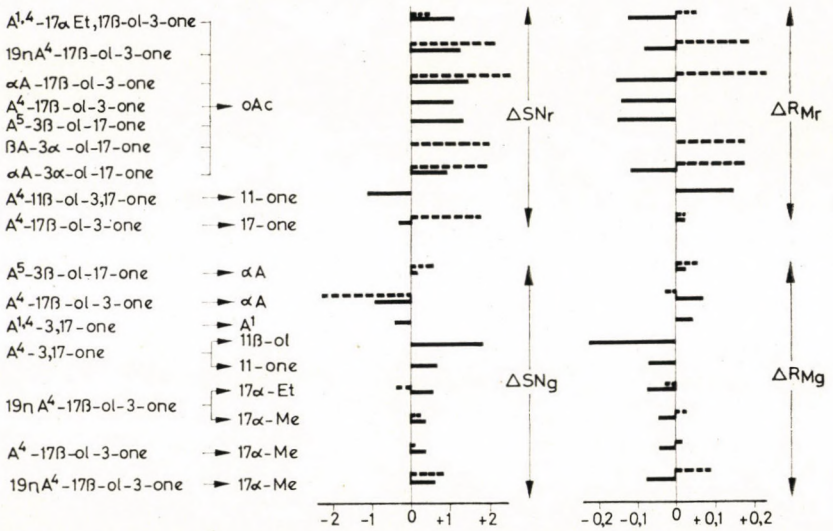

Fig. 4. ΔSN_r, ΔSN , ΔR_{Mr} and ΔR_{Mg} values for functional groups of androstanes (Nomenclature of steroids is given according to BUSH, I. E.: The Chromatography of Steroids, Pergamon Press, London 1961)

i) the steroid-number (SN) determined according to a calibration made with 5α-cholestane and 5α-androstane standards;

ii) the group retention factor (ΔR_M) calculated from changes in log-values of the relative retention time; and

iii) the group-number (ΔSN) concept introduced by us [3] as the changes in steroid-number values following the chemical alteration of functional groups, were used.

Fig. 4 shows the parameters of structure-chromatography relationships determined on SE-30 and QF-1 columns by the analysis of androgenic hormones, their metabolites and synthetic C_{19}-steroids.

As it can be seen from Fig. 4, changes in the retention behaviour are characteristic of the functional groups of molecule under study and on the stationary phase chosen for measurements. Data obtained by this type of analysis may be informative in the identification of steroids.

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A CRITICAL APPRAISAL OF THE METHODOLOGY OF DEHYDROEPIANDROSTERONE AND DEHYDROEPIANDROSTERONE SULPHATE DYNAMICS

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The secretion or daily production of dehydroepiandrosterone (D) dehydroepiandrosterone sulphate (DS), or any steroid hormone can be determined by the injection or infusion of the labelled hormone in tracer amount and estimation of the specific activity (SA) of its unique metabolite in urine, or of the isotope concentration of the tracer in blood, on the basis of the isotope dilution principle (SR = R/SA.t).

According to the general model of steroid dynamics, a chemical compartment is defined as a particular hormone (tracer) distributed in a single anatomical space. Two different hormones distributed in the same space, or one hormone in two anatomical spaces, represent two compartments.

The first attempts at determining the secretion rate of D by the adrenal (gonad) as a one-compartment model consisted of injecting tracer D and measuring the SA of DS in urine (Fig. 1) [1].

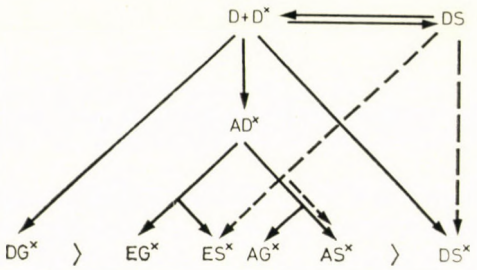

Fig. 1. Pathways of dehydroepiandrosterone and dehydroepiandrosterone sulphate metabolism (AD androstenedione, DG dehydroepiandrosterone glucuronoside, EG etiocholanolone glucuronoside, ES etiocholanolone sulphate, AG androsterone glucuronoside, AS androsterone sulphate)

It has been pointed out later that following the injection of the tracer D, the lowest SA value was obtained for DS and the highest one for DG of the SA values of the known D metabolites (EG, ES, AG, AS, DG and DS; for abbreviations, see Fig. 1). Meanwhile, other reports revealed the production by the adrenals of not only D but also of DS and the existence of a direct "sulphate-pathway" in their metabolism [3]. It became apparent that

urinary DS originated not only from D but also from the glandular DS, and the two steroids are peripherally interconvertible (D $\rightleftarrows$ DS), which could account for the low SA_{DS} in urine. Since urinary DG could not be produced from DS without prior conversion to D, DG can be considered a unique metabolite of D, and DS of the glandular DS [4].

A mathematical model of a two-compartmental analysis taking into account these considerations has been worked out by GURPIDE et al. [5]. Their approach appeared to be suitable for the calculation of kinetic parameters following the simultaneous injection of the two tracers with different label ([4-^{14}C]D and [7α^3H]DS) and subsequent determination of the cumulative specific activity of DG and DS in urine (Fig. 2) [5].

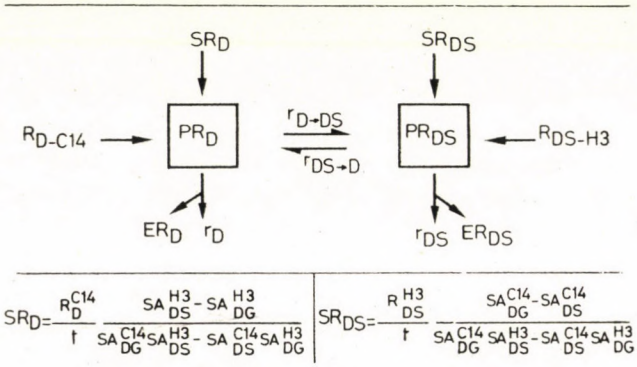

Fig. 2. The two-compartment model of dehydroepiandrosterone and dehydroepiandrosterone sulphate dynamics ("urinary method")

In view of the peripheral interconversion of D and DS, the glandular secretion of D (SR_D) is not equal with the total daily production of D (PR_D) it must be a lower value ($SR_D < PR_D$), and this is also the case for DS. The rate of interconversion is characterized by a transfer factor ϱ, from which the mg/die values for the conversion rates $r_{D\to DS}$ and $r_{DS\to D}$ are calculated. The mathematical model allows a determination of rates of the irreversible metabolism, r_D and r_{DS}, as well as of the rates of production of the two hormones, PR_D and PR_{DS}. This "urinary method" has been applied by MACDONALD et al. for determination of the parameters of D and DS dynamics in two control subjects and two patients with disturbances in hormone metabolism [6].

The characteristics of steroid hormone dynamics can be studied by estimating the changes in the radioactive concentration of hormone under consideration after a single injection or infusion of the tracer(s) [7]. The most important parameter in this "blood method" is the metabolic clearance rate (MCR) defined most generally as that theoretical volume of plasma from which

the steroid hormone (its radioactivity) is completely and irreversibly removed in unit time. Even for a single hormone the situation is considered a two-compartment model (Fig. 3 for D). According to this assumption, the hormone is distributed in two anatomical spaces, *viz.* an inner pool (V_1) which includes plasma, liver and extracellular volumes, and an outer pool (V_2) which represents the other tissues. There is evidence that metabolism occurs mainly in liver, therefore, the rate constant for D in the outer pool r_{D_0}, can be neglected. Hormone secretion is imitated by the administration of the tracer (R_D). Following its single injection, MCR is calculated by plotting the disappearance curve of radioactive concentration in plasma. The slope of the curve is determined by the biological half-live of the hormone, and MCR is related to the integrated area under the curve. In the case of a constant infusion, an equi-

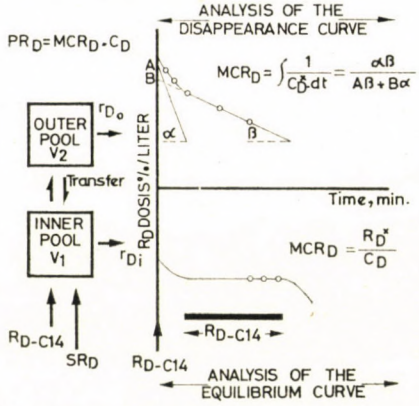

Fig. 3. The two-compartment model of dehydroepiandrosterone dynamics ("blood method")

librium curve is obtained and here the MCR is regarded as a quotient of the radioactive concentration of the tracer administered and of the steroid isolated from blood. In steady-state, the blood PR_D is equal to MCR multiplied by the non-radioactive steroid concentration ($PR_D = MCR_D \cdot C_D$). If the adrenal secretion of a peripherally interconvertible hormone-pair is considered, after simultaneous administration of the ^{14}C and ^{3}H tracer-pair and the determination of their interconversion rate allow a calculation of the glandular SR_D from PR_D.

The urinary and blood approach of D and DS dynamics both seem to have certain advantages and disadvantages. These are methodological in their nature or originate from the chances or limits of the mathematical model.

Based upon a critical review of the pertaining literature and our own experience with the urinary and blood approach applied simultaneously in healthy and obese subjects, some aspects of D and DS dynamics will be discussed.

For methodological (steroid-analytical) reasons, the urinary method is the one more advantageous.

i) Dehydroepiandrosterone sulphate and glucuronoside can be determined in urine with a more simple technique and more reliably than DS or especially the unconjugated D in plasma, the latter in the range below 1 μg/100 ml.

ii) The urinary method necessitates a single injection of the tracers and a 3-day pool of urine. In contrast, the blood method, in view of the significant differences in the biological half-time of D and DS requires at least four blood samplings at different intervals after the isotope administration. In the case of constant infusion, the technical schedule is more complex and means an increased strain for the patient.

iii) With the plasma method, errors in methodology and construction may result in a greater distortion of the parameters than with the urinary approach. In addition, it is from a short working-period that one has to extrapolate on the daily balance (diurnal rhythm, periodic secretion of hormones, etc.).

The two methods represent the dynamics of hormone metabolism in different ways. Both are in certain respects arbitrary, and disregard some known characteristics of metabolism. From this point of view, the blood method seems to be a better approach of the occurrences in vivo.

i) As a decisive criterium of the urinary method, the specific urinary metabolite (i.e., DG) must originate exclusively from the plasma steroid pool in which the injected tracer (i.e., 4-^{14}C D) is distributed, and must not be synthesized in the outer anatomical compartment of the same hormone or from another hormone produced in the inner compartment. This supposition is not entirely valid for the D-DS pair. It has been assumed that D produced from DS in the liver is partly transformed to androstenedione or DG and irreversibly metabolized before its mixing with the D pool. Consequently, DG is partly the metabolite of DS, and the validity of the "specific urinary metabolite" concept is not entirely answered.

ii) A reliable control of the two methods has hardly been performed in cases with disturbances in hormone metabolism and in not more than a few normal subjects. LORAS and MIGEON [8] have found identical SA_{DG} and SA_{DS} values in the urine of patients with virilizing adrenal tumour, and from the equation of GURPIDE et al. [5] this would result in a SR_{DS} of zero. In contrast to this conclusion, adrenal venous blood analysis revealed a secretion of DS. Our experiments on normal subjects [9] and obese patients [10] showed a normal urinary PR_{DS} and an increased blood PR_{DS} in the latter patients. This observation speaks for an increased hepatic extraction of the hormone in obesity. The finding, although it draws attention to limitations of the urinary method, indicates an aberration of liver function in obesity hardly detectable by other methods.

iii) The blood method is based on the MCR. The restriction of its determination is a steady-state. This, however, is not valid in every situation. For instance, several steroids such as DS, do not readily reach an equilibrium during infusion or after injection of the labelled steroid, or a change in the secretion rate during its measurement is not be strictly followed by similar changes in the plasma concentration of the steroid. Recent reports have revealed a periodical D secretion by the adrenals [11].

As a conclusion of the critical considerations discussed here we refer to HORTON and TAIT [12]: "At the present time it appears that the blood approach gives rise to more accurate estimates of secretion rates and more meaningful production rates. However, the application of both methods, if possible, will give the maximum amount of information."

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DETERMINATION OF TESTOSTERONE IN SERUM ON THE BASIS OF A PROTEIN-BINDING METHOD

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In the early sixties, a new microanalytical procedure based on saturation analysis was elaborated for the determination of thyroxin (EKINS, 1960), insulin (YALOW and BERSON, 1960) and of various steroid hormones (MURPHY et al., 1963) (Fig. 1).

The hormone to be estimated forms a complex with a protein as a result of specific binding. The binding protein must be added in a concentration which allows its saturation to its full binding capacity by the hormone. This gives a protein-bound and a free hormone fraction. The protein concentration being constant, it is the quantity of the hormone in question which determines the ratio of the protein-bound to free fraction. For the estimation of this ratio, radioactive hormone, identical with the cold hormone in every respect, including distribution of its protein-bound to free fraction, is added to the system in a known amount. In this manner, as the cold hormone is added to the system in increasing amounts, it displaces the protein-bound radioactive hormone from its complex binding to the profit of the free fraction. The proportion of the two fractions can be determined by measuring either the protein-bound or the free fraction of the radioactive hormone. A calibration curve from which the unknown hormone concentration of the assayed sample can be read, is constructed by plotting the proportion of protein-bound to free fractions at known concentrations of the hormone under test.

In our Institute we have been engaged in studying the clinical aspects of androgen metabolism for many years. Serum testosterone estimation is pertinent to the line of study. Saturation analysis makes it possible to determine free testosterone in the blood, it having been demonstrated by MERCIER (1966) that besides a nonspecific and weak binding to albumin, there exists a strong, specific binding of testosterone to a beta globulin fraction the binding capacity of which increases during pregnancy and in response to oestrogen therapy (PEARLMAN et al., 1967). Fig. 2 shows the design of our procedure.

From male serum 1 ml, from female serum 4 ml are used. Approximately 10,000 dpm tritiated testosterone (Ao) is added to the serum so as to trace the testosterone losses involved by the extraction and the purification procedures.

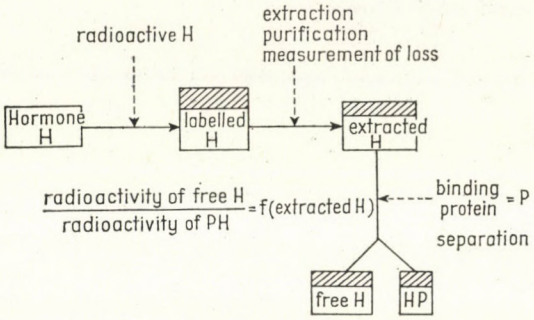

Fig. 1. **Principle of saturation analysis**

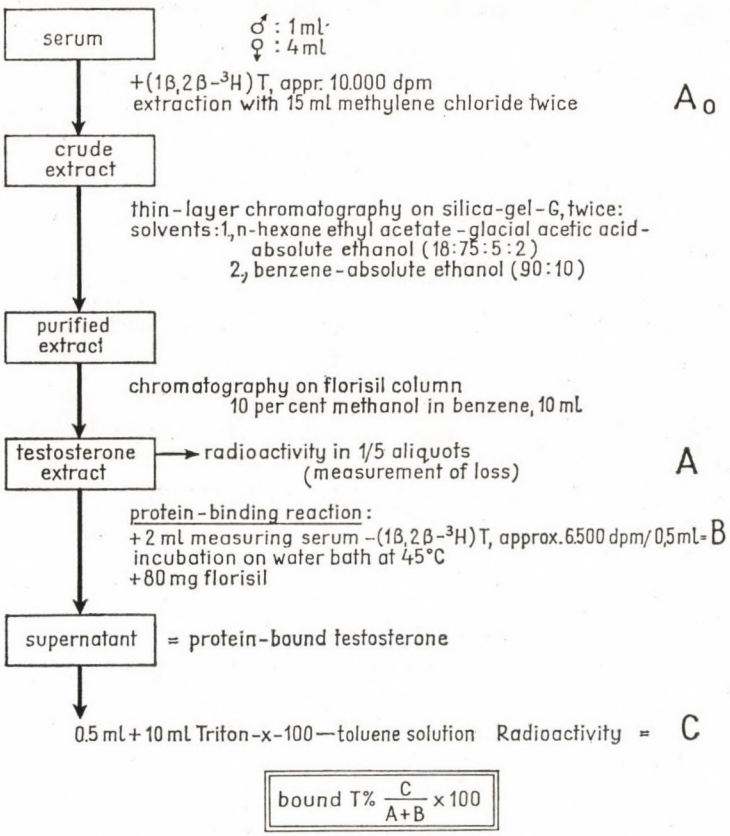

Fig. 2. **Estimation of testosterone. Schematic representation**

After alkalization the serum is extracted twice with 15 ml methylene chloride, washed with distilled water, and the extract is evaporated in vacuo to dryness at 40 °C. The crude extract thus obtained is chromatographed twice successively on silica-gel-G thin-layer for the separation of testosterone from other steroids and from interfering lipids. By the first chromatographic procedure the testosterone is separated from the nonpolar 17-ketosteroids in a solvent system of n-hexane—ethyl acetate—glacial acetic acid—absolute ethanol, 16 : 75 : 5 : 2. By the second procedure, testosterone is separated from the more polar 17-beta-hydroxy-C_{19}-steroids, the androstanediols and the androstenediols in a benzene—absolute ethanol, 90 : 10 system.

After each chromatography the testosterone-spot is located with the aid of a Packard Radiochromatogram Scanner and each time testosterone is eluted from the layer with methanol. The extract thus purified is chromatographed on a Nymco florisil column of 1.0 g in order to purify it from the contaminations due to the silica-gel-G thin-layer adsorbent. The eluate (10 ml 10% methanol in benzene) is evaporated in vacuo at 40 °C to dryness, dissolved in 1 ml methanol and the activity of the residual ^{3}H-testosterone (A) is measured in aliquots of 0.1—0.1 ml. In this manner the testosterone losses inherent in the procedures of purification and isolation are checked. The remaining 0.8 ml of the eluate is again evaporated in vacuo to dryness and in the sample the testosterone is estimated on the basis of its protein binding.

Serum of the third trimester of pregnancy at 200 fold dilution served as measuring protein. In our experience, at testosterone levels in the range of 0.5 to 6.0 ng this protein dilution gives calibration curves of the required steepness. Though higher dilutions allow estimations of higher sensitivity if the concentration of testosterone is low, they give flat and less sensitive curves at higher testosterone concentrations. To the diluted pregnancy serum tritiated testosterone is added and its radioactivity is checked on each occasion (in 0.5 ml approximately 6,500 dpm = B).

The measuring serum thus prepared is added to the extract and incubated in a water bath at 45 °C for 10 minutes during which time an equilibrium between the free and the protein-bound testosterone fractions is established.

For the separation of free testosterone, 80 mg florisil is added to the solution, shaken for two minutes, allowed to stand for 6 minutes and centrifuged. From the supernatant which contains exclusively protein-bound testosterone, 0.5 ml in a solution of 10 ml Triton-X-100 toluol is measured for its radioactivity (C) using a Packard Tri Carb Spectrometer. From the results the percentage of radioactive testosterone left bound to protein after incubation with the cold testosterone sample of unknown quantity is calculated via the formula

$$\text{bound T per cent} = \frac{C}{(A+B)} \times 100$$

A = radioactivity in the system after extraction and purification
B = radioactivity of the measuring serum
C = radioactivity of the protein-bound testosterone fraction

The percentage value of the T read from the calibration curve gives the amount of testosterone in ng. This is corrected for the extraction losses and referred to 100 ml serum.

Each serum sample is measured against blanks which are treated in exactly the same manner as the samples and the values are subtracted from the results.

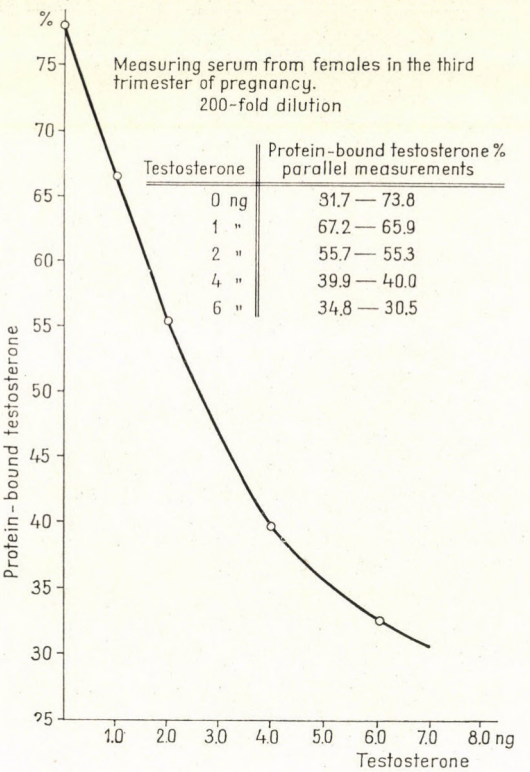

Fig. 3. Testosterone calibration curve

(Fig. 3) The calibration curve is constructed on the basis of parallel measurements with a cold testosterone series of 0.5—1.0—2.0—4.0—6.0 ng. These standard testosterone amounts are incubated with the measuring serum in the same manner as the actual samples. Each serial measurement requires a fresh calibration curve.

On the evidence of these measurements performed in a relatively small number of cases, the serum testosterone level ranges between 350 and 410 ng/100 ml in normal males and between 40 and 50 ng/100 ml in normal females.

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THE HORMONE SECRETION RATE, METHODS OF ASSAY

By

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The most reliable manner of estimating hormone production by endocrine organs, thus also by the adrenals, is the estimation of the hormone in the venous blood of the hormone-producing organ. Since in the human being, apart from exceptional occasions, there is no possibility of determining the hormones directly in the blood of the adrenal vein, studies of this kind have been largely confined to the laboratory animal until the introduction of the ingenious secretion rate procedures for the steroid hormones based on the idea of the biological dilution of labelled steroids (PETERSON, 1959).

The secretion rate studies by means of steroids labelled with ^{3}H or ^{14}C are based on the following principle. A labelled steroid of high isotope activity is introduced into the body in minute absolute amounts and left to mix with the body fluids. Within a certain time it becomes distributed over the fluid compartments in the same manner as the endogenous hormones. Actually, the hormone, whether endogenous or isotope-labelled, is indiscriminately metabolized and excreted by the organism. If after complete mixing, a sample is taken from blood or urine and after specific isolation, its hormone content is determined, in other words the specific activity of the isolated hormone (i.e., the ratio of radioactivity to the absolute amount of hormone in μg) is estimated, the secretion rate of the given hormone can be computed with reference to the specific activity of the steroid originally introduced in the organism. A fall in specific activity, the dilution of the original specific activity of the steroid, is directly related to the endogenous hormone content of the organism. Determination of the changes in specific activity from the hormone or from the metabolite of the examined hormone must give basically the same result, with the understanding that the hormone or its metabolites selected for this purpose can have no other possible source in the body than the investigated steroid for this study. For instance, the urinary 17-keto-steroids are unsuited for the measurement of the secretion rate, because they have multiple sources in the organism. On the other hand, there are numerous steroids of exclusive origin and, by this fact, suitable for secretion-rate studies. These include delta-5-pregnene-3-beta-20-alpha-diol for pregnenolone; tetra-

hydro-DOC for desoxycorticosterone; tetrahydrocorticosterone for corticosterone; tetrahydrocortisol and tetrahydrocortisone for cortisol; only testosterone for testosterone; dehydroepiandrosterone for dehydroepiandrosterone; pregnanetriol for 17-alpha-hydroxyprogesterone; 18-oxo-glucuronide- and tetrahydro-aldosterone for aldosterone, etc.

Calculation of the secretion rate is a simple task. The dose of the injected steroid being known, it can be derived from the equation in Fig. 1 where l_1 represents the specific activity of the steroid administered, a its amount, (this is, however, generally a negligible quantity, since for secretion-rate measurements hormones only of relatively high specific activity, i. e., of

$$\frac{l_1}{l_0} \; a \; c$$

where: l_1 = specific activity of the steroid administered
l_0 = specific activity of the steroid in the urine
a = amount ef steroid administered
c = correction factor

Fig. 1. Formula for the calculation of steroid production estimated from the secretion rate

minimum absolute amount are suitable). l_0 represents the specific activity of the isolated hormone, i. e., the metabolite of the steroid in urine; c is the correction factor, since the procedure can only be used if the radioactivity of the administered steroid is excreted in its totality during the period of urine collection, i. e., if the organism has attained a steady state. In this case, all metabolites formed from the administered labelled steroid must be recovered from the 24-hour urine which equals the amount of hormone being formed in the organism in 24 hours, thus corresponding to an equilibrium between production and elimination.

c = U/D
U = radioactivity excreted during collection
D = radioactivity of the administered steroid

A possible difference in molecular weight between the administered steroid and the isolated steroid metabolite requires a further correction factor for the final calculation of the secretion rate. Thus, delayed steroid excretion in consequence of renal impairment on the one hand, and inaccurate collection of urine, on the other, make the correction factor indispensable. Inaccurate administration of the labelled steroid or its incomplete absorption after oral administration as well as other special problems will be dealt with later in this study in the context of the aldosterone secretion rate.

Determination of aldosterone secretion rate

The secretion rate of aldosterone is estimated on the same principle as that of other steroids, by the intravenous administration of a few μC of ^{3}H-labelled aldosterone of a high specific activity. Since the tritiated aldosterone is metabolized and excreted by the organism in 24 to 48 hours in the same manner as endogenous aldosterone, secretion can be calculated from the fall in the specific activity, i. e., from the dilution of free aldosterone or of its 18-oxoglucuronide- or tetra-hydro-fraction isolated from the urine.

Fig. 2 adapted from the study of KLIMAN (1963), sets out the general principles of the determination of aldosterone secretion rate.

Principal considerations for the measurement of aldosterone secretion rate in man

I. Injected dose of radioactive aldosterone:
   1. High purity, "D" isomer.
   2. High specific activity.
   3. Known amount.
   4. Known specific activity, when calculation is based on specific activity dilution.

II. Mixing and metabolism:
   1. Tracer dose must be small in relation to miscible pool.
   2. No loss of radioactivity from the steroid.
   3. Complete metabolism during time interval studied.
   4. Complete excretion of specific metabolite.

III. Metabolite isolation:
   1. Metabolite derived only from aldosterone.
   2. Specific identification.
   3. Adequate purification.
   4. Accurate measurement.
   5. Correction for tracer dose, when indicated.

Fig. 2. Principles of aldosterone secretion assay (KLIMAN, 1963)

I. The isotope-labelled aldosterone used for this purpose must meet the following requirements: it must be d-isomer in order to be biologically active, to be metabolized in the same manner as endogenous aldosterone. It must be of maximum purity, i. e., its radio-purity must be in the neighbourhood of 99%. It must be of high specific activity so as to make the absolute quantity to be administered negligible.

II. As a precautionary measure in respect of distribution and metabolization of aldosterone, the hormone must be of exactly known minute amount and high specific activity. In order to ensure complete mixing of the radioactive aldosterone, its absolute amount in relation to the aldosterone content of the extracellular compartment must be negligibly small. Furthermore, the labelling must be stable in order to prevent any alteration in ^{3}H content of the administered aldosterone during its metabolization and excretion. In addition to complete metabolization, the excretion of the metabolite under study must also be complete. In contrast to the rapidly formed 18-oxo-glucuronide metabolite, the tetrahydro-metabolite produced relatively slowly and requires a 48-hour collection of urine.

III. A prerequisite of the isolation of the metabolite is that the metabolite to be isolated must have aldosterone as its only possible source. Specific identification, adequate purification to the constancy of $^{3}H/^{14}C$ and reliable measurement of specific activity are further requirements of importance.

Fig. 3 gives a schematic representation of the determination of the aldo-

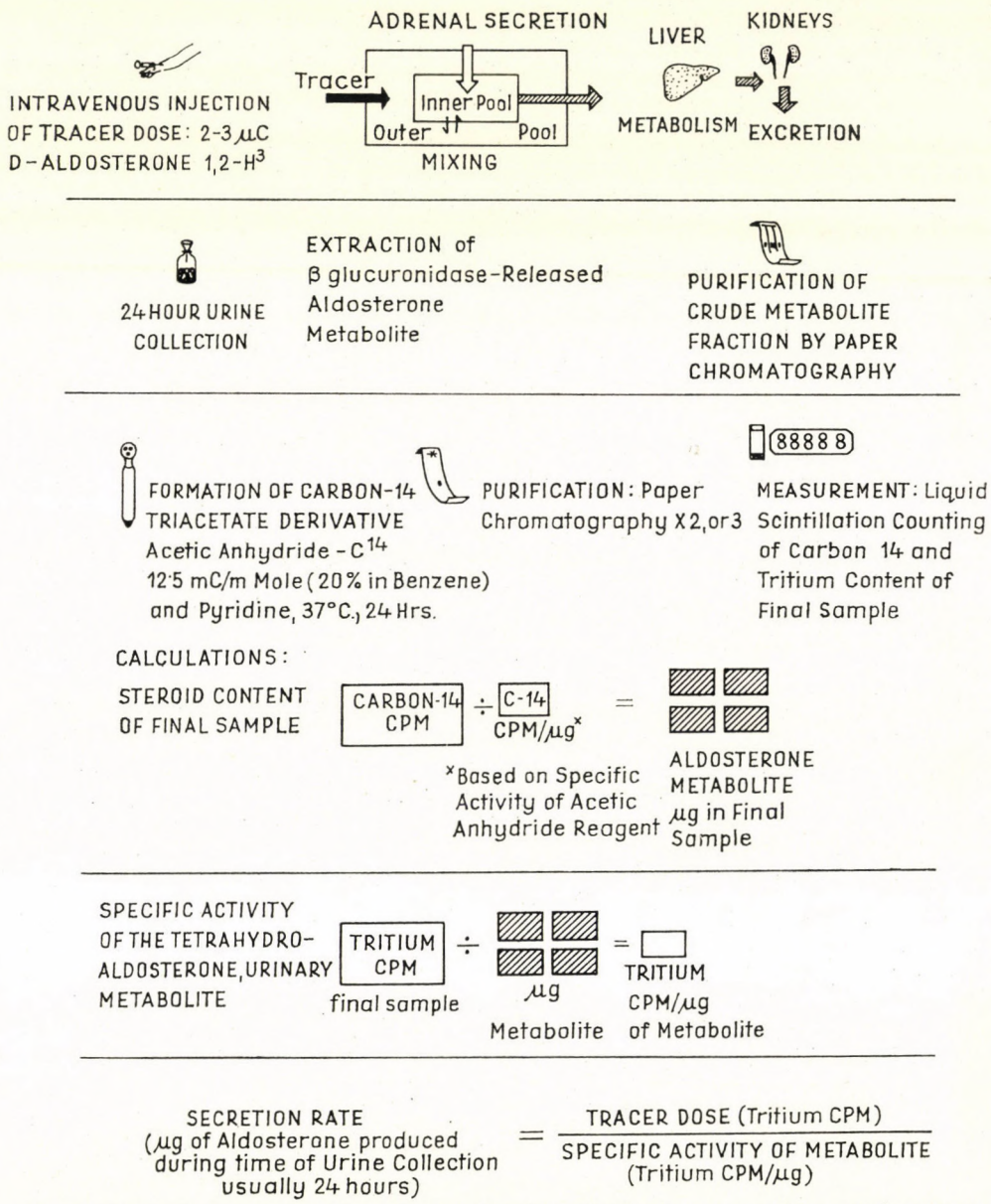

Fig. 3. Schematic representation of the estimation of aldosterone secretion rate by means of tetrahydroaldosterone isolation

sterone secretion rate from tetrahydro-aldosterone by the method of COPE (1964) and of the calculations. Fig. 4 presents the procedure of aldosterone secretion assay by making use of the free and the 18-oxo-glucuronide fractions of aldosterone (FODOR and GLÁZ 1971), and Fig. 5 shows the values thus obtained. Replacement of ^{14}C labelled anhydrous acetic acid by "cold" anhydrous acetic acid

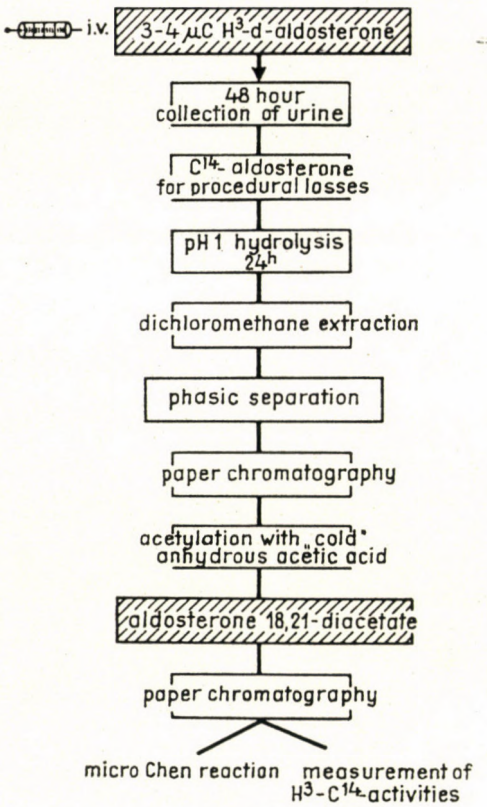

Fig. 4. Parallel estimation of aldosterone secretion rate and of aldosterone excretion rate, according to FODOR and GLÁZ (1971)

Aldosterone excretion on the evidence of 180 assays
Normal range: 13.0 (6—20) μg/24 hrs
Conn's syndrome: 45—120 μg/24 hrs
Adrenalectomy: 2—3 μg/24 hrs
Aldosterone secretion rate on the evidence of 180 assays
Normal range: 120 (60—200) μg/24 hrs
Conn's syndrome: 300—1200 μg/24 hrs
Adrenalectomy: 20—30 μg/24 hrs
Recovery from 500 μg d-aldosterone injected: 471 μg

Fig. 5. Aldosterone values obtained by the secretion-rate and excretion-rate methods (GLÁZ et al.)

greatly simplifies the procedure and makes it less expensive. Fig. 6 shows the normal values for aldosterone secretion rate obtained by various workers including ourselves, on the grounds of the estimation of the tetrahydroaldosterone and of the 18-oxo-conjugate metabolite (Fig. 6). There is no significant difference between the results of the two procedures. While the determination of tetrahydroaldosterone confronts us with problems of the specificity of isolation, in the case of the 18-oxo-conjugate we have to cope with difficulties involved by the smaller amounts and by the instability of the compound. In both cases it is essential that the total radioactivity excreted during the period of urine collection should be measured. Inaccurate collection of urine or impaired renal function greatly alter the results. Delayed urinary excretion of ^3H in the case of impaired renal function is best demonstrated by the graph

References	Estimation of specific radioactivity from 18-oxo-glucuronide, μg/24 hrs
Ayres et al. (1958)	170—190
Jones et al. (1959)	82—190 (male)
	73—315 (female)
Peterson (1959)	330—400
Hurter and Nabarro (1960)	72—313
Laidlaw et al. (1962)	231—522
Siegenthaler et al. (1962)	40—180
Muller (1962)	100
Kliman (1963)	50—270 (adults)
	25 and 112 (children)
Kono and Miyake (1965)	54.1—162.0
Luetscher et al. (1965)	103—130
Lommer et al. (1965)	126
Henkin et al. (1965)	104 (children)
Lund-Johansen et al. (1966)	74—155
Vecsei et al. (1967)	80—220
Flood et al. (1967)	77 (adults)
	34 (aged)
Gláz et al. (1971)	60—200

References	Estimation of specific radioactivity from tetrahydroaldosterone, μg/24 hrs
Ulick (1959)	230—350
Laragh (1960)	150—330
Ulick (1961)	100—300
Cope et al. (1961)	62—275
Flood et al. (1961)	50—118
Coppage et al. (1962)	95—249
Watanabe et al. (1963)	85—216 (females)
	88—180 (males)
Ulick et al. (1964)	135 (70—210)
Gláz and Pearson (1966)	60—203
Gláz et al. (1968)	80—200

Fig. 6. Aldosterone secretion values on the basis of 18-oxoglucuronide and tetrahydroaldosterone isolation, as observed by different authors in normal subjects (from Gláz and Vecsei, 1971)

from the study of COPE (1964) reproduced in Fig. 7. The correction factor proposed by this author is calculated on the basis of the radioactivity of urine excreted in the second and first 24 hours after the injection of ^{3}H-aldosterone. In our studies the correction factor was carefully registered in every case and has been taken into consideration in the calculations of all our values.

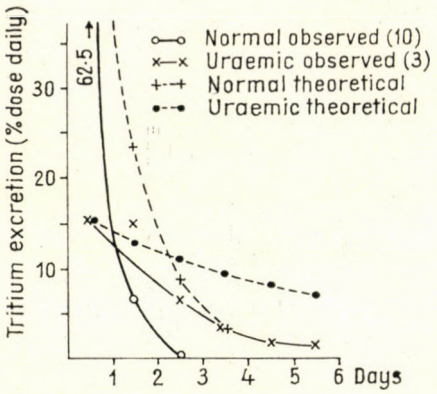

Fig. 7. Urinary excretion of ^{3}H in the case of impaired renal function. Incomplete urinary excretion of tritium from labelled aldosterone metabolites (from COPE, 1964)

In view of the fact that the plasma aldosterone concentration constitutes the most reliable indicator of the organism's aldosterone supply, more precisely of the level of biologically active aldosterone, the estimation of the aldosterone secretion rates and also of aldosterone excretion, must be regarded as inadequate (Fig. 8). The results of our studies in a total of 180 subjects presented in the graph conclusively demonstrate that the excretion and secretion rates of aldosterone, though providing sufficient information in the majority of cases concerning the state of aldosterone supply of the body, are insufficient in a number of cases. On confronting the plasma aldosterone concentrations with the values for aldosterone secretion rate and excretion in the same individuals, we find a discrepancy in certain cases. This is attributable to the fact that the secretion and metabolization of aldosterone are not necessarily of the same rate. As the graph clearly demonstrates, despite an increased aldosterone secretion the plasma aldosterone level may remain normal as a result of increased metabolization, on the other hand, even in the case of a normal aldosterone secretion, an impaired metabolization may result in increased plasma aldosterone levels, that is, in an enhanced aldosterone activity.

From the data presented it clearly emerges that the estimation of the aldosterone secretion rate permits a far more reliable assessment of the state of aldosterone supply of the body than does the estimation of aldosterone

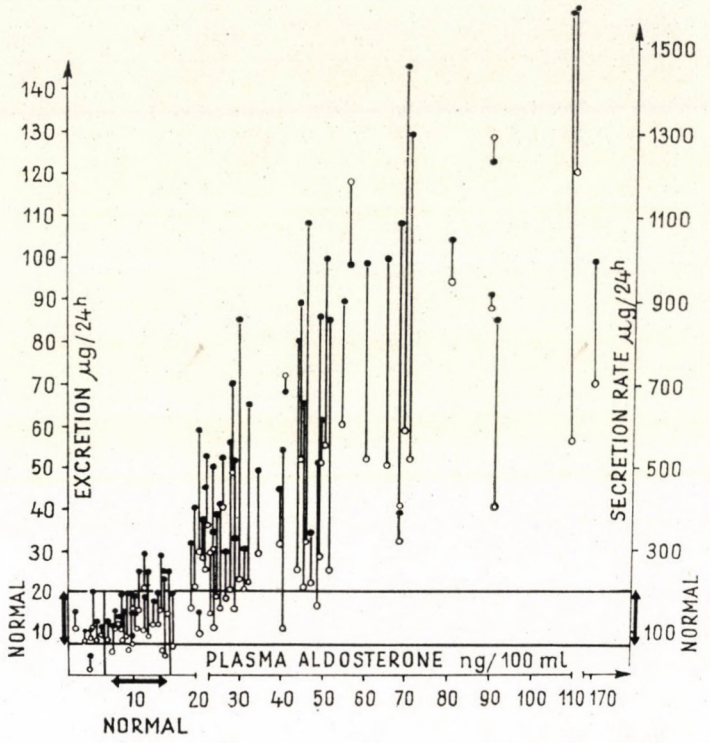

Fig. 8. **Aldosterone excretion and secretion rates plotted against plasma aldosterone values under various conditions of aldosterone production (GLÁZ et al.)**

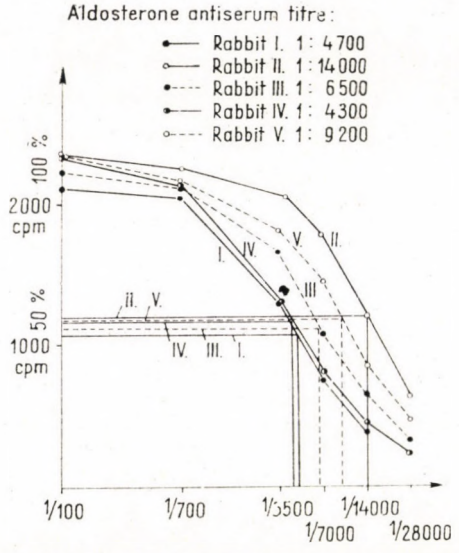

Fig. 9. **Aldosterone antiserum titre in five different rabbits (GLÁZ et al.)**

excretion. Notwithstanding this, serial determination of the plasma aldosterone level seems to offer the only promising avenue for future research concerning aldosterone, whether in animal experiments or in clinical studies. This would permit to replace the costly, tedious double-isotope techniques by radioimmunoassay.

As regards the radioimmunoassay in general, including those of aldosterone, we are still at the beginning, but the results thus far obtained seem encouraging. As shown in Fig. 9, we have been able to prepare antialdosterone immune serum in the rabbit, using aldosterone-21-bis-hemisuccinate bovine serum albumin (BSA)-complex produced by VECSEI and PENKE (unpublished data) for the immunization which was carried out by the procedure of LADA (unpublished data). Though the titre of our aldosterone antiserum thus obtained is far in excess of that required by the procedure, we have still a long way to go to make the method applicable for serial determination of the plasma aldosterone level.

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STEROID HORMONE DETERMINATION ON THE BASIS OF RADIO-IMMUNOASSAY

By

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Radioimmunoassay allows a reliable determination of very small quantities of various substances, in the first place of proteins, in terms of picograms or nanograms. This is of invaluable advantage in the case of estimations in biological fluids. Accuracy and reproducibility belong to the chief merits of the method.

Basically, every substance of immunogenic properties, in other words, against which antibody can be raised in an appropriate species, lends itself to quantitative radioimmunoassay. This primarily applies to proteins. The method was originally developed for the assay of insulin by YALOW and BERSON [1], later its principle was adapted by the same authors as well as by other workers to all substances capable of forming a specific binding with some other substance within an immunological or nonimmunological system.

The principle of immunoassay is presented in Fig. 1.

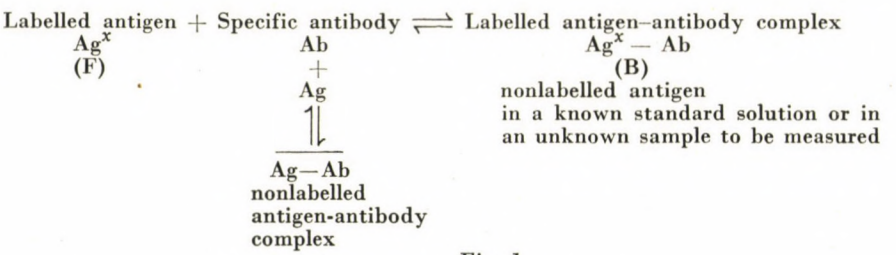

Fig. 1

The principle of radioimmunoassay is also valid for nonimmune systems; this is the competitive radioassay.

The results of radioimmunoassays turn on the issue of finding an appropriate procedure for the separation of the free tracer from that bound to the antibody.

The wide diversity of possibilities of separation allows to select the most convenient procedure. One of the most currently used techniques is that of

immunological double antibody precipitation. In view of its simplicity and its rapidity, it is the plastic-coated antibody tube procedure described by CATT and TREGEAR [2] which seems the most promising. Recently, the polymerised antiserum procedure has been adopted for the assay of hormones with a sterane nucleus.

Estimation of oestrone and oestradiol

The procedure owes its existence to the possibility of binding steroid hormones to proteins in which state they induce the formation of antibody specific of the steroid hapten. LIEBERMAN et al. [3], GOODFRIEND and SEHON [4] have developed radioimmunoassays for the estimation of progesterone, testosterone, oestrone and of other hormones, using bovine serum albumin (BSA) as carrier protein.

The immunizing antigen was a 17β-oestradiol-hemisuccinate-BSA complex, the antiserum was raised in sheep. The antiserum cross-reacts with oestrone, therefore oestrone and oestradiol can be determined separately with the aid of a suitable separation technique.

The advantage of the method is its high sensitivity. It measures the steroids in samples as small as 1 to 2 ml and also lends itself to serial tests in man, unlike other procedures requiring large amounts of plasma, such as gas chromatography [5, 6] or the double isotope derivation technique [7, 8].

The plasma oestrogens may be separated from ether extracts on Sephadex LH-20 by column chromatography, for which MIKHAIL et al. [9] use the polymerized antibody-colloid described by AVRAMEAS and TERNYNCK [10]. Tritiated oestradiol and oestriol may serve as tracers.

According to ABRAHAM [11] oestradiol can be estimated directly in follicular fluid without requiring previous purification.

The direct methods give, however, no reliable results in estimations in plasma.

MIDGLEY et al. [12] immunized rabbits with 13 different steroid-BSA complexes, of which only 9 proved immunogenic: oestradiol-3-BSA, progesterone-3-BSA, progesterone-20-BSA, progesterone-11-BSA, pregnanolone-3-BSA. The following four preparations failed to produce immune response: aldosterone-21-BSA, corticosterone-21-BSA, cortisone-21-BSA and desoxycorticosterone-21-BSA. Each protein molecule of these preparations contained only 10 or less steroid molecules. Since ERLANGER and BEISER [13, 14] were able to produce immunization even with these four preparations, the explanation can only be that each BSA molecule must contain at least 20 or more steroid molecules.

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The Vth Hungarian Endocrine Congress was held in Szeged,

Szeptember 1—4, 1971.

This volume combines the lectures delivered in the plenary sessions of the Congress. It further contains the lectures given at the Symposium on the regulation of ACTH secretion and modern trends in steroid analysis

INDEX

ANNOUNCEMENT OF AN IAEA SYMPOSIUM

Title: Radioimmunoassay and Related Procedures in Clinical Medicine and Research

Date: 10—14 September, 1973

Location: Istanbul, Turkey

Organizers: International Atomic Energy Agency,
Kärntner Ring 11—13, A-1010 Vienna, Austria

Scientific Secretaries: Dr. E. J. Garcia and Dr. E. H. Belcher
Medical Applications Section

The programme of this Symposium, the second to be organized by the International Atomic Energy Agency in the subject field, will cover all aspects of radioimmunoassay and related procedures and their applications. Particular attention will be given to the *in vitro* determination of substances of clinical interest such as hormones, vitamins and drugs. Sections of the programme will be devoted to recently developed techniques for the detection of tumour, viral and bacterial antigens, and to assays employing labelled antibodies and naturally occurring specific receptors.

Further information, participation forms and forms for submission of a paper intended for presentation at the Symposium will be obtainable from national authorities for atomic energy matters. Abstracts of such papers must be submitted to the International Atomic Energy Agency through these authorities.

SYMPOSIUM ANNOUNCEMENT

Title: New Developments in Radiopharmaceuticals and Labelled Compounds.

Date: 26—30 March 1973

Location: Copenhagen, Denmark

Organizes: International Atomic Energy Agency
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in collaboration with the World Health Organization
CH-1211 Geneva 27, Switzerland

Scientific Secretaries: Dr. S. Rothchild and Dr. E. Garcia

The Symposium will primarily be concerned with the chemical and pharmaceutical aspects of radiopharmaceuticals. A major objective will be to summarize by means of invited papers some of the recent developments in the areas listed below, to be followed by contributions on new work. Topics to be covered include nuclide generators, kits for preparing radiopharmaceuticals from short-lived nuclides, accelerator production of medically useful nuclides, synthesis of radiopharmaceuticals and labelled compounds from short-lived nuclides, labelled compounds for *in vitro* clinical tests, and stable isotopes in medicine.

A panel will discuss various aspects of regulatory and pharmaceutical responsibilities for radiopharmaceuticals, after which discussion will be invited from the floor. Another panel will attempt to project the directions which nuclear medicine is likely to take in the future, and the demands that this would place on the development of new radiopharmaceuticals.

Participation in the Symposium, whether or not a paper is presented, must be through nomination by the Government of a Member State of the I.A.E.A. or W.H.O. Each participant should complete a Participation Form, and a Form for Submission of a Paper if a paper is to be submitted, and send it (them) to the competent official authority for transmission to the Agency. The Form for Submission of a Paper should be received by the Agency before 16 October 1972. Further information and forms may be obtained from national authorities for atomic energy matters.

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Padua, Italy — 7—9 June, 1973

An International Congress on "Immunology in Obstetrics and Gynaecology" will be arranged at Padua, Italy, on 7 through 9 June, 1973, under the auspices of C.I.O.M.S.

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6th session: immunology of gynaecological tumours

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by T. BAKÁCS

The basic biological problems of urbanization are treated in this book, such as the direct factors responsible for the disruption of balance in ecology witnessed today, how far the increasingly artificial urban environment and chemicalization are responsible for this disbalance, and further the measures necessary to prevent untoward longterm reactions. The most important urbanization hazards, as well as the complex health injuries connected with the urban environment are analysed. The author also tries to find a solution to these urgent problems.

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(Ein Rundtischgespräch)

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SUBCLINICAL FORMS OF MYOCARDIAL INFARCTION

By

I. KENEDI, JULIA DÉVAI and F. GONDA

HUNGARIAN ARMY MEDICAL CORPS

(Received July 26, 1971)

Earlier myocardial infarction was ascertained on the basis of the Minnesota Code in 36 patients in whose history there was no typical heart attack.

The post-infarction ECG pattern may be produced in the following ways. 1) True, silent painless infarction; 2) transitory retrosternal pain of anginal character; 3) acute cardiac syndrome in the absence of pain (circulatory failure, ventricular tachycardia); 4) presence of some other disease masking the signs of acute myocardial infarction.

In the present series, the syndrome termed subclinical infarction was prevalent beyond the age of 55 years, and infarctions of the posterior wall were more frequent than those of the anterior wall.

The forms of myocardial infarction marked by the absence of an acute typical syndrome, to which the collective term "subclinical infarction" may be applied, are of major practical significance and ever since the beginnings of epidemiologic studies of coronary disease they have been given much attention in literature (GORDON et al. 1959, LINDBERG et al. 1960, KANNEL et al. 1970). The problem has been viewed from two different angles of approach, according to its clinical and pathological aspects. Myocardial infarction detected at autopsy is outside the subject of the present study.

The forms of myocardial infarction to be discussed here pose major diagnostic problems. The scar formed at the site of infarction interferes with the muscular performance of the heart and becomes an inexplicable handicap to the patient, thus imposing a search for its cause by every possible means.

Material and method

The 36 subjects forming the present series have been under our care as inpatients and followed up after discharge, or referred to our ECG-laboratory, since 1966. All had residual signs of myocardial infarction according to the criteria of the Minnesota code, confirmed on discharge or during follow-up or as an accidental ECG finding. In order to determine the exact location and extent of earlier infarction and to record the motions of the cardiac contour, all patients were submitted to a complex schedule of investigation including a 15-lead ECG, a PCG derived from the apex and the base in four frequency bands, an apex cardiogram (ACG) recorded from 9 sites between the sternal border and the midclavicular lines in the third, fourth and fifth intercostal spaces, and an X-ray kymogram in five or six positions. The abnormal circumscribed movements revealed by the ACG were verified in two ways, *viz.* by returning from other areas to the abnormal site in the course of the same study, and by a comparison with later recordings.

Results

Thirty-six patients, 13% of the 270 postinfarction cases submitted to the complex schedule of investigations referred to above, were found to have experienced myocardial infarction in the past in the absence of a classical acute coronary attack. It is deemed convenient to divide the cases of subclinical infarction into the following four types.

1) *True silent myocardial infarction.* Detection of its residual ECG-signs was exclusively the result of routine clinical investigation, even close questioning having failed to reveal any indication of earlier cardiac episodes. The 12-lead ECG belongs to our preoperative routine in all patients over 45 years of age before major surgery including urological or traumatological interventions, and recently also before minor surgery.

On the evidence of the ECG, the location of "silent" infarction was the anterior wall in 5 and the posterior wall or the postero-lateral region in 8 of the 13 cases. Fluoroscopy revealed cardiac enlargement and/or pericardial adhesions in four cases and circumscribed aneurysmal bulging in one case. The ACG showed paradoxical pulsation at some circumscribed segment of the cardiac contour in 3 cases. The essentials of an illustrative case are given in the following.

The patient was a 65-year-old female. Pulmonary screening performed in 1970 revealed a solitary round shadow suggestive of bronchial carcinoma in the left lower field. Thoracotomy was performed on these grounds in a unit for pulmonary surgery. On exposure, a cardiac aneurysm accounting for the round solitary lesion was found. The patient was referred for resection of the aneurysm to the First Clinic of Surgery of the Postgraduate Medical School. The patient denied ever having experienced retrosternal pain or any symptom of heart failure in the past but had been hospitalized for heart disorders five years earlier. The X-ray kymogram (Fig. 1A) showed a markedly enlarged heart, its base showed a bulging hemispheroid aneurysm with calcified borders. Interpretation of the abnormal pulsations was not possible owing to the presence of broad pleuro-pericardial adhesions partly of surgical origin. The presence of a Q and of an inverted T in standard lead I and in aVL and of a QS in V_{1-4} as well as in the Nehb-J-lead was typical of an old infarction scar involving the apex. ACG revealed paradoxical pulsations at the apex (locations 0 and 2) with normal movements of the contours at the base (Fig. 1B).

In one of the cases of silent infarction, a consecutive ECG series revealed an extension of the cicatricial area of the anterior wall in the absence of any pain (latent progression).

2) ECG-recordings taken after a *short attack of effort angina* in 19 cases revealed, quite surprisingly, signs of an acute myocardial infarction

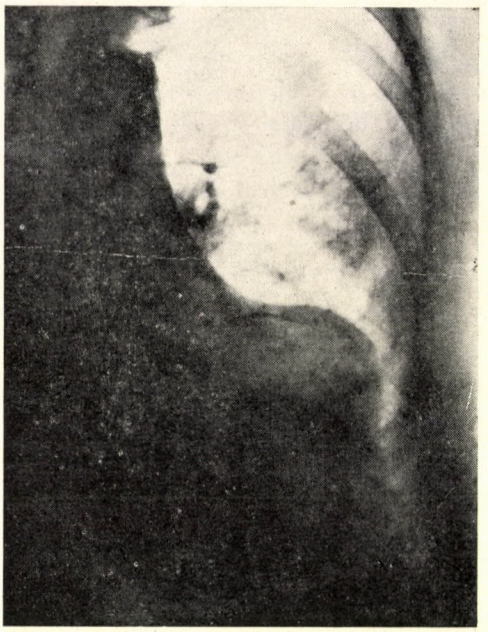

Fig. 1A. Aneurysm with calcified borders at the base of the heart

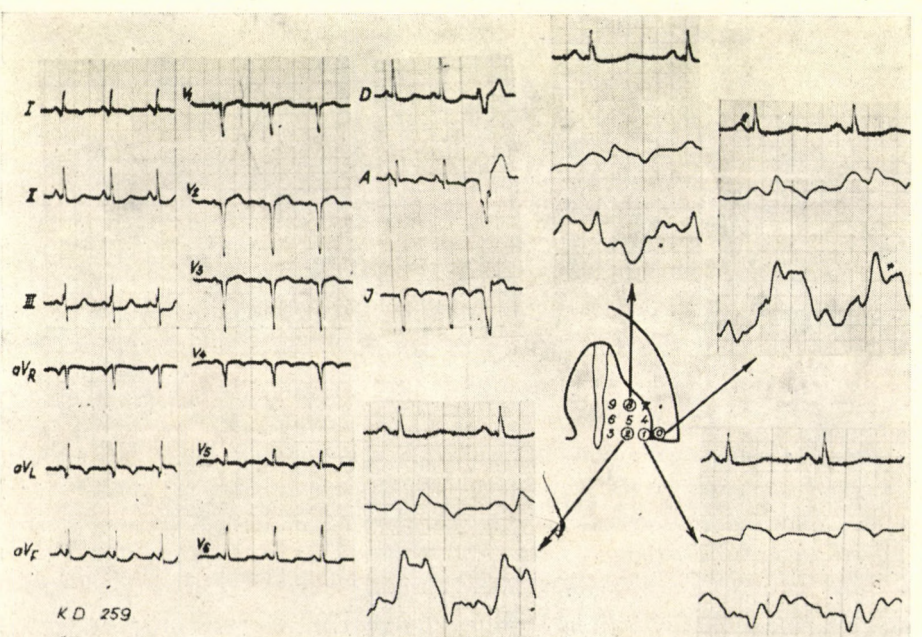

Fig. 1B. ECG typical of an infarction scar on the anterior wall (QS in V_{1-4}). ACG shows paradoxical pulsations at the apex (locations 0 and 2)

after the first attack in 8 cases and of an old infarction after recurrent transient pain in 11 cases. A posterior wall pattern was found in 12 and an anterior wall pattern in 7 cases. The ACG showed in six cases paradoxical pulsations at some well-defined area. There was X-ray evidence of an aneurysm in the form of a circumscribed bulge in one case, of cardiac enlargement and/or of pericardical adhesions in 5 cases. The following case was illustrative.

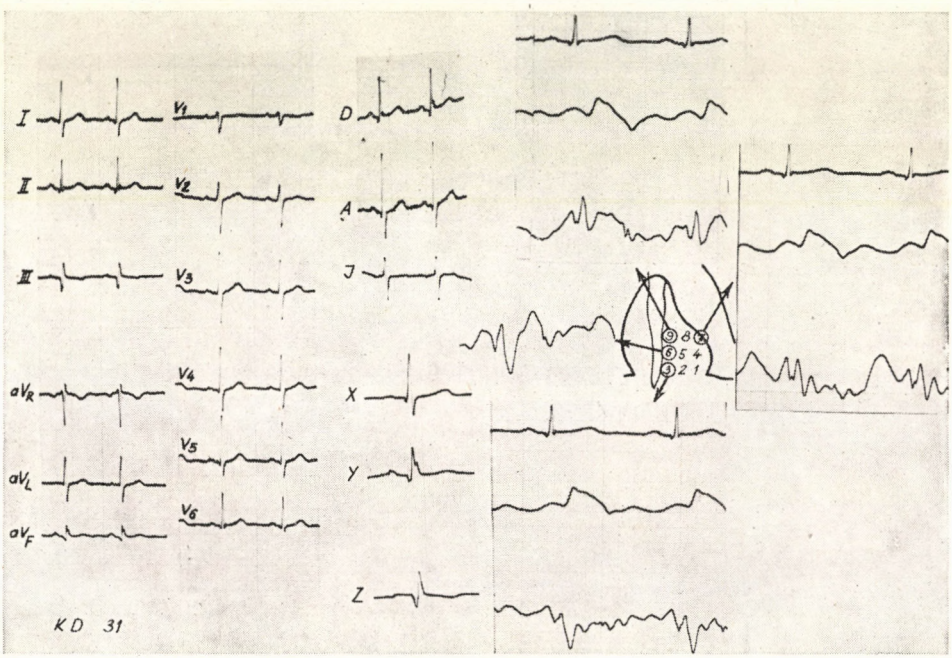

Fig. 2. ECG and VCG: infarction scar of the posterior wall. ACG: the upper medial contour and the lateral wall show abnormal movements

The patient was a 41-year-old male having experienced recurrent transient anginal symptoms while serving a prison sentence. The ECG performed after his release showed a Q in standard leads II, III, aVF and Nehb-D, as well as on the y-axis of VCG as a sign of an old posterior infarction scar. ACG revealed a semiparadoxical pulsation in the upper medial part (location 9) and jagged contours laterally at location 7 (Fig. 2).

3) *Acute cardiac symptoms* in the absence of pain. Grave dyspnoea, acute left heart failure (Friedberg 1950, Papp 1952, Jochweds 1969), ventricular tachycardia, grave arrhythmia (Evans–Sutton 1956) are suggestive of acute myocardial infarction. Painless myocardial infarction has been found responsible for acute circulatory failure in two of our patients, shown by the following case record.

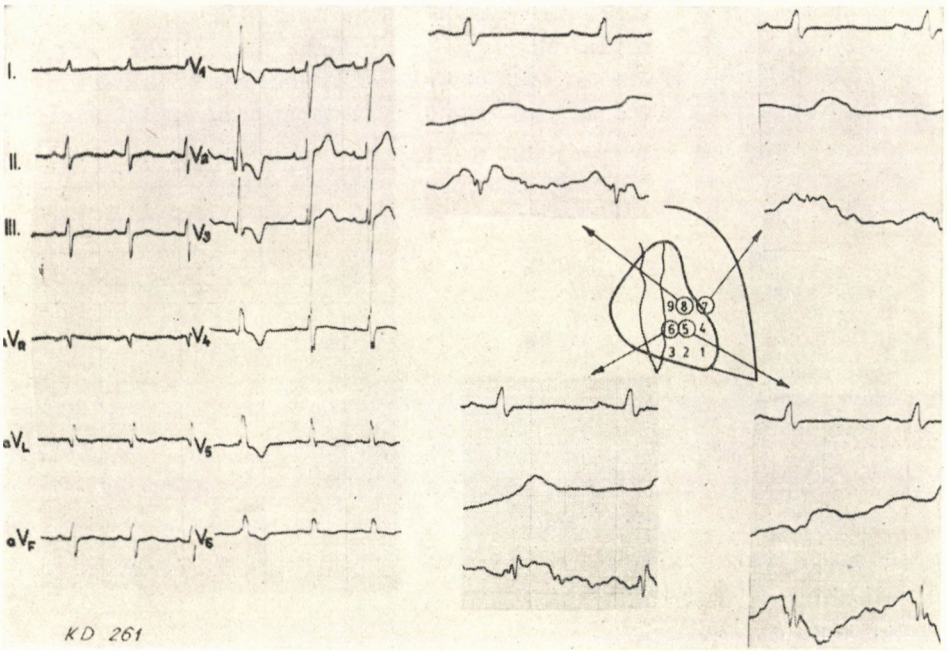

Fig. 3A. ECG: infarction scar of the anterior wall (see text.); ACG: the base of the heart exhibits semiparadoxical pulsations

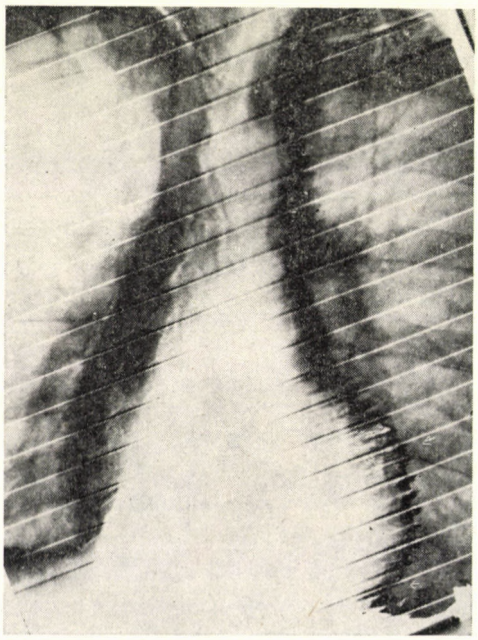

Fig. 3B. X-ray kymogram: extensive paradoxical pulsation at the apical region and the posterior wall

The patient, a 46-year-old male, hitherto in good health, had developed acute pulmonary oedema during heavy physical exercise in the absence of any retrosternal pain not only at the time of the attack but also during a subsequent observation in a local hospital for 21 days. A later ECG performed at our unit (Fig. 3A) showed a deep broad Q in aVL and a Q appearing during ventricular premature beats in V_{1-4}, thus indicating an anteroseptal infarction. ACG revealed a semiparadoxical pulsation at the base (locations 6, 7 and 8) while remaining normal at the apex and the supraapical segment (location 5). X-ray kymography: the heart was vertical; the area of infarction involving the apex and the dorsal surface revealed itself by a paradoxical pulsation which was brought out very sharply with the aid of the oblique grid employed for the procedure (Fig. 3B).

4) The signs of acute myocardial infarction may be *masked by the presence of some other disease* such as acute cerebrovascular accidents (GOULD–CAWLEY 1958, MELICHAR et al. 1963), postoperative complications (MASTER et al. 1938), psychoses (LIEBERMAN 1955, MARCHAND 1955), diabetes (MARBLE 1955, AGAR 1962, BRADLEY–SCHONFELD 1962), cholelithiasis, etc. This is the group which accounts for the majority of cases where myocardial infarction eludes diagnosis and remains undetected during lifetime. It is thus understandable that we had no more than two cases falling into this category, the signs of acute myocardial infarction having been masked in both by those of gallstones. One of the cases is reported here in brief.

The patient was a 66-year-old female, with a history of cholecystectomy for gallstones. She was admitted for pain in the right upper abdominal region and transient jaundice. Cholangiography revealed a calculus 12 mm in diameter over the Vater's ampulla. She had never had any retrosternal pain. ECG showed a Q in standard leads II and III and a deep Q in aVF and in Nehb-D as a sign of a posterior wall scar. ACG: (Fig. 4) small-amplitude movements of serrated character at the base and the apex. X-ray kymogram revealed a dorsal silhouette with a jagged outline as a sign of slight muscular dysfunction.

Cor pulmonale with grave acute heart failure also involves diagnostic difficulties, inasmuch as the clockwise rotation of the heart may produce a QS in V_{1-4}. On the other hand, acute respiratory failure may be associated with retrosternal oppression and even with anginal pain. This ECG-pattern was found in one of our patients at admission, a few days later an extension of the QS as far as V_6 was, however, demonstrable.

From the global analysis of the four groups of subclinical myocardial infarction under discussion it emerges that in 28 patients the lowest age limit was 55 years and only 8 patients belonged to younger age groups. As regards the site of infarction, posterior wall infarctions of the left ventricle (22 cases) showed a certain prevalence over anterior wall infarctions (14 cases).

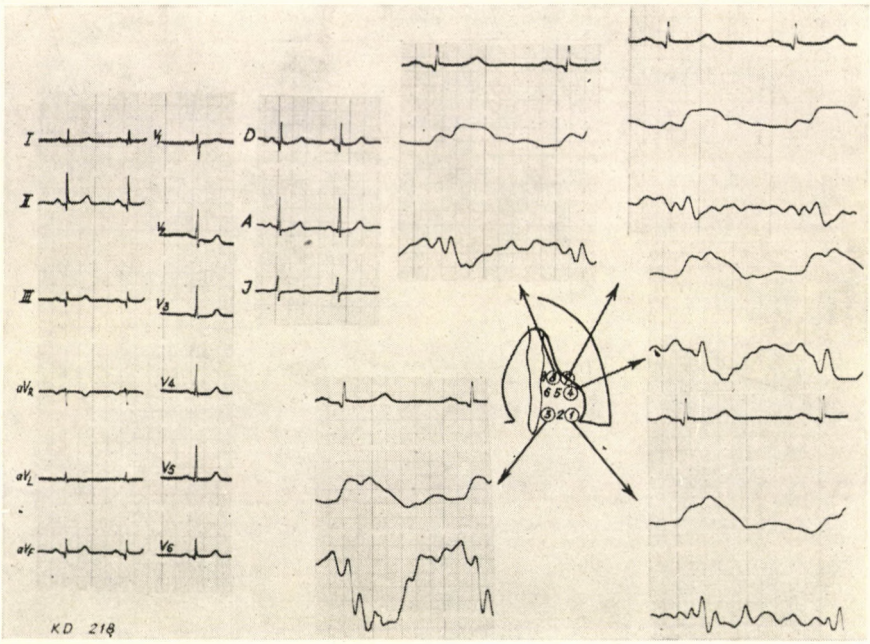

Fig. 4. ECG: infarction scar of the posterior wall (see text); ACG: abnormal pulsation at the base of the heart and at the apex

Discussion

Classification of subclinical myocardial infarction on the above discussed grounds is justified by the fact that each of the individual subgroups has a significance of its own and requires a different diagnostic approach.

The prevalence of true silent myocardial infarction is convincingly illustrated by the results of a prospective study by KANNEL et al. (1970) extending over 14 years in which 50% of unrecognized infarctions were marked by a total absence of chest pain. This is consistent with the observations of the present authors (KENEDI et al. 1964) according to which 7 out of 8 patients, partly with diagnosed, partly with undiagnosed earlier infarcts, died either during operation or postoperatively as a result of reinfarction.

ECG-screening in the age-groups being at risk is advocated by various authors (FAGIN and CHAPNIK 1950, LINDBERG et al. 1960, MELICHAR 1966). Owing to lack of adequate technical facilities including equipment and staff, it is not possible for the time being to set a mass screening campaign into operation which would involve the respective age-groups of the general population. It is, however, essential to make a 12-lead ACG, a routine test in every patient over 55 years who comes to be hospitalized for any reason, in the first place for surgery under intratracheal anaesthesia.

Diagnosis of myocardial infarction associated with effort angina is less difficult. Close investigation after each anginal attack is, however, imperative so as to identify and, if possible, to minimize the precipitating factors (hypertensive disease, diabetes, cholesterolaemia, adverse conditions of life, mental stress, etc.). Increase in the frequency of anginal attacks or the appearance of chest pain at rest also call for investigations of the same extent.

The fact that acute myocardial infarction is a common cause of acute left heart failure marked by sudden attacks of dypnoea has been duly emphasized in the literature in the last two decades (EVANS—SUTTON 1956, FRIEDBERG 1950, PAPP 1952, JOCHWEDS 1969). This confirms the necessity for consecutive ECG-recordings in case of painless acute cardiac syndromes so as to confirm or to rule out the presence of myocardial infarction.

Acute myocardial infarction poses the greatest diagnostic difficulty when masked by the stormy signs of some acute disease. The most common conditions of this kind have been reviewed by GOULD–CAWLEY (1958) and MELICHAR et al. (1963). It is obvious that myocardial infarction eludes detection in unconscious patients with cerebrovascular accidents or in psychotics who may not complain of pain (MARCHAND 1955, LIEBERMAN 1955). It is more difficult to account for the observation that anginal pain may be slight or absent in diabetes (MARBLE 1955, AGAR 1962, BRADLEY 1962). Acute myocardial infarction belongs to the most common postoperative complications of major surgery; retrosternal pain, fever, fall in blood pressure, signs of tissue necrosis may, however, also have other causes. The ECG brings conclusive evidence if the possibility of acute myocardial infarction is borne in mind (MASTER et al. 1938).

Myocardial infarction associated with some grave primary disease involves great diagnostic difficulties. The patient generally dies of the primary disease. Unless he survives the acute phase, the infarction usually remains undetected until necropsy.

Evidence of the present postinfarction study including ECG, PCG, ACG and X-ray kymography (DÉVAI 1963) and our subsequent observations are consistent with the data of numerous authors from which it emerges that the outcome of subclinical myocardial infarction marked by a misleading paucity of symptoms is in no way more favourable than in the case of a sudden typical onset.

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PHOSPHOLIPID COMPOSITION OF BLOOD IN MULTIPLE SCLEROSIS

By

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The level and composition of phospholipids in the plasma and blood cells of multiple sclerotics as well as control patients were investigated by column and thin-layer chromatography.

Neither the total nor the individual phospholipid content of the blood showed any significant difference.

It could, however, be observed that the level of individual phospholipids oscillated in the plasma and in the blood cells of both control and MS patients, and the resulting oscillations may be greater in the case of MS. This finding may explain the fact that some authors have reported contradictory data concerning the phospholipids in the blood of MS patients.

Regarding the special damage of myelin the study of lipid metabolism in multiple sclerosis (MS) represents one of the most important tasks in the research of the mechanism of demyelination. However, owing to the human character of the disease, the only possibility for gaining information on the disturbances of lipid metabolism is by investigating the blood or cerebrospinal fluid.

In relation to lipid metabolism we have examined the changes of the total lipids, lipid-P and cholesterol in blood after loading the patients orally or intravenously with different lipids (Huszák and Heiner, 1966). No significant differences could be observed.

Concerning the plasma phospholipids in MS, contradictory experimental data have been published. An elevated total phospholipid level was described by Dobin and Switzer (1954), Persson (1958), Freeman and Siegel (1959); a lower level was found by Bernsohn and Namajuska (1955), Chiavacci and Sperry (1952) and Sercl et al. (1961); while no alteration was found by Fog (1951), Jones et al. (1954), Plum (1959), Gerstl et al. (1965), Cumings et al. (1965), Montfoort et al. (1966), Tichy et al. (1969), Andreoli and Cazzullo (1969).

In order to throw light upon the contradictions in literary data, we have investigated the total and individual phospholipid pattern of the blood.

Material and methods

The examinations were performed on 20 to 40-year-old male and female patients suffering from multiple sclerosis. Neurotic patients were used as controls. The patients were

kept on a standard diet. From oxalated blood samples serum and blood cells were separated by centrifugation. Lipids were extracted by 20 volumes of chloroform–methanol (2 : 1 v/v), and filtered quantitatively through fat-free filter paper. The filtrate was evaporated at 40°C under vacuum in a rotary evaporator. Lipids were redissolved in 25 ml of chloroform—methanol (2 : 1) and washed with 5 ml of 0.1 n KCl to remove non-lipid water-soluble substances. After separation of the two phases, the lower one was withdrawn and evaporated. The residue was dissolved in 1—2 ml of chloroform and repeatedly evaporated, until the lipid extract had become transparently clear. Lipids were separated into four fractions as described by WEBSTER (1960) and DOMONKOS and HEINER (1968) on an aluminium oxide column 1 cm in diameter (aluminium oxide standardized according to Brockmann, Merck). Lipids were eluted successively with 20 ml of chloroform (Fraction 1), 30 ml chloroform—methanol (1 : 1) (Fraction 2), 15 ml of chloroform—methanol—water (7 : 7 : 1) (Fraction 3), and with 30 ml of chloroform—ethanol—water (2 : 5 : 2) (Fraction 4). Fraction 1 involves neutral lipids, Fraction 2 the choline phosphatides (lecithin, sphingomyelin and lysolecithin), Fraction 3 involved the non-phospholipids (cerebrosides, sulphatides) and Fraction 4 the other phosphatides (ethanolamine, serine, inositol phosphatides and the corresponding lyso derivates). Fractions 1 and 3 were discarded. Fraction 2 was evaporated to dryness under vacuum by a rotary evaporator. Fraction 4 was mixed with an equal volume of chloroform, the mixture was shaken, the lower phase was withdrawn and the upper one discarded. The lower phase containing the lipids was evaporated to dryness. From part of the phospholipid fractions the total lipid-P content was determined according to FISKE and SUBBAROW (1925). From another part of these fractions the individual phospholipids were separated by thin-layer chromatography (Kieselgel G nach Stahl, Merck). Solvent systems for choline phosphatides were chloroform—methanol—water (65 : 25 : 4), for cephalin phosphatide chloroform—methanol : concentrated NH_3 (65 : 25 : 4 v/v). To attain better separation of the cephalin phosphatides, the procedure was repeated with the same solvent system. The separated lipids were visualized by iodine. Spots were quantitatively collected and phosphorus was determined by the Bartlett method as modified by DOMONKOS and HEINER (1968) in the following way: a lipid sample containing a maximum 0.2 μg atom P was ashed with 1.2 ml 10 n H_2SO_4: 2 drops of 30% H_2O_2 were added and ashing was continued for 1 hr at 160 °C. Then 2.7 ml of distilled water, 0.8 ml of 5% ammonium molybdate and 0.2 ml of Fiske and Subbarow's reagent were added. It was kept in a water bath at 100 C° for 7 min and the extinction was read at 800 mμ in a spectrophotometer. Fatty aldehyde was estimated by the method of WARNER and LANDS (1961).

Results

Results concerning the level of lipid phosphorus in the plasma and blood cells of multiple sclerotics and controls are demonstrated in Table I. The amount of choline and cephalin phosphatides separated on an aluminium oxide column did not show any significant difference either in plasma or blood cells.

Table I

Lipid phosphorus in plasma and blood cells of control and multiple sclerotic patients (μg atoms/100 ml of plasma and blood cells in 100 ml of blood)

	Choline phosphatides		Cephalin phosphatides	
	control	MS	control	MS
Plasma	253±32 (11)	253±32 (12)	11.3±6.6 (13)	10.5±5.2 (10)
Blood cells	222±17 (13)	212±20 (13)	135±16 (14)	142±16 (14)

The values are means ± S. D.
Figures in brackets give number of cases.

In Table II the fatty aldehyde content of choline and cephalin phosphatides in the plasma and blood cells of the same patients is demonstrated. There were no significant differences between them.

Table II

Fatty aldehyde in plasma and blood cells of control and multiple sclerotic patients (μ moles/100 ml of plasma and blood cells in 100 ml of blood)

	Choline phosphatides		Cephalin phosphatides	
	control	MS	control	MS
Plasma	1.62±0.74 (11)	1.68±0.82 (12)	2.46±0.77 (13)	2.45±0.96 (10)
Blood cells	0.56±0.25 (10)	0.63±0.24 (10)	28.7±6.4 (13)	25.8±5.5 (13)

The values are means ± S. D.
Figures in brackets give number of cases.

Table III

Individual phosphatides in plasma and blood cells of control and multiple sclerotic patients (μg atoms/100 ml of plasma and blood cells in 100 ml of blood)

	Plasma		Blood cells	
	control	MS	control	MS
Lysolecithin	24.0±5.9 (8)	23.4±5.7 (11)	7.8±2.2 (11)	8.6±2.0 (11)
Sphingomyelin	51.9±7.7 (10)	57.0±12.1 (11)	91.5±12.9 (12)	92.5±10.2 (12)
Lecithin	166.8±22.8 (10)	166.3±7.8 (10)	104.3±15.8 (14)	114.3±16.1 (14)
Phosphatidyl-serine	0.76±0.58 (12)	0.56±3.25 (10)	7.67±1.05 (6)	8.45±3.38 (6)
Phosphatidyl-inositol	3.09±1.65 (11)	3.01±1.11 (11)	4.56±1.39 (13)	5.65±1.25 (13)
Lysophosphatidyl-ethanolamine	1.22±0.82 (13)	1.06±0.52 (13)	5.05±2.32 (9)	6.21±1.27 (9)
Phosphatidyl-ethanolamine	4.7±1.9 (11)	4.3±1.5 (11)	92.9±10.5 (12)	86.1±5.0 (12)

The values are means ± S. D.
Figures in brackets give number of cases.

Table III demonstrates that investigation of the individual phospholipid fractions by the thin-layer silicagel method revealed that in the amount of the lysolecithin, sphingomyelin, lecithin, phosphatidyl-serine, phosphatidyl-inositol, lysophosphatidyl-ethanolamine and phosphatidyl-ethanolamine there were no significant differences between the MS and control group, either in blood cells or in plasma. There were, however, considerable individual variations in the amount of different fractions.

Discussion

In conclusion we may state, as some other authors have done [Fog (1951), Jones et al. (1954), Plum (1959), Montfoort et al. (1966), Gerstl et al. (1965), Cumings et al. (1965), Tichy et al. (1969), Andreoli and Cazzullo (1969)] that there are no significant differences in the content of total or individual phospholipids of the plasma, or that of the blood cells. The total and the individual phospholipid content of the plasma showed remarkable variations in the controls, too; these individual variations were, however, more pronounced in MS.

These greater oscillations of the individual and total phospholipid content of the plasma in MS may be the consequence of the homeostatic dysregulation. The contradictory data obtained by some other authors may be explained by these facts.

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SERUM IMMUNOGLOBULINS IN CHILDREN WITH BILHARZIAL INFECTION

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1. This study included 23 children infected with *S. haematobium* and 15 with *S. mansoni* complicated with hepato-splenomegaly, and 15 controls.

2. There was a significant reduction in albumin in both types of schistosomiasis either due to nutritional factor (in *S. haematobium*) or to impaired albumin synthesis as a result of liver damage (in *S. mansoni*).

3. The hypo-albuminaemia was accompanied by a significant increase in gamma-globulin which was caused by the remarkable elevation of IgG. Both total gamma globulin and IgG component were significantly higher in cases with intestinal schisto-somiasis complicated with hepato-splenomegaly than in urinary schistosomiasis. This is attributed to a higher activity in reticuloendothelial cells in *S. mansoni* infection.

4. Care should be taken not to overlook the nutritional factors since these may partly be responsible for some reduction in the albumin value and in a compensatory increase in gamma globulin.

5. Elevation in IgG fraction was detected as early as six months after bilharzial infection.

Introduction

There are many reports on the plasma protein abnormalities in both humans and experimental animals with schistosomiasis (ARRU and PARRICIATU, 1961, RAMIREZ et al. 1961, KAGAN and GOODCHILD, 1961, SHAMMA and AZZO, 1966). Most authors agree in that these abnormalities are principally represented by an increase in total plasma protein and gamma globulin accompanied by a loss of albumin. The increase of the globulin level occurs in the beta–gamma region (DE WITT and WARREN, 1959, FIORILLO, 1966).

KAGAN and GOODCHILD (1961) studied the sera of 61 patients with chronic *S. mansoni* infection and reported that the circumoval precipitation test was more markedly positive in cases with significantly higher gamma globulin fraction.

Ouchterlony double diffusion test (SILVA DA and FERRI 1965) disclosed one precipitin line in 11 of 29 sera from patients with hepato-intestinal form and from 1—4 lines in 29 of 37 sera from those with hepato-splenic form of bilharzial infection. They also proved that one of these precipitin lines was IgG.

Sephadex chromatography and ultracentrifugal studies showed that the fraction of serum protein that gave a positive circumoval precipitin test, was gamma globulin with a sedimentation coefficent of 6.6 S and a molecular weight of 200,000 (TORO-GOYCO, 1964).

FIORILLO (1966) reported that the observed abnormalities of serum proteins in the beta–gamma area were the results of the increased level of gamma M macroglobulin of humans infected with *S. mansoni*. These results were further supported by DEL REY CALERO et al. (1968) who mentioned that the immuno-fluorescent reaction was given only by a fraction of serum proteins of bilharzial patients which contained IgM antibodies. However, the latter result was not in accordance with those of KURATA and NODA (1966) who reported that IgG increased greatly and IgM only slightly. They presented evidence strongly suggestive of the presence of autoimmunity in rabbits infected with *S. japonicum*.

On the other hand, immuno-electrophoretic experiments yielded evidences of the presence of specific circulating antigens in laboratory animals infected with *S. mansoni*. This antigen was of parasitic origin and not of host origin (BERGGREN and WELLER, 1967).

The equivocal information cited above has prompted us to study the level of the three main classes of immunoglobulins in children with schistosomal infection.

Material and methods

Clinical investigation

Thirty-eight children with bilharzial infection were admitted to Tanta University Hospital. Fifteen non-bilharzial apparently healthy children of matched age were used as the control group. All children were aged from 5 to 12 years. The bilharzial group had the infection for a period ranging from about 6 months to 3 years. All children including the control group were subjected to a complete and thorough clinical investigation in addition to laboratory tests.

Diagnosis was based on clinical examination and the presence of schistosoma ova in urine or faeces. The history of previous bilharzial infection was recorded. Patients who received anti-bilharzial drug within the last three months before examination were excluded from the study.

Of the bilharzial patients, 15 had *S. mansoni* infection which led to hepato-splenomegaly while the remaining 23 patients had *S. haematobium*.

Laboratory examinations

All cases were subjected to microscopic examination of urine and faeces for the detection of ova, in addition to the following laboratory tests.

1. Blood counts and sedimentation rate.
2. Total serum proteins were estimated colorimetrically by the biuret method. Differential serum protein fractions were determined electrophoretically by the conventional method using barbitone buffer pH 8.6, 0.05 M (BRIERE and MULL 1964).
3. Liver function tests according to the facilities available included thymol turbidity, zinc sulphate turbidity, serum bilirubin, icterus index and serum proteins.
4. Standard immunoglobulins: human serum gamma A and gamma M were obtained from Hyland Laboratories, California, U.S.A., gamma G was prepared and administered to rabbits in an emulsified form after mixing with complete Freund adjuvant according to HASE and MAHIN (1965) with a slight modification. Booster doses were usually given if the antibody titre was not high.
5. Estimation of immunoglobulins. Immunoglobulins were determined by the radial diffusion technique (FAHEY and MCKELVEY, 1965). A graph (concentration against precipitation ring diameter) was constructed on semilog paper using a series of dilutions of appropriate concentration for each plate. The protein concentration in a test sample was then determined by comparing its ring diameter to the graph obtained from the reference protein of known concentration.

Results

1. Urinary bilharziasis

Tables I and II show no significant change in either the total protein, or the gamma, beta and IgA serum protein fractions. However, the average

Table I

Serum protein fractions in controls and children with schistosomiasis

Group	Total protein g/100 ml	Serum protein fractions, in g/100 ml			
		Albumin	α-globulin	β-globulin	γ-globulin
1. *Control*					
Range	6.30—7.98	2.64—4.44	0.67—1.67	0.61—1.19	0.75—2.56
Mean	7.22	3.56	1.01	1.02	1.63
S. D.	0.52	0.48	0.27	0.16	0.46
2. *Urinary bilharziasis*					
Range	6.38—8.15	2.58—4.22	0.35—1.54	0.64—1.42	1.35—3.42
Mean	7.29	3.11	1.04	0.96	2.18
S. D.	0.54	0.56	0.30	0.17	0.56
3. *Intestinal bilharziasis*					
Range	6.96—8.92	2.44—4.14	0.35—1.43	0.84—1.34	1.6—4.29
Mean	7.96	3.00	0.90	1.08	2.98
S. D.	0.62	0.54	0.28	0.17	0.85

Table II

Immunoglobin components in controls and children with schistosomiasis

Group	IgG (mg/100 ml)	IgA (mg/100 ml)	IgM (mg/100 ml)
1. *Control*			
Range	927—1556	300—385	119—146
Mean	1276	352	133
S. D.	197	30.50	6.82
2. *Urinary bilharziasis*			
Range	1255—2994	282—375	90—142
Mean	1818	337	121
S. D.	523	24.15	13.15
3. *Intestinal bilharziasis*			
Range	1365—4179	268—402	122—141
Mean	2734	335	133
S. D.	724	31.86	5.57

for albumin (3.11 ± 0.56 g/100 ml) was significantly reduced (t = 2.65) as compared to that of controls (3.56 ± 0.48 g/100 ml).

Electrophoretic analysis disclosed that the total gamma globulin fraction (2.18 ± 0.56 g/100 ml) was significantly increased (t = 3.24) versus the control values (1.63 ± 0.46 g/100 ml) and that this elevation was mainly due to the increase (1818 ± 523 mg/100 ml) in IgG component (t = 4.41). However, radial diffusion tests showed a significant reduction in the IgM component as indicated in Table II (t = 3.62).

Table III

Blood counts and liver function tests in controls and children with schistosomiasis

	Control Mean ± S. D.	Urinary bilharziasis Mean ± S. D.	Intestinal bilharziasis with hepatosplenomegaly Mean ± S. D.
1. *Blood*			
Hb per cent	82 ± 2.00	69 ± 7.21	56 ± 6.5
RBC	4,155,000 ± 13,963	3,607,500 ± 1,681	3,015,500 ± 432,100
WBC	5,960 ± 1,246	6,590 ± 400	5,425 ± 201
ESR	1st hour: 7.3 ± 1.7	11.95 ± 8.33	32.70 ± 3.92
	2nd hour: 15.7 ± 2.8	24.15 ± 5.57	36.40 ± 5.60
2. *Liver function tests:*			
Thymol turbidity	2.30 ± 0.20	2.75 ± 0.15	8.20 ± 3.34
Zinc sulphate turbidity	4.40 ± 1.04	5.65 ± 0.95	15.25 ± 5.60
Serum bilirubin mg/100 ml	0.35 ± 0.11	0.47 ± 0.10	0.59 ± 0.29
Icterus index	3.30 ± 0.81	3.90 ± 1.06	6.25 ± 1.40

Liver function test did not reveal any significant change. The ESR was slightly higher than in the control group (Table III). Blood counts showed anaemia in the children with urinary bilharziasis. Their Hb and erythrocyte count was usually decreased when compared to the control level (Table III).

2. *Intestinal bilharziasis with hepatosplenomegaly*

No significant changes in serum total protein, alpha, beta, IgA and IgM were detected (Tables I, II). A significant reduction occurred in the albumin fraction (3.00 ± 0.54 g/100 ml), (t = 3.04). The difference between intestinal and urinary schistosomiasis for albumin was not significant (t = 0.61).

Electrophoresis showed a significant increase in gamma globulin (2.98 ± 0.85 g/100 ml). The gel diffusion test indicated that the significant rise in gamma globulin was the result of a marked elevation of IgG (2734 ± 724 m/100 ml), as compared to the control value of 1276 ± 197 m/100 ml (t = 7.51).

The other immunoglobulin classes studied (IgA and IgM) did not show any significant change in children with *S. mansoni* infection.

In this group clinical and laboratory tests showed a severe impairment of liver function (Table III).

The mean haemoglobin level was low even in the controls (82%), markedly reduced to 69% in *S. haematobium* and decreased to 56% in hepatosplenomegalic cases.

The IgG value was significantly more increased in intestinal schistosomiasis with hepatosplenomegaly than in urinary *schistosomiasis* ($t = 4.22$). This was the cause of the more significant increase in gamma globulin in the intestinal cases than in urinary bilharziasis ($t = 3.26$).

Discussion

There are many reports on the plasma protein pattern in bilharzial patients (RAGHEB, 1956, ISMAIL and SIDKY, 1962, and SABOUR and SALIB, 1966). However, equivocal information and only few data have been recorded concerning the role of immunoglobulins in schistosomiasis (FIORILLO, 1966, TORO-GOYCO, 1964, and DE WITT and WARREN, 1959).

The significant reduction in albumin (Table I) in children with urinary bilharziasis may be attributed to nutritional factors or to an impaired albumin synthesis. This is in agreement with the previous finding of HANNINO (1954) who stated that the poorer the food in protein, the lower the serum albumin and the higher the increase in gamma globulin.

The remarkable elevation in gamma globulin in urinary bilharziasis may have been due to nutritional factors (HANNINO, 1954) and to parasitic infection leading to a hyperactivity of the reticuloendothelial system (RAMIREZ et al. 1961 and GHANEM et al. 1970). Immuno-chemical quantitation showed that this elevation in gamma globulin was due mainly to the increased level of the IgG component. This observation confirms the results of TORO-GOYCO (1964).

The reduction in the albumin fraction in *S. mansoni* infection was more considerable than in *S. haematobium* infection and though it was not significant at the 5% level, yet one tends to attribute this greater reduction to a severer degree of impaired synthesis. This may be due to liver damage as confirmed by the positive liver function tests (Table III). These findings agree with those of GHANEM et al. 1970.

The average gamma-globulin level in hepatosplenomegalic cases was significantly higher than that in cases with urinary bilharziasis and the increase was due to marked elevation of the IgG component. This increase in IgG may be ascribed to the increased reticuloendothelial activity (ANDRADE and ANDRADE, 1966) in the synthesis of IgG since the other two immunoglobulin

classes (IgA and IgM) studied showed no such increase. This supports the earlier finding of TORO-GOYCO (1964). However, this is not in agreement with the finding of FIORILLO (1966) who reported an elevation in the beta–gamma region and attributed this to the increase of the IgM component.

The highly increased gamma globulin level in *S. mansoni* was the cause of the increased ESR and zinc sulphate turbidity (Table III), in agreement with the observation of ISMAIL and SIDKY (1962).

It should be stressed that children in the control group had relatively high gamma globulin levels. The same finding was reported by GHANEM et al. (1970). STAINER (1953) also mentioned that African students have more gamma globulin level than Europeans living in Africa. He concluded that the poorer the food in protein the lower the serum albumin and the higher the gamma globulin. On this assumption nutritional factors (HANNINO, 1954) should not be overlooked. It is suggested that in our study, nutritional deficiency may have been at a subclinical level as evidenced by the haemoglobin value which was only 82% in the control group (Table III). It was further reduced to 69% in cases with urinary schistosomiasis and to 56% in children with intestinal schistosomiasis associated with hepatosplenomegaly.

It has to be added that all the present cases including the controls were of low socio-economic classes and other types of subclinical deficiency.

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ESTIMATION OF IMMUNOREACTIVE INSULIN IN SERUM, HEPARINIZED AND EDTA-PLASMA

By

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Parallel estimations of immunoreactive insulin (IRI) in serum, heparinized and EDTA-plasma of identical human blood samples were undertaken. It was found that neither heparin (12.5 and 50 IU/ml blood) nor EDTA (5 mg/ml blood) applied for the prevention of clotting, produced any significant change in the IRI-plasma values, compared with those of sera derived from the respective samples. On these grounds EDTA- or heparin-plasma at the concentrations referred to above, as well as blood serum, are equally suited for measurements of IRI. Since, however, the immunoassays involve a certain, though insignificant difference between the serum-plasma values, it is advisable to prepare the blood samples serving for the same series of immunoassay under identical conditions.

High concentrations of heparin in buffer exceeding those used for the preparation of plasma (500 IU) yielded higher values for IRI. The levels of IRI measured in plasma in the presence of relatively high concentrations of EDTA (25 mg/ml blood) were found significantly lower. On the other hand, recovery of insulin from albumin-containing buffer was not affected by EDTA.

It is suggested that while heparin interferes with the interaction between insulin and insulin-binding antibody, a reaction which does not involve the presence of plasma, EDTA promotes this antigen-antibody reaction by the agency of some factor of the plasma.

Immunoassay of insulin, a method described by YALOW and BERSON [9] and further developed by HALES and RANDLE [3] was carried out by the original authors in heparinized plasma. As the procedure has been generally adopted, the estimates were done by the various authors in blood serum [1, 2, 6] or EDTA-plasma [6.] This is one of the factors accounting for the differences found by the various research teams at the level of immunoreactive insulin (IRI) before as well as after loading.

HENDERSON [4] observed that the concentration of IRI in heparinized plasma was higher than that in the same serum, moreover, that the addition of heparin to serum raised the original concentration of IRI. An IRI concentration of 40 μU/ml would give a mean difference of 13 μU/ml and a concentration over 40 μU/ml a difference of approximately 20 μU/ml if the plasma concentrations of the same blood samples were measured. In contrast, SOELDNER and SLONE [8] found a fall in the amount of IRI as the concentration of heparin was increased.

SHELDON and TAYLOR [7] examined the influence of EDTA on the IRI level and found that addition of EDTA to the serum prior to assay decreased

the apparent concentration of IRI, though not to the degree of significance Upon addition of 25 and 50 μU/ml insulin to human serum, EDTA failed to affect the respective insulin concentration. It has been suggested by these authors that EDTA might produce this effect by interfering with the complement.

In view of the inconsistency of these data, it was deemed of interest to examine whether measurements of IRI in serum, heparinized and EDTA-plasma of the same blood sample show any substantial difference.

Material and method

Heparin: 5000 IU/ml (Chemical Works Gedeon Richter Ltd., Budapest).

EDTA: disodium diamine-ethan-tetraacetate, analytical grade, Reanal, Budapest.

Human insulin: Wellcome Research Laboratories, Beckenham, England, Control No. K. 9228.

The blood samples of 2 ml were obtained from inpatients of the Department.

IRI was estimated by the double-antibody assay of HALES and RANDLE [3]. The complete kit of reactants for the assay was supplied by the Radiochemical Centre, Amersham, England. The original procedure was modified inasmuch as for the separation of the precipitate centrifugation was used instead of filtration and the precipitate was measured for ^{125}I-insulin activity in test tubes.

The results obtained in the individual groups were compared by Student's two sample *t* test.

Results

In the first part of the study, IRI was estimated in 60 samples of human blood sera parallel with plasma of the same blood containing 12.5 IU or 50 IU heparin per ml. The heparin concentrations referred to in this, as well as in the subsequent, part of the study are understood in terms of ml of whole blood sample. This is also valid for the EDTA concentrations. The present material was divided into three groups according to the concentration of IRI. This was prompted by earlier findings of HENDERSON [4], as well as by the fact that the double antibody immunoassays give the most reliable values at concentrations ranging below 40 μU/ml, fairly accurate values at levels of 41 to 80 μU/ml, whereas over the level of 81 μU/ml the results are no longer reliable. This is reflected by the considerable scatter of the figures seen in Table I.

These studies failed to reveal any significant difference between the IRI values estimated in serum and in 12.5 IU/ml or 50 IU/ml heparinized plasma at any of the concentration levels referred to above.

In order to eliminate the presence of plasma proteins, in the further course of the study 40, 79 and 116 μU/ml human insulin was measured into the buffer used for the immunoassay of IRI in the presence of 12.5, 50, 100 and 500 IU/ml heparin, respectively (Table II).

Table I

Simultaneous concentrations of IRI in μU/ml ($\bar{x} + s$) found in blood serum, and heparinized plasma in 60 patients. Data grouped on the basis of three concentration ranges of IRI

Insulin concentration	Serum	Heparin concentration of blood samples	
		12.5 IU/ml	50 IU/ml
< 40 μU/ml	24.2±7.7	25.1±9.1	26.1±12.4
n = 16		p < 0.7	p < 0.8
		p < 0.7	
41–80 μU/ml	56.3±12.2	59.7±16.6	55.9±10.3
n = 22		p < 0.4	p < 0.4
		p < 0.9	
> 81 μU/ml	130.2±39.9	142.8±50.6	140.5±42.2
n = 22		p < 0.3	p < 0.8
		p < 0.4	

With insulin at concentrations of 40 μU/ml, it was only with 500 IU/ml of heparin that significantly higher IRI values were found as compared with the heparin level of 12.5 IU/ml. With insulin concentrations of 79 μU/ml, too, significantly higher IRI values were found in the presence of 500 IU/ml heparin. This significant difference was also demonstrable in comparison with all lower heparin concentrations studied. In this group the IRI values were significantly higher in the presence of 100 IU/ml heparin, too, as compared with those found in the presence of 12.5 IU/ml heparin. With insulin concentrations of 116 μU/ml, the IRI values found in the presence of 500 IU/ml heparin were significantly higher as compared to all samples with heparin at lower concentrations.

In the further course of the study it was examined whether the figures of IRI were affected in any way by EDTA used for the prevention of clotting. The values of immunoassayable insulin in serum, 50 IU/ml heparinized plasma and 5 mg/ml and 25 mg/ml EDTA-plasma of the same venous samples taken 2 to 3 hours after the first morning meal were compared (Table III).

A reduction in the IRI concentration was demonstrable in the 25 mg/ml plasma samples, a difference which was found significant in comparison with the IRI levels of blood sera and of 50 IU/ml heparinized plasma. In order

Table II

40, 79 and 116 μU/ml human insulin, estimated in the presence of various concentrations of heparin. Heparin concentrations refer to the solutions of human insulin IRI values given in μU/ml ($\bar{x} \pm s$)

Insulin concentration	Heparin concentration			
	12.5 IU/ml	50 IU/ml	100 IU/ml	500 IU/ml
40 μU/ml	n = 12	n = 12	n = 16	n = 12
		p < 0.2		
			p < 0.2	
			p < 0.9	
		p < 0.1		p < 0.1
	41.0±3.8	43.1±3.8	42.9±3.9	44.7±2.9
			p < 0.01	
79 μU/ml	n = 12	n = 13	n = 13	n = 13
		p < 0.5	p < 0.05	
	83.0±6.6	85.0±6.2	90.4±9.3	100.4±7.0
		p < 0.02		p < 0.001
			p < 0.001	
		p < 0.001		
116 μU/ml	n = 12	n = 12	n = 14	n = 12
		p < 0.6		
		p < 0.7	p < 0.4	
	123.3±10.7	125.5±18.5	121.5±9.1	143.8±15.8
				p < 0.001
			p < 0.01	
		p < 0.001		

to find out whether EDTA inhibition takes effect through the plasma proteins, we examined the recovery of 40 and 80 μU/ml insulin from buffer at EDTA concentrations of 1, 5 and 25 mg/ml (Table IV). None of the concentrations studied were found to affect the recovery of insulin from the buffer solution.

Discussion

For the estimation of IRI, blood serum, heparinized or EDTA-plasma has hitherto been employed. The differences in normal IRI in the fasting state as well as after glucose tolerance tests might be connected with the

Table III

Simultaneous concentrations of IRI measured in serum, 50 IU heparinized plasma, 5 and 25 mg/ml EDTA-plasma of identical blood samples obtained from clinical patients (μU/ml, $\bar{x} \pm s$, $n = 15$ in each group)

Serum	Heparinized plasma 50 IU/ml	EDTA-plasma	
		5 mg/ml	25 mg/ml
	$p < 0.4$		
$p < 0.4$		$p < 0.1$	$p < 0.1$
41.2 ± 16.1	45.2 ± 10.3	37.0 ± 16.3	27.9 ± 13.2
		$p < 0.001$	
	$p < 0.01$		

Table IV

Recovery of 40 and 80 μU/ml insulin from its solutions in buffer at EDTA concentrations of 1, 5 and 25 mg/ml, respectively (μU/ml, $\bar{x} \pm s$, $n = 20$ in each group). There is no significant difference within the groups

Insulin concentration	EDTA concentration		
	1 mg/ml	5 mg/ml	25 mg/ml
40 μU/ml	40.3 ± 3.9	40.4 ± 4.2	40.2 ± 2.9
80 μU/ml	79.2 ± 3.4	79.1 ± 3.4	80.0 ± 3.5

procedure itself or with the very properties of the material (serum or plasma) subjected to the process.

For the interpretation of the higher values of immunoassayable insulin in blood plasma as compared with those in serum, HENDERSON [4] alleged the possibility of "trapping" of a proportion of the insulin during the conversion of fibrinogen to fibrin. A comparison of the serum-plasma values obtained in the present study failed to support this possibility. It seemed more probable that heparin, as a molecule of negative charge, inhibits the binding of insulin to insulin-antibody which would result in a shift to higher values inherent in the procedure itself. However, in the literature on heparin we have found no indication of a possible inhibitory effect of the substance on the antigen-antibody reaction.

From the finding that heparin at minor concentrations (12.5 IU/ml, 50 IU/ml) failed to affect the IRI level either in plasma or in the buffer solution to any significant extent, it may be inferred that the effect of heparin is unre-

lated to the plasma proteins. On the evidence of the present study, heparin gives no false positive IRI values unless present at excessive concentrations which are not used for the preparation of heparinized plasma. The interpretation that in the course of IRI bioassay, heparin acts by interfering with the binding of insulin to insulin-antibody finds support in the present data, too.

In the study of HENDERSON [4], addition of EDTA to plasma resulted in slightly lower IRI values. In the present study, lower values attaining the level of significance were confined to EDTA concentrations of 25 mg/ml. EDTA being a potent chelating agent, may act by stabilizing the binding between insulin and insulin-antibody, which would shift the results of the measurements to lower values. Since, however, in case of buffer solutions, i.e. in the absence of plasma proteins, EDTA failed to affect the IRI bioassay even at concentrations of 25 mg/ml, we tend to subscribe to the interpretation offered by SHELDON and TAYLOR [7]. On the evidence of our results we find it more likely that reduction of the IRI values by EDTA takes effect indirectly through some plasma factor rather than by a direct inhibition of the reaction between insulin and insulin-binding antibody.

To the question "Serum insulin or plasma insulin?" raised by HENDERSON [4] the results of the present study provide the following answer: heparin-plasma (maximum concentration 50 IU/ml blood), or EDTA-plasma (maximum concentration 5 mg/ml blood) are equally suited for the bioassay of IRI, with the understanding that EDTA-plasma yields slightly lower, heparinized plasma slightly higher values than does blood serum. These differences have, however, not proved significant. Heparin concentrations of blood plasma over 50 IU/ml give significantly higher, EDTA concentrations over 5 mg/ml significantly lower, IRI values than in reality

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EFFECT OF CLOFIBRATE ON AORTIC GLYCOSAMINOGLYCANS AND PROTEINS AND ON SERUM LIPID LEVELS IN EXPERIMENTAL ATHEROSCLEROSIS

By

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The effect of clofibrate has been studied in cholesterol-fed rabbits.

1. A very highly significant reduction of the serum lipid level could be established after 2 weeks of clofibrate treatment.

2. In the aorta of cholesterol-fed rabbits a slight increase of glycosaminoglycan concentration and intensive lipid deposition was found by histological, histochemical and electronmicroscopical methods. By thermal analysis the presence of aortic glycosaminoglycan–serum beta-lipoprotein complexes and the accumulation of "abnormal" crosslinks within the fibrillar tissue-proteins could be demonstrated in the intima of the animals.

3. As a result of clofibrate treatment a significantly increased glycosaminoglycan content was found in the aorta of rabbits with experimental atherosclerosis. At the same time a decrease could be established in the amount of aortic glycosaminoglycan–beta-lipoprotein complexes. The anti-atherogenic action of clofibrate influenced the pathologically changed protein structure as well: the cholesterol induced alterations proved to be partly reversible.

Clofibrate is known to have a blood lipid lowering effect (in cholesterol-sclerosis e.g. [1–4]) but few data are available on its possible action on the tissue level. In the present investigation the effect of clofibrate has been studied on the cholesterol-induced gross, microscopical and biochemical alterations taking place in the arteries of rabbits.

Material and methods

Rabbit serum and tissue

Effect of clofibrate on cholesterol feeding. In 10 male rabbits aged about 10 months, experimental atherosclerosis was produced by feeding 2 g of cholesterol daily for 18 weeks. The animals received 50 mg/kg body weight of clofibrate (Miscleron, Chinoin, Budapest) orally three times weekly, starting 12 weeks after feeding the high cholesterol diet and continuing for 6 weeks (group I).

Control experiments. Five rabbits were fed on a cholesterol rich diet, but did not receive clofibrate (group II), 15 rabbits of the same age were kept on stock diet; 10 animals were given clofibrate for 6 weeks as described above (group III), 5 served as controls without any treatment (group IV).

Collection of samples. Blood was drawn from the ear vein of all the animals at the beginning of clofibrate administration and 2 and 6 weeks thereafter.

Arterial specimens from the rabbits were collected immediately after killing the animals.

Table I

Mean serum lipid values for cholesterol-fed rabbits before and

Duration of exp. (weeks)	Triglyceride			Total cholesterol			Ester
	mg per 100 ml	change per cent	P_t	mg per 100 ml	change per cent	P_t	mg per 100 ml
0	408.0±70.3			494.0±227.8			313.0±94.5
2	211.0±60.1	−48.4	< 0.001	226.0±138.5	−54.4	< 0.01	123.3±83.6
6	166.4±35.8	−59.2	< 0.001	115.0±47.5	−76.7	< 0.001	92.6±43.4

* Mean ± S. D.

Chemical analysis

The lipids were extracted from the sera with chloroform–methanol 2 : 1 (v/v) according to the method of FOLCH [5]. Total cholesterol, ester cholesterol and free cholesterol were determined by the method of SCHÖNHEIMER and SPERRY [6]. The extract for phospholipids was ashed and the phosphorus determined by the method of FISKE and SUBBAROW [7]. Triglyceride was determined by the method of VAN HANDEL and ZILVERSMIT [8].

Thermal analysis

For the determination of glycosaminoglycan (GAG) concentration in aortic tissue [9], as well as for the characterization of the stability of structural proteins [10] a thermoanalytical method, derivatography [11], was used. Intima samples, cut into small pieces with a scalpel, were prepared for analysis by drying for 3 hrs in 50% saturated air with constant flow rate. Parallel samples were delipidated by extraction in 25% ether in ethanol for 2 days, followed by 50% chloroform in methanol for 2 days, both at room temperature, and then dried. Lipids, bound to the fibrillar protein-moiety of the material, were extracted with chloroform—methanol—hydrochloric acid (2 : 1 : 0.03).

Thermoanalytical studies were carried out using a Paulik–Paulik–Erdey MOM derivatograph. The instrument measured and recorded simultaneously the weight change (TG curve), rate of weight change (DTG curve), and enthalpy change (DTA curve) as a function of temperature. The samples were weighed (approximately 100 mg) into a platinum crucible. The heating rate was 10°/min. up to 900 °C.

Statistical analysis was performed using Student's *t* test.

Histological and histochemical investigations

A general examination was made of internal organs to exclude any disease other than that induced by the experiment. Detailed examination of the heart, kidneys, aorta and the great arteries was carried out. After cutting the aorta on its whole surface the per cent of sclerotic alterations was estimated. The material for histological and histochemical examination was removed from the arcus, thoracal and abdominal part of the aorta. The samples were fixed in 10% neutral formol and embedded in paraffin. Sections 6 μ thick were cut. The following staining methods were used: haematoxylin-eosin, van Gieson, alcian blue, Hale-periodic acid Schiff reaction (Hale-PAS), trichrom staining. For demonstrating the elastic elements, orcein reaction was used partly without oxidation, partly after potassium monopersulphate ($KHSO_5$) — Oxone^R — pretreatment, as described by RANNIE [12].

Electronmicroscopical studies

Aortic samples were fixed in 1% buffered osmium tetroxide, pH 7.2. Ultrathin sections embedded in Durcupan were cut at 0.1 μ, stained with 6% uranyl acetate and lead citrate, and observed with a Tesla BS 242 E type electronmicroscope.

during clofibrate treatment and their statistical evaluation

cholesterol		Free cholesterol			Phospholipid		
change per cent	P_t	mg per 100 ml	change per cent	P_t	mg per 100 ml	change per cent	P_t
		211.0±81.9			240.6±74.9		
−60.8	< 0.001	102.8±81.0	−51.4	< 0.001	146.6± 7.6	−39.0	< 0.001
−70.5	< 0.001	22.5±10.6	−83.5	< 0.001	143.5±20.8	−40.5	< 0.001

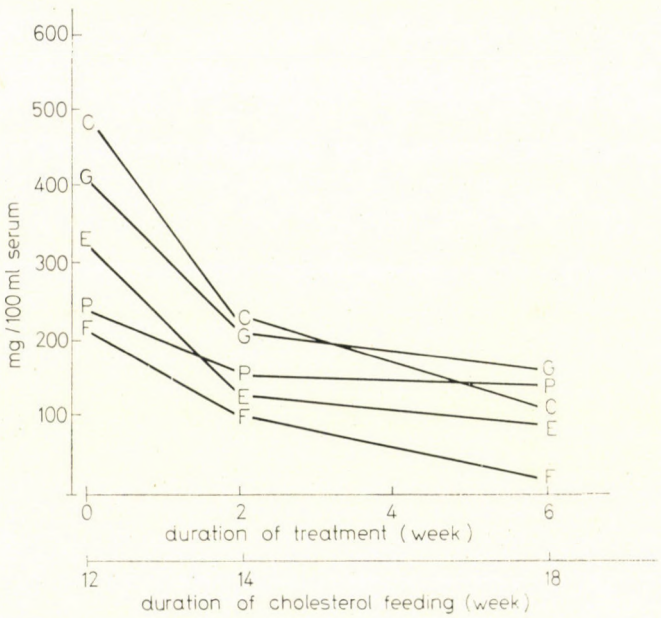

Fig. 1. Average concentration of serum lipids in clofibrate-treated cholesterol-fed rabbits. G = triglyceride; C = total cholesterol; E = ester cholesterol; F = free cholesterol; P = phospholipid

Results

Sera

Fig. 1 and Table I show the effect of clofibrate treatment on the average serum lipid concentration in cholesterol-fed rabbits. After 2 weeks of repeated administration of the drug, a mathematically very highly significant decrease in the concentration of all the investigated serum lipid fractions was observed. Continued treatment resulted in a further, somewhat less expressed, but still very highly significant decrease of the lipid level.

In group II (cholesterol-fed rabbits without clofibrate treatment) a slight but not significant further increase of lipid content could be demonstrated.

In control rabbits on stock diet, clofibrate administration did not influence the serum lipid composition.

Aortic specimens

Results of thermal analysis. In Fig. 2 typical thermoanalytical curves obtained for rabbit aortas are presented.

The first process, indicated by the DTG curves was the departure of the water content of the air-dried sample between 20° and 180°C, resulting in a 11.0%, 10.0% and 8.0% weight loss in the aorta samples of control, cholesterol-fed and cholesterol-fed + clofibrate-treated rabbits, respectively, as calculated from the TG curves. The process was endothermal (negative DTA peak). The samples were thermostable up to 200°C.

Between 200° and 300°C, as reported previously [13], thermal decomposition of structural GAG-s took place, characterized by well defined peaks on the DTG curves. (In the healthy rabbit aorta intima at 240°C, as demonstrated in Fig. 2a.) The cleavage of chemical bonds in GAG components was always accompanied by a weight change of the sample. The percentual weight loss, calculated from the TG curves, was proportional to the concentration of the corresponding GAG in the investigated tissue [9]. It was reported previously [14] that when GAG-s are bound in the form of beta-lipoprotein (beta-LP) complex, the 240°C maximum is not observable on the DTG curves. In the arteries of rabbits with cholesterol induced atherosclerosis a part of intimal GAG-s had presumably become bound to lipoproteins during the atherogenic process; the significant decrease of the characteristic peak on the DTG curve (Fig. 2b) was possibly due to this phenomenon. The diminution of the maximum (i.e. the formation of the GAG-beta-LP containing lesion) was definitely smaller in the aorta of cholesterol-fed + clofibrate-treated animals (Fig. 2c). Lipids bound to structural GAG-s could be extracted with organic solvents.

Decomposition of the GAG-s was complete at 290°C and a next thermal process started, the decomposition of the structural proteins. This was found to take place in two successive temperature intervals. The first process reached its maximum rate at about 300°C and resulted in the partial cleavage of peptide bonds [15] and in the formation of peptide subunits of low molecular weight and of various pyrrol-type compounds. The (very exotherm) decomposition of these newly formed compounds took place between 450° and 800°C. It could be proved in a series of experiments that the thermostability of the sample within this temperature interval is correlated with the amount of covalent crosslinks involved in the formation of the original protein mole-

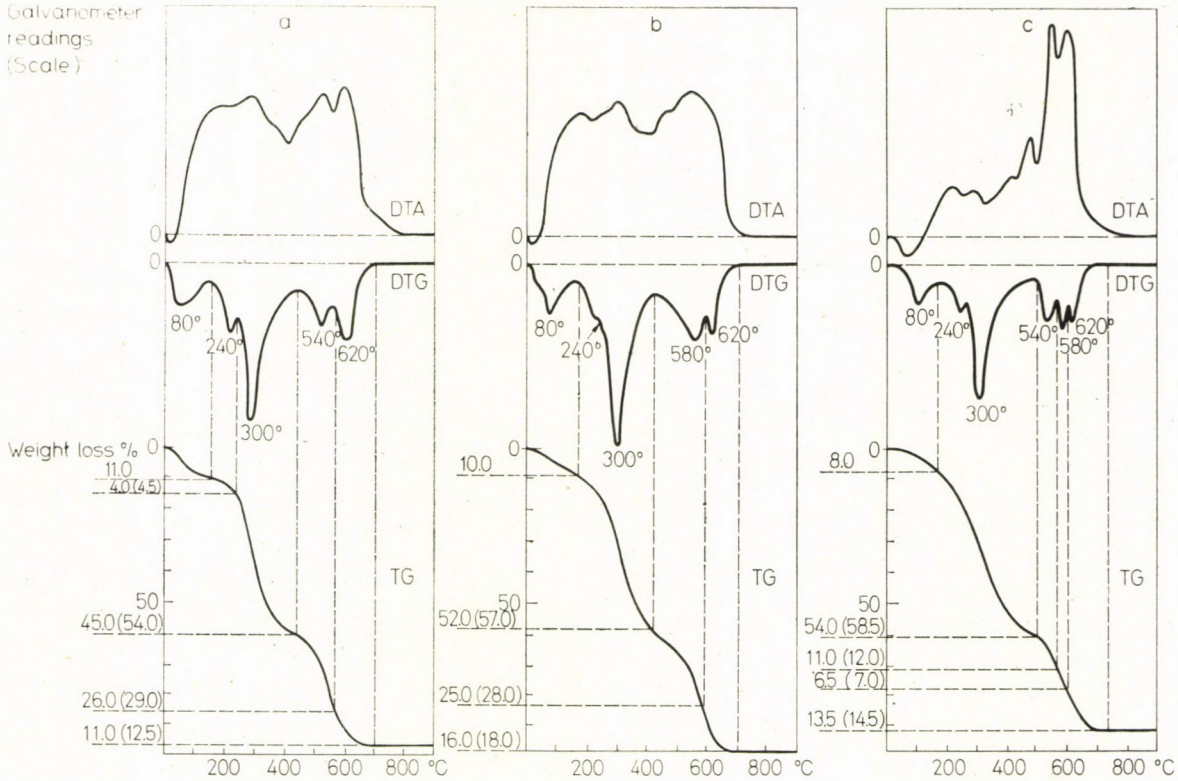

Fig. 2. Thermal decomposition curves of rabbit aortas: *a)* healthy intimal tissue; *b)* intima of cholesterol-fed rabbit; *c)* intima of clofibrate-treated cholesterol-fed animal

cules [10]. On the DTG curves of adult healthy rabbit aorta 2 maxima could be distinguished in this interval, at 540° and at 620°C (Fig. 2a). Proteins in the aorta of cholesterol-fed rabbits were of higher thermostability, DTG peak-values were shifted to 580° and 620°C (Fig. 2b). As a result of clofibrate treatment this cholesterol-induced increase of thermostability was partly reversible: on the DTG curves of the aorta of this group of animals all the three DTG peaks (540°, 580°, 620°C) were detectable (Fig. 2c). According to our assumption, the pathological increase of protein stability might be partly due to a deposition of some lipid compounds into the aortic fibrillar protein molecules. This hypothesis was supported by the result of an experiment *in vitro:* the intimal tissue obtained from cholesterol-fed rabbits was treated with an acid organic solvent known to extract protein-bound lipids. Thermoanalytical curves of these samples resembled those of the healthy aortas.

Calculations performed on the basis of the TG curves permitted quantitative evaluation of the results of the above described thermoanalytical studies.

Table II

Glycosaminoglycan content of rabbit aorta

Group	GAG concentration	
	Mean	± S. D.
Control	1.9	0.44
Cholesterol-fed	2.1	0.49
Cholesterol-fed + clofibrate-treated	3.6	0.54

Table II contains the average concentration of aortic GAG-s, calculated from the decomposition curves of delipidated aortas. In the intima of cholesterol-fed rabbits a slight but not significant increase could be observed. Clofibrate treatment of cholesterol-fed rabbits resulted in a further, significant increase ($p < 0.01$) of the aortic GAG content.

Table III

Percentual distribution of protein subunits

Group	Characteristic DTG peak		
	540°C	580°C	620°C
Control	70.0	—	30.0
Cholesterol-fed	—	72.0	28.0
Cholesterol-fed + clofibrate-treated	39.7	32.4	27.9

The amount of the components decomposed between 450° and 800°C characteristic for the stability of structural proteins could also be calculated on the basis of the TG curves. The average distribution of the material within this temperature interval is summarized in Table III.

In the aorta of rabbits without cholesterol feeding, clofibrate treatment did not induce any alteration.

Histological and histochemical findings

Gross alterations. Data illustrating the grade of sclerotic changes are presented in Table IV.

The aorta of the rabbits fed with cholesterol showed severe sclerotic alterations. The most remarkable changes covering almost the whole surface were observed at the aortic arch in all cases. The alterations at the thoracic part of the aorta were somewhat less extensive but still remarkable. The degree of sclerosis was slightest at the abdominal part of the aorta. At the origin of the intercostal, renal, and mesenteric arteries, the damaged endothelium was covered in some places by thrombotic changes. The grade of sclerosis was only in one case less than 45% of the whole aortic surface.

Table IV

Sclerotic alterations on the intimal surface of rabbit aortas

Group	Case No.	Proportion of area involved, per cent
I. Cholesterol-fed + clofibrate-treated	1	5
	2	20
	3	60
	4	5
	5	45
	6	60
	7	5
	8	5
	9	5
II. Cholesterol-fed	1	75
	2	80
	3	60
	4	45
	5	25

In the clofibrate-treated cholesterol-fed group it was conspicuous that only in three cases out of nine the sclerotic alterations covered more than 45% of the aortic surface. The sclerotic changes in this group were again most marked at the arch, the thoracic part showed a moderate sclerosis while in the abdominal part only slight alterations could be observed.

The aortas in groups No. III and IV did not show any histological alteration.

Microscopy

In cholesterol-fed rabbits the aortic intima was thickened in the whole surface of the arch, in a considerable part of the thoracal aorta and in a small part of the abdominal aorta. The intimal thickening was produced by foam cells arranged in 8—10 rows (Fig. 3). The elastic membrane was broken up, in some places duplicated. Near the elastic membrane a cellular proliferation could be observed (fibroblast-like cells, in some places cellular elements similar to smooth muscle cells). At the basis of these alterations an increase of GAG-s could be seen. The staining reaction was especially remarkable on the basis of the less severe alterations. Hale-positivity was extended to the inner part of the media. Orcein staining showed the increase of the amount of the elastic fibres on the basis of the sclerotic changes. The number of the fibres stained with orcein following oxidation was even greater. In the media under the

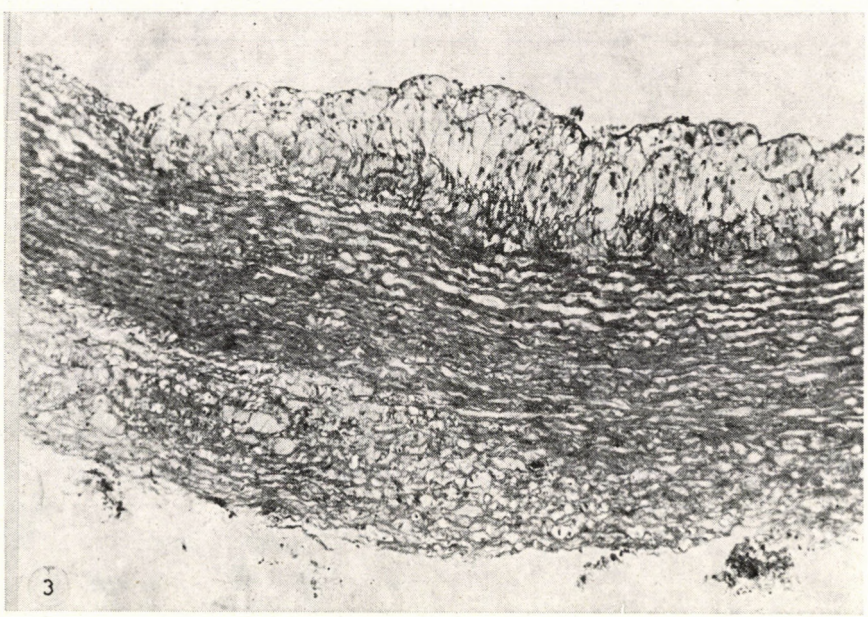

Fig. 3. Atheromatous lesion from cholesterol-fed rabbit aorta. The intimal foam cells are arranged in 8—10 rows. (Haematoxylin-eosin, ×96)

severe sclerotic alterations there were in some places necroses, with a marked cellular reaction, in other places small scars and an increase of collagen fibres were observed.

In group No. I the aorta (except 3 cases) showed only slight histological alterations. The intimal thickenings consisted also here of foamy cells situated in not more than 3—4 rows. At the basis of the changes near the media the amount of the mesenchymal cells was increased. The elastic membrane was crumbled and duplicated. The staining reaction for GAG-s was more expressed than in the cholesterol-fed group not treated with clofibrate. In the more severe cases a scar tissue with increased amount of collagen could be observed.

Electronmicroscopical observations

The endothelial cells in groups No. III and IV were 3—4 μ thick and revealed many microvilli, marginal folds, pinocytotic vesicles, vacuoles, lysosomes, endoplasmic reticulum, microfibrils, intercellular junctional complex and intercellular contact layer.

In the rabbits fed on cholesterol (group No. II) the intimal foamy cells contained a large quantity of lipid distributed in vesicles approximately 1 μ in diameter. Most of the intracellular lipid particles were surrounded by a limiting membrane (Fig. 4a). Within these cells four morphologic forms of lipid inclusions could be identified: ring, solid, reticulated and lysosomic forms. In the subendothelial layer in addition to the foamy cells a marked accumulation of plasma elements and infiltration of mesenchymal cells (fibroblasts, smooth muscle cells) was found; infrequently mononuclear cells, probably lymphocytes and monocytes also occurred. The fibrillar elements, occupying the intercellular space, were readily classified into several types. Most abundant was collagen, easily recognized by its characteristic banding. The amount of collagen was very remarkable (Fig. 4b). Bundles of fibrin were usually present in small amounts mixed with other elements, principally collagen. Some areas were occasionally occupied by a fine fibrillar material, often blending the basement membrane around the smooth muscle cells. Elastic tissue could also be recognized. Extracellular lipid could be identified in the form of small, irregularly shaped, deeply osmiophilic particles roughly outlined by clear areas, presumably representing spaces from where lipid has been removed by the techniques involved in tissue preparation.

In the aorta of rabbits treated with cholesterol and clofibrate (group No. I) the endothelial cells showed a slightly marked dilatation of the roughly surfaced endoplasmic reticulum, moderate increase of microvilli and vacuoles. The number of foamy cells was significantly less than in group No. II. The lipid inclusions in these cells could be observed only in a minor quantity. Fibroblast was the most characteristic cell type in this group. These cells had slightly

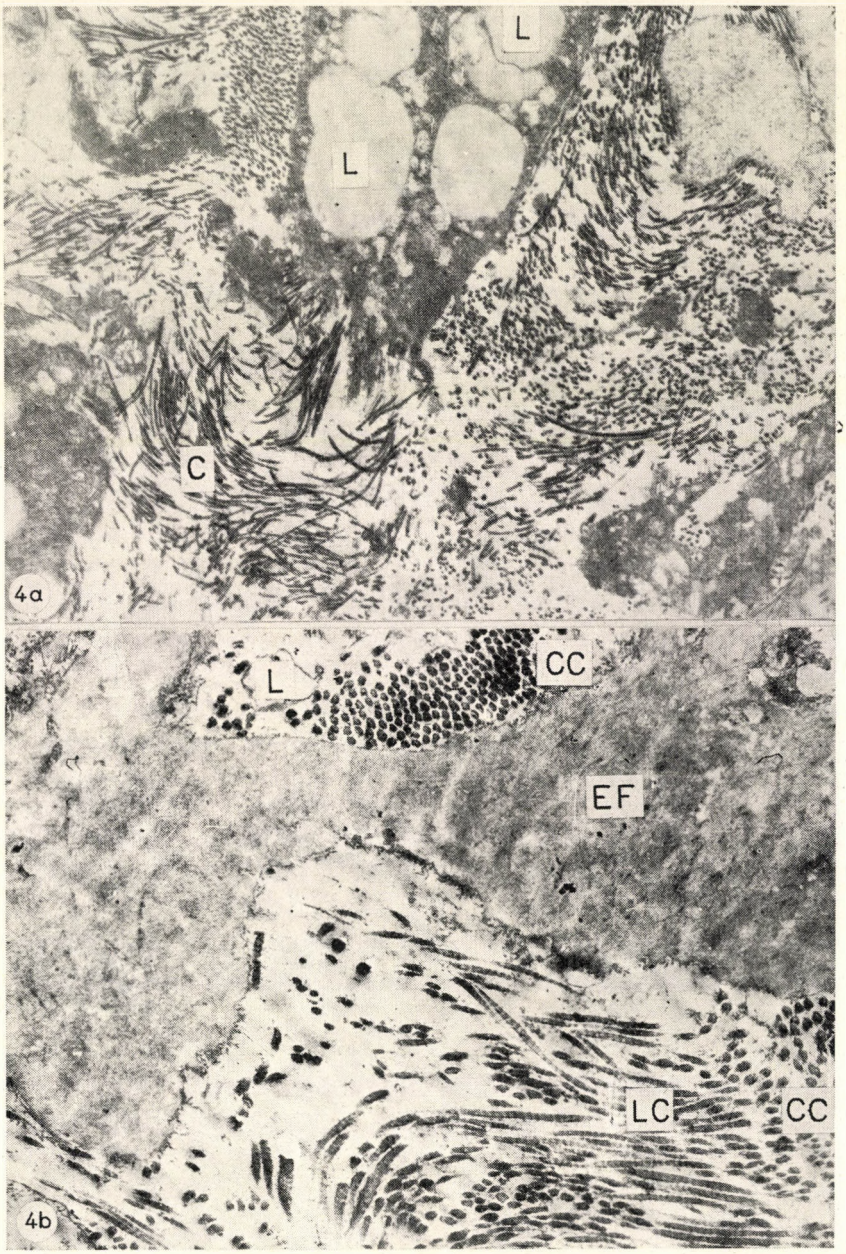

Fig. 4. Electron micrographs of intimal sections from cholesterol-fed rabbit aorta. *a)* Portions of foam cells with association of collagen (C) fibres. The ring of osmiophilic material with a vacuolated centre surrounded by a membrane is the type of lipid (L) occurring most frequently (×14,580). *b)* An acellular area. Two types of intercellular material can be recognized. The collagen fibrils appear in cross section (CC) and in longitudinal section (LC) beside the elastic fibres (EF). The small vacuoles represent extracellular lipid (L) material. (×19,200)

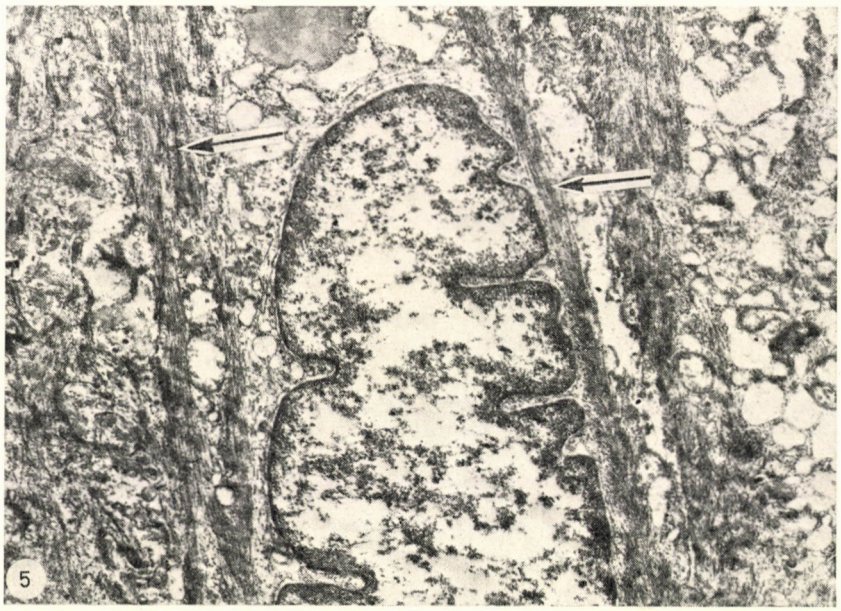

Fig. 5. Electron micrograph of a section of intimal tissue obtained from the aorta of a rabbit treated with cholesterol+clofibrate. Portion of typical fibroblast with a slightly dilated endoplasmic reticulum. The arrows show the microfibrillar material. (×14,580)

delated endoplasmic reticulum and they contained a fine microfibrillar material near the nucleus (Fig. 5). In some areas smooth muscle cells were observed. In the intercellular space slight accumulation of lipid droplets and plasma components occurred, furthermore the presence of small amounts of collagen and elastic tissue could be demonstrated.

Discussion

The reports on the serum lipid lowering effect of orally administered clofibrate could be confirmed in cholesterol-fed rabbits.

Furthermore, some information was gained concerning the influence of clofibrate on the arterial lipid deposits and on the structural changes taking place in the intimal tissue during the atherogenic process.

In our experiments, results of thermal analysis showed a slight increase in the GAG content of the aortas in cholesterol-fed rabbits. These findings could also be demonstrated by histochemical methods and were in good agreement with literary data [16—18]. Lipid accumulation, observed macroscopically and microscopically as well as electronmicroscopically might partly be due to the formation of complexes between the aortic GAG-s and serum beta-lipoproteins. This concept seems to be confirmed by the thermoanalytical demonstration of GAG-beta-LP complexes in the atherosclerotic aorta. A

further alteration of significance took place in the fibrillar protein components of the intimal tissue of cholesterol-fed animals: the quantity of elastic and collagen fibres increased (as demonstrated by the orcein-stained histochemical and by the electronmicroscopical sections, respectively). Within the collagen and elastic fibrils an accumulation of intermolecular crosslinks could be established by the thermoanalytical investigations. The appearance of "abnormal" crosslinks characterized by the 580°C DTG peak could be distinguished from the covalent crosslinks known to be formed in the course of the physiological ageing process. This difference at the molecular level between senescence and atherogenesis was previously assumed by other authors [19—20]. The thermoanalytically demonstrated effect of the *in vitro* extraction of the intima with an acid organic solvent seems to support the hypothesis that bound lipids associated with the collagen fibres might be responsible for the above discussed increase of the structural stability of the latter [19—21]. The parallelism observed between the increase of GAG concentration and of the intensity of protein fibril formation, supports the idea of the alleged correlation between GAG and collagen synthesis [22].

Following clofibrate administration a significantly increased GAG content was found in the rabbit aorta, probably as part of a repair mechanism. At the same time a decrease could be established in the amount of GAG-beta-LP complexes. The anti-atherogenic action of clofibrate influenced the pathologically changed protein structure as well: the cholesterol-induced alterations proved to be partly reversible.

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WASTING DISEASE AND TETANY FOLLOWING NEONATAL PINEALECTOMY

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The physiological parameters of development and the microscopic appearance of thymus, thyroid, parathyroids, ultimobranchial body and bursa of Fabricius have been studied in chicks (Gallus domesticus) after pinealectomy performed on the first day after hatching. Arrested development and excessive shedding of the plumage were found as a sign of wasting disease resulting from atrophy of the thymus and bursa. Tetany ensued in 50% of the chicks and proved fatal to 35% in the absence of any abnormality of the parathyroids. The possible mechanism of the phenomenon and the role of the pineal body in the regulation of the glands of entodermal origin are discussed.

The relationship between the pineal body and the other endocrine organs has been a subject of much debate. The activity of the pineal hormone, melatonin, in higher animals is uncertain, and even in amphibia it is confined to the pigment cells. Therefore, in spite of being provided with connections to the central nervous system, the central regulatory role of the pineal body is still unclear [1, 20]. The significance of the pineal body and the effect of pinealectomy on the endocrine organs of entodermal origin (thyroid, parathyroids, thymus, islets of Langerhans) have been borne out by recent research [1, 13, 20] including the studies of the present authors [6, 8, 9, 10, 11, 14] which have furnished *in vitro* evidence of a feedback mechanism [11]. All these allow to regard the pineal body as a regulator of all those endocrine glands which are outside the control of the pituitary, the more so as these glands form a particular group in the embryological respect, inasmuch as they are of ecto- or entodermal origin and their hormones are not of the steroid type. The thyroid might be regarded as the only exception since it seems to have been "reintegrated" into the pituitary system through the TSH, though being subject to the control of the pineal body [6, 8, 13].

While in mammals the branchiogenic organs migrate to different sites of the body during development, in birds they line up within the region of the neck [7, 19]. Moreover, the ultimobranchial body developing from the fifth gill-pouch is of easier access in birds owing to its separate site, in contrast to its situation in mammals where it fuses with the thyroid or with the thymus, thus interfering with the interpretation of studies concerned with the endocrine organs originating from these structures [3, 17, 18, 19]. This makes birds particularly suitable for studies on the relationships between the pineal body

and the branchiogenic endocrine organs. It is expected to obtain a clearer insight into these relationships by performing pinealectomy in the early neonatal period and to register its effects at some later time, so as to gain information on the development of the organs which have not yet reached full maturity at the time of the intervention.

Material and methods

Twenty test and ten sham-operated Leghorn chicks have been studied. The test subjects were operated upon in ether anaesthesia under stereomicroscopic control within 48 hours of hatching. Through an incision of the deplumed skin after excision of the marginal portions of the parietal and occipital bones the pineal body was removed together with its stem. For haemostasis fibrin foam was used, but no intracranial pack was left after the operation. Surgical lethality was 15 to 20%. Except for the pinealectomy, the sham-operation was identical in all respects.

The chicks were kept under observation and the surviving 13 pinealectomized and 10 control birds were killed by exsanguination between six weeks and eight months. The branchiogenous organs, adrenals, ovaries and bursa of Fabricius were processed for histological study. The organs were fixed in Bouin's fluid, embedded in paraffin, cut at four levels, stained with haematoxylin-eosin, iron-alum alcianblue safranin, Giemsa's stain, Gomori's aldehyde-fuchsin and PAS, respectively.

Efficiency of the intervention of involuntary surgical injuries was checked after sacrificed intracranial inspection. The intervention proved successful and the gross appearance of the brain was found normal in all cases.

Results

Seven of the 20 pinealectomized animals died with tetanic seizures six weeks to six months after the intervention. Three further test animals also developed tetany (Fig. 1).

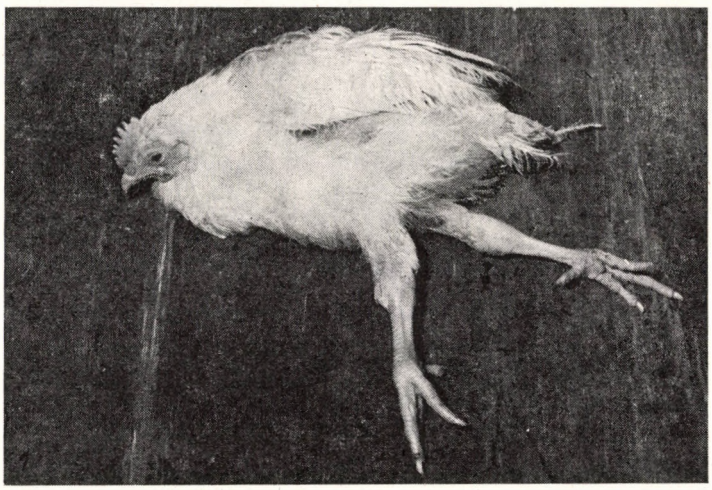

Fig. 1. Tetany in a chick six weeks after pinealectomy. Typical tetanic attitude, sparse plumage

The animals which died spontaneously in the end, failed to grow normally and exhibited atrophy. In the pinealectomized animals, particularly in those

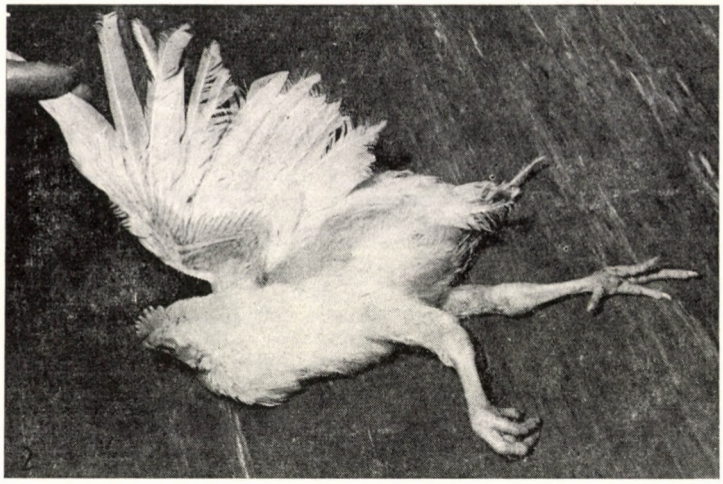

Fig. 2. Tetanic chick. On the inner side of the wing the feathers are thinly scattered, most of the quill-feathers of the tail have fallen out

Fig. 3. Ovary of excessive size with marked furrowing of its surface in a pinealectomized animal

which died spontaneously, excessive shedding of feathers with naked patches in the plumage was noted. Shedding started at the inner surface of the wings and on the adjoining surface of the body, involving the quill feathers in grave cases (Fig. 2).

In one of the birds the ovary attained many times its normal size and differed also in the gross appearance of its surface from the ovaries of the other animals (Figs 3, 4).

Development of the controls was entirely normal, with no sign of tetany and no abnormal shedding of feathers.

In the pinealectomized animals the thymus was atrophic. Its medullary substance was broadened at the expense of the cortex. There was an excessive,

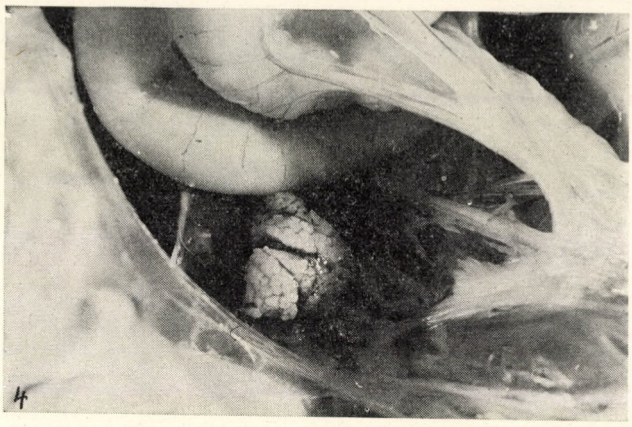

Fig. 4. Ovary of control animal of the same age

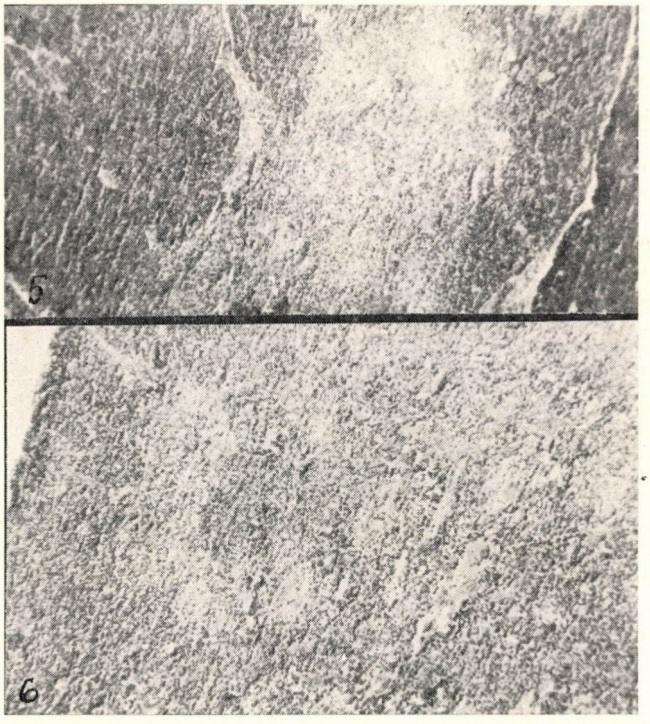

Fig. 5. Thymus of control animal. Note the broadened cortical area and the sharp corticomedullary boundary. H + E, ×100

Fig. 6. Thymus of pinealectomized animal. Narrowing of the cortical area, its boundary is no longer distinct. H + E, ×100

in some cases nearly total, depletion of lymphoid elements. The medullary substance had lost its normal pattern, there were numerous epithelial structures similar to Hassal's corpuscles and cysts of smaller size (Figs 5, 6, 7, 8).

The epithelium of the thyroid was found taller and the colloid of the acini sparser than in the controls. The epithelium was often of a stratified appearance and the interacinic connective tissue was scanty (Fig. 9).

The parathyroids, apart from appearing larger than in the controls, displayed no abnormality.

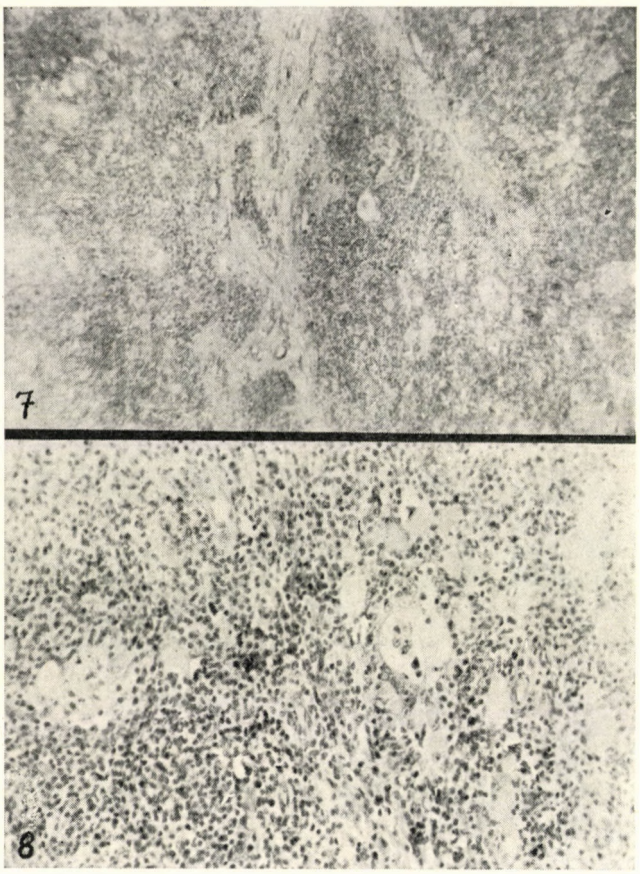

Fig. 7. Thymus of pinealectomized animal. Broadening of the medullary substance, depletion of lymphoid elements, disruption of the architecture. H + E, ×100
Fig. 8. Thymus of pinealectomized animal. Hassal's corpuscles consisting of a few cells and cysts in the medulla. H + E, ×200

In the pinealectomized animals the ultimobranchial body contained numerous cysts, in many cases with parathyroid elements (Figs 10, 11). Histochemical procedures failed to reveal any significant cytological difference from the controls.

Th bursa of Fabricius was atrophied, its lymphoid population was considerably reduced, its follicular structure was broken up (Fig. 12).

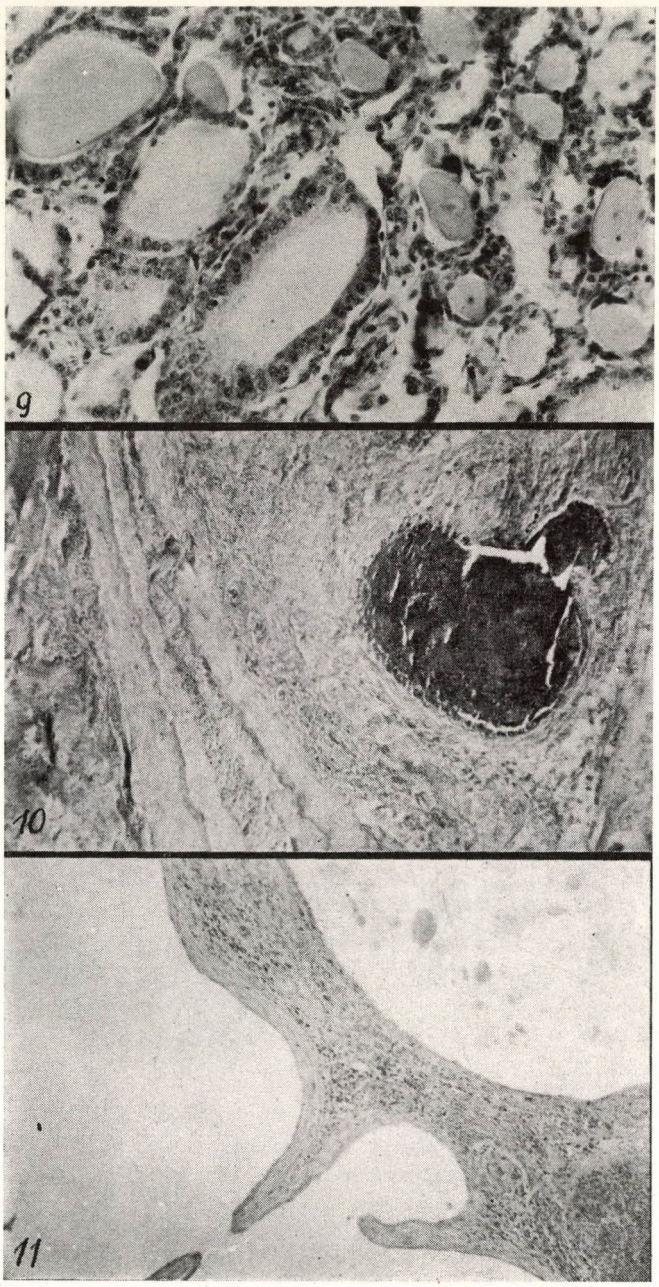

Fig. 9. Thyroid of pinealectomized animal. Tall epithelial cells, forming two layers at sites. Diminished colloid. H + E, ×200

Fig. 10. Ultimobranchial body of pinealectomized animal. Cyst lined with columnar epithelium, filled with PAS-positive material. PAS, ×100

Fig. 11. Ultimobranchial body of pinealectomized animal. Distended cysts lined with flattened epithelial cells. H + E, ×100

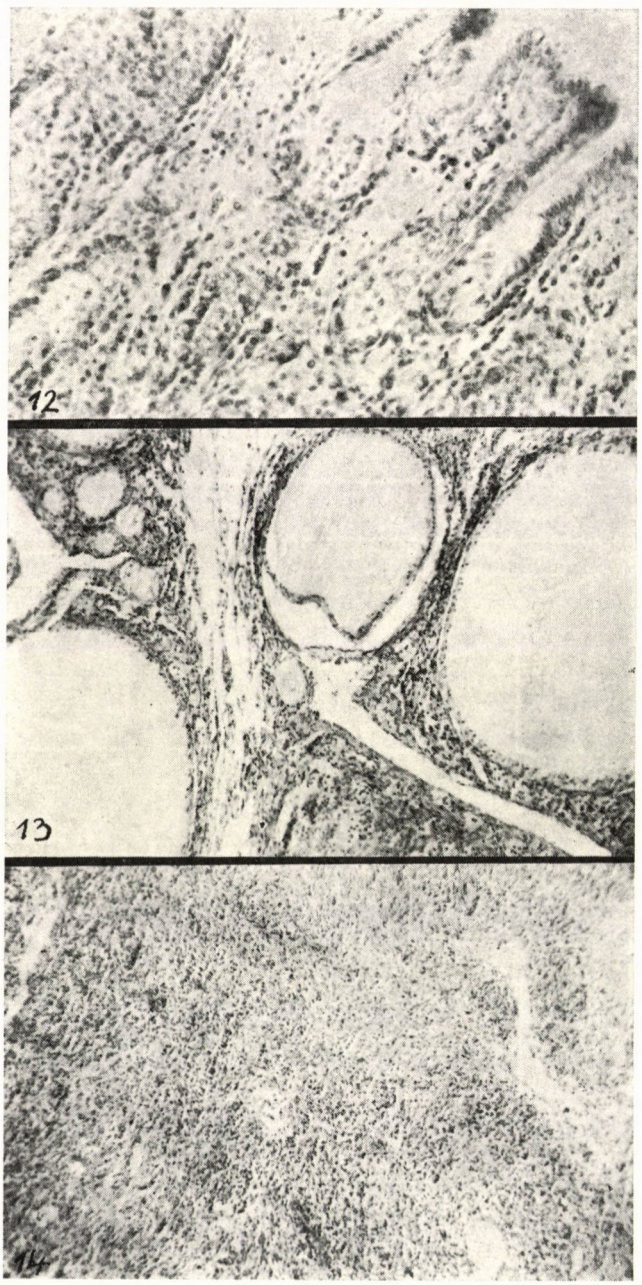

Fig. 12. Bursa of Fabricius of a pinealectomized animal. The follicular structure is unrecognizable, the lymphoid cell population has diminished. H + E, ×200
Fig. 13. Ovary of control animal. Large number of developing follicles. H + E, ×100
Fig. 14. Ovary of pinealectomized animal. No follicle detectable; hyperplasia of connective tissue. H + E, ×100

On subjective assessment, the ovarian follicles seemed to be less numerous in the pinealectomized animals than in the controls. In the single instance where the ovary had attained an excessive size, no intact follicle was found and the entire ovary was occupied by fibrous tissue (Figs 13, 14).

The sham-operated animals revealed no abnormality as compared with the normal controls.

Discussion

From the results it clearly emerges that some of the functional alterations consequent upon early pinealectomy may be connected with the morphological changes in the organs, some others are still unaccounted for.

Retarded growth and shedding of feathers in the pinealectomized birds correspond to the wasting syndrome induced in mice and in other mammals by thymectomy in the neonatal period [15]. The histological picture of the thymus and of the bursa of Fabricius were in fact conclusive of an inadequacy of these two organs essential to the achievement of immunocompetence. In earlier experiments we have shown in adult rats that while pinealectomy had an adverse effect on immune responsiveness, the pineal body has a positive influence on the thymus [8]. This makes it understandable that neonatal pinealectomy results in an atrophy of the thymus and consequently in wasting disease. An interesting point is connected with the fact that avian immunity comprises two independent immune systems, one dependent on the bursa and another dependent on the thymus [2]. Yet, pinealectomy was followed by atrophy of both systems which would seem to suggest that the presence of the pineal body is essential to the full development of both organs. In the present study not all of the signs of the wasting syndrome have appeared and death was not due to the wasting in any of the cases. This was probably due to the fact that pinealectomy was performed as late as 24 to 48 hours after hatching. The chick reaches a fairly advanced stage of maturity with signs of immune responsiveness by the time of hatching [12], and therefore it seems likely that in this species for the introduction of the full-blown wasting syndrome pinealectomy should be done during embryonal life.

Judged by the morphological features, the thyroid response seems to be of the same type as in mammals. In fact, it has been possible to demonstrate in rats that iodine uptake by the thyroid, while being inhibited by the hormone of the pineal body, is enhanced by pinealectomy [6, 13]. This is consistent with the present findings of a tall acinar epithelium and of sparse colloid.

The most striking complication of prevalently fatal outcome was tetany; 50% of the pinealectomized animals developed such seizures which were lethal in 33%. It is therefore safe to assume that the other 17% would also have succumbed to tetany had they not been sacrificed before. It has been pointed

out earlier [5] that the pineal body seems to be involved in calcium metabolism and that it has a positive influence on the function of the parathyroids [14]. Yet, the present histological findings failed to account for the production of tetany. In fact, the parathyroids of the pinealectomized animals tended to be larger than those of the controls without revealing any structural abnormality. This would seem to suggest that the ultimobranchial body or other branchigenous structures [15] have undergone a C-cell transformation which would be responsible for the fall in the blood Ca level. This, however, awaits experimental proof, as this time the C cells have not been studied. At any rate, the tendency of the ultimobranchial body to cystic transformation might be regarded as a sign of enhanced endocrine activity. The possibility that in chicks the thymus might similarly be involved in Ca metabolism as it is actually in rats [7] would provide a new angle of approach to the problem, the more so as thymic atrophy was demonstrable in the present study, too. A different line of approach, though of difficult access to cytological study, would be to seek the primary factor of tetany in an impaired function of the parathyroids. In sum, clarification of these problems calls for further studies.

The relationship between pineal body and the gonads has been a major subject of research. Though it is outside the scope of the present study, we have to call attention to the fact that the ovary of extreme size noted in one case contained no normal follicles, a finding which does not support the observation that a tumour or destructions of the pineal body results in precocious puberty. Incidentally, the fact that the finding was confined to a sole animal raises the possibility of an accidental injury to some region of the brain during the intervention.

The present results may be summed up as being confirmatory of a regulatory influence of the pineal body on the endocrine organs of entodermal origin, thus supporting our earlier view [9]. A closer insight into the mechanism requires further studies.

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CEREBRAL HAEMODYNAMICS AND METABOLISM IN ISCHAEMIC DISTURBANCES OF CEREBRAL CIRCULATION

By

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Certain parameters of cerebral haemodynamics (cerebral blood flow, cerebral vascular resistance, perfusion pressure of the brain) and cerebral metabolism (oxygen consumption, glucose uptake, carbon dioxide output, respiratory quotient, glucose–oxygen ratio) were studied in 30 patients with ischaemic disturbances of cerebral circulation. Cerebral blood flow was measured by a radioisotope venous dilution method, respiratory gases by a microgasometric technique. A decrease was observed in the hemispheral blood flow (268.76 ml/min), oxygen consumption (35.10 ml/min) and glucose uptake (28.90 mg/min), carbon dioxide output (18.72 ml/min), respiratory quotient (0.58) and glucose–oxygen ratio (0.89) of the hemisphere affected by the pathologic process. Cerebral perfusion pressure was elevated (106.33 mm Hg). The correlations between the parameters of cerebral haemodynamics and cerebral metabolism were analyzed. The correlation coefficients were compared with those calculated on the basis of data obtained with the ^{131}I-iodoantipyrine method of REINMUTH et al. in normal subjects and in an analogous group of patients. Disturbances were observed in the haemodynamic–metabolic correlations in patients except for the close relation between cerebral blood flow and oxygen consumption by the brain. New haemodynamic–metabolic interrelations were found in cerebral ischaemia which elucidated some pathogenetic mechanisms and may thus contribute to a more rational therapeutic approach in disturbances of cerebral circulation.

In the recent decade numerous studies have been carried out on the total and regional cerebral haemodynamics [1—3]. Few papers have, however, been devoted to the correlations between disturbances of cerebral haemodynamics and cerebral metabolism. Reduced oxygen and glucose consumption by the brain has been established in the ischaemic cerebral lesions [4, 5]. Experimental studies disclosed metabolic disturbances in the ischaemic focus manifesting itself by a reduced oxygen tension, pH and electrolyte changes [6]. On the other hand, MEYER et al. [5] did not find substantial dependence between some haemodynamic and metabolic parameters.

The scarcity of the data and the lack of full concordance between them require further investigations into the correlations between cerebral haemodynamics and metabolism in a variety of physiological and pathological conditions. The aim of the present study was an analysis of some essential parameters of cerebral haemodynamics and metabolism and their correlation in patients with cerebral ischaemic disturbances.

Material and methods

Blood flow in the hemisphere affected by a pathologic process was estimated by the radioisotope venous dilution technique developed by SOLTI et al. [7], as modified by SHILLINGFORD et al. [8]. ^{131}I was used as indicator. Estimation was accomplished according to the formula

$$CBF\ (ml/min) = I_i \left(\frac{C_i}{C_v} - 1 \right)$$

where I_i = infusion flow in ml/min, C_i = isotope solution infusion and C_v = isotope recovered from venous blood.

Mean arterial pressure was measured by a bloodless method, followed by estimation of cerebral vascular resistance. We measured the venous pressure in the upper bulb of the internal jugular vein and determined the perfusion pressure of the brain as a difference between mean arterial pressure and jugular pressure. With a microgasometric technique we estimated the oxygen and carbon dioxide content in arterial and cerebral venous blood samples and then calculated the oxygen consumption and carbon dioxide output of the hemisphere and its respiratory quotient. Using a reductometric technique in the same blood samples we determined the glucose concentration in mg per 100 ml and calculated the glucose uptake and glucose–oxygen ratio. The analyzed haemodynamic and metabolic parameters are presented in Table I.

Table I

Haemodynamic and metabolic parameters

I. Haemodynamic parameters	
1. Hemispheral cerebral blood flow	ml/min
2. Cerebral vascular resistance	dyne/sec/cm^{-5}
3. Perfusion pressure of brain	mm Hg
II. Metabolic paramesers	
1. Oxygen consumption of hemisphere	ml/min
2. Carbon dioxide output by hemisphere	ml/min
3. Glucose uptake by hemisphere	mg/min
4. Respiratory quotient of hemisphere	
5. Glucose–oxygen ratio of hemisphere	

The studies were undertaken in 30 patients (10 women and 20 men) from 44 to 87 years of age with cerebral ischaemic disturbances, caused by atherosclerosis and arterial hypertension. The main clinical parameters in the patients studied are presented in Table II.

Statistical data processing was performed by estimating the rectilinear correlation coefficients for all possible pair combinations (21 in all) of the studied parameters. We used the data of REINMUTH et al. [9—10] for cerebral haemodynamics and metabolism obtained with the ^{131}I-iodoantipyrine method in an analogous group of patients and in normal individuals, and calculated the same correlation coefficients. The significance of the differences was analyzed between the correlation coefficients in the group of patients studied (r_1) and the coefficients in patients (r_2) and in normal individuals (r_3) studied by the iodoantipyrine technique.

Results

Mean values, standard deviation, standard error and reliability interval of the indices studied in our patients are given in Table III.

As seen from Table III, hemispheral blood flow, oxygen and glucose consumption, carbon dioxide elimination, respiratory quotient and glucose–oxygen ratio were reduced, whereas perfusion pressure was raised. Moreover, the standard deviation is significant which is suggestive of broad individual variations in the examined group.

Table II

Basic clinical parameters

	No. of patients
Females	10
Males	20
Age, years	44—87 (63.3)
Aetiology	
Cerebral atherosclerosis	7
Arterial hypertension with cerebral atherosclerosis	23
Type of impaired cerebral circulation	
Chronic failure	8
Transient ischaemic disturbance	3
Non-obstructive cerebral infarction	17
Obstructive cerebral infarction	2
Degree of brain lesion	
Mild	9
Moderate	8
Severe	13

Table III

Statistical data

Parameters	n	$\bar{x}$	S_x	$S_{\bar{x}}$	$\bar{x} \pm tS_{\bar{x}}$
Hemispheral cerebral blood flow	30	268.76	80.80	14.75	238.59—298.93
Perfusion pressure	30	106.33	11.93	2.18	101.87—110.79
Oxygen consumption	23	35.10	17.05	3.55	27.74— 42.47
Glucose uptake	22	28.90	22.22	4.74	19.05— 38.76
Carbon-dioxide output	23	18.72	9.50	1.98	14.61— 22.83
Respiratory quotient	23	0.58	0.27	0.06	0.46— 0.70
Glucose–oxygen ratio	21	0.89	0.65	0.14	0.60— 1.18

In Table IV we present the significance of the differences between the mean values of the parameters analyzed in our patients ($\bar{x}_1$) and the data obtained in patients ($\bar{x}_2$) and in normal ($\bar{x}_3$) individuals with the iodoantipyrine method.

Obviously, mean hemispheral blood flow, perfusion pressure and glucose uptake values in the studied group of patients did not differ from those obtained with the iodoantipyrine method in other patients, but essentially differ from the values obtained in normal individuals. Therefore, the haemodynamics and metabolic parameters studied in patients with cerebral infarction showed essentially lower values in comparison with normal individuals, with the exception of perfusion pressure, for which the relations were opposite.

Table IV

Statistical data

Parameters	Mean values			Significance of differences between					
				x_1 and x_2		x_1 and x_3		x_2 and x_3	
	x_1	x_2	x_3	t	P	t	P	t	P
Hemispheral cerebral blood flow	268.76	256.07	486.29	0.51	0.610	15.20	0.000	7.98	0.000
Perfusion pressure	106.33	109.68	83.39	0.52	0.603	4.59	0.000	5.61	0.000
Oxygen consumption	35.10	15.57	29.64	4.89	0.000	2.76	0.006	0.74	0.459
Carbon dioxide output	18.72	12.68	29.12	2.23	0.027	6.10	0.000	9.44	0.000
Glucose uptake	28.90	22.06	47.07	1.26	0.207	6.95	0.000	9.20	0.000
Respiratory quotient	0.58	0.81	0.96	3.85	0.000	6.29	0.000	2.34	0.029
Glucose-oxygen ratio	0.89	1.49	1.50	2.88	0.04	5.26	0.000	0.08	0.986

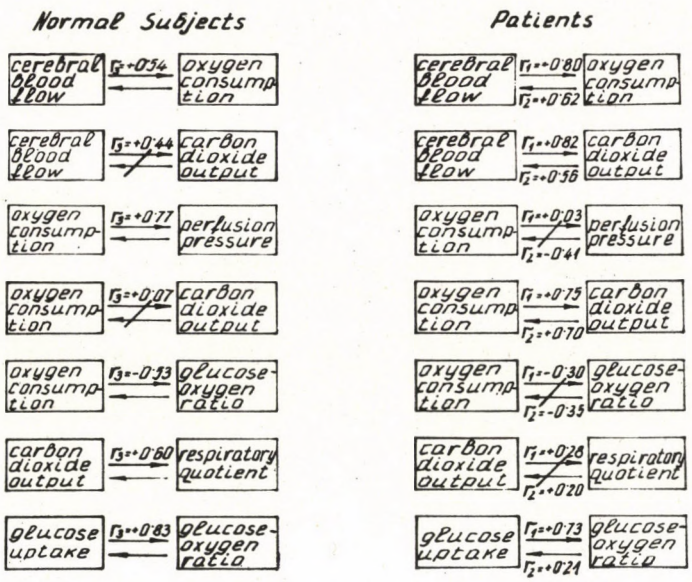

Fig. 1. Correlation coefficients of the basic haemodynamic–metabolic interrelations in normal subjects and in patients with cerebral ischaemia according to data of the iodoantipyrine method (r_3 and r_2) and according to the radioisotope venous dilution technique (r_1)

Moreover, these differences were established by using the radioisotope venous dilution technique and the iodoantipyrine method.

The connections between the haemodynamic and metabolic parameters analyzed in normal subjects and in the patients are presented in Fig. 1.

In normal individuals, positive correlations were obtained between cerebral blood flow and oxygen consumption, between oxygen consumption and perfusion pressure, between carbon dioxide output and respiratory quotient, and between glucose uptake and glucose oxygen ratio. A negative correlation was present between oxygen consumption and glucose–oxygen ratio. There was no significant relation between cerebral blood flow and carbon dioxide output and between oxygen consumption and carbon dioxide output.

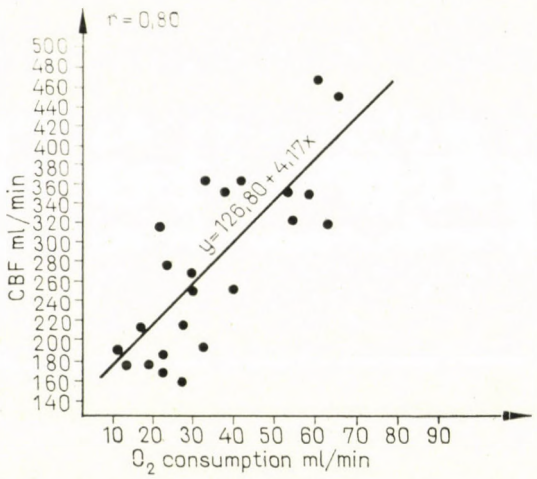

Fig. 2. Relationship between blood flow and oxygen consumption of cerebral hemisphere affected by the pathologic process

In ischaemic disturbances of cerebral circulation, a positive correlation appeared to exist between cerebral blood flow and oxygen consumption, between oxygen consumption and carbon dioxide output, and between glucose uptake and glucose–oxygen ratio. In contrast to the group of normal individuals there was no substantial connection between oxygen consumption and perfusion pressure, between oxygen consumption and glucose–oxygen ratio, and between carbon dioxide output and respiratory quotient.

In Figs 2 and 3 we present the correlations between cerebral blood flow and oxygen consumption and between cerebral blood flow and carbon dioxide output in the patients, as established by the radioisotope venous dilution technique.

In patients with cerebral ischaemic disturbance some relationships were observed between haemodynamic and metabolic parameters (cerebral blood flow and carbon dioxide output) and between some metabolic parameters (oxygen consumption and carbon dioxide output) which could not be observed in the examined normal individuals. Conversely, in the patients we

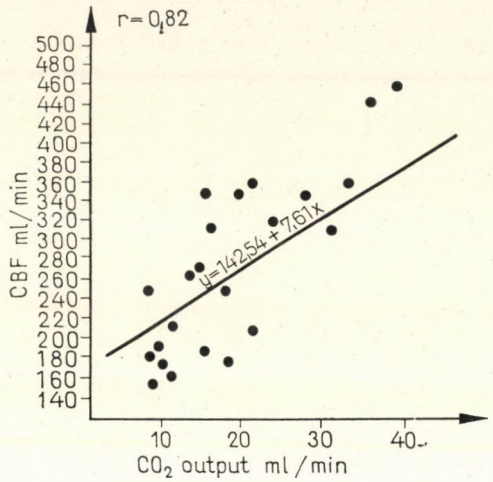

Fig. 3. Relationship between blood flow and carbon dioxide output of cerebral hemisphere affected by the pathologic process

did not observe some relationships found in normal individuals (oxygen consumption and perfusion pressure; oxygen consumption and glucose–oxygen ratio; carbon dioxide output and respiratory quotient). In cerebral ischaemia there persisted only the close relationship between cerebral blood flow and oxygen consumption existing in normal individuals as well.

Discussion

Our studies showed a significant reduction of blood flow, of oxygen and glucose consumption, carbon dioxide output and of the respiratory quotient and glucose–oxygen ratio in the cerebral hemisphere affected by the pathologic process. A similar reduction of blood flow, of oxygen consumption and carbohydrate metabolism in ischaemic disturbances of cerebral circulation has been observed by other authors as well [5].

The elevated perfusion pressure in cerebral ischaemia may be regarded both as a manifestation of the basic pathologic process and as a compensation of the increasing cerebral vascular resistance, the reduced oxygen consumption and deranged cerebral metabolism. These observations correlate well with the experimental, pathomorphologic and clinical evidence concerning the role of cerebral ischaemia in arterial hypertension [11]. Obviously, any lowering of this "compensating" arterial hypertension is inexpedient. The limited facility of the pathologic vascular process for a persistent increase in cerebral blood flow in brain ischaemia warrants the use of therapeutic agents directly affecting the impaired cerebral metabolism. The complex therapeutic approach

with improvement of cerebral haemodynamics and metabolism finds its theoretical grounds in the simultaneous derangement in cerebral ischaemia.

Our studies showed that in cerebral ischaemic disturbances a number of haemodynamic–metabolic relationships observed in normal individuals are disturbed. The positive correlations are lost between cerebral oxygen consumption and perfusion pressure, and between carbon dioxide output from the brain and its respiratory quotient. This fact suggests that solely by raising the perfusion pressure of the brain its oxygen consumption on cerebral ischaemia cannot be compensated. On the other hand, absence of a correlation between carbon dioxide output and the respiratory quotient in the brain suggestsothat cerebral metabolism is disturbed and the energy requirements of this rgan are probably met by other substrates as well. The essential differences between the correlation coefficients for oxygen consumption and carbon dioxide output and the glucose uptake and glucose–oxygen ratio for patients with ischaemic disturbances of cerebral circulation and for normal individuals favour the assumption that there exist qualitative metabolic differences in cerebral ischaemia and, also, that it is possible for disorders of glucose metabolism to be compensated by the breakdown of other substrates. This is substantiated by the studies of GOTTSTEIN et al. [12] suggesting in severe atherosclerosis the possibility for an active involvement in cerebral metabolism of some amino acids and lipoproteins.

The only preserved correlation in cerebral ischaemia is the one between cerebral blood flow and cerebral oxygen consumption. This fact is indicative of the expedience of additional blood oxygenation in cases of excessive reduction of cerebral blood flow, including the use of hyperbaric oxygen therapy.

These haemodynamic–metabolic interrelations may contribute to a more complete pathogenetic analysis and well-grounded treatment of cerebral ischaemia.

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SIDE EFFECTS OF CHRONIC MEPHENYTOIN TREATMENT IN THE RAT

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Rats subjected to chronic mephenytoin treatment have been studied for immunoserological and morphological changes. Various immunological abnormalities including antinucleoprotein and antigammaglobulin antibodies appeared in the course of treatment and parallel with its duration increased their rate of incidence. Hyperproteinaemia and hypergammaglobulinaemia were also demonstrable. After prolonged administration of the drug the lymph nodes of the animals exhibited various microscopic changes. Among these, a blastomatoid proliferation of plasmocytes and pleomorphic cells was remarkable, without neoplastic proliferation. The possible mechanisms underlying the induction of autoimmune disease by hydantoin preparations is discussed. It is suggested that the morphological changes, attributed to a direct effect of the drug, accounted for the serological abnormalities.

Antiepileptic medication involves various hazards. Drugs of this kind may precipitate the production of systemic lupus erythematosus (SLE), affect the blood-forming system (megaloblastic anaemia, leukopenia, agranulocytosis, panmyelopathy) or give rise to hypersensitivity reactions of allergic nature. Animal experiments and human observations raised the possibility of blastomogenic properties of hydantoin derivatives (GAMS et al. [9], HYMAN and SOMERS [12], JUHÁSZ et al. [13]). Though secondary effects are fairly common in subjects under chronic antiepileptic treatment, the effects of hydantoin derivatives in the laboratory animal have received little attention [14, 16, 17].

The aim of the present study was to ascertain whether and how often chronic mephenytoin administration to rats caused immunological abnormalities, and to examine the lymph nodes for morphological changes.

Material and methods

Albino Wistar rats of either sex were used in the experiments. Three groups, each of 20 animals (10 males, 10 females) were set up against control groups formed by the same number of animals receiving 1 ml tap water through a tube. Mephenytoin was administered through a gastric tube in doses of 20 mg/kg body weight daily, for a period of four weeks to the first, for 20 weeks to the second, and for 44 weeks to the third group. From the animals of the third group blood was withdrawn during treatment at the end of 28 weeks (the values thus obtained being referred to as Group III) and the studies were repeated at the end of 44 weeks (the values being referred to as Group IV). The animals of all groups were sacrificed to exsanguination by heart puncture. The studies included blood counts (WBC, RBC,

platelets, reticulocytes, haemoglobin, differential counts), femoral bone-marrow smear, serum total proteins, paper electrophoresis, LE-study (using heparin as anticoagulant and gelatin for sedimentation), rheumatoid factor by the Waaler–Rose test, antinucleoprotein-antibody using BOYDEN's passive haemagglutination procedure [4] in the modification of GAJDUSEK [8]. Microscopic study included the following organs: lymph nodes, liver, hilus, kidney, thymus, heart, gut. The lymph nodes of each region (cervical, axillary, pulmonary ileocoecal, retroperitoneal) were studied separately. One animal of Group I and three animals of Group III died during treatment. There was no death in Group II.

Results

During chronic mephenytoin treatment, the body weight of the animals showed an increase. The weight curves of the test animals were similar to those of the controls all throughout (Table I). Four weeks treatment left the blood counts practically unaffected (Table II). The differential count revealed

Table I

Treated groups	Duration of treatment (weeks)	Body weight, g		Spleen weight, mg	Spleen-weight/body-weigth index g/100 g
		before treatment	after treatment		
Group I n = 19	4	230±24	248±22	1100±200	0.44
Controls n = 19		220±22	240±26	950±150	0.39
Group II n = 20	20	220±8	270±34	1200±240	0.44
Controls n = 20		225±20	275±38	1050±180	0.38
Group III n = 19	28	240±24	342±38		
Controls n = 20		230±22	330±36		
Group IV n = 18	44	240±24	370±44	1700±350	0.46
Controls n = 18		230±22	368±42	1200±200	0.32

Table II

Treated groups	Duration of treatment (weeks)	RBC	Hg g per 100 ml	WBC	Platelet count	Reticulocyte count per cent
Group I n = 19	4	7.5	16	11,140	430,000	1
Controls		7.48	16.2	10,870	390,000	1
Group II n = 20	20	7.2	15.6	10,300	400,000	1.2
Controls		7.3	15.8	11,600	440,000	1.1
Group III n = 19	28	6.8	15.1	9,800	460,000	1.4
Controls		7.3	16	12,100	480,000	1.2
Group IV n = 18	44	6.4	13.8	9,000	420,000	3
Controls n = 18		7.1	15.8	11,600	450,000	1.3

an increase in the proportion of lymphocytes, and the bone marrow a moderate increase in that of plasma cells. Treatment for 20 weeks resulted in moderate lymphocytosis with no evidence of anaemia, leukopenia or thrombopenia. The bone marrow exhibited a 6 to 7% increase in the plasma cell population, as against 1% in the controls. The M : E ratio was identical in the test animals and in the controls. At the end of 28 weeks there was a moderate fall in RBC and WBC as well as in haemoglobin, with the platelet and reticulocyte counts remaining unchanged. The differential counts revealed a relative lymphocytosis. In this group the bone marrow was examined after the completion of treatment only. At the end of 44 weeks there was a reduction in RBC and haemoglobin values, an increase in the reticulocyte count, no change in the platelet count; leukopenia was slightly more distinct, with the same degree of relative lymphocytosis as previously. The bone marrow revealed a further increase to 9 to 10% in the number of plasma cells, as against 1% in the controls. The reticulum cell population had also increased, the M : E ratio was practically unaffected.

After four weeks treatment with mephenytoin there was an increase in the total serum proteins resulting from an increase in the gamma globulin level (Table III). After 20, 28 and 44 weeks treatment, the total serum protein

Table III

Treatment	Se. total protein g/100 ml	Albumin g/100 ml	α-globulin g/100 ml	β-globulin g/100 ml	γ-globulin g/100 ml
4 weeks (I) n = 19	7.5±0.29	2.57±0.13	1.40±0.12	1.42±0.12	2.11±0.27
Controls n = 19	7.5±0.5	2.50±0.22	1.34±0.12	1.47±0.11	1.69±0.21
	p 0.1%	p 5%	p 5%	p 5%	p 0.1%
20 weeks (II) n = 20	8.1±0.70	2.51±0.31	1.54±0.17	1.62±0.19	2.43±0.30
Controls n = 20	7.2±0.46	2.45±0.17	1.30±0.23	1.58±0.17	1.87±0.30
	p 0.1%	p 5%	p 0.1%	p 5%	p 0.1%
28 weeks (III) n = 19	8.59±0.75	2.61±0.20	1.61±0.14	1.60±0.32	2.77±0.49
Controls n = 20	7.1±0.22	2.27±0.18	1.55±0.07	1.55±0.12	1.73±0.23
	p 0.1%	p 0.1%	p 5%	p 5%	p 0.1%
44 weeks (IV) n = 18	8.8±0.6	2.67±0.35	1.57±0.15	1.52±0.22	3.04±0.57
Controls n = 18	7.2±0.32	2.45±0.20	1.58±0.13	1.58±0.17	1.59±0.31
	p 0.1%	p 5%	p 5%	p 5%	p 0.1%

Comparison of the values of the treated groups:

Se. total protein			γ-globulin		
Group	I : II	$p < 1$		I : II	$p < 1$
Group	I : III	$p < 0.1$		I : III	$p < 1$
Group	I : IV	$p < 0.1$		I : IV	$p < 0.1$
Group	II : III	$p < 2$		II : III	$p < 5$
Group	II : IV	$p < 0.1$		II : IV	$p < 0.1$
Group	III : IV	$p < 5$		III : IV	$p < 5$

and gamma globulin levels increased further. This increase parallel with the duration of treatment was found significant statistically, with the exception of the 28 and 44 week values correlated to each other. The same was valid for the increase in the gamma globulin level. Its rise was of polyclonal character, no M-component was demonstrable. The sera of some animals with an uncommonly high gamma globulin level were subjected to immunoelectrophoresis which revealed no paraproteinaemia.

LE-cells were absent from the sera of all animals. The number of animals revealing antinucleoprotein antibodies increased parallel with the duration of treatment (one animal at the end of 4, four animals at the end of 20, five animals at the end of 28, and nine animals at the end of 44 weeks). No anti-

Table IV

Duration of treatment	LE-cell positive	Antinuclear factor positive	Rose-test positive
4 weeks n = 19	—	1	2
Controls n = 19	—	—	—
20 weeks n = 20	—	4	6
Controls n = 20	—	—	—
28 weeks n = 19	—	5	8
Controls n = 20	—	—	—
44 weeks n = 18	—	9	13
Controls n = 18	—	—	—

nucleoprotein antibodies were demonstrable in any of the control groups. In the 4 week group there were two, in the 20 week group six, in the 28 week group eight, in the 44 week group 13 animals with RF-positive sera, as opposed to the controls where all the sera were RF-negative. The results are shown in Table IV.

The lymph nodes were enlarged in all the five regions studied. The spleen increased its weight parallel with the duration of treatment. This was clearly reflected in the spleen-weight/body-weight index.

The structure of the lymph nodes was affected in some areas. These changes could be grouped as follows.

1) Follicular hyperplasia. Enlarged, hypercellular follicles with broad germinal centre, prevalent in a minority of lymph nodes, the majority being free from any of these features (Fig. 1).

2) Lympho-reticulocellular hyperplasia. Appearance of poorly demarcated focal accumulations of lymphoid cells and of reticulum cells with pale cytoplasm in the cortical region, extending at sites to the medulla or merging with unsharply demarcated follicles (Fig. 2).

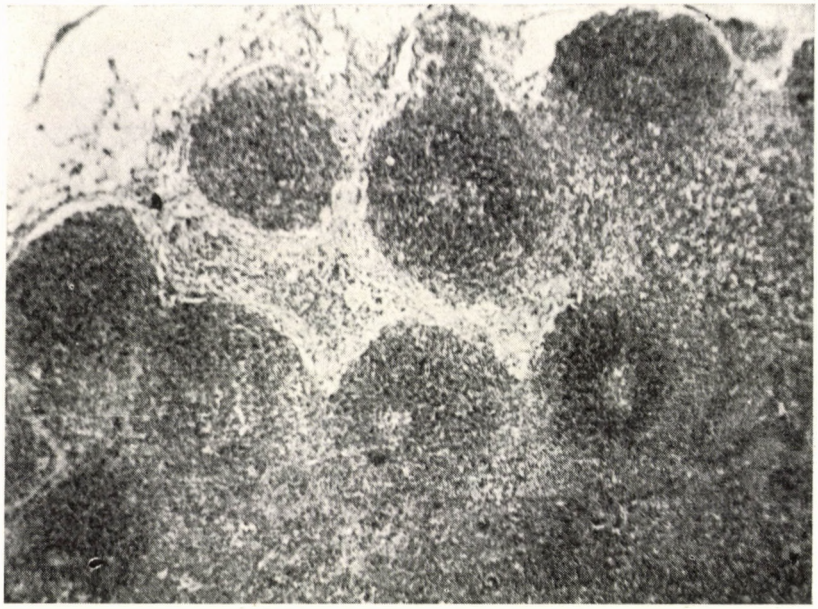

Fig. 1. Follicular hyperplasia. H.E., ×150

Fig. 2. Lymphoreticular hyperplasia. H. E., ×200

3) Sinus catarrh. Cellular reaction, prevalently with detached endothel cells, plasma cells, lymphoid cells, sparse lymphocytes, involving the marginal and intermediary portions of the sinus system (Fig. 3).

4) Marked vascularization of the cortical and medullary areas.

5) Perilymphadenitis with diffuse and perivascular lymphoid cell infiltration of the capsule and the pericapsular connective tissue.

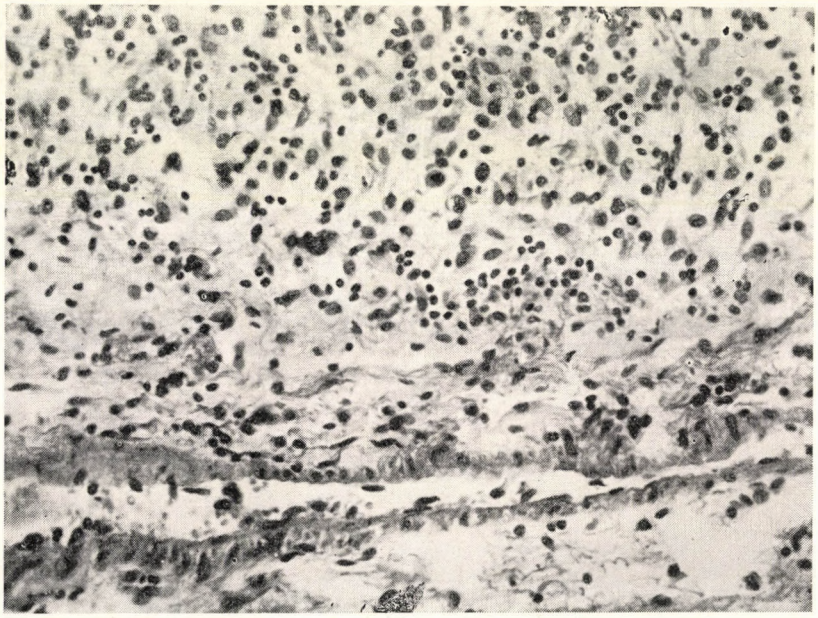

Fig. 3. Sinus catarrh. H. E., ×300

6) Confluent plasmacellular foci in the medullary area interspersed with numerous Russel's bodies. The process involves all lymph nodes, either respecting the borderlines of the medullary trabecules or assuming a blastomatoid appearance in the form of large confluent cell-rich areas extending to the capsular region (Figs 4, 5).

7) Blastomatoid foci of pleomorphic cells in the medulla, different from the above changes. The poorly demarcated cell-rich foci are made up of lymphoid cells, reticulum cells with large nuclei and plasma cells with numerous dividing forms, sporadic eosinophils and neutrophils. The majority of cells exhibit morphologic characters similar to those of immunoblasts. The foci of blastomatoid appearance are reminiscent of those described in drug-induced human lymphadenopathies [15, 23] (Figs 6, 7).

The changes of the last two types were found to affect chiefly the mesenteric and the iliocoecal lymph nodes, blastomatoid plasmacellular prolifera-

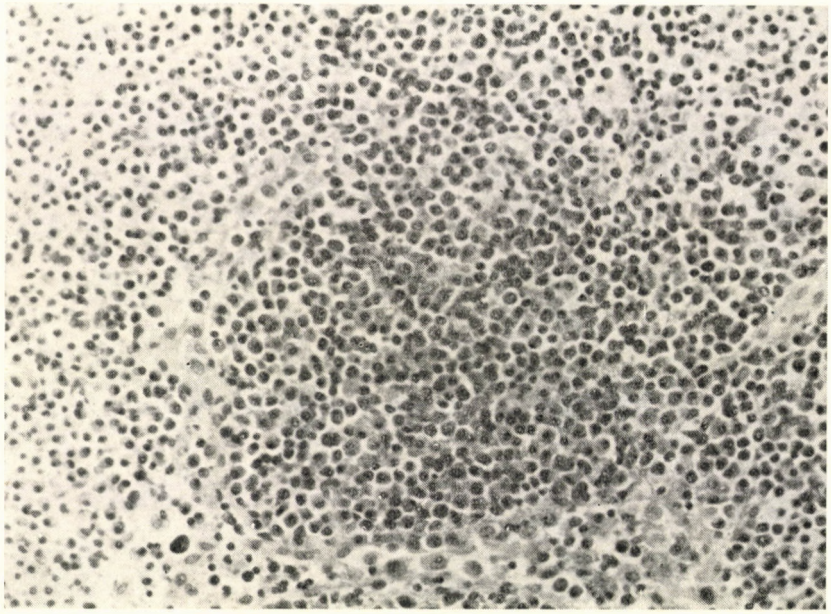

Fig. 4. Plasmocellular accumulations in the medullary substance. H. E., ×250

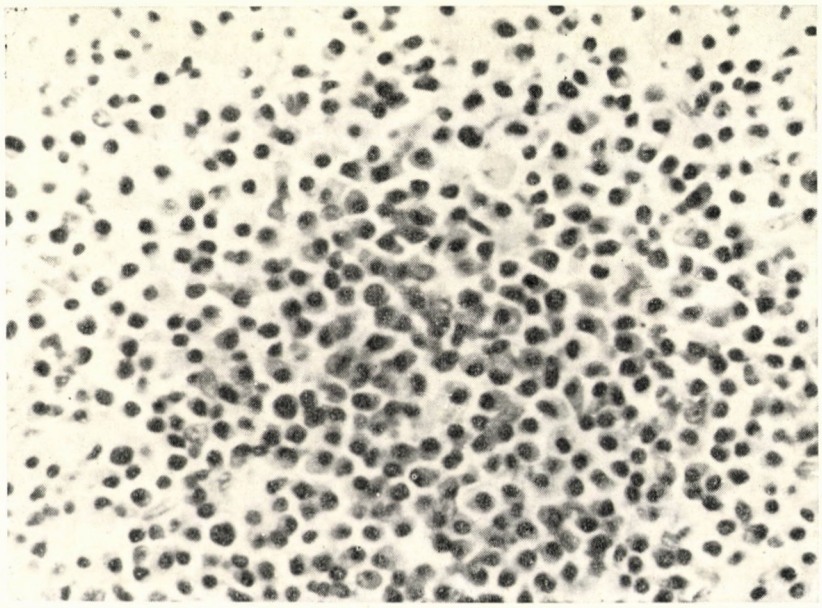

Fig. 5. Plasmocellular foci in the medullary substance H. E., ×350

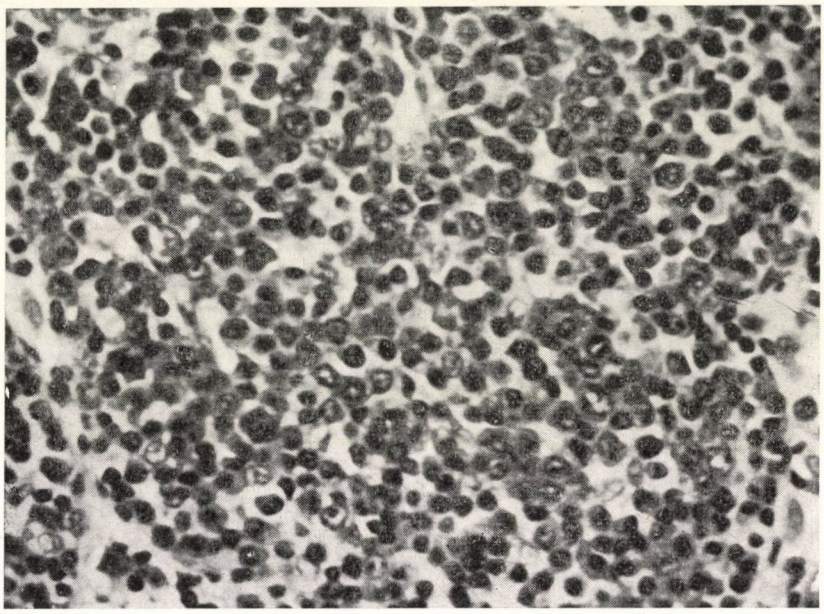

Fig. 6. Blastomatoid proliferation of pleomorphic cells. H. E., ×350

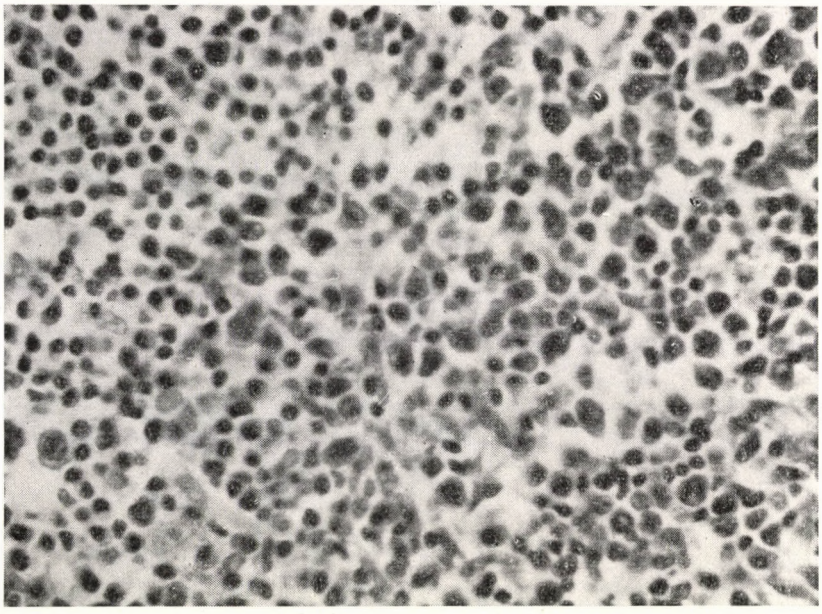

Fig. 7. Blastomatoid proliferation of pleomorphic cells. H. E., ×350

tion being noted in 15% and blastomatoid pleomorphic cell proliferation in 5% of the cases. Thus, follicular and lympho-reticulocellular hyperplasia accompanied by an inflammatory reaction represent the slightest degree of the drug-induced effects under study. The frequency of blastomatoid changes increases with, while the severity of the other changes was unrelated to, the duration of treatment. No true neoplastic transformation was demonstrable in any of the cases, and the typical microscopic features described in drug allergy [15] were also absent. The other organs examined revealed no microscopic changes.

Discussion

The effects of chronic administration of antiepileptic drugs in laboratory animals have received little attention. Hydantoin-induced lymphoreticular proliferation in cats has been described by KASLARIS [14]. Production of lymphoid tumours and of leukaemia in mice after the chronic use of phenytoin has been observed by JUHÁSZ et al. [13]. In our earlier studies administration of mephenytoin to rats and guinea pigs for 10 weeks produced no LE-phenomenon [17].

The present study revealed definite immunological abnormalities and these were related to the duration of treatment. However, no LE-phenomenon had developed even on 44 weeks treatment. On the other hand, antinucleoprotein antibody was demonstrable with increasing frequency with the duration of treatment. Whereas at the end of four weeks its presence was confined to a sole animal, at the end of 20 weeks, 20% and at the end of 44 weeks, 50% of the group had antinucleoprotein antibody in the serum.

Antigammaglobulin-antibody was found in a still larger proportion, the respective figures being 30% at the end of 20 weeks, 42% at the end of 28 weeks, and 72% at the end of 44 weeks. On the other hand, the serum of the controls revealed neither antinucleoprotein, nor antigammaglobulin antibody.

Anaemia developing parallel with the length of treatment was moderate and was associated with slight reticulocytosis by the end of 44 weeks, pointing to the possibility of haemolysis. Leukopenia was likewise of minor degree; the initial absolute lymphocytosis in the differential count was gradually replaced by relative lymphocytosis during the 28th to 44th weeks.

Parallel with the plasma reticulum-cell and immunoblast proliferation in the bone marrow and the lymph nodes, serum total protein was gradually rising, the elevation as compared with the controls as also within the test groups, with the exception of the 28- and 44-week values, proved statistically significant. The serum total protein and gamma globulin levels were still increasing beyond the 28th week of treatment, however at a slower rate.

Hyperproteinaemia and hypergammaglobulinaemia are typical of the animal strains, with autoimmune disease including the NZB mouse-strain, and of the Aleutian mink disease [3, 11]. In addition to these features, antinuclear antibody, LE-cell and Coombs-positivity are fairly common in NZB-mice. This is also true for antigammaglobulin-antibody in the Aleutian mink disease [11].

In our earlier studies, antinuclear antibody was induced in 27% and antigammaglobulin antibody (Rose-test) in 20% of guinea-pigs by the administration of hydralazine [18]. CANNAT and SELIGMANN [6] produced antinuclear antibody in BALB/c and C_{57}Bl/6 mouse strains by the administration of isoniazid and hydralazine [6].

Human subjects using these drugs may also possess various antibodies. CANNAT and SELIGMANN [7] detected antinuclear factors in 20% of tuberculous patients being on maintenance treatment with isoniazid, as against 4% in untreated subjects. Sera of hypertensive subjects taking hydralazine also exhibit antinuclear factors, often even in the absence of hydralazine-provoked S—E [2, 21]. Hypergammaglobulinaemia, increased immunoconglutinin titres, antigammaglobulin and antinuclear antibodies have been found by us in a large proportion of patients taking antiepileptic drugs, particularly hydantoin derivatives [24].

What is the possible mechanism accounting for the production of autoantibodies by these drugs?

As far as the hydantoin derivatives are concerned, there is no evidence to suggest that preparations of this kind might give rise to modifications of antigenicity.

No signs of a toxic injury of any of the parenchymatous organs have been noted in the course of long-term studies, nor do observations in epileptic subjects point to any such effect of these drugs.

The mechanism responsible for the production of autoimmune disease by these drugs admits of two possible interpretations.

1) The drug may act as an antigen or a hapten and elicit antibody formation, thus producing a specific drug-hypersensitivity which, by a mechanism still poorly understood, results in the manifestation of autoimmune disease.

On the grounds of clinical observations we have come to the conclusion, also shared by PETRÁNYI [22], that the production of SLE as a result of hydantoin treatment is scarcely ever associated with hypersensitivity reactions [25].

2) The other theory connects the manifestation of autoimmune disease with certain potential pharmacological properties of the drugs in question. We believe, in accordance with PETRÁNYI [22], that these drugs affect the immune system directly and cause there morphological changes which then are responsible for the various immunological abnormalities. If the patient is susceptible to lupus [1], hydantoin-derivatives are apt to provoke the SLE

phenomenon [25]. On the other hand, in the absence of such a susceptibility, the drugs in question, though involving the production of various immunological abnormalities, will never lead to an actual SLE [24].

In the lymph nodes of epileptic subjects we have demonstrated certain morphological changes which might have been connected with the long-term use of hydantoin derivatives [26]. The essential microscopic features of lymphonodular enlargement induced in the laboratory animal include lymphoreticulocellular, plasmacellular and immunoblast proliferation, at sites of blastomatoid focal character. No real neoplastic features were demonstrable in any of the cases. Signs of an allergic hydantoin lymphadenopathy, such as necrosis, thrombotic microangiopathy, eosinophile cell infiltration, were likewise absent.

The microscopic changes of the lymph nodes result from a direct pharmacodynamic action of the drug rather than from a drug-induced allergic reaction.

KRÜGER [16] noted a reticulo-plasmacellular proliferation in the lymph nodes of mice after the administration of phenytoin in medium and massive doses, and attributed the changes partly to a direct action of the drug and partly to a hypersensitivity-reaction. The fact that mephenytoin attains high concentrations in the lymph nodes, indicates the probability of a direct pharmacodynamic mechanism (SZEGEDI and KARSAY [27]).

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MODEL MYOCARDITIS IN THE RAT

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Rats fed a standard diet supplemented with ethyl laurate were found to develop diffuse myocardial changes meeting the criteria of myocarditis. The severity of the microscopic lesions has been correlated with that of the ECG abnormalities. The conclusions derived from 242 ECG-studies are presented.

Myocarditis constitutes a sadly neglected field of pathological and clinical studies. Though there is no lack in papers calling attention to the high frequency of the condition, the difficulty of its detection still leaves a wide scope for diagnostic and therapeutic studies [2, 9, 12, 14, 24]. Despite numerous attempts at classification on morphological, aetiological or clinical grounds [1, 3, 7, 10, 13, 15, 16, 20, 22, 25, 29, 31], no agreement has been reached as regards its terminology. Some authors confine the definition of primary myocarditis to processes of unknown aetiology while qualifying all other diseases of the myocardium as secondary. Others consider myocarditis to be primary if it is limited to the myocardium, without involving either the endocardium or the pericardium, a classification comprising idiopathic myocarditis, i.e. of unknown aetiology, and postinfective myocarditis with its subtypes. Other classifications have been based on the pathology of myocarditis, taking its morphological, instead of its clinical, types into consideration [3, 22, 27].

WHITEHEAD [37] divides myocarditis into acute, subacute and chronic types according to the clinical course. The microscopic pattern of the acute type is marked by interstitial oedema, inflammatory infiltration and secondary damage to the muscle fibres. The chronic type is characterized by a diffuse fibrosis lacking inflammatory elements. The subacute form constitutes a borderline between the two other types. However, the three types seem to correspond to three stages of the same process.

The wide range of experimental procedure for the production of myocarditis in the laboratory animal includes lesioning of the myocardium, application of bacteria, or of bacterial endotoxin, production of serum sickness or of autoimmunity, administration of steroids, electrolytes, feeding of triglyc-

eride containing diets, etc. These procedures generally resulted in focal lesions of the heart muscle with a cellular reaction followed by proliferative changes.

KESTEN et al. [cit. 26] were the first to induce fatal myocarditis in laboratory animals by the administration of synthetic triglycerides (1945), rats fed a diet containing 30 to 40% ethyl laurate having been found to develop lesions similar to those observed in human myocarditis. Abnormalities of this type would appear in three-week-old, just weaned rats in 1 to 3 weeks, to end fatally in 2 to 7 days. Administration of choline, betaine or of methionine was found to prevent the injury the severity of which was related to the amount of ethyl laurate contained in the diet.

The present study has been aimed at the production of myocardial lesions which might be regarded as a model of myocarditis in man. It was sought to gain information on the state of the myocardium in the course of the process by ECG studies which were to provide an answer to the questions whether there are consistent ECG changes characteristic of the given stage and whether there is any relationship between the ECG abnormalities and the outcome of the process.

Material and methods

Three-week-old Wistar rats of either sex were studied in six groups, making up a total of 90 animals. Each group (I to VI) included five controls and 10 test animals, the latter receiving 33 to 36% ethyl laurate admixed to standard rat food. Water was allowed ad libitum. The animals of groups II and IV, having died two days after beginning the diet (probably as a result of the direct toxic effect of the fatty acid ester) are disregarded in this report. In groups I and VI, the first signs of disease appeared during the third week of diet, death ensuing in 8 to 21 days. In groups III and V, the first manifestations were noted after 7 to 9 days of diet and all animals died in 2 to 4 days. The first signs were a changed behaviour, ruffled fur, sluggish motions, indifference to external stimuli. These were followed by irregular, rapid, gasping respiration. The final stage was marked by unsteady, staggering movements, aversion to food, apathy, death ensuing in a few hours after the appearance of these signs. In the animals of group VI, upon the manifestation of the first signs of disease the diet was replaced by standard rat food; this resulted in a more protracted course of the process without averting its fatal outcome.

Autopsy failed to reveal any change in the gross appearance either of the heart or of the other organs. The heart was fixed in 4% neutral formalin solution, embedded in paraffin, cut into 3 to 4 μ thick serial sections and stained with haematoxylin-eosin, Azan and Farkas–Mallory's dye. In each group, the liver and striated muscles of one animal were processed for microscopic study.

In all test groups ECG-recordings were made prior to the start of diet (six-channel Hellige-apparatus, paper speed of 50 and 100 mm/sec) [36] under ether anaesthesia. The recordings included standard and unipolar limb leads and three chest leads, the positions of the chest electrodes being the left and right parasternal and the anterior axillary lines. Partly these basal recordings, partly the recordings of the controls receiving normal food served as reference for comparative purposes. As soon as the first signs of disease had appeared, ECG recordings were made daily at identical times.

Results and discussion

The normal varieties of the rat ECG [17] served as a basis of evaluation. Changes in heart rate in themselves were not considered abnormal, the animals

having been under anaesthesia. The variations in voltage were also attributed to anaesthesia, deep anaesthesia being often associated with low voltage. On the other hand, a persistent low voltage or the progressive decline of voltage was regarded as definitely abnormal. The incidence of nodal rhythm was the same in the controls as in the test animals. No other disturbances of rhythm or conduction were seen in the healthy animals. Modifications of the ST-segment were considered normal unless they attained double the original height and this persisted or increased in the further course.

A total of 242 recordings was obtained from 30 animals of 4 series (10 animals having died in the course of the experiment); 109 of these were considered abnormal on the basis outlined above. Their main features are set out in Table I.

Table I

Group	Number of ECG recordings	Extrasystole	Other types of arrhythmia	Low voltage	Prolonged PQ-interval	AV-block	Intraventricular conduction disturbances	ST elevated	Total number of abnormal ECG
I	56	2	2	4	—	3	2	5	18
III	16	—	—	2	—	—	—	8	10
V	22	1	1	3	—	—	—	8	13
VI	148	5	2	18	12	21	3	7	68
	242	8	5	27	12	24	5	28	109

The most marked changes were noted in Groups V and VI. While in Group V a marked ST elevation was prevalent, in Group VI disorders of rhythm and conduction were more common.

In the animals of Group I mean survival was 8 days after manifestation of the first signs. The ECG-changes noted in this group were scanty: extrasystoles from multiple foci (Fig. 1) in 2, other disturbances of rhythm (Fig. 2) in 2, low voltage in 4, AV-block in 3, intraventricular block in 2, and marked ST-elevation in 5 out of 56 cases. In the animals of series III and V, after the manifestations of the signs, of extreme severity all throughout, survival was 2 to 4 days. The most conspicuous ECG-abnormality was a gross ST-elevation in these groups (Fig. 3). In the animals of Group VI, the protracted course of the disease allowed to make as many as 148 recordings 5 of which revealed extrasystoles from multiple foci, 18 (obtained from 4 animals) progressive low voltage, usually a premonitory sign of death. In this group conduction distur-

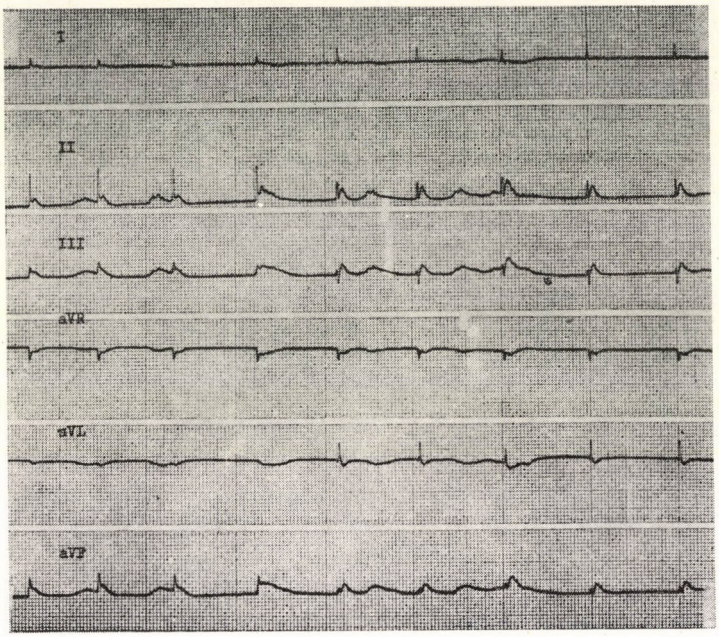

Fig. 1. Extrasystoles from multiple foci (animal I/5). Paper speed, 100 mm/sec

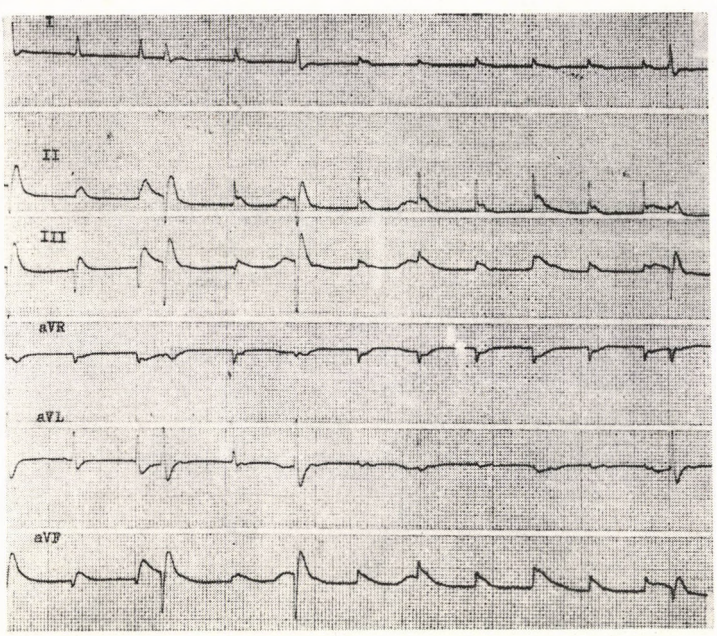

Fig. 2. Disturbances in impulse production (animal I/2). Paper speed, 100 mm/sec

bances of variable severity were prevalent, the mild forms being marked by a delayed conduction and those of major severity by a 2 : 1, 3 : 1 or complete AV-block or by intraventricular conduction disturbances. In one animal, all the tracings recorded on 17 successive days revealed an AV-block (Fig. 4).

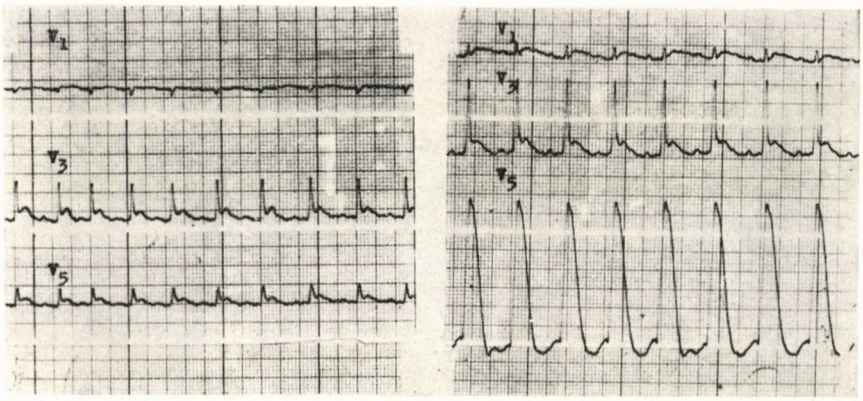

Fig. 3. Left side: ECG before the experiment. Right side: ECG on 10th day of diet; gross ST-elevation (animal V/1). Paper speed, 50 mm/sec

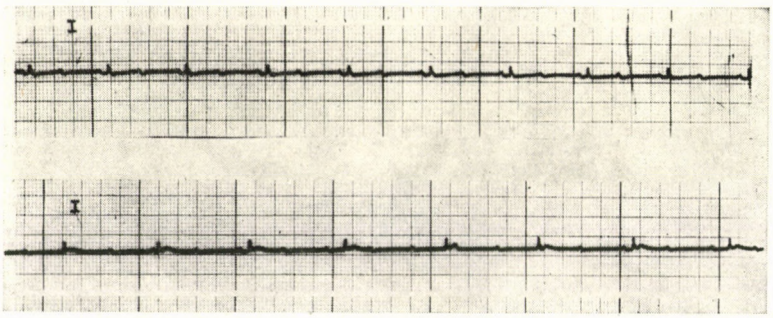

Fig. 4. The upper strip shows a 2 : 1, the lower, a complete auriculoventricular block in the same animal (VI/2). Paper speed, 100 mm/sec

Ventricular flutter (Fig. 5) and fibrillation were noted immediately before death.

Histology revealed myocardial changes of a consistent pattern but of variable severity. The myocardium of both ventricles was equally involved, the lesions were, however, invariably confined to the myocardial area without affecting the endocardium, the pericardium or the coronaries. The histological finding was in accordance with WHITEHEAD's definition of "acute isolated myocarditis" [37] and included the following features:

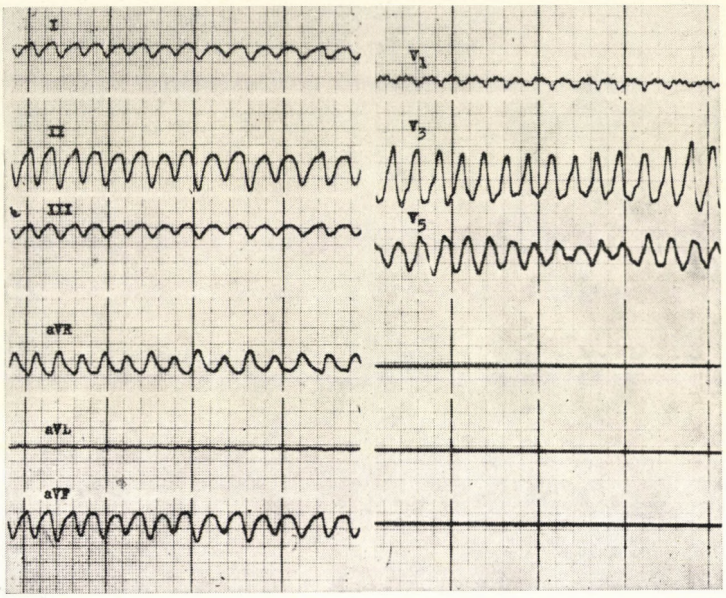

Fig. 5. Ventricular flutter and electric alternant (animal VI/3). Paper speed, 100 mm/sec

1) interstitial oedema;

2) grossly dilated, giant capillaries in the myocardium;

3) focal or diffuse interstitial infiltration of inflammatory character, prevalently with lymphocytes, histiocytes and fibroblasts;

4) oedema of muscle fibres and parenchymal degeneration;

5) necrosis of muscle fibres, appearance of myogenic giant cells.

Grading of the histological abnormalities was based on the criteria which had been adopted for the assessment of morphological abnormalities in human material, crosses + to +++ representing the successive grades of severity (Table II).

Table II

Group	Duration of disease, days	Interstitial oedema	Capillary dilatation, congestion	Inflammatory infiltration	Oedema of muscle fibres	Necrosis of muscle fibres
I	8—12	+++	+++	+++	+	++
III	2— 4	+++	++	+	++	+
V	2— 4	+++	++	+	++	+
VI	8—21	+++	+++	+++	+	+++

From Table II it emerges that interstitial oedema was of similar severity in the animals surviving 2 to 4 or 8 to 21 days, a finding consistent with the observations of EPPINGER [8] which seem to indicate that the primary process is the production of an interstitial serous exudate, followed by the appearance of inflammatory cells. It is a relevant fact that migration of inflammatory cells goes hand in hand with that of a large population of fibroblasts and histiocytes providing the elements for consecutive interstitial fibrosis.

Interstitial oedema affects myocardial blood supply. The ischaemia thus produced is accompanied by a dilatation of the myocardial capillary bed, assuming the appearance of "giant capillaries" regarded by BOIKAN [4] as pathognomonic of isolated myocarditis.

Inflammatory infiltration is slower in its production, as reflected by groups I and VI (Fig. 6).

The changes involving the interstitial tissue result in gross myofibrillar, i.e. parenchymatous, damage with diffuse oedema (disappearance of striation), granular disintegration, nuclear pycnosis and lysis, formation of transverse hyaline fibrous bundles and finally segmental necrosis. The destruction of myocardial fibres goes hand in hand with the appearance of myogenic giant cells. These changes, though being of secondary nature, are none the less of vital significance. The hearts of the animals of group VI revealed extensive, partly confluent areas of necrosis (Figs 7, 8, 9). The nature of the morphological abnormalities clearly accounts for the prevalence of the conduction disturbances in this group.

The abnormalities described in the foregoing were present in all animals of the test groups, differing only in extent and in the prevalence of the individual features, in accordance with the duration of the disease.

No microscopic abnormalities were demonstrable in the striated muscles and in the liver.

Modification of the diet resulted in a more protracted course of the process with a slightly attenuated symptomatology, without, however, affecting the fatal outcome. On the other hand, the survival thus attained was long enough for the production of microscopic changes marking the later stages, i.e. inflammatory infiltration and degenerative myofibrillar lesion. These findings agree well with the high incidence of diverse ECG abnormalities, in particular with the prevalence of conduction disturbances [23].

Our further objectives include the production of myocarditis in full-grown rats too by means of the present diet combined with physical exercise which would allow to study the process as well as its therapeutic aspects in the adult animal.

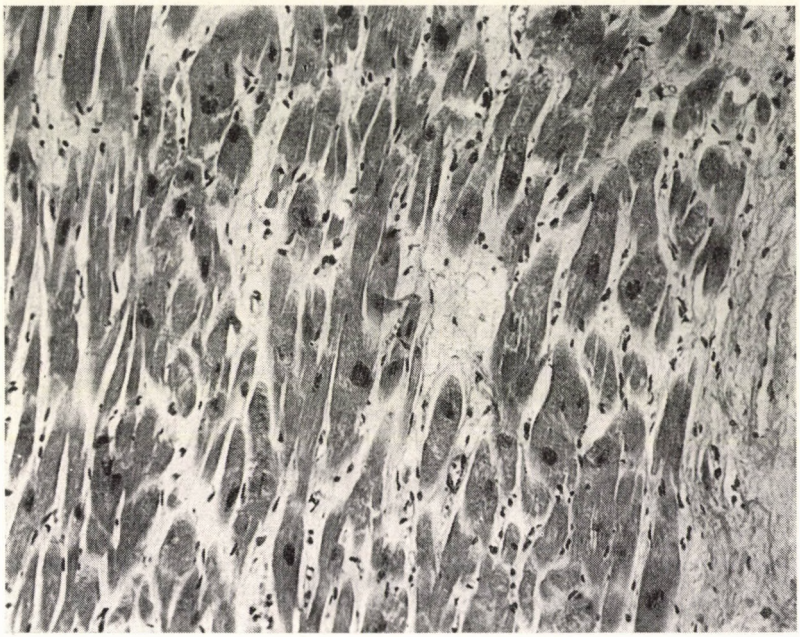

Fig. 6. Inflammatory cells in oedematous interstitial tissue. Myocardial hypoxia: nuclear swelling, myofibrillar oedema and disintegration. (H. E., ×240)

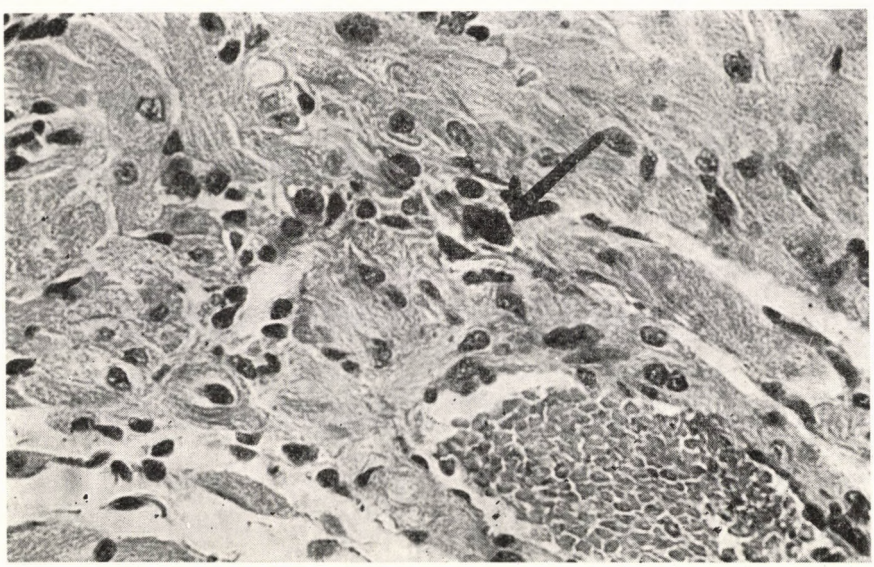

Fig. 7. Swelling of myocardial fibres, myofibrillar disintegration; perivascular inflammatory infiltration with myogenic giant cell (arrow). (H. E., ×380)

Fig. 8. Typical histology of acute isolated myocarditis. *A:* Giant capillary; *B:* medium-sized intramural coronary branch with perivascular infiltration; *C:* groups of necrotized myocardial fibres with homogeneously staining dark cytoplams. (Farkas–Mallory, ×96)

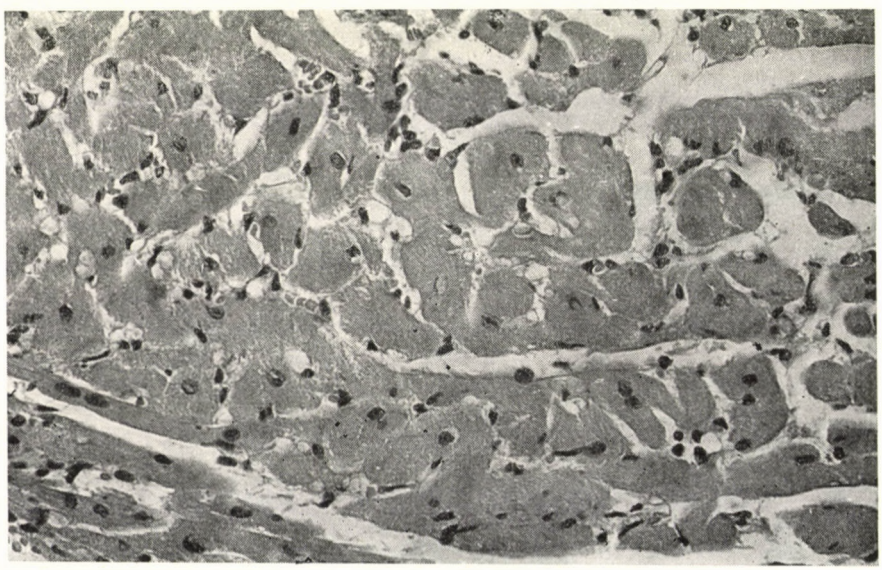

Fig. 9. Extensive myofibrillar necrosis. Homogenization of eosinophilic cytoplasm, nuclear pycnosis. Delicate connective tissue fibres in oedematous interstitial tissue. (H. E., ×240)

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ULTRASONIC PROVOCATION OF LOCAL SHWARTZMAN-REACTION

By

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A thrombohaemorrhagic skin reaction with necrosis was induced in rabbits by exposure to ultrasound (2 W/cm² for 10 min) of a skin area to which a preparatory intradermal injection of *E. coli* O111 endotoxin had been applied. The kinetic pattern as well as the gross appearance and the microscopic features of the reaction were similar to those of the Shwartzman-reaction produced by intradermal and subsequent intravenous administration of endotoxin.

Local provocation of the Shwartzman-phenomenon is attributed to the complex effect of ultrasound in which local damage to the tissues and to the leukocytes on the one hand and circulatory changes on the other, possibly also activation of blood coagulation factors, seem to be involved.

A local Shwartzman-rection (LSR) is induced by the intradermal injection of endotoxin to rabbit, for the phase of preparation and, 24 hours later, an injection of endotoxin into the circulation, for the phase of provocation. In the following 4 to 6 hours a typical cutaneous lesion, marked by capillary and small-vessel thrombosis, extravasation of erythrocytes and necrosis makes its appearance [13]. The fact that the phase of intradermal preparation is separated from that of intravenous provocation by an interval of 24 hours, permits to study the conditions and the production of the two phases independently from each other. We have learned in this manner that, apart from endotoxin, numerous other agents of a leukotactic property *in vivo* lend themselves to the preparation of LSR [17]. This is also true for the provocation of the actual reaction, as agar [11], starch [4], tissue extracts [1], kaolin, glycogen [15] and other macromolecular substances administered by the intravenous route have been found to provoke the LSR on the endotoxin-prepared skin area in the same manner as does intravenous endotoxin.

On the other hand, a local administration of endotoxin to the prepared skin area induces no typical LSR [10].

In view of this fact it was surprising to note a typical LSR in some cases where the conventionally prepared skin area had been affected mechanically at the optimum point of time for provocation. This observation had prompted us to apply ultrasound irradiation to the prepared skin area for the provocation of LSR, a procedure enabling a far more accurate adjustment

of the doses than does mechanical injury. The pioneer studies by POHLMAN et al. [8] have revealed the effects of ultrasound on the tissues, and these observations have been confirmed by extensive observations [2].

Material and methods

Eighteen rabbits of either sex weighing 2000 ± 200 g were used. They were kept under standard conditions and on a standard diet supplemented with vegetables, water being provided ad libitum. Endotoxin was prepared from the E. coli O111-strain by the procedure of BOIVIN routinely used at our institute. The stock solution was made up to contain DL_{50} referred to rabbits (summer months) in 2 ml. The skin of the animals was depilated on the day before the experiment. For the preparation of the reaction, 0.2 ml of an eightfold dilution of the stock solution was applied intradermally to two different areas of the skin of the back. Twenty-four hours later, one of the areas thus prepared together with the other area to which no preparatory injection had been applied, were irradiated with ultrasound, by means of a generator calibrated according to TARNÓCZY [14]. With a working frequency of 800 kHz of the instrument, ultrasound was applied with an intensity of 2 W/cm² for 10 min. To eliminate the thermal effect, a water-filled rubber bag was placed between the headpiece and the skin. Reactions were read at 6 and 24 hours, at the same time tissue specimens were taken for microscopic study. The material was fixed in Susa's solution, embedded, the sections were stained with haematoxylin-eosin and examined under the light microscope.

Results

A few minutes after irradiation, the prepared areas exposed to ultrasound exhibited a marked hyperaemia as compared with the prepared areas to which no ultrasound had been applied. Occasional petechiae were also seen. In the absence of previous preparation the changes noted after irradiation were confined to hyperaemia. On the other hand, six hours after irradiation, the areas which had been prepared and irradiated were affected in 14 out of 18 rabbits by a lesion reminiscent of LSR. Twenty-four hours later the reaction had reached its peak. The prepared areas which had not been exposed to ultrasound exhibited only hyperaemia and swelling. Without previous preparation, the ultrasound induced petechiae in the first 24 hours after exposure in four instances.

The microscopical findings are shown in Fig. 1. Without preparation, 6 hours after irradiation the area of exposure to ultrasound, in particular the fibrous, vascular layer poor in cells, situated between the epidermis and the cutaneous muscles, i.e. the part most closely involved in the LSR, exhibited a condensation of fibrous elements and a dilatation of the small vessels with congestion (Fig. 1).

In the case of preparation without provocation, the areas exposed to ultrasound displayed a conspicuous leukocytic infiltration; the fibrous structure was, however, preserved (Fig. 2).

In the case of preparation and subsequent ultrasonic provocation an intensive LSR was seen at 6 hours, still more so at 24 hours (Fig. 3).

Epidermal necrosis and crust formation were more marked than after the conventional provocation procedures. This must have been the result of

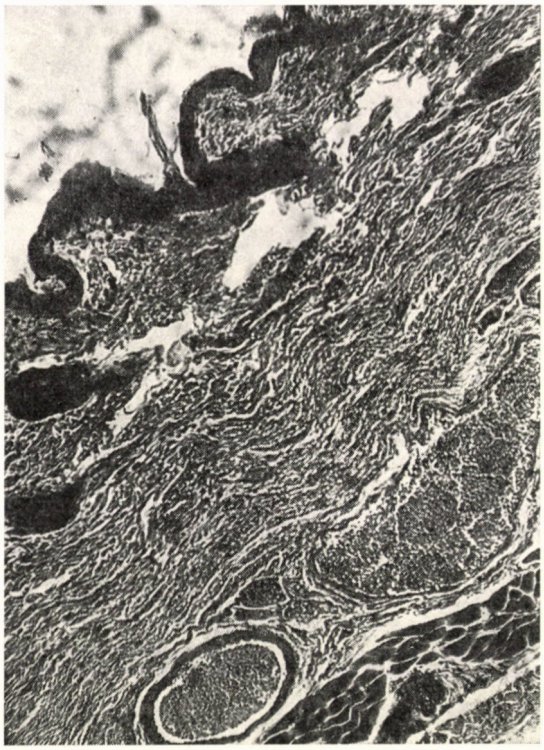

Fig. 1. Rabbit skin 6 hours after ultrasonic irradiation. Insignificant epidermal lesion, condensation of connective tissue fibres. Vascular dilatation, congestion. Haematoxylin-eosin, ×210

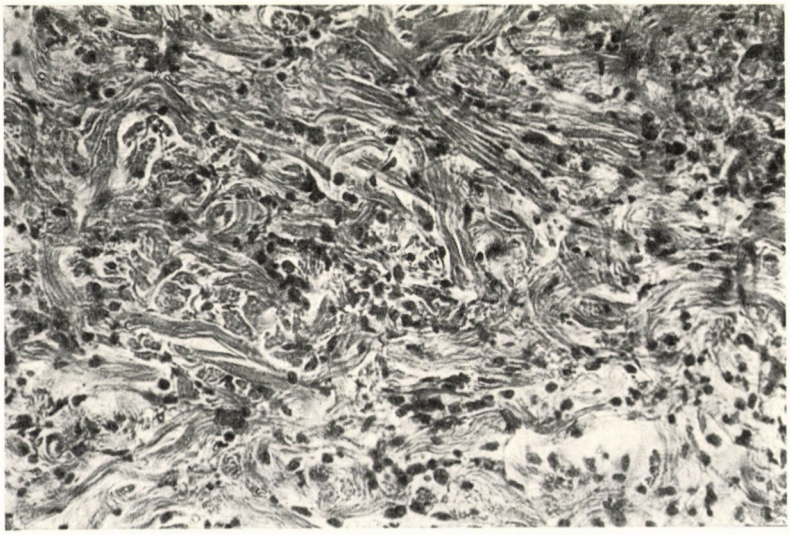

Fig. 2. Rabbit skin 24 hours after intradermal injection of endotoxin. Leukocytic infiltration; preserved fibrous structure. Haematoxylin-eosin, ×540

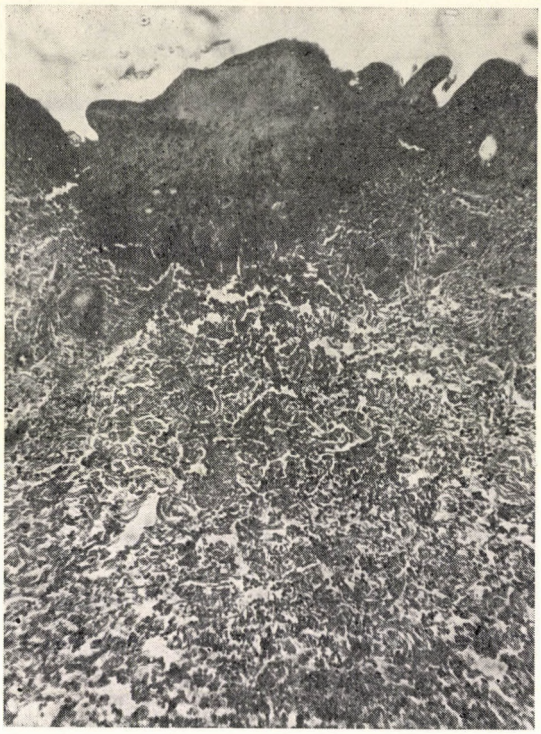

Fig. 3. Endotoxin-prepared and irradiated rabbit skin 24 hours after ultrasonic irradiation. Epidermal necrosis and crust formation, grave connective tissue necrosis. Haematoxylin-eosin, ×210

a direct ultrasonic damage to the tissues and would seem to indicate the direction of ultrasound effect. The changes seen in the deeper layers were identical with the usual features of LSR. The connective tissue fibres were damaged (Fig. 4), and there was an extensive extravasation of erythrocytes (Fig. 5).

Discussion

The aim of the preparatory phase of LSR is basically the production of a leucocytic infiltration of the skin; this change is then associated with various histochemical changes [13]. On the other hand, the "final common pathway" represented by the provocation phase is marked by the release of leukocytic lysosomal enzymes including proteases [7, 13]. Gold salts applied locally in this phase are able to suppress the LSR, probably by inactivating the release of SH-enzymes by the leukocytes [12].

Coagulation factors are closely involved in the thrombohaemorrhagic reaction. While HALPERN et al. [5] inhibited the development of LSR by hepa-

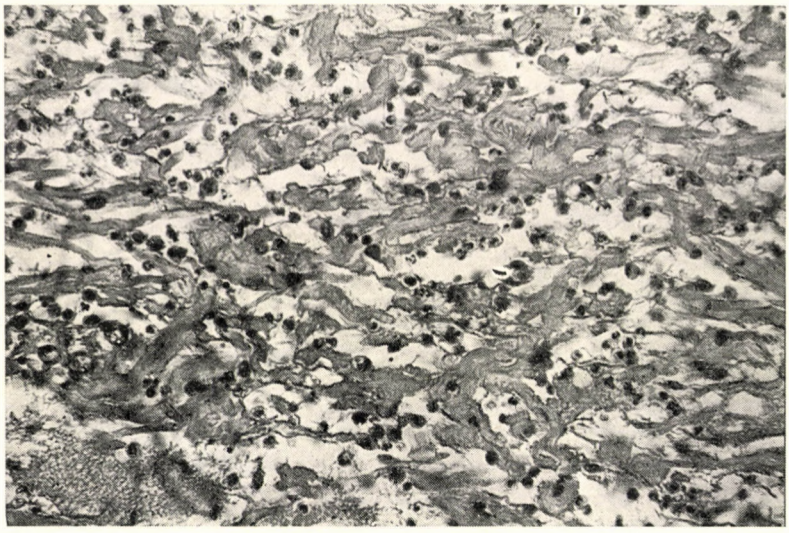

Fig. 4. Endotoxin-prepared rabbit skin 6 hours after ultrasonic irradiation. The fibrous structure is gravely affected (see Fig. 2). Haematoxylin-eosin, ×540

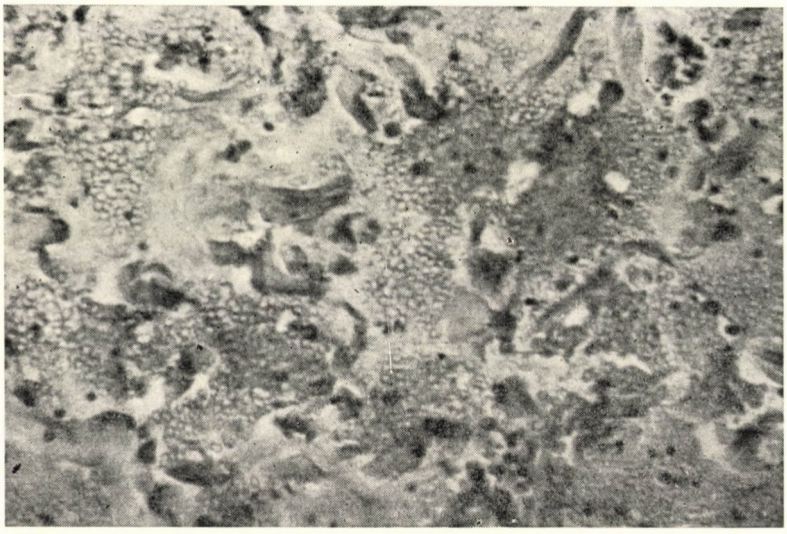

Fig. 5. Endotoxin-prepared rabbit skin 6 hours after ultrasonic irradiation. Large numbers of erythrocytes among degenerated fibrocytes and leukocytes. Haematoxylin-eosin, ×840

rin, the present authors noted an intensification of the reaction by reptilase, a preparation of thrombin-like activity [16].

In the light of these facts it is remarkable that the endotoxin-prepared skin should require only a local exposure to ultrasound for the production of LSR and that the reaction thus produced differs neither in its gross appear-

ance nor in its microscopic features from the LSR induced in the conventional fashion, by means of intravenous endotoxin. Minor damage to the tissues marked by dilatation and stasis of the capillaries and small vessels, was produced by exposure to ultrasound even without preparation, but this procedure never induced a typical LSR. On the other hand, with previous preparation of the skin, ultrasonic provocation was followed by a typical LSR which appeared 6 hours after exposure and attained its peak at 24 hours.

There have been various, for the greatest part unsuccessful, attempts at the preparation and provocation of LSR by means of physical factors [9]. In the present study a typical reaction has been produced by local ultrasonic irradiation. This seems to be due to the complex effect of ultrasound. Enhanced activity of the lysosomal enzymes in the cellular elements of the exudate produced by ultrasonic irradiation has been confirmed by histological evidence [6]. The enzymes, particularly proteases, released from the leukocytes may well play a decisive part in the production of necrosis.

As it has been seen, in the absence of preparation ultrasonic irradiation is followed by capillary dilatation and stasis within the area of exposure which clearly points to a functional impairment of microcirculation as well. One of the simplest procedures for the disintegration of platelets *in vitro* is their ultrasonic irradiation [3]. This may well happen *in vivo* too, capillary dilatation and congestion providing important additional clot-forming factors.

Though it has been sought to attenuate the thermal effect of the ultrasound by cooling the skin, the possible provocative role of a thermal factor in the production of the reaction cannot be ruled out.

In sum, we attribute the ultrasonic provocation of LSR in the endotoxin-prepared rabbit skin to the complex effect of this radiation which combines the essential factors of the reaction, i.e. local injury to the tissues, damage to the leukocytes accompanied by a release of lysosomal enzymes, microcirculatory disturbances, and an activation of blood coagulation factors.

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PROTECTIVE EFFECT OF ANTI-MOUSE-SKIN-EPITHELIUM RABBIT-IgG ON SURVIVAL OF MOUSE-SKIN HOMOGRAFTS

By

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Rabbit-IgG raised against BALB/c-mouse-skin epithelium has been found to prolong the survival of BALB/c-mouse-skin grafts in CBA-mice. Anti-skin-epithelium IgG and antithymocyte-IgG have many features in common. It is suggested that the protective effect of anti-skin-epithelium rabbit-IgG involves three mutually related factors, i.e. 1) antithymocyte effect; 2) protective coating of the surface; 3) antibody-suppression affecting the macrophages.

The principal safeguard for the survival of grafts is the identity of histocompatibility antigens. The fact that, with the exception of monozygotic twins, there exists virtually no complete identity of histocompatibility antigens, gives a prime importance to all preventive or curative procedures promoting the survival of allogeneic organs or tissues in the recipient, and prompts extensive research work in order to explore new possibilities in addition to the conventional immunosuppressive therapy making use of steroids, cytostatic agents and antilymphocyte globulin.

One of the procedures for the prolongation of graft survival is a treatment of the recipient with heterologous immunoglobulins directed at the donor's antigens [3, 7, 9, 25]. The data concerning this question are, however, conflicting, there being no general agreement on the possible mechanism accounting for the effect [2, 5, 6, 9, 12, 15, 17, 19, 21].

In the present study it has been examined whether immune serum raised against donor antigens has any graft-protective effect and if so, what mechanism is responsible for it. The experimental model selected for this purpose was skin grafting between H_2-incompatible mouse-strains.

Material and methods

Production of anti-skin-epithelium and antithymocyte-antisera: The tail-skin of BALB/c-mice was treated with trypsin at 37 °C for 60 min. The epidermal layer was detached, minced, aspirated several times through a fine-bore cannula, washed thrice in Parker's culture medium. The intact epithelial cells were resuspended in Ringer's solution. Two male rabbits weighing 2000 g each were immunized by an intravenous injection of 5×10^7 epithel cells, and the procedure was repeated 14 days later. On the seventh day after the second injection the animals were killed by exsanguination.

The antigen for antilymphocyte-globulin was derived from thymocytes of two week-old BALB/c-mice. The thymus was minced, suspended in Parker's medium, and aspirated

several times through a fine-bore cannula. After three washings, the thymocytes were resuspended in Ringer's solution. Two rabbits were immunized, each with 5×10 thymocytes administered intravenously on two occasions at an interval of 14 days, and the animals were bled 7 days after the second injection. The sera were inactivated at 56 °C for 30 min and exhausted with BALB/c mouse-erythrocytes. The IgG-fraction was separated by anion-exchange DEAE-cellulose column chromatography [6]. Purity of the fractions was checked by immunoelectrophoresis. The protein fractions of pure IgG content were lyophilized. For the tests *in vitro*, freshly prepared IgG solutions of 10 mg/ml concentration were used.

Lymphocytotoxicity in vitro. The anti-mouse-epithelium and antithymus rabbit-IgG were studied for lymphocytotoxicity by the method of TERASAKI et al. [22].

Lymphocyte agglutination in vitro. The lymphocyte agglutination titres of anti-mouse-skin epithelium and antithymus rabbit IgG fractions were estimated by the method of ABAZA and WOODRUFF [1].

Opsonization. Mouse peritoneal macrophage cultures were raised in tissue culture chambers. Mouse thymocytes, incubated with anti-mouse-skin-epithelium and antithymus rabbit-IgG, in the presence of complement, were added to macrophage monolayers. The opsonized thymocytes adhered to the macrophages. The titre of opsonization was taken as the dilution giving a minimum lymphocyte–macrophage ratio of 5 : 1 [10].

Distribution of anti-skin-epithelium and antithymus rabbit-IgG in the mouse organism. Two per cent solutions were prepared from the two kinds of specific IgG and from IgG derived from a serum pool of untreated (control) rabbits. The solutions were labelled with ^{125}I by an electrolytic procedure. Labelling proved of 70% efficiency. The unbound ^{125}I was adsorbed on activated palladium powder. Labelling was checked on the following basis. 1) After electrophoretic migration, the activity of the preparation on contact radioautography was confined to the precipitation band corresponding to IgG, as a sign that the procedure had left the protein unaffected and that no iodine containing by-products had formed. 2) Freedom of the preparation from free iodine was confirmed in a bioassay; no concentration of ^{125}I by the thyroid of the test animals was noted. The mice were given intraperitoneally 2 mg of 5 μCi ^{125}I-IgG (anti-mouse-epithelium and antithymus rabbit-IgG and normal rabbit IgG). Radioactivity was measured in skin, thymus, muscles, blood, kidney and brain, 5, 15 and 30 days after administration of the isotope, using a model NK-108 Gamma (Budapest) energy-selective counter [13].

Antibodies. Precipitation by the antiepithelium and antithymus rabbit-IgG with the individual mouse organ extracts was identified by the procedure of OUCHTERLONY [18].

Skin homograft survival. Skin from the ear of BALB/c-mice was grafted into skin windows prepared on the back of CBA-mice. The wound was covered with a gauze pad soaked in mineral oil and provided with a plaster of Paris dressing which was removed on the 8th day after grafting.

The first group (n = 26) received IgG from untreated (control) rabbits, the second group (n = 26) anti-mouse-skin-epithelium rabbit-IgG, and the third group (n = 24), anti-mouse-thymus rabbit-IgG, two days before transplantation, intraperitoneally, in 2 mg doses. For statistical analysis of the results, the *t* test was used.

Results

Lymphocytotoxicity in vitro. Both anti-mouse-epithelium rabbit-IgG and anti-mouse-thymocyte rabbit-IgG proved cytotoxic to BALB/c-mouse thymocytes; the titre of the former attained 1 : 4000; while that of the latter, 1 : 25 000, as against 1 : 128 of the control rabbit sera.

Lymphocyte agglutination in vitro. Thymocytes of BALB/c-mice were agglutinated by anti-mouse-epithelium IgG to a titre of 1 : 250, and by antithymocyte rabbit-IgG, to 1 : 18 000, as against 1 : 64 of IgG of the control rabbit sera.

Opsonization. The opsonization titre (BALB/c mouse thymocytes) of anti-epithelium rabbit-IgG was 1 : 6000; that of antithymocyte rabbit-IgG, 1 : 30 000, as against 1 : 256 of the IgG of untreated rabbits. The values for

lymphocytotoxicity, lymphocyte-agglutination and opsonization of anti-mouse-skin and antithymus rabbit-IgG have been summed up in Table I.

Table I

In-vitro titres of anti-mouse-skin-epithelium rabbit-IgG and anti-mouse-thymocyte rabbit-IgG

	Lymphocytotoxicity titre 1	Lymphocyte agglutionation titre 1	Opsonization titre 1
Anti-mouse-skin-epithelium rabbit-IgG	4,000	250	6,000
Anti-mouse-thymocyte rabbit-IgG	25,000	18,000	30,000
IgG-pool of untreated rabbits	128	64	256

The radial double immune diffusion precipitation procedure yielded the following results. The anti-epithelium rabbit-IgG gave the most marked precipitation band with the skin extract, but was found to precipitate liver, kidney, muscle, and brain extracts, too, though faintly. Antithymocyte rabbit-IgG gave precipitation bands, in addition with the thymus extracts, also with those of liver, skin and kidney.

Distribution of anti-mouse-epithelium rabbit-IgG in the murine organism is presented in Fig. 1. It can be seen that the highest IgG activity was bound by the skin, thymus and kidney.

Distribution of anti-mouse-thymocyte rabbit-IgG is presented in Fig. 2. Here too, the maximum activities were found in skin, thymus and kidney.

Distribution of normal (untreated) rabbit-IgG was in conformity with data in the literature [11, 23].

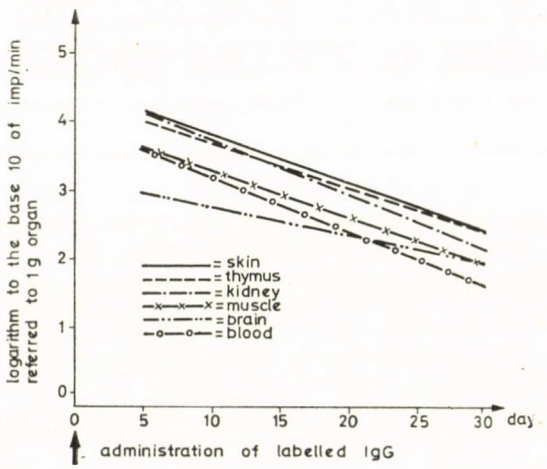

Fig. 1. Distribution of labelled anti-mouse-epithelium-IgG in the murine organism

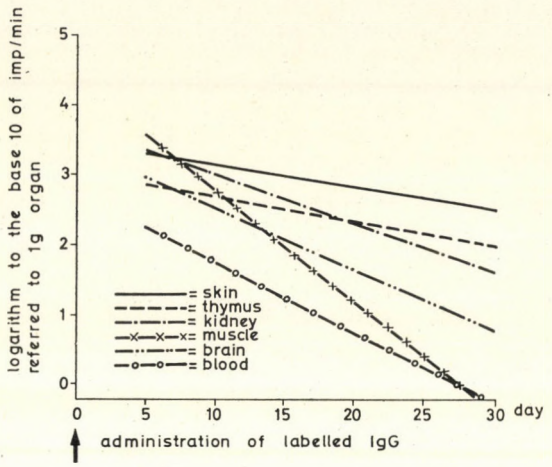

Fig. 2. Distribution of labelled anti-mouse-thymocyte-IgG in the murine organism

Survival of skin grafts. In the CBA-BALB/c transplantation system of H_2-locus incompatibility, survival of skin-grafts was 11.7 ± 1.3 days without treatment, and was found practically identical in the case of IgG treatment of untreated rabbits (12.1 ± 1.4 days). On the other hand, it extended to 15.6 ± 1.1 days in the mice treated with anti-mouse-epithelium rabbit-IgG, and to 16.8 ± 1.9 days in those treated with anti-mouse-thymocyte rabbit-IgG. The results were significant statistically ($p < 0.05$) (Table II).

Table II

Skin graft survival in mice treated with anti-mouse-skin-epithelium rabbit-IgG and anti-mouse-thymocyte rabbit-IgG

	Untreated mice	Mice treated with		
		IgG-pool of untreated rabbits	Anti-mouse-skin-epithelium rabbit-IgG	Anti-mouse-thymocyte rabbit-IgG
Skin graft survival, days	11.7 ± 1.3 (SE) $n = 45$	12.1 ± 1.4 $n = 26$	15.4 ± 1.1 $n = 26$	16.8 ± 1.6 $n = 26$

Discussion

Both anti-mouse-epithelium as well as antithymocyte rabbit-IgG were found to prolong the survival of skin grafts.

Anti-mouse-epithelium rabbit-IgG bound by skin, thymus and kidney showed high activity. This would seem to suggest that a coating of the antigen determinants by the specific antibodies might also be involved in the graft-

protective effect of anti-mouse-epithelium rabbit-IgG. It is assumed that xenogeneic rabbit immunoglobulin is coating the allogeneic murine epithelial cells, thus barring the access of the recipient's immunocytes to the allogeneic murine tissue. To gain access to it, the recipient's cells would have to break down first the outer wall of the fortification, i.e. the xenogeneic immunoglobulin coating which certainly implicates a delay in the graft's rejection. Even a longer graft survival might be expected on theoretical grounds by using allogeneic mice instead of rabbits for antibody production, considering the fact that rabbit-IgG forming contact with skin-epithelium antigens elicits a stronger immune response than does the IgG derived from allogeneic mice. Uptake of large amounts of anti-mouse-skin rabbit-IgG by the thymus, too, suggests that there are identical antigen determinants on the surface of skin-epithelial cells and of thymocytes. This may be the other basis of the protective effect of sera derived from nonlymphoid tissues. The high activity of the kidneys might be due to the large number of tubular epithelial cells [24].

Thus, it follows from the above that the immunoglobulins of two kinds of specificity have various features in common, viz.

1) both are primarily bound by skin, thymus and kidney;
2) they are lymphocytotoxic even at high dilutions;
3) they produce opsonization of lymphocytes even at high dilutions;
4) they form precipitation bands with various antigens.

This raises the possibility that the anti-mouse-epithelium rabbit-IgG has also an antithymocyte (globulin)-activity in addition to its graft-coating capacity. If this is so, it also holds true for the reverse, that is, the graft-protective effect of antilymphocyte globulin must involve a protective, coating surface mechanism, too. This is suggested also by the results of our earlier studies according to which the production of antilymphocyte sera is accompanied by the appearance of various high-titre anti-autogenic antibodies in the sera [4, 8, 14].

In addition to the antithymocyte- and to the protective, surface-coating effects involved in the graft-protective activity of anti-mouse-epithelium rabbit-IgG, the passive antibody suppression asserting itself within the macrophage cell system also seems to provide an essential factor of this mechanism [20]. The inconsistency of data concerning the *in vivo* immunosuppressive effect of specific antibodies may be attributed to 1) difficulties in finding suitable experimental models for the complex effects in question; 2) variability of the results according to species; 3) substantial differences in the antigenicity of organ and tissue extracts serving as sources for the antisera; 4) differences in the immunosuppression provided by antibodies of high avidity as opposed to those of poor avidity [20].

Standardization constitutes the most important task in the production of antibodies of suppressive activity directed at the surface-antigens of grafts,

and it calls for the elaboration of a test-system providing answers to the following questions:

1) which antigen (or antigens) should be used, in what amounts and on how many occasions?

2) which stage of immune response is optimal for the harvest of antibodies being bound with maximum avidity onto the surface of the allograft?

3) under which conditions are the specific antibodies apt to block the macrophages and thymocytes?

The present study has failed to provide a complete answer to these questions. Further procedures for the production of reliable immunosuppressive sera based on their specific antibody content have yet to be developed. Sera of this nature would have the advantage over nonspecific immunosuppressive measures to confer a certain specific protective effect on the allograft.

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IN-VITRO SENSITIVITY TO CYTOSTATICS IN CHRONIC MYELOID LEUKAEMIA

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Peripheral leukocyte cultures obtained from patients with chronic myeloid leukaemia were examined for sensitivity to various cytostatic agents. Administration of the drugs which had been found efficient *in vitro*, brought about a clinical remission in 7 out of 8 cases.

Chemotherapy of malignant diseases offers certain possibilities of choice. Though, with the exception of l-asparaginase the cytostatic drugs are of little selectivity, their clinical effects differ in many respects. There may be a primary resistance to a particular drug, or a secondary resistance may develop during the treatment, an eventuality which still adds to the limitations of the haphazard use of these products.

The idea of a predictive assessment of cytostatic sensitivity *in vitro* is by no means new; various attempts have been made with cultures of solid tumours as well as of peripheral leukocytes in chronic myeloid leukaemia [1, 14], chronic lymphoid leukaemia [19, 30–33] and acute leukaemia [5, 6, 14, 18]. Assessment of the cytotoxic effect *in vitro* is far from simple and that it is fraught with a wide margin of uncertainty is reflected by the great variety of procedures which have been devised for this purpose, e.g. glycolysis, inhibition of protein synthesis, uptake of labelled precursors, methylene blue reduction, uptake of supravital stains, cytomorphology, etc. These procedures have been reviewed in detail by ISSEKUTZ [15].

Other lines of study, such as *in-vivo* testing of human tumours transplanted to animals [13, 35] have been attempted with little success.

Evidence of *in-vitro* tests has been found to correlate well with the clinical results in the majority of cases [1, 6, 7, 9, 20]. On the other hand, certain authors [10, 36] find such results of little use for clinical purposes, not only because they provide no information on the systemic effect of the drug, but also because the tumour may change its original character in the course of treatment, a claim which finds no support in recent literature [29].

Chronic myeloid leukaemia (CML) cells are easy to obtain and lend themselves to *in-vitro* testing of antileukaemic drugs. The need for cytotoxicity tests of this kind arises in view of the frequently assuring resistance to some

drug in the course of long-term treatment, which would thus result in an early recurrence or in a failure of attaining a remission. Insistence on further use of a drug to which the patient has become resistant would obviously do more harm than good. Though the very nature of the disease makes it easy to assess the degree of responsiveness, or, conversely, of resistance, to the given drug after each short-term course of intermittent treatment [12], it would still be preferable to avoid the use of drugs which have merely toxic effects without ensuring any benefit.

Sensitivity tests *in vitro* have been carried out before prescribing the treatment, on peripheral leukocyte cultures obtained from 8 CML patients during periods of relapse (WBC between 34,000 and 220,000) and from 8 normal controls.

Material and methods

The following method, adapted from the literature [4, 8, 11, 16, 19, 21, 23—26] was used.

Heparinized (100 I.U.) blood drawn under sterile conditions was allowed to sediment at 37 °C for 45 min, and, after removing the supernatant, it was centrifuged at 500 r.p.m. for 15 min. Then the supernatant was discarded, the cells were resuspended in TC 199 medium and again centrifuged. This washing procedure was repeated, finally the cells were resuspended in TC 199 with 10% calf serum; the medium contained in addition 200 I.U. per ml of penicillin and 100 μg per ml of streptomycin and was made up to a concentration of one million cells per ml. 1.5 ml samples of the cell suspension were pipetted into siliconized penicillin vials of 10 ml and the cytostatics were added in the corresponding dilutions. The vials were tightly closed with rubber stoppers and incubated at 37 °C for 48 hours. The drugs under study were hydroxyurea (Biogal), dibromomannitol, mannomustin (Chinoin), Zitostop^R = tetramesylmannitol (EGYT), R-74 (GyKI), methotrexate (Lederle), Cérubidine^R = rubidomycine (Specia), and busulfan (Burroughs Wellcome); they were diluted in accordance with the usual human doses. The dilutions were calculated according to HIRSCHAUT et al. [14], using tenfold values of the serum concentrations.

After incubation, the cells were brought together with a 1% trypan blue solution and the viable (i.e. unstained) cells were counted in a counting chamber (Bürker). Toxicity was calculated on the basis of the index $100\left(1-\frac{A}{B}\right)$, where A represents the number of viable cells per ml in the treated (drug-containing) vials, and B the surviving cells per ml in the control (drug free) vials.

Results

Results are summarized in Table I. Though we must evaluate every finding under the individual clinical aspects of the case, yet some of the features emerging from the observations show a striking consistency. 1) The toxicity pattern was different in each case. 2) None of the drugs under study proved equally cytotoxic in every case. 3) The drugs qualified as myelotropic were found cytotoxic in the majority of the cases of CML, in contrast to the normal controls where they were not cytotoxic.

Confrontation of *in-vitro* evidence with the clinical results seems still more informative. The results of sensitivity tests were not yet known at the start of cytostatic treatment (Zitostop, Myelobromol, Hydroxyurea), and it

Table I

Drug sensitivity of peripheral leukocytes of patients with chronic myeloid leukaemia on the basis of the cytotoxicity-index (c: complete, I: incomplete remission, Ø: on remission; HU: hydroxyurea, Z: Zitostop, DBM: dibromomannitol)

	Patient No.	hydroxyurea 400 μg/ml	methotrexate 1 μg/ml	Zitostop® 200 μg/ml	dibromo-mannitol 100 μg/ml	rubidomycin 10 μg/ml	mannomustin 10 μg/ml	R-74 10 μg/ml	busulfan 1 μg/ml	Therapy	Remission
CML	1.	14.5	14.5	16.2	20.9	31.2				DBM	C
	2.	17.0		17.0	38.1	40.7				DBM	C
	3.	24.8		19.2	43.5	34.1				DBM	C
	4.	32.0		20.0	53.5	31.7				HU+Z	C
	5.	16.0	17.0	26.2	12.0	32.9	14.0	7.0	24.0	Z	C
	6.	27.0	12.0	10.0	14.5		15.2	14.0	12.2	HU+Z	C
	7.	30.1	11.5	20.7	13.2		18.2	10.2	20.2	HU+Z	Ø
	8.	28.0		16.6			10.5	14.0	15.0	HU+Z	I
Controls	1.	12.1		2.1	6.9	5.5	27.2	18.3			
	2.	7.9		10.1	8.9	3.9	21.2	20.4			
	3.	0.5		3.9	15.5	5.2	39.6	17.0			
	4.	3.9		0.0	16.0	29.0	29.0	27.5			
	5.	0.5	3.9	5.9	17.5	4.3	42.0	25.0			
	6.	8.0	9.0	15.0	22.0	22.0	31.0	45.7			
	7.	12.0	17.0	7.9	10.5	17.3					
	8.	2.3	10.5	12.0	17.0	43.7					

was only in retrospect that the outcome of therapy was checked with *in-vitro* sensitivity. The results of the tests were disregarded until we could convince ourselves of their practical value. Short-term massive-dose type of treatment was applied all throughout. Actually, in 7 out of 8 cases studied the drug of empirical choice proved efficient *in vitro* and the course of treatment was followed by remission of two months duration. In one case where the process had reached the stage of blast-cell crisis, the combined administration of Zitostop + hydroxyurea, though having proved adequately cytostatic *in vitro*, was unable to induce a full remission, and the patient died soon thereafter.

Discussion

The results of *in-vitro* sensitivity test correlate well with those of short-term massive-dose therapy with a sole exception. Still, it seems unlikely that the correlation will prove nearly as good in cases of maintenance therapy by the use of smaller doses. It is well known that drugs of adequate

cytotoxic properties fail to induce a clinical remission in every case [6]. The predictive value of *in-vitro* tests is confined to the actual cytotoxic effect of the drug in question leaving us in ignorance about the diverse factors of remission or resistance.

The question how far the peripheral leukocytes in CML represent the malignant cell population has also to be raised. In view of the different culturing possibilities of normal and leukaemic cell populations [25] it is safe to assume that the peripheral cells are prevalently of leukaemic nature. It is, however, quite a different question, how far this population corresponds in respect of sensitivity to cytostatics to the leukaemic stem cells which are the carriers of malignancy, and, by this fact, also the actual targets of cytostatic therapy. It is most unlikely that a total destruction of the entire leukaemogenic cell population in CML should be possible even by intensive chemotherapy. The reason for this must be sought partly in the differences in sensitivity of the individual malignant cells, partly in a metabolic dissimilarity resulting from the diversity of developmental stages in the leukaemic cell population [3, 22, 28, 34]. This amply accounts for our doubts whether and how far the sensitivity of the peripheral leukaemic cells characterizes that of the totality of the malignant cell population. Despite these theoretical objections, published evidence [1] including our own observations offer encouraging lines of orientation.

The techniques of *in-vitro* procedures are still uncertain. As mentioned in the introductory part, for the measurement of the cytotoxic effect various methods have been used. Though supravital staining allows to separate viable from nonviable cells, it fails to detect the irreversible processes which might have taken place within the apparently viable cell and result later, in the phase of cell division, in cell death due to cytotoxic effect.

Further tasks include a closer adjustment of the time of incubation and of the drug concentrations applied. While, on the one hand, the practicability of the tests is limited by the length of time required, on the other, their reliability, in other words their predictive value, is affected by unduly short exposure to the cytostatic agent, though some authors have found a good correlation even in the case of short exposures [2, 6, 9, 17, 27]. Since concentration and duration of effect definitely belong to the factors deciding the efficiency of a given cytostatic drug, we have used concentrations tenfold the average blood levels attained in man.

It has been sought to simplify the procedure as far as possible so as to make it practicable for clinical use, despite its limitations which have to be taken into consideration in the interest of a proper evaluation of the results. However, the actual usefulness and predictive value of *in-vitro* sensitivity tests have yet to be established by correlating their results with further clinical observations.

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HISTOLOGY OF GASTRIC MUCOSA AND ITS SECRETORY ACTIVITY IN MAN

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The histological picture of the fundic mucosa obtained by blind biopsy has been compared with the HCl secretory capacity of the same stomach. HCl production after histamine injection was noticeably greater in subjects with normal mucosa than in patients with different forms of gastritis. In the latter the production of HCl decreased parallelly with the severity of gastritis.

The thickness of the active parenchymal layer reflects the degree of the morphological and functional damage of the mucosa more characteristically than do the values of the full thickness of the mucosa. The decrease in the number of active parenchymal cells follows the severity of the morphological lesions. Among the glandular constituents, the chief cells seem to be relatively most sensitive to progressive gastritis.

The relation between the histological structure and the acid secretory capacity of the gastric mucosa has been the subject of several studies [1—6]. The present paper is an attempt to contribute a few new data to the problem.

Material and methods

The investigations were made in 144 male and 120 female clinical patients selected at random. Aspiration of the gastric juice was done according to the original method of LAMBLING [7]; 0.5 mg histamine dihydrochloride was used to stimulate acid secretion. The secretory groups were distinguished according to the degree of free acidity. In the anacid group there was no free HCl, in subacid patients free acidity was <40 meq/l, in normacid patients it was 40—90, and in hyperacid patients >90 meq/l. Maximum acid output per hour was calculated according to the method of BARON [8], i.e. the amount of the two successive largest 15 minute outputs was multiplied by two and the amount of acid expressed in meq/h.

Biopsy was made with a Wood type instrument on the morning after the gastric analysis. At each biopsy two or three pieces of mucosa were taken from the fundus. Specimens from the antrum were not evaluated.

The bioptic material was fixed in formalin and after embedding in paraffin stained with haematoxylin-eosin, and with Zimmermann's differential dye.

Results

1. Relation between the secretory and the morphological groups

The subjects with normal mucosa were predominantly hyperacid or normacid, a small percentage of subjects were subacid; no anacid persons were found in this group. In superficial gastritis the ratio of subacid subjects

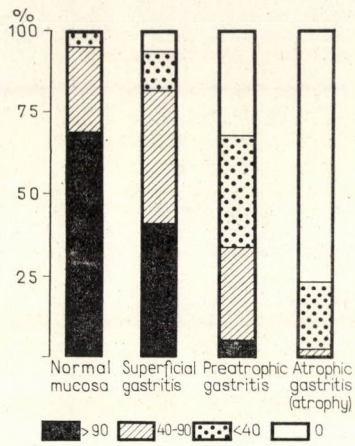

Fig. 1. Relation between morphological picture and acidity values

was greater and there were a few anacid persons as well. In preatrophic gastritis sub- and anacidity constituted the majority, while atrophic gastritis was mostly associated with anacidity; subacidity could be demonstrated in a smaller percentage; normacidity was found in a few cases but there was not a single hyperacid subject in this last group (Fig. 1).

2. Acid output in the various morphological groups

Calculated maximum HCl output (meq/h) was compared in subjects with normal mucosa, in patients with superficial, and preatrophic gastritis. Cases of atrophic gastritis were not evaluated from this point of view, because a large part of these were achlorhydric and so had no acid output. According to Table I, HCl output was greater in subjects with normal mucosa than in

Table I

Calculated maximum free HCl output, meq/h

	I. Normal mucosa n = 88	II. Superficial gastritis n = 71	III. Preatrophic gastritis n = 38
Mean	14.054	9.153	1.878
20th percentile	5.260	1.515	0.351
Median	11.885	6.400	0.924
80th percentile	21.872	15.520	5.150
		$p < 0.01$	$p < 0.01$

patients with superficial gastritis; the difference was significant statistically. HCl output in the group with preatrophic gastritis was significantly reduced as compared with the two other groups.

3. *Thickness of the mucosa*

The thickness of the mucosa was determined in 149 specimens; it ranged from 0.556 to 1.207 mm. The thickness of the active parenchyma, i.e. the glandular part from the base of the glands to the foveola was examined in 152 cases. The results of these examinations are shown in Tables II and III.

Table II

Whole mucosal thickness, mm

Normal mucosa	Superficial gastritis	Preatrophic gastritis	Atrophic gastritis		Hyperacidity	Normacidity	Subacidity	Anacidity
n = 62	n = 38	n = 26	n = 23		n = 63	n = 36	n = 22	n = 27
0.858	0.850	0.779	0.671	Mean	0.867	0.815	0.782	0.706
0.755	0.726	0.626	0.593	20th percentile	0.763	0.697	0.675	0.598
0.849	0.853	0.741	0.669	Median	0.874	0.785	0.761	0.680
0.959	0.947	0.979	0.765	80th percentile	0.970	0.917	0.941	0.786
$p > 0.1$	$p > 0.1$	$p < 0.01$			$p < 0.05$	$p > 0.1$	$p > 0.1$	

Table III

Active parenchymal thickness, mm

Normal mucosa	Superficial gastritis	Preatrophic gastritis	Atrophic gastritis		Hyperacidity	Normacidity	Subacidity	Anacidity
n = 63	n = 39	n = 27	n = 23		n = 63	n = 39	n = 22	n = 23
0.623	0.517	0.327	0.151	Mean	0.590	0.542	0.337	0.213
0.550	0.416	0.262	0.057	20th percentile	0.416	0.409	0.203	0.103
0.609	0.490	0.320	0.148	Median	0.593	0.557	0.313	0.215
0.714	0.628	0.428	0.241	80th percentile	0.700	0.655	0.457	0.291
$p < 0.01$	$p < 0.01$	$p < 0.01$			$p > 0.1$	$p < 0.01$	$p > 0.1$	

The thickness of the active glandular layer reflected both the morphological changes and the functional capacity of the mucosa more characteristically than did the thickness of the whole mucosa.

4. *Distribution of the active parenchymatous cells*

The quantitative data of the composition of glandular cells of 152 subjects are summarized in Table IV according to the morphological picture and

Table IV

Distribution of active parenchymal cells in the various morphological and secretory groups, cell count (sq. mm)

Normal mucosa n = 63	Superficial gastritis n = 39	Preatrophic gastritis n = 27	Atrophic gastritis n = 23		Hyperacidity n = 65	Normacidity n = 37	Subacidity n = 22	Anacidity n = 28
1799.7	1449.8	775.6	515.1	Mean	1721.5	1531.5	792.5	623.3
1562	1234.4	480	75.2	20th percentiles	1398	1244	425.6	188.4
1816	1432	788	354	Median	1747	1590	741	538
2020.8	1691.2	1066	736	80th percentiles	2005	1783	1244	846.4
$p < 0.01$	$p < 0.01$	$p < 0.01$			$p < 0.05$	$p < 0.01$	$p > 0.1$	

the secretory groups. The active parenchymal cell count decreased parallelly with the morphological and functional mucosal damage. Unlike the significant differences in morphology, the functional difference between the hyperacid and normacid groups was slight. On the other hand, the values for the sub- and anacid groups did not differ essentially from each other.

Table V

Distribution of the parietal cells in the various morphological and secretory groups, cell count (sq. mm)

Normal mucosa n = 63	Superficial gastritis n = 39	Preatrophic gastritis n = 27	Atrophic gastritis n = 23		Hyperacidity n = 65	Normacidity n = 37	Subacidity n = 22	Anacidity n = 28
391.3	340.3	179.0	32.3	Mean	389.2	333.7	144.8	97.7
298	275.2	93.2	0	20th percentile	286	262	42	0
394	324	182	0	Median	397	328	106	65
476.8	425.6	231.2	66.8	80th percentile	474	428	265.6	194
$p < 0.05$	$p < 0.01$	$p < 0.01$			$p < 0.01$	$p < 0.01$	$p > 0.1$	

In Tables V and VI the changes in the number of parietal and chief cells respectively are shown in a similar arrangement. These types of cell changed in the same direction as those mentioned above. The glandular mucous cells occurred in the greatest number in the atrophic group, while their ratio was lowest in the normal mucosa.

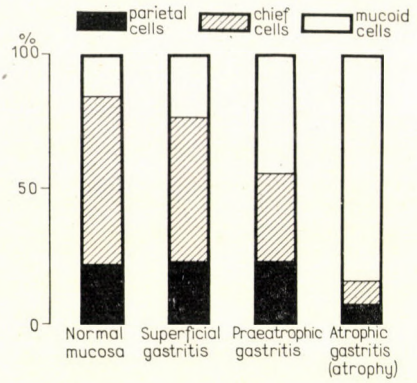

Fig. 2. Cellular composition of the active parenchyma in percentage in the various morphological groups

Finally, the percentual cell composition of the active parenchyma is shown in Fig. 2. Within the gradual decrease of the active parenchymal cells the proportion of parietal cells was practically constant in individuals with normal mucosa, superficial gastritis, or preatrophic gastritis. Again, the gradual decrease of the ratio of chief cells was compensated by the increase of the mucoid elements.

Table VI

Distribution of chief cells in the various morphological and secretory groups, cell count (sq. mm)

Normal mucosa	Superficial gastritis	Preatrophic gastritis	Atrophic gastritis		Hyperacidity	Normacidity	Subacidity	Anacidity
n = 63	n = 39	n = 27	n = 23		n = 65	n = 37	n = 22	n = 28
1132.6	769.5	251.1	37.8	Mean	1044.6	847.7	303.8	126.6
923.2	568	94.4	0	20th percentile	782	606	58	0
1110	770	202	0	Median	1034	905	124	50
1303.2	1026.4	403.6	64.4	80th percentile	1266	1110	618.4	220.8
$p < 0.01$	$p < 0.01$	$p < 0.01$			$p < 0.01$	$p < 0.01$	$p > 0.1$	

Discussion

The investigations demonstrated a well-evaluable relation between the morphological changes and the intensity of acid secretion. The relatively frequent occurrence of hyperacid individuals in the material was explained by the fact that many patients with duodenal ulcer were examined: their mucosa could mostly be classed in the normal group or the group with super-

ficial gastritis. Acid output in patients with superficial gastritis was less than in those with normal mucosa. According to CHELI [10], proliferation of the periglandular connective tissue is frequent in superficial gastritis; on this basis he suggested that the blood supply of the glands is disturbed; this would be the cause of the slight functional disturbance.

The data differing from the group characteristics underline the importance of taking biopsy material from at least two or three places of the fundus simultaneously. Thus the probability of obtaining a false picture of the whole of the fundus owing to patchy changes will be reduced. Since the mucosa of the antrum was not examined, the question arose whether an isolated antral gastritis may — through decreased gastrin production — influence the acid output. OTTENJANN et al. [11] observed a reduced secretion in their cases of antral atrophy even in the presence of intact fundic glands.

The data of the full thickness of the mucosa present no true picture of its functional capacity because in atrophy the growth of the foveola layer [12] as well as proliferation of the ground substance may compensate the disappearance of the active parenchyma. On the other hand, changes in the thickness of the active parenchyma go parallel with the intensity of the morphological lesions influencing the function.

The mucosal damage runs parallel with the decrease of the number of glandular cells per unit of surface area. Within this, however, the number of chief cells decreases more than that of the parietal cells, for while in preatrophic gastritis the number of parietal cells is reduced to its half as compared with the normal mucosa, less than one fifth of the chief cells are to be found here. This fact must be mentioned particularly because so far the decrease of the parietal cells has been considered the most sensitive indicator of mucosal damage. In our study it appears that the chief cells suffer more in the course of mucosal damage than the parietal cells. Without simultaneous pepsin determinations, however, it cannot be proved that this morphological phenomenon is accompanied by a similar functional change and so the ratio of acid-pepsin production also changes. Again, the study supplied no data as to whether the remaining parietal and chief cells continue to be intact functionally.

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CLINICAL AND HISTOPATHOLOGICAL STUDIES IN HUMAN RENAL DISEASE

III. THE PROBLEM OF HAEMATURIA AND FOCAL GLOMERULONEPHRITIS IN THE ADULT

By

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Clinical and histopathological data of 41 adult subjects allowed the following conclusions concerning the diagnostic value of haematuria and the clinical significance of focal glomerulonephritis.

1. Recurrent gross haematuria connected with catarrhal infections of the upper respiratory tract or with physical stress or permanent microhaematuria is rarely a sign of focal nephritis in adults; the symptom is mostly due to diffuse glomerulonephritis. The symptoms of glomerulonephritis of a focal pattern are not consistent, therefore the condition eludes clinical detection. Focal nephritis is a histological definition.

2. Similarly to diffuse glomerulonephritis, focal nephritis is an inflammatory reaction associated with an immunopathologic process. The immunoglobulin deposits on the glomerular basement membrane are too extensive to be compatible with a focal reaction inflammatory in nature. In contrast with streptococcal infection, the etiologic role of which has not been substantiated, viral or other bacterial factors may be involved in the pathogenesis of the process.

3. Focal glomerulonephritis represents a relatively benign type of glomerulonephritis. It generally takes a chronic course in adults where its acute recurrence may affect renal function to the degree of uraemia. It is assumed to indicate the initial stage of glomerulonephritis in individual cases, this requires further evidence though. In addition to the necessity for antiinfective measures and the avoidance of physical strain, immunosuppression is a potential therapeutic factor.

Haematuria is regarded as the leading sign of focal nephritis. This type of nephritis is marked by episodes of gross haematuria in connection with some infective process, usually of the upper airways. Proteinuria is slight, oedema and hypertension are absent. Since BAEHR's description of the syndrome (1926) it has been generally looked upon as a well defined clinical entity of mild clinical course, which spontaneously recovers parallel with the cure of the primary infective process. Still, it had already been emphasized by VOLHARD and FAHR (1914) that the process is subject to recurrences, that often it is clinically indistinguishable from diffuse glomerulonephritis and that chronic forms are fairly common. According to these authors, the morphologic glomerular abnormalities differ in degree rather than in kind, depending on the extent of the areas affected by the toxin of the responsible agent.

Before the common use of renal biopsy there was obviously little possibility to form a clear view on the pathological and clinical significance of focal glomerulonephritis. Today certain basic questions bearing on the pathogenesis

and on the diagnostic value of the clinical manifestations as well as on the therapeutic aspects of the syndrome, are seen in a new light.

In our renal material comprising 289 biopsy cases, the clinical and histopathological data of 41 subjects have been found illustrative of the problems to be discussed.

Diagnostic value of haematuria

The prevalent cause of haematuria is some abnormality of the urinary tract or haemorrhagic diathesis, and it can be clarified in the majority of cases by conventional diagnostic procedures including clinical, laboratory, radiological and urological examinations. It is, however, by no means uncommon that all these findings remain negative. This applies in the first place to a particular form marked by recurrent episodes of gross haematuria. During the apparently symptom-free intervals there is only microscopic haematuria or none at all. Proteinuria, if present, is slight, and no oedema appears. Renal function and blood pressure are unaffected. Prognostic and therapeutic considerations make it no longer warranted to leave it at the diagnosis or at the suspicion of focal nephritis or of acute diffuse nephritis in cases of this type without the evidence of biopsy. Unsubstantiated conjectures of this kind would be nearly as meaningless as to qualify the haematuria as "essential", "benign", or "idiopathic", labels attached all too often to haematuria of unidentified origin.

In 30 out of the 41 cases forming the basis of the present study there had been recurrent episodes of gross haematuria in the past. Since 14 of these subjects had also other signs indicative of diffuse glomerulonephritis (heavy proteinuria, oedema or hypertension) closer analysis will be confined to the other 16 patients. From the history of these subjects it emerges that in view of the recurrent episodes of haematuria their attending physicians had considered the possibility of focal glomerulonephritis, in 7 instances after the urological examinations had proved negative. Close follow-up during the "symptom-free" periods had revealed permanent microscopic haematuria and slight proteinuria, not beyond the degree of faint opalescence with sulphosalicylic acid, in all but two cases, the daily protein excretion measured on the basis of the biuret reaction varying between 100 and 300 g and being usually related to the extent of haematuria. Renal function was unaffected, hypertension and oedema were absent. The male-to-female ratio was 13 : 3. Mean age was 24.3 years, the limits being 14 and 59 years, respectively.

Haematuria either appeared simultaneously with certain signs typical of focal glomerulonephritis or followed close upon them. The history revealed the following factors, in all probability in direct causal relationship with the haematuria.

1. Catarrhal processes of the upper airways, tonsillectomy	7 cases
2. Physical stress	4 ,,
3. Abdominal symptoms (colics, gastroenteritis)	2 ,,
4. 1, 2, or 3, at various times in the same case	2 ,,
5. No identifiable factor	1 case

On the ground of earlier knowledge all these seemed to support the diagnosis of focal glomerulonephritis. However, contrary to expectations, the histological finding revealed a diffuse, immunohistologically active, glomerulonephritis in the majority of cases. The histological findings were as follows.

1. Membraneous glomerulonephritis	10 cases
2. Membraneous glomerulonephritis combined with pyelonephritis	3 ,,
3. Focal glomerulonephritis	1 case
4. Pyelonephritis	1 ,,
5. Nephroangiopathy	1 ,,

Cystoscopy performed at the time of gross haematuria may be of prime diagnostic importance by revealing some unilateral urological process as the source of haematuria, but it may be also misleading, as illustrated by one of our cases where diffuse glomerulonephritis had been ascertained on the ground of biopsy six months earlier. On the evidence of cystoscopy performed at the time of gross haematuria, haemorrhagic actions were confined to one side, but no actual urological abnormality accounting for them was demonstrable. The patient died with uraemia three months later and autopsy also failed to reveal any urological abnormality which might have given rise to haematuria. This observation supports the view of JOEKES (1962) that, despite its unilateral cystoscopic appearance, haematuria may well be due to bilateral renal disease.

Though diffuse glomerulonephritis may be responsible for recurrent haematuria in children, too (GLASGOW et al. 1970., ARNEIL et al. 1969), at this time of life it is in fact a focal glomerulonephritis which accounts for the majority of cases (BODIAN et al. 1965, SINGER et al. 1968, FERRIS et al. 1967). On the other hand, focal glomerulonephritis is rarely the primary cause of haematuria in adults. This emerges from the literature (JOEKES 1962, LITTLE et al. 1967, NATUSCH et al. 1969, HAMBURGER 1968) as well as from our own observations. In the majority of cases, the responsible factor is diffuse glomerulonephritis or interstitial nephritis, possibly also some renovascular abnormality. The clinical pattern defined by BAEHR (1926) as "the benign form of acute haemorrhagic nephritis" thus hardly ever corresponds in adults

to the pathological features of focal nephritis described by VOLHARD and FAHR (1914).

In numerous cases of monosymptomatic microscopic haematuria the only urinary abnormality is the presence of a few erythrocytes per visual field without any indication of infection or nephritogenic injury in the history. Cases of this type also involve the necessity for renal biopsy, should the urological and haematological investigations prove negative. Application of the earlier term "essential" to this kind of haematuria is no longer justified. In fact, biopsy performed in six of our cases of this type revealed a diffuse active glomerulonephritis in two, and focal nephritis in one patient, a normal histological finding being confined to three of these subjects. According to HAMBURGER, today the diagnosis of "essential" haematuria scarcely ever (approximately in 2%) can be upheld after biopsy. But even a negative biopsy finding does not exclude the possibility of a later manifestation of organic disease, parallel with the progress of the primary process (tuberculosis, malignancy, etc.).

Moreover, it has to be borne in mind that the diagnostic value of needle biopsy is greatly limited by the very nature of focal nephritis. This makes it necessary to keep the patient under close observation and to envisage a further biopsy at some later date.

Clinical significance of focal nephritis

The term was first used by LÖHLEIN (1910) for glomerular lesions of embolic nature associated with subacute bacterial endocarditis. The same term was applied by VOLHARD and FAHR to non-diffuse glomerulonephritis appearing after infections of the upper respiratory tract in the absence of endocarditis. According to their description, the glomeruli involved by the typical lesion, i.e. endothelial proliferation, swelling of the glomerular capillary walls, accumulation of leukocytes, fibrin deposits, greatly vary in number. Occasionally, there are scarcely more than one or two affected glomeruli. We owe the definition of "segmental glomerulitis" to VERNIER et al. (1958), a term referring to the presence of affected as well as unaffected loops within the same tuft involved by the process.

Changes of focal or segmental nature are common in systemic lupus erythematosus (MUEHRCKE et al. 1957), in Wegener's granulomatosis (GODMAN et al. 1954), in Goodpasture's syndrome (RUSBY and WILSON 1960), in Schoenlein-Henoch purpura (VERNIER et al. 1961) and in subacute bacterial endocarditis (BAEHR 1912). The essential feature distinguishing focal nephritis, as understood by VOLHARD and FAHR, from the conditions referred to above is the absence of necrosis of the glomerular loops, in opposition to the other forms where lesions of this kind are often typical. In the present material,

biopsy performed in three subjects with focal glomerulonephritis revealed necrotizing glomerulitis. In two of these cases clinical evidence was also conclusive of SLE.

In the following four cases the microscopic appearance of the kidneys was consistent in all respects with the classical description of focal glomerulonephritis:

No.	Sex	Age	Clinical manifestation	Immunohistological findings
1.	Female	24	Monosymptomatic haematuria	Not available
2.	Female	31	Monosymptomatic proteinuria	Fibrin
3.	Female	27	Nephrotic syndrome	IgG, C′ fibrin
4.	Female	14	Haematuria, proteinuria	IgG, IgA, C′ fibrin; 19 months later negative

In this small series, each patient represents a different clinical syndrome. It is in the youngest of these patients (Case 4) where we found the closest correspondence with the clinical features regarded as typical of focal glomerulonephritis, therefore this case seems to illustrate best the problems involved by the process under study.

The patient, a 14-year-old girl, had experienced a sudden episode of gross haematuria six months before admission, on the second day of an acute febrile condition ascribed to a cold, and was found to have an increased blood pressure. She was referred to a paediatric unit where, on the basis of a decreased GFR, moderate azotaemia and increased blood pressure, the haematuria was attributed to an acute recurrence of chronic glomerulonephritis. Though the urine had become clear and blood pressure normal by the time of her discharge, microhaematuria and proteinuria were still present. In the course of the following six months two episodes of gross haematuria had ensued, on both occasions after physical strain (excursion).

At admission in November 1969, blood pressure was between 175/95 and 120/75 mmHg, BSR 26 mm/h, blood counts were normal. The urine was sterile, specific gravity 1022, proteinuria 1.09 g/24 hr, the sediment was packed with erythrocytes and contained 8 to 10 leukocytes as well as granular and mixed (leukocyte + erythrocyte) casts per visual field. Creatinine clearance was 95 ml/min; serum total protein, 6.8 g per 100 ml. Electrophoretic pattern: albumin 69.5%, alpha$_1$ globulin 4.8%, alpha$_2$ globulin 8.8%, beta globulin 10.1%, gamma globulin 6.8%. AST: 140 U. Serum complement titres normal. The eyeground vessels had a spastic appearance. In the renal biopsy specimen 17 glomeruli were suitable for study. Six of these were enlarged and rich in

cells. The other glomeruli were medium-sized and poor in cells. In the enlarged glomeruli the proliferation of endothelial and epithelial cells are present. The lumen of one of the glomerular loops were filled by an eosinophilic homogeneous material. The basement membrane of these glomeruli showed focal thickening, where increased amounts of PAS-positive and of argentophile material is visible (Fig. 1). The other glomeruli revealed no abnormality. In some Bowman's capsular spaces we found homogeneous material. The tubular

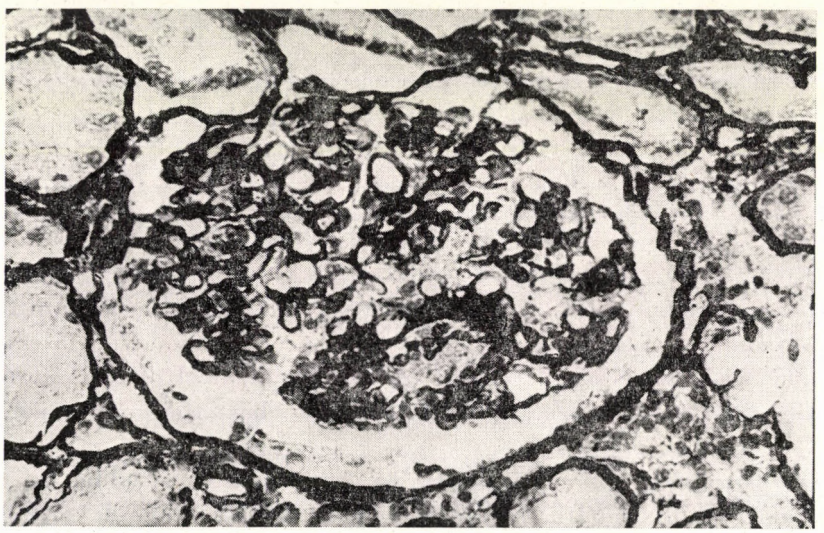

Fig. 1. The glomerular basement membrane shows focal thickening with marked argentophilia (Jones's methenamine silver staining)

epithelial cells showed a fine granulation, the lumen contained scattered erythrocytes and granular material. The interstitial tissue was scanty and contained a few lymphocytes and leukocytes. Immunofluorescence investigation revealed the presence of IgG (Fig. 2) as well as of IgA, not only in the glomeruli with light-microscopic changes but also in those of normal light-microscopic appearance. Fibrin deposits were found in a few glomerular loops (Fig. 3), IgM was not demonstrable. Focal thickening of the glomerular basement membrane was demonstrable by electronmicroscopic investigation, deposits of electron-dense precipitate were identifiable on the endothelial site of the thickened basement membrane and occasional fusion of the foot processes of the epithelial cells was noted (Fig. 4). Histological diagnosis was focal glomerulonephritis.

Tonsillectomy was performed three weeks after admission. On the first postoperative day gross haematuria appeared in association with abdominal colics, diarrhoea, fever and oliguria. Haematuria subsided parallel with an increase in urinary output by the end of the third day. On the 10th day, BUN

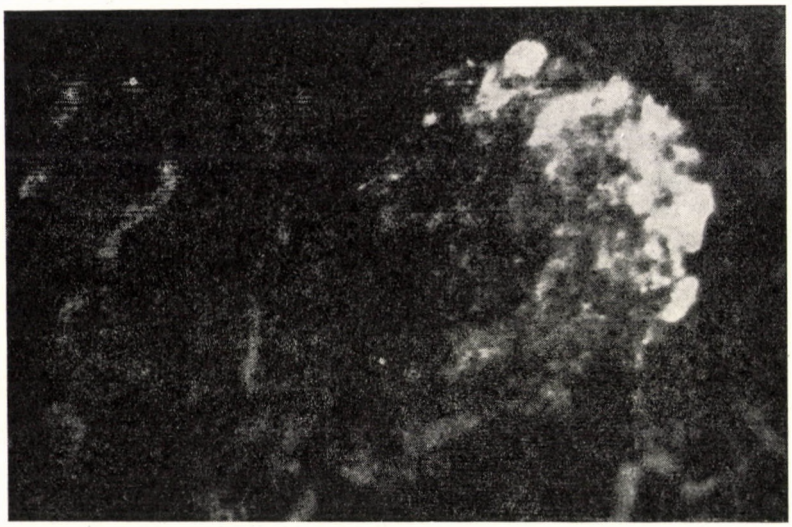

Fig. 2. On the glomerular basement membrane in spots large amount of gamma globulin (Section incubated with anti-human gamma globulin conjugated to fluorescein-isothiocyanate)

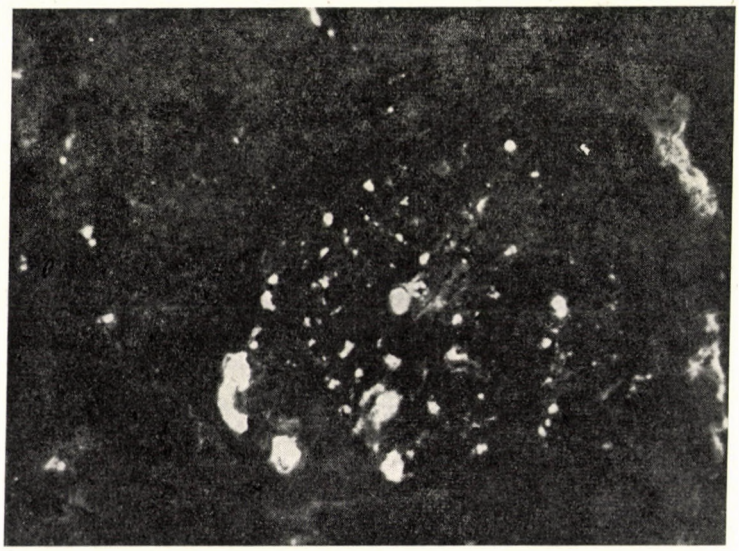

Fig. 3. Spots of fibrin on the glomerular basement membrane. At sites, intraluminal fibrin thrombi (Section incubated with anti-human fibrin conjugated to fluorescein-isothiocyanate)

was none the less 116 mg per 100 ml; GFR, 17.1 ml/min; specific gravity of urine, 1011; BSR, 100 mm/h; RBC, 3,2 million; Hb, 8.5 g per 100 ml; WBC, 9000. Blood pressure remained in the neighbourhood of 155/95 mmHg. Tonsillectomy had been performed under tetracycline protection, which was continued for two days and followed by the administration of penicillin, streptomy-

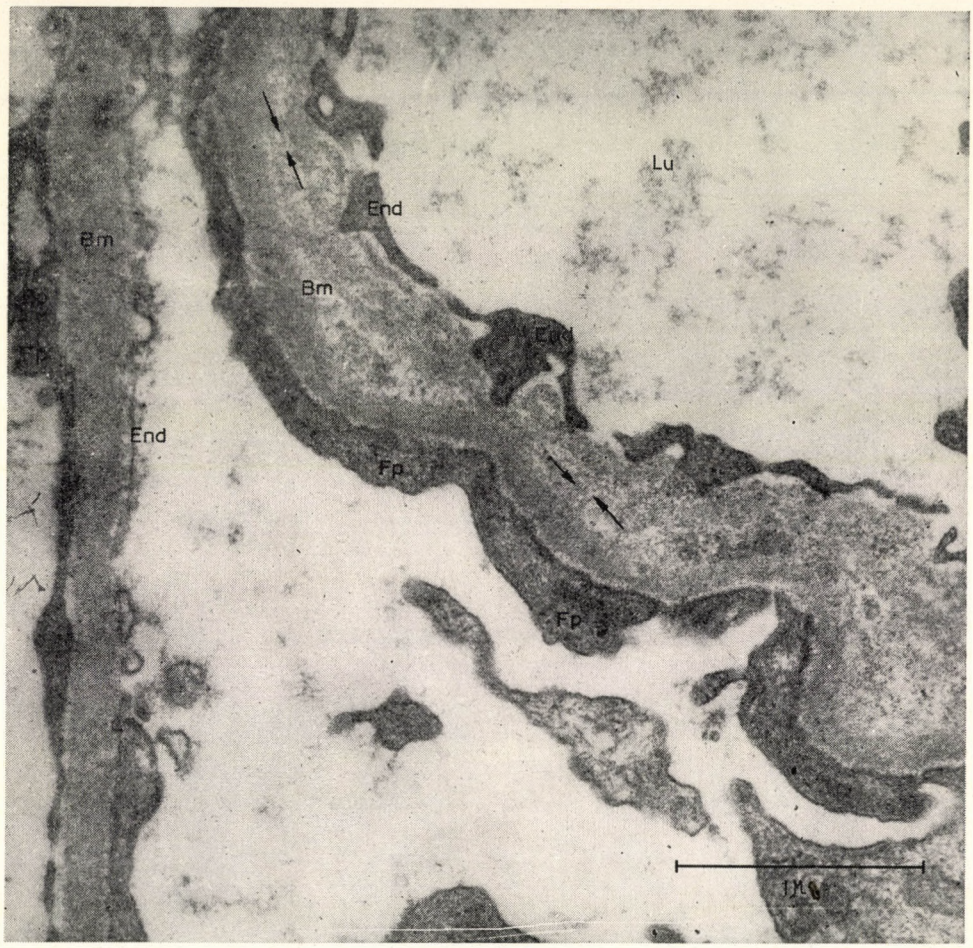

Fig. 4. The electronmicroscopic picture shows that the basement membrane (Bm) of the glomerular loops is thickened in some places, and has normal thickness in some other places. We found subendothelial (End) deposits of electron-dense precipitate (→ ←) on the broadened basement membrane. The fusion of the foot process of the epithelial cells is conspicuous. (HITACHI HU-10 electron microscope, ×32,245)

cin and iodochloroxychinoline. Owing to persistent diarrhoea and slightly increased temperatures the patient was started on chloramphenicol on the 10th day; this was changed to erythromycin on the 21st day. This resulted finally in a full control of the enterocolitis. Faecal cultures during antibiotic treatment were negative for salmonellae, shigellae and staphylococci. The signs of azotaemia subsided in three weeks after the normalization of diuresis, GFR gradually increased. When the patient was discharged eight weeks after tonsillectomy, GFR was 70 ml/min; maximum specific gravity of urine, 1017; proteinuria, 0.83 g/24 hr, the sediment contained 20 to 30 erythrocytes per high power field; RBC was 3.7 million; Hb, 10.6 g per 100 ml; WBC, 5200.

After discharge she continued on erythromycin for another three months. This was combined later with immunosuppressive therapy, azathioprine being given in doses of 2 mg/kg body weight daily and prednisolone, 30 mg daily. After six weeks immunosuppressive therapy she again experienced gross haematuria consequent upon an infection of the upper airways. However, the episode subsided in 24 hours without affecting the general condition or renal function, and during another year of antibiotic and immunosuppressive treatment the patient remained symptom-free.

She was readmitted for follow-up studies in June, 1971. Daily protein excretion was 275 mg, the urinary sediment contained 10 to 12 erythrocytes per high-power field. Renal biopsy revealed at this time no focal proliferation or basement membrane thickening. The focal hyaline changes in a few glomerular loops and the little amount of fibrin deposits were regarded as signs of earlier focal glomerulonephritis. IgG and IgM were absent. Electronmicroscopically no thickening of the basement membrane was demonstrable and the epithelial cells as well as the foot processes seemed unaffected. However, the mesangial matrix showed scattered areas of focal widening (Fig. 5).

For the last six months which have elapsed since the second biopsy the patient remained well. Microhaematuria and slight proteinuria are present as before. Her blood pressure is normal.

This case clearly exemplifies the clinical and pathological features of focal glomerulonephritis. We have, however, to regard this process as one of definitely chronic nature the acute recurrences of which have been precipitated by infections of the upper airways, physical stress, surgical intervention, and enterocolitis. The presence of immunoglobulins in the glomeruli suggest that in the pathogenesis of focal glomerulonephritis immunological factors have a role. It was remarkable that immunoglobulins were demonstrable diffusely and in larger amounts than it might have been expected on the basis of the focal histological reaction. Moreover, similarly in cases Nos 2 and 3, there were substantial fibrin deposits in the glomerular loops too and they persisted after the disappearance of immunoglobulins. Though the primary injury is obviously not identifiable, the fact that episodes of gross haematuria occurred under antibiotic protection makes it improbable that streptococcal infection should have been the responsible factor, moreover during these recurrences which went parallel with impaired renal function the AST-titres were not increased.

At the time of the second biopsy, the clinical signs were those of a chronic process. The histological changes marking this condition were confined, apart from the presence of fibrin, to signs of a previous focal inflammation, without any evidence of immunological activity in the glomeruli. Although, in view of the focal character of the process, allowance must be made for the limitations of needle biopsy owing to the smallness of the specimens, an actual

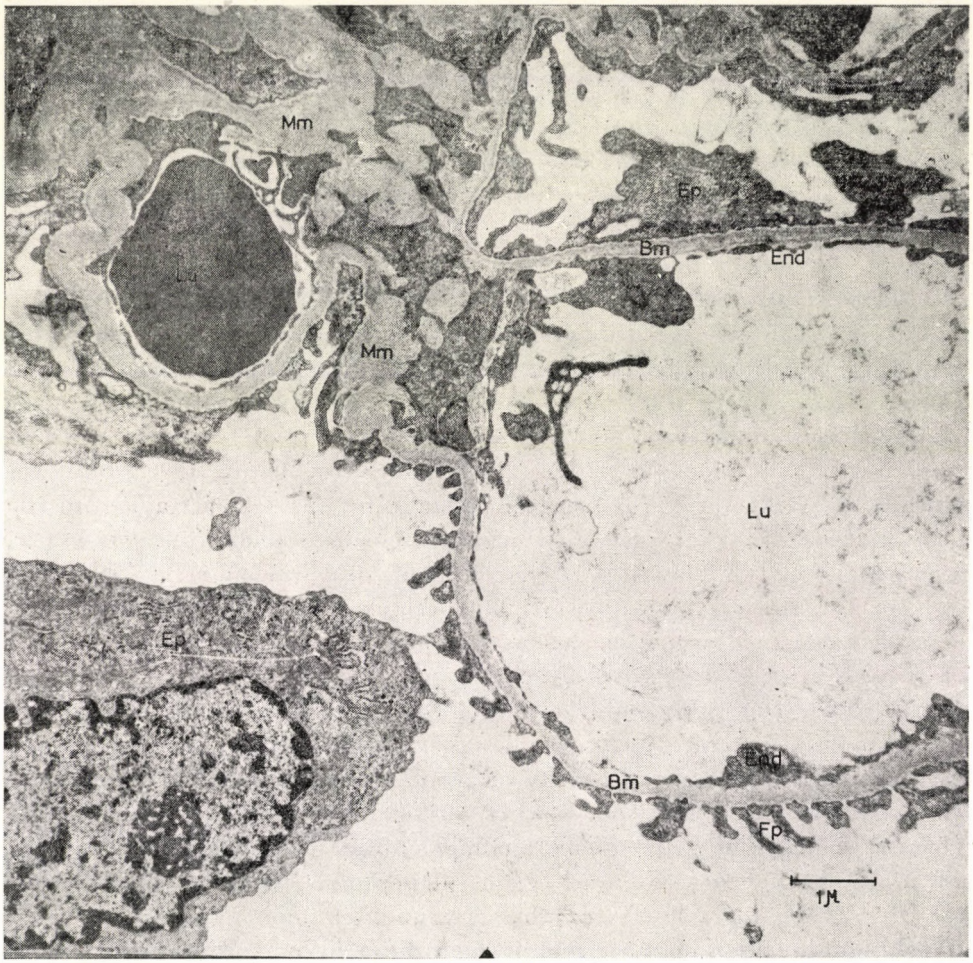

Fig. 5. Electronmicroscopical picture after repeated biopsy. The glomerular basement membrane (Bm) is thin, the epithel cells (Ep) as well as their foot processes (Fp) are of normal structure. In some places the quantity of mesangial matrix (Mm) is increased. (HITACHI HU-10 electron microscope, × 14,000)

regression of the process as a result of long-term antibiotic and immunosuppressive treatment is none the less well within the possibilities. It must however be emphasized that there can be no question of a true cure, neither here nor in the three other cases followed up for more than 20 months.

In the overwhelming majority of cases, the cause of the haematuric nephritis syndrome is not a focal glomerulonephritis. As concerns the cases of histologically confirmed focal glomerulonephritis, the clinical data indicate that, in the absence of haematuria, proteinuria may be the only sign of the condition, as it has been noted by MUTH et al. (1965) too. That focal nephritis may result in a nephrotic syndrome (WEST et al. 1968) and that it may be

associated with hypertension, oedema, nitrogen retention and a reduction in GFR (WILSON 1967), are observations to which we must subscribe. Thus, focal glomerulonephritis is merely a pathological definition implicating no special clinical syndrome in adult age. All that can be said about its clinical features is that it generally tends to be oligo or monosymptomatic in its manifestations (ARNEIL et al. 1969, HAMBURGER et al. 1968).

Before the advent of renal biopsy, evidence in respect of the significance of the syndrome was scanty and confusing (BAEHR 1926, PAYNE and ILLINGWORTH 1940). We owe the most illuminating facts on the syndrome, ever since VOLHARD and FAHR (1914), to BATES and JENNINGS (1957) who observed among mariners stationed in an isolated basis an outbreak of focal nephritis, despite the preventive use of penicillin. Haematuria had been preceded by pharyngitis, the latent period between infection and haematuria having been as short as 24 hours in most cases. Streptococcal infection could be ruled out on the ground of serial throat cultures and of serum antibody studies. The clinical signs and symptoms were consistent with those of viral infection. Renal biopsy revealed focal glomerulonephritis in a number of cases, it showed no structural abnormality in some others. ROSS (1960) presented biopsy evidence in support of the relatively favourable outcome of the process, any progress having been absent on the evidence of follow-up studies extending over several years. SINGER et al. (1968) demonstrated by electronmicroscopical method electron-dense deposits on the basement membrane of glomerular capillaries which, on light microscopic investigation, were segmental glomerulitis. BODIAN et al. (1965) identified by immunofluorescence method immunoglobulin deposits in diffuse arrangement in the glomeruli; immunoglobulins were demonstrable in far more glomeruli than it might have been expected on grounds of conventional light microscopic staining procedures. These authors too believe that while the inflammatory reaction is focal or segmental in character, the immunological abnormalities in focal nephritis affect the total of the glomeruli. Their observation that the presence of demonstrable immunoglobulins is confined to the early stages and that they are no longer detectable after two years duration of the process is also of interest. VOLHARD and FAHR suggested that the histological appearance of focal glomerulonephritis may represent a diffuse glomerulonephritis in its initial stage as well as in some phase of its resolution. Although, as a result of the simultaneous presence of normal and still diseased glomeruli, the resolution in diffuse glomerulonephritis may assume a focal pattern (POLLAK et al. 1958), here, in resolving diffuse glomerulonephritis, in contrast with focal glomerulonephritis, proliferation of the mesangial cells persists for months (HEPTINSTALL and JOEKES 1959). The beginning of diffuse glomerulonephritis may, however, be definitely marked by a focal-segmental pattern, probably in cases of insidious onset (SINGER et al. 1968).

The literature on the pathogenesis of glomerulonephritis alleges a wide range of factors being apt to give rise to the process; the most common are streptococci, basement membrane and nuclear fractions, malaria, syphilis, heterologous sera, drugs. It has not been possible thus far to demonstrate any involvement of streptococcal antigens in the immune reactions associated with focal nephritis (BATES and JENNINGS 1957), on the other hand, viruses or bacteria seem to play a part, particularly tuberculosis, as it did in the case of VOLHARD and FAHR and by which these authors illustrated the pathogenesis of focal nephritis (1914). The observation that laboratory animals develop focal glomerulitis with proteinuria as a result of repeated intramuscular injections of nonbacterial substances giving rise to local tissue reactions (MANALIGOD et al. 1969) also deserves interest since it seems to suggest that various substances released from extrarenal inflammatory tissues may also be involved in the mechanism responsible for glomerular inflammatory processes of focal character.

The process offers a more favourable outlook than does streptococcal nephritis, owing to better prospects of spontaneous recovery. Chronic forms are, however, fairly common and for the time being we are unable to form any definite view about their final outcome since the contracted kidneys of patients died of uraemia are no longer suitable for differentiation. At any rate, as the observations indicate, the process is slow in its progress, even though the possibility of recurrences has always to be taken into account, not only because of their life-threatening significance but also because of a further impairment of renal function.

Elimination of the nephritogenic factor would amount to causal therapy, provided the role of the microorganism accessible to antibiotic treatment could be ascertained beyond doubt. The probability that this type of nephritis belongs to the immunopathological reactions, still adds to the therapeutic difficulties, because it means that the very first episode seen and correctly interpreted by the physician is actually an acute flare-up of a latent process which has been in existence before. Catarrhal infections of the upper respiratory tract, enteral infections, physical stress, may precipitate acute recurrences and impose the need for eradication of foci, long-term antibiotic treatment, avoidance of physical overstrain. Immunosuppression has its theoretical justification, but our observation in a single case where it has been used with benefit is no adequate basis for the assessment of its value.

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CLINICAL STUDIES ON THE PATHOGENESIS OF PERNICIOUS ANAEMIA

IV. IMMUNOLOGICAL STUDY OF THE DUODENAL JUICE

By

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Immunoassays have been performed for the identification of a substance detected in duodenal juice and found similar to the intrinsic factor. Activity of this duodenal factor was inhibited by serum from pernicious anaemia patients, owing to the presence of antibody to intrinsic factor. These findings support the earlier claim that the duodenal factor is closely related to the intrinsic factor.

A substance similar to Castle's intrinsic factor (IF) has been demonstrated bacteriologically in the duodenal juice of patients with pernicious anaemia (PA) [1]. In some cases of untreated pernicious anaemia the factor was found in reduced amounts, but administration of vitamin B_{12} restored its normal level [2]. Reliability of the procedure was controlled by the reproduction of Castle's clinical assays with identical results [3].

Detection of intrinsic factor or of a substance closely related to it in the duodenal juice of pernicious anaemia patients was found of sufficient importance to call for further studies. Research of pernicious anaemia in recent years has been centering on its immunological aspects. This trend proved fruitful in clinical respects too and seemed to offer a promising angle of ap proach to the present study.

The presence of antibody (*ab*) to intrinsic factor in the serum, gastric juice and saliva of most patients with PA has been borne out by ample experimental evidence [4—8]. Antibodies of other kind, irrelevant to the subject of the present study [9—14] have also been identified. Antibodies to IF of two types, i.e. blocking and precipitating antibodies, have been demonstrated in the serum by different procedures. These revealed that the antibodies inhibited absorption to and binding of labelled vitamin B_{12} electrophoretic migration, immunoelectrophoretic binding and immunodiffusion. Lymphocyte transformation, luorescein fixation and RBC-agglutination tests are also suited for their demonstration [15—17]. It is a point of interest that absorption of B_{12} in pernicious anaemia is inhibited even when no antibody is demonstrable [18, 19]. The identifiable gastric antibodies are partly directed at the cytoplasm of the parietal cells, partly at the microsomal antigens. Theoretically, these antibodies should be of pathogenetic significance. Their

formation seems to be connected with the presence in the gastric mucosa of immunocompetent elements (plasma cells, lymphocytes), though there are views [20, 21] that they originate from the blood. While the antibodies of serum are of IgG-character, those of the gastric juice belong to the IgA-immunoglobulins and have been located by electrophoresis to the IgG fraction.

Immunoassays belong to the most sensitive procedures for the detection and identification even of vanishingly small amounts of proteins. For the identification of the *E. coli* mutant factor found in the duodenal juice, serum of patients with pernicious anaemia, or more correctly, the antibody to intrinsic factor contained in it, was used and this, in turn, was checked by the growth and inhibition of an *E. coli* mutant strain.

Material and methods

The factors of serum and duodenal juice under study are unstable, therefore the measurements were performed either on the day of sampling or within 3 days. The samples were kept in a refrigerator at 4 °C until processing; deep-freezing was avoided. The normal serum-antibodies of complement dependent activity have an inhibitory effect on the growth of the *E. coli* mutant test strain, therefore the sera were previously inactivated in a water bath at 56 °C for 30 min. This inactivation procedure did not affect the activity of the complement-independent immunoglobulins of higher thermostability [24]. For the collection and preservation of duodenal juice, paraffin-coated test-tubes were used in view of the adsorption of intrinsic factor to glass [25]. The duodenal juice was filtered through sterile gauze in order to free it from mucus. Particular care was given to correct duodenal drainage, reaction of the contents being checked repeatedly by Congo red paper and the position of the tube, if necessary, by fluoroscopy. The inactivated serum was brought together with equal amounts of duodenal juice and the mixture was kept at 37°C for 30 min. Generally, 1 ml amounts of each were used but smaller quantities are also sufficient since the actual measurement requires 0.05 ml only. The subsequent steps of the procedure were identical with those described earlier and the mixture was kept with chopped meat at 37°C for 3 hours.

Test and control sera were measured for their activity side by side on the same plate on which vitamin B_{12} was also placed for reference purposes. All parallel plates yielded identical results. Here, too, the amount of the growth factor thus formed was judged on the basis of the growth rings of the *E. coli* mutant strain. Serum was obtained from 25, and duodenal juice from 5 patients with pernicious anaemia. Three of these subjects were untreated or in relapse, the others were in remission. The tabulated data belong to 21 patients of the series. In four cases the findings were unsuited for evaluation because of coexisting diabetes, ethylism or hyperthyroidism. All measurements were performed against controls consisting of serum or duodenal juice of subjects without any evidence of haemopoietic or digestive abnormalities. The sera of nine pernicious anaemia patients were submitted to repeated studies at intervals of a few days, on the average of one week, with the same results.

Results

Results are shown in Table I and Fig. 1. It can be seen that the pernicious anaemia sera gave smaller growth rings all throughout; from this it has been concluded that pernicious anaemia serum, in contrast to normal serum, contains an inhibitory factor. Duodenal juice obtained from five patients with pernicious anaemia was used for parallel assays. The results are shown in the column at the right side of Table I. Here too, the inhibitory effect is distinct and in some of the cases very marked. On the evidence of Student's *t* test the results have proved significant. Neither the untreated

Table I

Inhibitory effect of E. coli mutant growth factor formed in a mixture of duodenal juice and meat

Serial number	Effect of an incubated mixture of meat and duodenal juice from			
	normal subjects		patients with pernicious anaemia	
	on the growth of *E. coli* mutant strain (area of growth ring in sq. mm) serum from			
	normal subjects	PA patients	normal subjects	PA patients
	added to the mixture prior to assay			
1.	63	18	—	—
2.	54	16	—	—
3.	54	32	—	—
4.	72	29	—	—
5.	50	18	—	—
6.	39	16	63	50
7.	54	39	39	16
8.	50	29	39	18
9.	50	25	39	25
10.	50	39	50	25
11.	58	39	—	—
12.	58	35	—	—
13.	58	35	—	—
14.	46	32	—	—
15.	46	32	—	—
16.	63	50	—	—
17.	50	32	—	—
18.	54	39	—	—
19.	54	18	—	—
20.	54	39	—	—
21.	54	18	—	—
mean	54	30	46	27
SD	±7	±9.7	±10.6	±13.6
t	9.2		2.44	
p	0.05%		5%	

cases nor those in relapse or in remission have been listed separately, because this factor did not affect the result. In the light of these facts it seems of interest to recall the observation [26] that cases of pernicious anaemia showed the same degree of responsiveness to and the same absorption of vitamin B_{12}, regardless whether or not antibody to intrinsic factor was demonstrable in

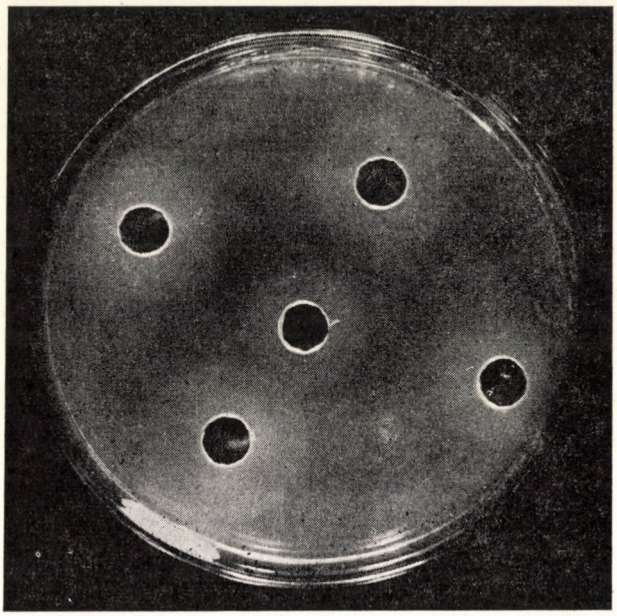

Fig. 1. In the middle of the plate the effect of vitamin B_{12} used as reference substance can be seen on the growth of the *E. coli* mutant strain incorporated in the culture medium. The largest of the four growth rings represents the effect of normal serum, and each of the three others that of serum of different pernicious anaemia patients, added to the incubated mixture of meat and duodenal juice

that particular case. With the exception of the five patients referred to above, all cases were outpatients on maintenance therapy prescribed in the last two years.

Discussion

The present study has been focused on the assumption that if the *E. coli* mutant growth factor detected in duodenal juice is of similar nature as the intrinsic factor, then it must be necessarily inhibited by the serum of patients with pernicious anaemia, owing to the presence of antibody to intrinsic factor. By the demonstration of an inhibitory effect of this kind new evidence has been furnished in support of the claim that the *E. coli* mutant growth factor identified in duodenal juice corresponds in fact to the intrinsic factor. The high sensitivity and specificity of the immune reactions makes them particularly suited for objective assessment. Moreover, the described findings seem to account for the seeming contradiction that duodenal intrinsic factor may be present in spite of pernicious anaemia. Obviously, this intrinsic factor is liable to inactivation by the antibody demonstrated at the same time.

The above results permit to attribute an important role to intrinsic factor-antibody in the pathogenesis of pernicious anaemia. It is assumed that when the production of gastric intrinsic factor has ceased, as reflected by

the atrophy of the gastric mucosa which is in fact inseparable from pernicious anaemia, the presence of antibody asserts itself by a blocking mechanism directed at the duodenal intrinsic factor. The presence of the gastric and of the postulated duodenal intrinsic factor implies that the utilization of the extrinsic factor involves a mechanism of two phases. In the light of these facts we no longer feel justified in reducing the pathogenesis of pernicious anaemia to a deficiency of gastric intrinsic factor. An additional role involved in the breakdown of the equilibrium must be attributed to a suppression of duodenal intrinsic factor resulting from the production of an inhibitory factor. The intricacy of the mechanism is still enhanced by the fact that the deficiency in vitamin B_{12} and probably also in other vitamins associated with the process may be inhibitory or even suppressive to the formation of antibody [27], thus resulting in a delay or in a temporary standstill of the progress of anaemia. This would account for the occurrence of spontaneous remissions reported chiefly in earlier literature.

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LONG-TERM STUDY OF SOME PARAMETERS OF THE POST-ISCHAEMIC KIDNEY

By

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Creatinine and PAH extraction have been studied two hours after renal ischaemia in dogs. While a gradual improvement was demonstrable in the group where ischaemia had been induced under general anaesthesia, a progressive deterioration was found in the other group where the same intervention had been performed in conscious dogs. The differences are attributed to a shifting of intrarenal blood flow.

Constriction of the renal artery for two hours or longer after unilateral nephrectomy in dogs has been found to result in a condition similar to human acute renal failure (HAMILTON et al. 1948, PHILLIPS et al. 1948, SELKURT, 1945, BÁLINT et al. 1960). The condition marked by azotaemia and oliguria is fatal in the majority of the laboratory animals. When renal ischaemia was induced in unanaesthetized dogs (TARABA et al. 1966) or under superficial anaesthesia (BÁLINT et al. 1964b), the animals died with acute renal failure in a few days. If, however, for the induction of ischaemia of the same duration the same technique was applied under deep general anaesthesia, azotaemia was only transitory, renal failure did not develop and the animals survived the intervention (BÁLINT et al. 1964b). Participation of the central nervous system in the condition was supposed, and we have produced evidence in support of this claim that, by denervation of the kidney (FEKETE et al. 1965, FEKETE and TARABA, 1965) or by suitable drugs (TARABA, 1965) we could prevent the fatal renal failure.

Since the duration of the renal ischaemia is practically the same in all model experiments of this kind, the grade of severity of renal failure, in other words, survival of the animals after transitory ligation of the renal artery, seems to depend on factors connected with the immediate post-traumatic period. As shown by the results of our acute experiments performed on successive days of the crucial postischaemic period (BÁLINT et al. 1964a), in the dogs where the intervention had been carried out under anaesthesia, diuresis set in and the NPN levels rose slightly or not at all, in the unanaesthetized

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dogs oliguria and azotaemia ensued. Though RBF decreased in both groups, a complete renal ischaemia was not observed.

In the present series of experiments, a continuous study of the post-ischaemic period has been undertaken under long-term condition in animals with implanted catheters, so as to be able to follow the postischaemic changes in the parameters of renal function over prolonged periods.

Material and methods

Adult dogs of both sex weighing 12 to 20 kg were studied. The catheters for blood sampling were inserted in the preliminary stage of the operation under aseptic conditions. One catheter was placed into the right carotid artery. The other catheter was introduced into the jugular vein, passed through the heart, directed into the left renal vein and sutured with a nontraumatic needle to the wall of the vena cava from the mid-line approach. A plastic thread was placed round the left renal artery, pulled into a plastic tube 3 mm in diameter which was brought out to the surface and fixed to the animal's flank, then the abdominal wound was closed. By pulling the plastic thread tight, the renal artery can be compressed by the tube and blood flow will be arrested (TARABA et al. 1966). Forty-eight hours after the preliminary operation the position of the catheter in the renal vein was checked by fluoroscopy. Then, first by preliminary doses, subsequently by continuous intravenous infusion, adequate plasma creatinine and PAH levels were maintained. Control values were obtained by withdrawal of two or three arterial and venous blood samples at five-minute intervals for studying creatinine and PAH extraction and O_2 values. The animals were divided into two groups. One group was anaesthetized with 0.1 g/kg of chloralose after sampling for the control values, immediately before the injury, whereas the other group was left without any anaesthesia. Then renal ischaemia was induced by pulling the thread tightly around the renal artery, and the state was maintained in both groups for two hours.

After the lapse of two hours, the loop around the renal artery was loosened from the outside and removed. During the postischaemic phase blood samples were obtained at 30 min intervals, on a total of six occasions. (The animals of the anaesthetized group were still under deep anaesthesia during these manipulations.) Then the animals were returned into their cages and were allowed food and water ad libitum. Subsequently, blood samples were obtained through the catheters in both groups in the waking state, at 1, 2, 3 and 4 days following the intervention.

For the estimation of plasma creatinine the method of POPPER, MENDEL and MAYER as modified by BÁLINT and VISY (1965), for that of PAH, the method of FINKELSTEIN was used. O_2 was measured in the blood samples with an Atlas oxymeter. Statistical evaluation was based on FISHER's tables.

Results

The results obtained in the unanaesthetized group (six dogs) are represented in Figs 1 and 2. Normal values for endogenous creatinine and PAH extraction were plotted at 0 min. At 1, 2, and 3 hours after release of the constriction, E_{Cr} and E_{PAH} had fallen to fractions of their original value and declined still further in the course of the following days. The regression coefficient for both E_{Cr} and E_{PAH} was negative, the regression line being $Y = 0.026 - (0.00028 \pm 0.0001)x$ for E_{Cr}, and $Y = 0.042 - (0.00078 \pm 0.0003)x$ for E_{PAH}.

The five animals of the anaesthetized group had been in deep anaesthesia not only during constriction of the renal artery but also in the first hours after release. In this group too, E_{Cr} fell to low values (Figs 3 and 4), but during

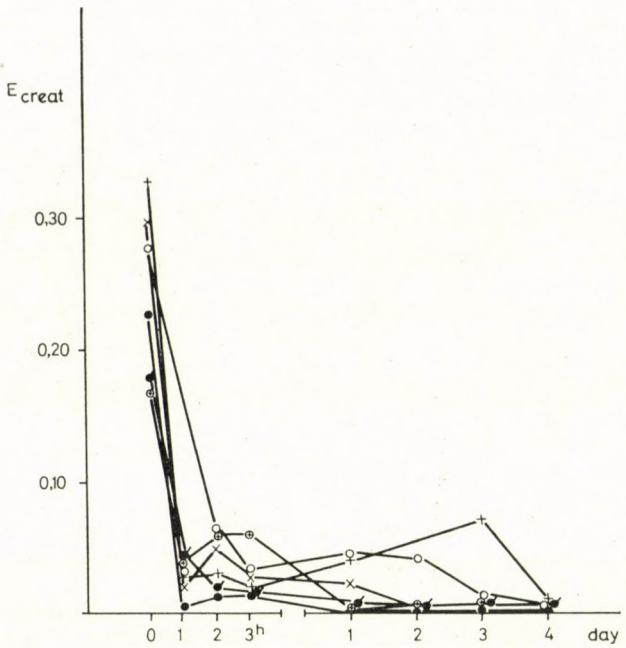

Fig. 1. E_{Cr} before and after renal ischaemia, in terms of hours and days, in the unanaesthetized group. Each line represents the data of an individual animal

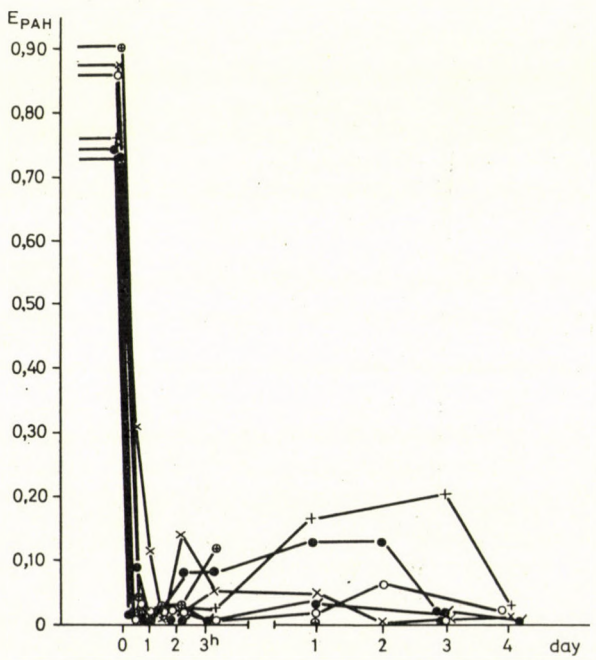

Fig. 2. E_{PAH} before and after renal ischaemia, in terms of hours and days, in the unanaesthetized group. Each line represents the data of an individual animal

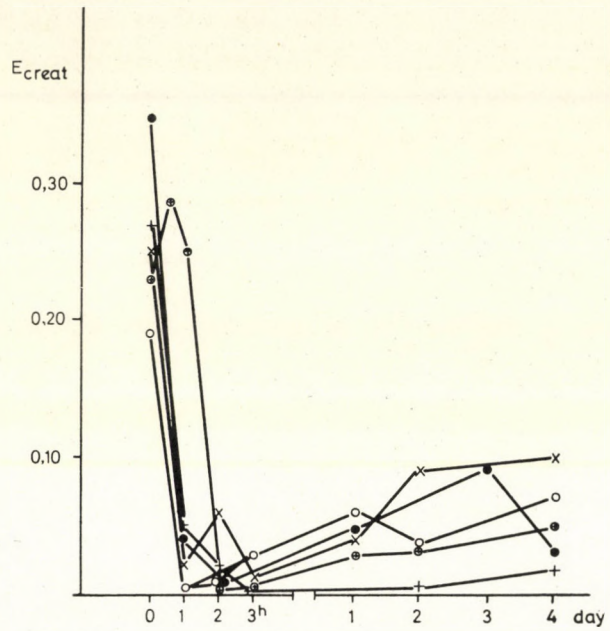

Fig. 3. E_{Cr} before and after renal ischaemia, in terms of hours and days, in the anaesthetized group. Each line represents the data of an individual animal

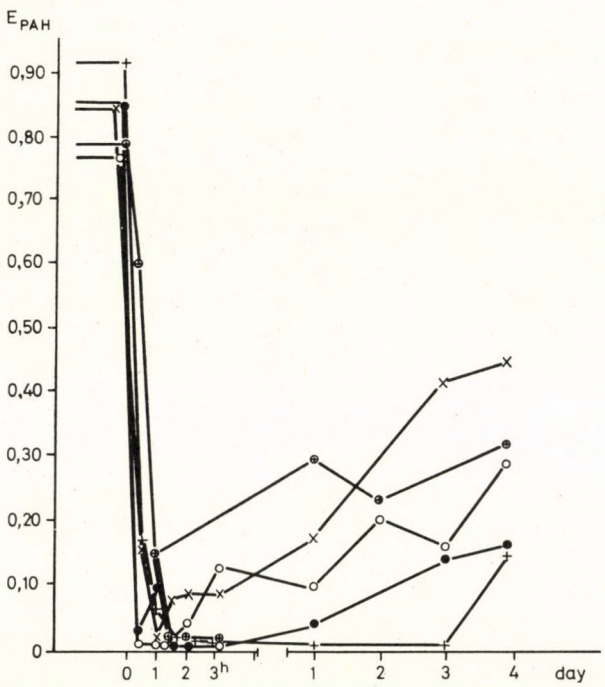

Fig. 4. E_{PAH} before and after renal ischaemia, in terms of hours and days, in the anaesthetized group. Each line represents the data of an individual animal

the successive days a slow increase was noted and the regression coefficient was positive [$Y = 0.048 + (0.00087 \pm 0.0003)x$]. These dogs displayed a considerable reduction also in E_{PAH}, but a significant improvement was demonstrable in the postischaemic period [$Y = 0.08 + (0.0021 \pm 0.00061)x$].

The values for renal arterio-venous O_2 difference in the two groups are shown in Fig. 5. While as compared with the controls, the O_2 difference increased during the postischaemic period in the unanaesthetized group, it

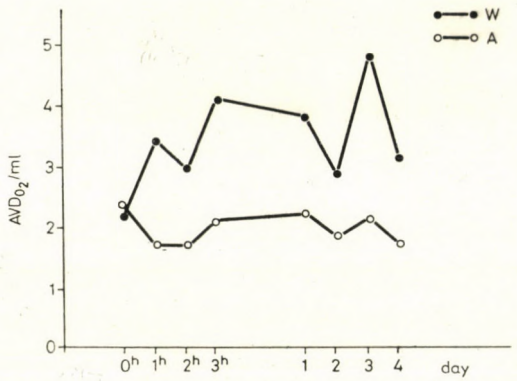

Fig. 5. Renal arterio-venous O_2 difference before production of renal ischaemia (0^h) and during the postischaemic period, in terms of hours and days. W = Wakened (unanaesthetized), A = Anaesthetized

remained practically unaffected in the anaesthetized group. The differences between the individual values for the two groups were considerable, but not invariably significant at all points of time.

Discussion

The present study extending over several days allowed to ascertain definite differences in the time-relationships of individual parameters of renal function, according to whether the dogs had been in conscious state or under anaesthesia at the time of the renal ischaemia. E_{Cr} and E_{PAH} fell in both groups to excessively low values. However, on the first day after the intervention there was already a distinct difference between the two groups and subsequently the anaesthetized one showed a continuous improvement of the extractions, attaining a significant level by the fourth day. In the unanaesthetized group there was no amelioration of these functions.

The low extraction values found in the postischaemic period seem to have been due to a reduction in RBF. The reduced extraction in the unan-

aesthetized group is suggestive of impaired glomerular and tubular functions, as a result of the reduced RBF, as it has been demonstrated earlier. The gradual increase in GFR as well as in E_{PAH} in the anaesthetized group may be connected with an increase in RBF.

The renal arterio-venous O_2 difference in the unanaesthetized group exceeded that found in the anaesthetized group. It has been demonstrated by BÁLINT et al. (1964a) that the correlations between filtration and O_2 consumption are linear in the post-ischaemic state, too. O_2 utilization by the kidney is ensured by RBF and AVD_{O_2}. The larger AVD_{O_2} in the unanaesthetized group seem to suggest that the kidney increases its O_2 utilization in order to counteract the postischaemic reduction in RBF.

The present as well as our earlier results suggest that the difference in RBF between the two groups was due to neurogenic factors. The existence of connections between the central nervous system and renal blood supply has been borne out by ample experimental evidence. The injury resulting in renal ischaemia is followed by a shifting of the intrarenal blood supply. Experimental proof of a readjustment of this kind under various conditions has been produced by several authors (GÖMÖRI and NAGY 1961, SHALDON et al. 1963, PILKINGTON et al. 1965, CARRIÈRE et al. 1966, GÖMÖRI et al. 1968, POMERANZ et al. 1968, BÁLINT et al. 1969, etc.). The functioning renal parenchyma is bypassed by the circulation for some length of time. This neurogenic effect has been shown to be suppressible by surgical or pharmacological denervation (FEKETE and TARABA 1965, TARABA 1965).

From the present study it emerges that improvement of creatinine- and PAH-extraction in the anaesthetized animals while being incontestable, is by no means complete. In our earlier studies where the tests had been performed two weeks after ischaemization, restitution of renal function had reached a far more advanced stage. Full normalization requires, however, a longer time extending several weeks or even months.

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INHIBITION OF IMMUNOSPECIFIC ROSETTE FORMATION FOR THE STUDY OF TWO PREDNISOLONE DERIVATIVES

By

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Methyl-piperazinyl prednisolone and prednisolone succinate have been compared for their immunosuppressive effect by means of the rosette inhibition test. The procedure based on the inhibition *in vivo* of immunospecific rosette formation has been found more suitable for this purpose than *in vitro* inhibition. Rosette formation was inhibited more intensively by massive doses of prednisolone succinate than by its derivative. With reduced doses, the difference between the two compounds tended to diminish.

Therapeutic observations with two steroids, prednisolone succinate (Di-Adresone-F aquosum, Organon, Oss, Netherlands) (PS in the following) and deoxy-methyl-piperazinyl prednisolone (Depersolone, Richter, Budapest) (MPP in the following) have made us to assume that the effect of the two compounds on the acute symptoms of autoimmune disease or on the asthmatic state involved a different dynamism. In order to substantiate this impression, we had to find a model suitable for the discrimination of two steroids of the same active groups, differing only in their side chains. The procedure based on the inhibition of immunospecific rosette formation seemed suitable for this purpose.

Immunospecific rosette formation is basically the following process: lymphonodular, splenic or peripheral lymphoid cells of some laboratory animal immunized with xenogeneic or allogeneic cells adhere *in vitro* to the antigenic cells by clustering into rosettes. Under suitable experimental conditions, the rosette-forming lymphocytes are prevalently thymus-dependent, antigen-sensitive lymphocytes, in other words they are carriers of cell-mediated immunity [1—4] and thus suited for its study.

The number of rosette-forming cells is decreased by immunosuppressive agents, by administration of these drugs *in vivo* or by adding them to lymphocyte suspensions *in vitro* [4]. The inhibition is related to the allograft-protective capacity of the agents in question [5], therefore the bioassay *in vitro* may be used as a model of the effects *in vivo*. The rosette model is of adequate sensitivity and lends itself to the comparative study of cytostatic drugs, steroids and antilymphocyte globulins which are similar in their range of activity but dissimilar in their structure and potency [5, 6].

Material and methods

Two months old Akr-mice were injected intraperitoneally with 2.2×10^8 sheep erythrocytes. For the inhibition of the rosette-forming cells *in vivo*, identical doses of PS and of MPP, i.e. 5, 1 and 0.25 mg per mouse were administered intraperitoneally on a single occasion on the day of immunization. Taking into account the population dynamics of the rosette forming [4], they were counted on the 9th day. After sacrifice of the mice, cell suspensions were prepared from their spleen in Parker's 199 medium. The cells were washed twice, the cell counts were adjusted to 25×10^6/ml. The same volume of washed sheep erythrocyte suspension at a concentration of 10^8/ml was admixed to the splenocytes. The following steps were centrifugation (500 r.p.m.) for 10 min, resuspension and counting. The number of rosette-forming cells per thousand lymphocytes was counted; results are given in terms of per thousand and in absolute figures.

Inhibition of rosette formation in vitro

From the spleen of mice which had been immunized but not drug-treated, a cell-suspension was prepared on the 9th day to which identical dilutions of PS and MPP were added. After incubation at 37 °C for 60 min, sheep red cells were added to the splenocytes. The further steps of the procedure were identical with those of the previous test.

Results

Rosette inhibition by the two steroids in mice *in vivo* is shown in Fig. 1.

The first full column represents the spontaneous rosette proportion in normal, untreated mice, the second full column that of immunospecific

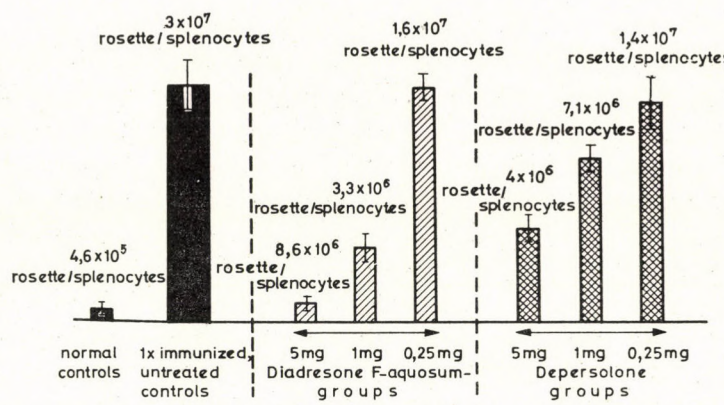

Fig. 1. Inhibitory effect of MPP and of PS on immunospecific rosette formation *in vivo*

rosette formation. The hatched columns represent the rosette formation by splenocytes of mice treated with identical doses of PS and MPP. It can be seen that the original number of 3.3×10^7 immune rosette-forming lymphocytes was decreased to 8.6×10^5 (2.6%) by 5 mg doses of PS and to 4×10^6 (12%) by the same doses of MPP. In 1 mg doses, MS reduced the original number of rosette-forming cells to 3.3×10^6 (10%) and MPP to 7.1×10^6 (21.5%). With 0.25 mg doses a reduction to 1.6×10^7 (approximately 50%) and of 1.4×10^7 (42.5%) respectively, was observed.

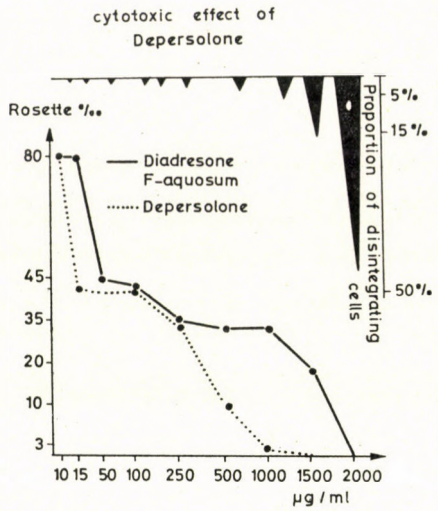

Fig. 2. Inhibitory effect of MPP and of PS on immunospecific rosette formation *in vitro*

The results of *in vitro* inhibition of immunospecific rosette formation are presented in Fig. 2.

Rosette formation by splenocytes of untreated mice with sheep red blood cells amounted to 80%. It can be seen that

(1) full inhibition of the immuno-rosette model has been achieved by 1500 µg/ml of MPP and by 2000 µg/ml of MS;

(2) in the range of 50 to 250 µg/ml of MPP, and in that of 100 to 250 µg/ml of PS, the rosette inhibitory efficacy did not change significantly;

(3) at higher concentrations (1 to 2 mg/ml) MPP exerted a direct lymphocytotoxic effect, in opposition to PS which exhibited no effect of this kind at similar concentrations.

Discussion

Both MPP and PS were found to inhibit rosette formation *in vivo* as well as *in vitro*.

Upon administration to immunized mice, massive doses of PS proved more suppressive to rosette formation than did similar doses of MPP. This difference dwindles, however, parallel with the reduction of the doses. At dose levels of 0.25 mg, the inhibitory effect of the two drugs is practically the same.

The graphs representing the rosette-inhibiting effect of PS and of MPP are of the same shape. However, as it can be seen in Fig. 2, the inhibitory effect of MPP *in vitro* was more intensive than that of MS, its graph showing a shift to the left as compared with PS. Evaluation of the inhibitory effect *in vitro* is blurred by the direct lymphocytotoxic activity of MPP.

Inhibition of rosette formation *in vivo* thus seemed more suitable for the comparative evaluation of the immunosuppressive properties of MPP and of PS than their inhibition *in vitro*. Clinical observations concerning the advantages of PS over MPP in respect of the potency and rapidity of their immunosuppressive effect are consistent with the present results obtained at high dose levels. For instance, the rosette-suppressive activity of 5 mg doses of PS is five times as intensive as that of MPP.

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IRON METABOLISM AND STORAGE IN GILBERT'S DISEASE, AND IN ROTOR'S AND DUBIN-JOHNSON'S SYNDROMES

By

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Iron kinetics have been studied in 3 patients with Dubin–Johnson's syndrome, in 2 with Rotor's syndrome, and in 7 with Gilbert's disease. The following abnormalities were found.

(1) Of the 12 patients with functional bilirubinaemia 4 were anaemic, and 1 of the 4 suffered from iron deficiency anaemia.

(2) The disappearance of radioiron from plasma was accelerated in 3 patients and normal in the others.

(3) In 7 patients iron turnover in the plasma was increased, and in the others it was normal.

(4) Utilization of radioiron in the erythrocytes was considerably decreased in 4 patients, and moderately decreased in 6 patients.

(5) Erythrocyte survival was normal in all but one of the patients studied.

(6) Measurement of radioactivity *in vivo* revealed an increased storage of iron in the liver.

Theoretically, jaundice is spoken of when the blood level of bilirubin is elevated. This may be due to any of four different causes: (1) increased deposition of bile pigment in the liver cells; (2) disturbance of bilirubin uptake and transport in the liver cells; (3) faulty bilirubin conjugation by the hepatic microsomes; and (4), impaired excretion of bilirubin into the canaliculi or mechanical obstruction of the bile ducts [12].

Since its description by Gilbert about six decades ago, a familial form of jaundice has been known in which there are no complaints or haemolysis and for which a disturbance of bilirubin metabolism is responsible. From the above aetiologic schema, the disturbance of intracellular transport is implicated in Gilbert's disease, enzymatic insufficiency of bilirubin conjugation in the Crigler–Najjar syndrome, and a disturbed excretion of bilirubin into the bile canaliculi in the Dubin–Johnson and Rotor syndromes [3, 4, 8, 10, 11]. A review of the differential diagnosis of functional hyperbilirubinaemias in adults is given in Table I. Although each type of functional hyperbilirubinaemia originates from the dysfunction of a different phase of bilirubin metabolism, all types have certain characteristics in common, which can be found in the bilirubinaemias due to a disturbance of transport, to enzymopathy, or impaired excretion. Such common features are the usually congenital origin of the disease,

Table I

Differential diagnosis of Gilbert's disease, Rotor's and Dubin–Johnson's syndrome

	Gilbert's disease	Rotor's syndrome	Dubin–Johnson's syndrome
Beginning	Before puberty	Before puberty	Before 25 years
Hepatomegaly	Infrequent	—	50% of cases
Serum bilirubin	Under 3 mg per 100 ml indirect	About 5 mg per 100 ml indirect 50%	About 5 mg per 100 ml dominantly direct
Bromsulphaleine	Normal	Pathological retention	Increasing retention after 45 min.
Histology	Fine gold-brown pigment	Normal liver tissue	Rough brown pigment

its familial character, its juvenile onset and benign course. Only the Crigler–Najjar syndrome is an exception, because in most cases its outcome is fatal.

The liver being the largest iron depot in the healthy human, its role in iron metabolism is of great importance. Under pathological conditions, iron storage may be increased and cause damage to the liver cells, but increased storing of iron may also be observed as a concomitant phenomenon in diseases of the liver. As we do not know of any study of iron kinetics in hyperbilirubinaemia, it seemed warranted to carry out examinations in patients suffering from the above described conditions.

Material and methods

The material comprised 3 patients with Dubin–Johnson's syndrome, 2 with Rotor's syndrome, and 7 with Gilbert's disease. In addition to routine laboratory examinations, a histological study of the liver was also made for diagnostic purposes. Examinations of iron kinetics was carried out by a modification [2] of HUFF and HENESSY's method [9].

Results

Four patients with functional hyperbilirubinaemia could be regarded as anaemic on the basis of their haematocrit values. Two of them displayed Dubin–Johnson's syndrome, and two Gilbert's disease. In 3 patients the fasting serum iron and the haemoglobin level were moderately decreased, the mean erythrocyte diameter was indicative of microcytosis. An increased serum iron level (256 μg per 100 ml) was found in one case (K. Sz.) A low serum iron level was recorded in 5 patients (M. K., L. S., F. A., I. B. and K. Sz.). Acceleration of the disappearance of radioiron was observed in three patients who had low serum iron levels (L. S., F. A. and K. Sz.). The other important parameter of iron kinetics, plasma iron turnover, was higher than normal in more than half of the patients. There was a considerable increase

mainly in the anaemic patients, of whom F. Sz. should be mentioned first, since in this patient the pathologically increased serum iron value (256 μg per 100 ml) was associated by a considerably increased iron turnover (Table II and Fig. 1). Incorporation of radioiron into red blood cells is presented graphically in Fig. 2. It can be seen that the rate of incorporation followed for 12 days was below the empirical limits obtained in subjects with normal haematopoiesis (Fig. 2). Only small differences could be found between the the groups of functional hyperbilirubinaemia; on this basis, apart from the uniformly decreased rate of incorporation, it was the group of patients with Gilbert's disease that was nearest to normal conditions.

Table II

Name age, sex	Haematocrit per cent	Se iron per 100 ml	Plasma iron clearance T1/2 min.	PIT* mg/24h	Utilization ^{59}Fe max/day	Se. bilirubin: Conjugated	Se. bilirubin: Unconjugated	Type of disease
M. K. 22 M	34	80	90	0.88	65/8	4.4	1.4	Dubin–Johnson's syndrome
J. P. 21 M	43	100	100	1.00	71.5/8	2.4	1.4	Dubin–Johnson's syndrome
L. S. 32 F	30	80	25	3.20	30/12	3.0	1.2	Dubin–Johnson's syndrome
M. B. 31 M	45	110	120	0.90	30/8	4.2	0.2	Rotor's syndrome
I. K. 14 M	42	130	80	1.62	85/8	slight	0.8	Rotor's syndrome
D. V. 38 M	45	130	90	1.44	55/12	slight	3.6	Gilbert's disease
L. Sz. 41 M	46	100	120	0.83	69/8	—	2.3	Gilbert's disease
F. A. 25 M	43	70	45	1.55	70/8	0.4	1.3	Gilbert's disease
I. B. 25 M	46	79	70	1.00	73/12	0.5	1.2	Gilbert's disease
K. Sz. 20 M	36	60	30	2.00	86/8	0.7	1.3	Gilbert's disease
F. Sz. 26 M	42	256	80	3.20	34/12	0.5	2.0	Gilbert's disease
Sz. F. 28 M	34	110	65	1.65	39/12	slight	4.6	Gilbert's disease
Normal value	41—45	85—125	70--120	0.45--0.75	78—98	0.1	0.9	—

* Plasma iron turnover

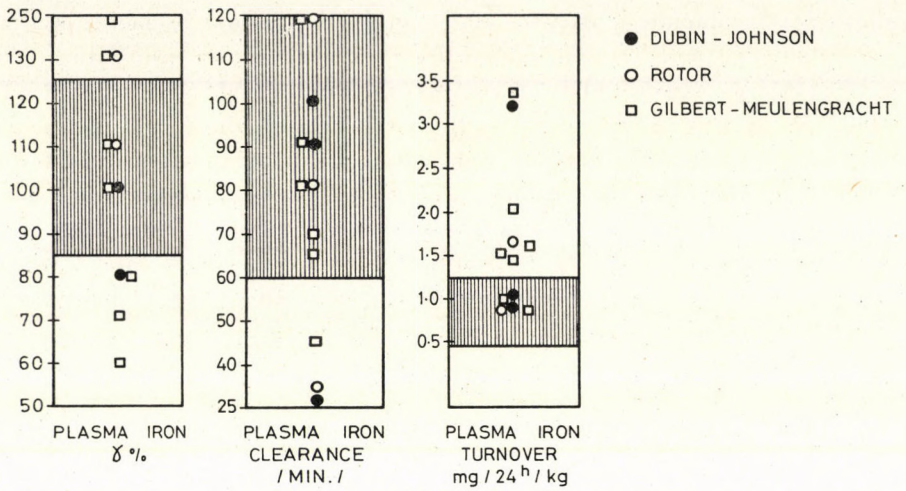

Fig. 1. Distribution clearance and turnover of plasma iron in relation to normal values (hatched areas indicate the limits of normal values)

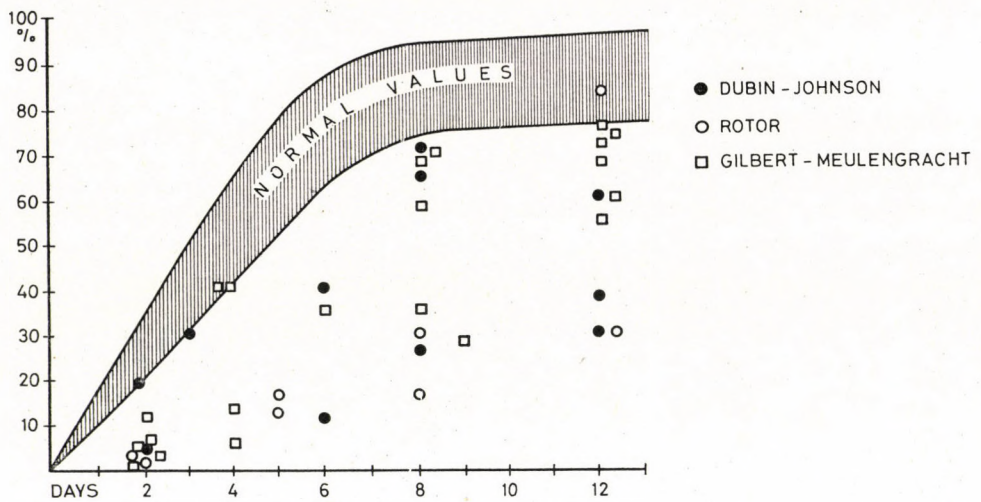

Fig. 2. Incorporation of ^{59}Fe (radioiron) into erythrocytes in three types of functional bilirubinaemia

The patients showed equally striking differences in organ surface activity. In relation to the controls in Fig. 3 the most striking disturbance was found in Dubin–Johnson's and Rotor's syndrome, while in the patients with Gilbert's disease the differences from normal were more modest.

The disturbance manifested itself mainly with a changed iron storage by the liver. In relation to the normal cases, the liver was the dominantly

active organ, and its activity did not decrease considerably during the 12 days of examination. Even in comparison with the sacrum, considered the representative of bone marrow, hepatic activity was dominating, and iron turnover in the bone marrow decreased. The difference appeared also in the decreased utilization of radioiron (Fig. 2).

Discussion

Radioiron bound to transferrin seemed to disappear rapidly and completely from plasma. Under normal conditions most of it migrates into the bone marrow and, representing the labile iron pool there, gradually takes part in the formation of haemoglobin. Transferrin molecules carry one or two iron

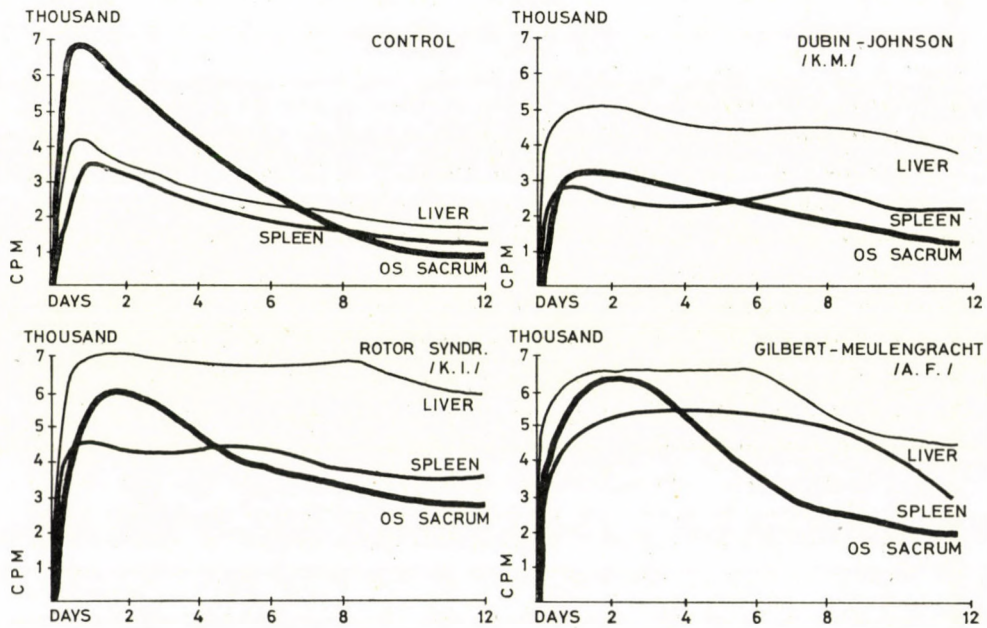

Fig. 3. Radioactivity over liver, spleen and sacrum in normal controls and in patients with Dubin–Johnson's, Rotor's and Gilbert–Meulengracht syndromes. (The values indicated on the ordinate represent counts per min)

atoms; the normal iron saturation of transferrin amounts to 30%. The affinity of erythrons and of hepatic cells to transferrin carrying two iron atoms is almost identical [6]. Just like bone marrow, the liver also takes up a certain amount of iron in the form of ferritin, but the liver gives it off rapidly partly through the circulation and partly through a lymphatic shunt. The ferritin iron found in the liver also forms part of the labile pool and takes part in haematopoiesis.

Ferritin is a protein compound with a molecular weight of 460,000 M in which there are ferrihydroxide bundles and 20—23% iron in crystallin form. It is colourless, and so finely dispersed in the tissues that it cannot be demonstrated with the light microscope, only by a special method by electron microscope [1]. The steric distribution and iron content of haemosiderin is larger, its demonstration is possible with the traditional staining methods and also with the light microscope.

In functional bilirubinaemias the uptake of iron by the bone marrow decreased and its uptake by the liver increased. It may be assumed that one of the causes of this is that the liver stores more ferritin than under normal conditions. The increased ferritin storage manifests itself in an increased radioactivity. In the course of the histological study the sections prepared by the usual methods and studied under the light microscope failed to show an increased storage of iron. This contradiction can only be resolved by assuming that the iron which had shown increased activity was contained in ferritin, which, as has been said, cannot be demonstrated by usual methods. The particular nature of this disturbance of storage, which occurs in all three functional hyperbilirubinaemias under consideration, has not been clarified. It may, however, be stated that, just like the disturbance of bilirubin metabolism, that of iron metabolism does not cause a severe change in haematopoiesis, since the clinical picture is not associated with grave anaemia. In only one of the four patients with anaemia (K. Sz.) did we find characteristics pointing to the aetiological role of iron deficiency, namely a shortened plasma iron clearance, increased iron-binding capacity and normal iron utilization by the erythrocytes. We have failed to clarify the origin of anaemia in the other three patients (L. S., F. Sz. and M. K.) during the time of examination and they did not report for reexamination.

The disturbance manifested itself first of all in a protracted iron utilization. There was a discrepancy between normal iron turnover, observed in about half of the patients, and the decreased iron utilization.

In several haematological conditions the above change is due to an increase in ineffective erythropoiesis. This can hardly be the case in functional hyperbilirubinaemia, but is in connection with the increased iron storage in the liver. The same phenomenon must be reckoned with, although to a lesser extent, in the spleen.

Literature contains several data on increased deposition of iron in the liver. FAWWAZ et al. [5] demonstrated an increased deposition of iron in cirrhosis, which they ascribed to an increased iron absorption and to a decreased iron-binding capacity of the serum. Another assumption of the above-mentioned authors explains the increased storage of iron by the cirrhotic liver as a result of abnormal iron binding by transferrin. In other pathological conditions (haemolytic anaemia, aplastic anaemia, haemochromatosis, etc.),

in the presence of an increased fasting serum iron level, and in the case of decreased free iron-binding capacity, an increased iron binding by the liver is met with. Estimation of the capacity to bind free iron carried out in some of the patients (M. K., 63%; M. B., 51%; F. Sz., 58%; normal, 70%), showed a slight decrease.

FRIEDMAN et al. [7] observed quantitative and qualitative changes of alpha-2-globulins, pointing to the possible role of the elevated coeruloplasmin level in increased deposition of iron. All these may cause a deposition of iron, and a protracted and in some cases decreased iron utilization in the functional hyperbilirubinaemia. A further possibility would be that affinity to transferrin of hepatic cells is changed in functional bilirubinaemia.

The splenic surface activity of the patients suffering from Dubin–Johnson's or Rotor's syndrome also indicated a slightly increased erythrocyte sequestration, which, however, was not observed in each case. In one patient with Dubin–Johnson's syndrome (I. P.) this was quite marked and on the 12th day the amount of utilized radioiron decreased in parallel with the increase in activity over the spleen. This finding was in agreement with the findings of a slight shortening of the erythrocyte life-span. In our own material, platelet survival was determined with ^{51}Cr in some cases; however, a pathological value was obtained in the single patient mentioned above ($T_{1/2} = 21$ days).

Clarification of the anomalies observed in iron kinetics awaits further studies. Thus, the conditions of iron absorption, and the definition of the whole iron pool of the body and the kind of protein binding of the iron which forms this pool must be clarified.

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INFLUENCE OF DIETARY PROTEIN INTAKE ON THE INCISORS OF RATS

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It has been observed that a protein-deficient diet results in general disturbances of dental development, as reflected by a slower rate of growth of the teeth, alterations in their chemical structure and a loss of resistance to acids. The findings indicate that inadequate dietary protein intake during the period of dental development affects the resistance of the teeth.

Though the importance of proteins to the whole organism including the individual organs and tissues has long been known, the influence of protein intake on the development and structure of the teeth and on their resistance to decay has received little attention. The significance of protein intake as well as of minerals as regards dental development, calcification and resistance of bones has been fully borne out by many experiments [5, 7]. Though the structure of the bone tissue differs in many respects from that of the hard dental material, it is none the less evident that dietary protein intake plays an essential part in the development of the teeth.

STEIN and ZISKIN [6] described alterations involving the odontoblast layer and the root surface of the molars and the dentinal surface surrounding the pulp of the incisors, in addition to other oral changes in albino rats kept on a protein-free but otherwise adequate diet for 3 to 4 weeks. SHAW [3] fed female albino rats on a protein deficient diet during gestation and lactation and noted a delay in the eruption of the third molar of the orogeny, moreover the dental cusps had an unusual shape and there was an increase in the incidence of caries.

In the light of these observations we have studied the effect of dietary protein intake on the incisors of albino rats.

Material and methods

A total of 120 female albino rats of our own breed was studied. The animals were kept on a semi-synthetic diet (Sós [4]) the protein content was: a) low-protein diet (3% casein), b) high-protein diet (30% casein), c) normal-protein diet (18% casein).

The animals of the first series were divided into three groups, each fed a different type of diet. Before beginning the diet and subsequently at 14-day intervals, the left lower incisor was cut off 1 to 2 mm from the gum. The distance between the edge of the intact

tooth and the cut surface of the amputated tooth was measured every 1 to 3 days until the tooth had attained its former size. The rate of growth was reflected by the decrease of this difference.

The second series included three groups, viz. 1) low-protein diet (30 animals); 2) high-protein diet (20 animals); 3) normal-protein diet (10 animals). After six weeks diet the first (low-protein) group was divided into three subgroups and the diets were modified according to the following schedule: the animals of group 1a remained on the low-protein diet, those of group 1b were fed the high-protein, and those of group 1c, the normal-protein diet. Group 2 (high-protein diet) was divided into two subgroups, group 2a being given the low-protein, and group 2b the normal-protein diet. The animals of group 3 continued on the normal-protein diet (Table I).

Table I

Animal groups and dietary schedules

At the start of experiments	After the sixth week
1. Low protein	1a. Low protein
	1b. High protein
	1c. Normal protein
2. High protein	2a. Low protein
	2b. Normal protein
3. Normal protein	3. Normal protein

The third series comprised two groups. The first (20 animals) were fed the low-protein, and the second (10 animals), the normal-protein diet. The left lower incisors were amputated, as indicated earlier, at two-week intervals. In 50% of the animals of each group the removed dental fragments were cleaned, washed in distilled water, dried at 90 °C for 24 hours and studied for nitrogen and phosphate contents. In the other 50% the dental tissue was subjected to a similar treatment and tested for calcium contents and resistance to acids.

Dental nitrogen was estimated by the micro-Kjeldahl method. Anorganic phosphates were determined in specimens destroyed and extracted with an organic solvent. The procedure was adapted by ZELLES [9] to estimations in biological material.

The resistance of the hard dental tissues to acids was estimated on the basis of the amount of calcium brought into solution by 2% hydrochloric acid in 24 hours. The dental fragments were placed in the diluted acid and 1 ml samples were taken at 24 hours. The concentration of the solution was then adjusted with concentrated hydrochloric acid to 10% and left to stand for seven days thus allowing the entire calcium content to be brought into solution (TERSIC and TERSIC [8]) which was estimated by complexometry (ERDEY [1], SCHWARZENBACH [2]).

Results

The gain in body weight and the growth of incisors have been found to decrease under the influence of the protein deficient diet. The high-protein diet had apparently no influence on these parameters (Fig. 1). Normalization of the protein intake or its increase beyond normal levels was found to accelerate the rate of body weight gain and of the growth of the incisors in the animals which earlier had been fed the low-protein diet. The rate of growth

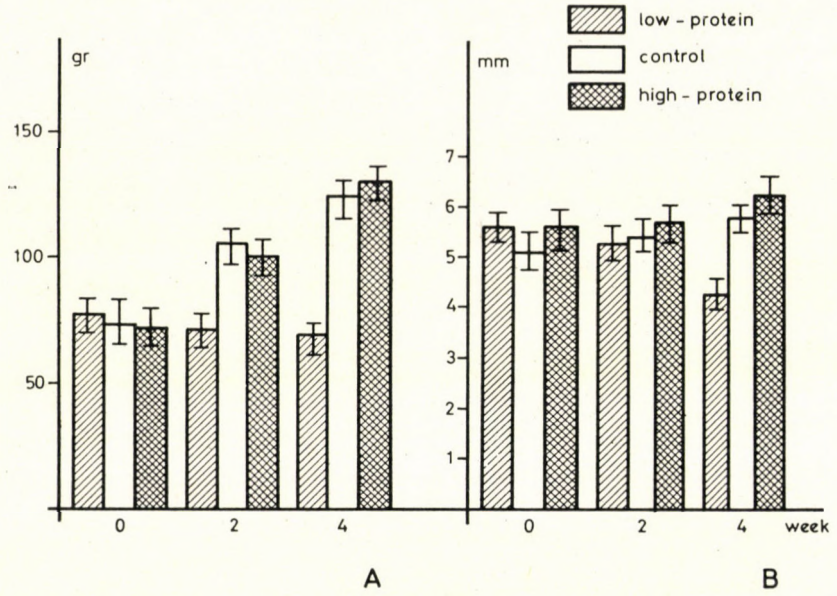

Fig 1. Body weight (A) and weekly rate of dental growth (B) under the influence of diets of different protein contents

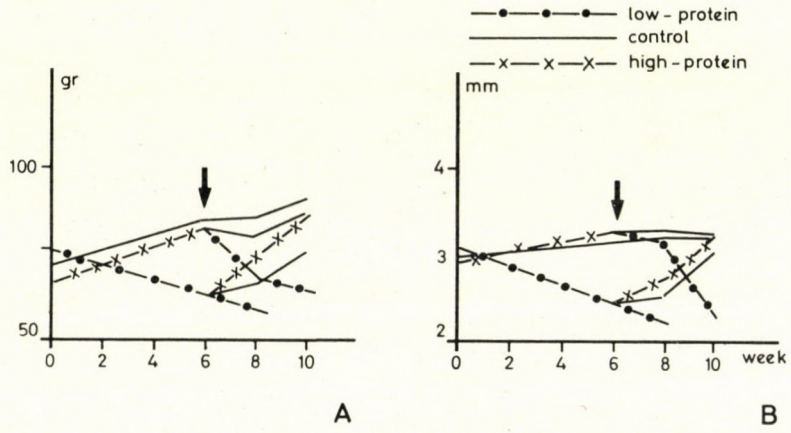

Fig. 2. Body weight (A) and three-daily rate of dental growth (B) under the influence of varying diets of different protein contents

was restored more rapidly by the high-protein than by the normal-protein diet. Change-over from the high-protein to the low-protein diet was soon followed by a loss of body weight and in four weeks growth of the incisors was also slowing down. Change-over from the high-protein to the normal-protein diet did not seem to have any distinct influence on the processes under study (Fig. 2).

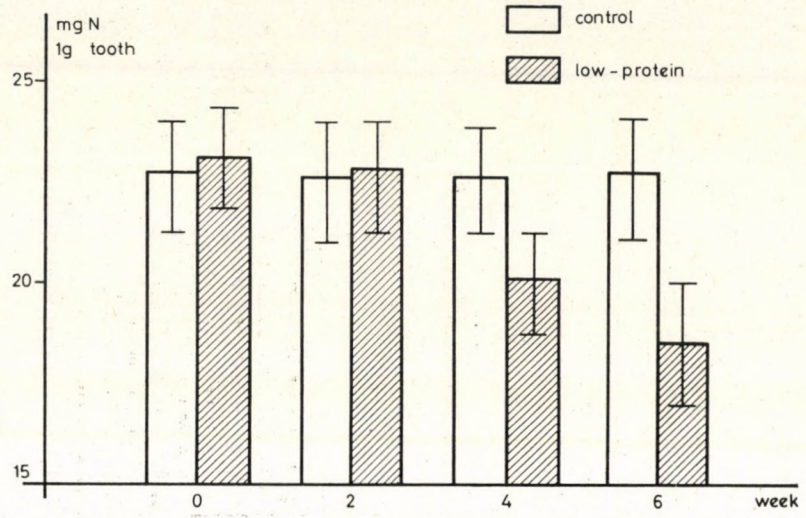

Fig. 3. N-content of the hard tissues of the incisors

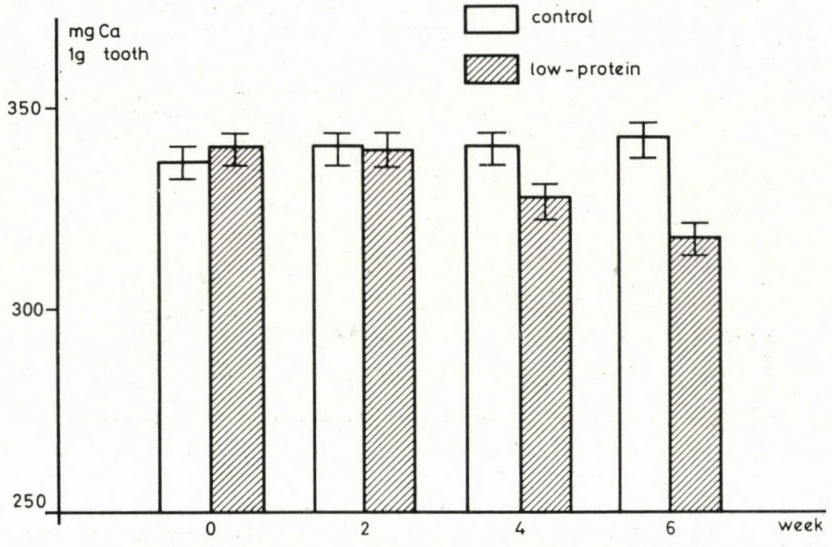

Fig. 4. Ca-content of the hard tissues of the incisors

The hard tissues of the incisors showed in response to the low-protein diet a loss of nitrogen and calcium (Figs 3, 4) and an increase in phosphorus (Fig. 5). As a result of calcium depletion and phosphorus retention, a shift in the calcium–phosphorus ratio from 2 : 0.97 to 2 : 1.26 was noted. The resistance to acid of the hard dental tissues diminished. The amount of calcium

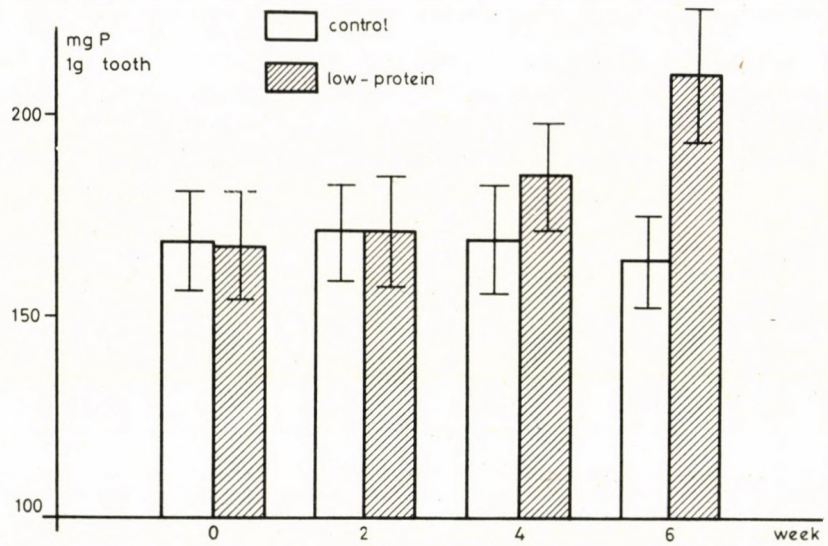

Fig. 5. P-content of the hard tissues of the incisors

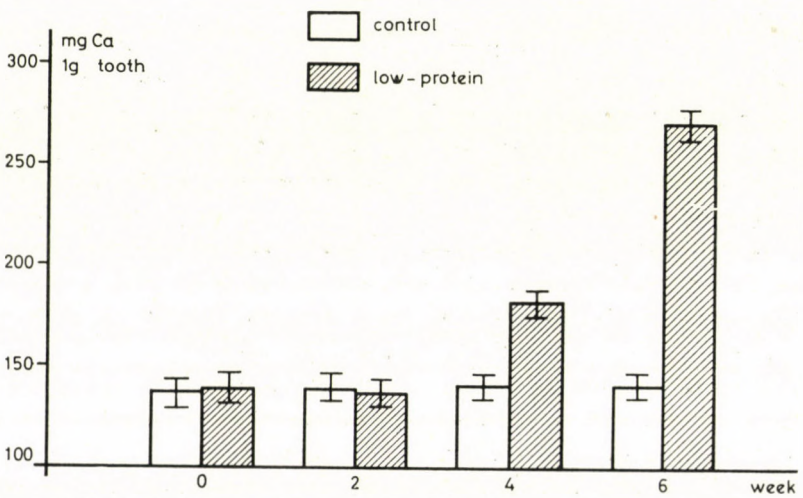

Fig. 6. Acid resistance of the incisors (amount of Ca in mg brought into solution by 2% HCl in 24 hours)

per g dental tissue dissolved by 2% hydrochloric acid in 24 hours increased to 270 mg at six weeks as against 140 mg in the controls (Fig. 6).

The changes in dental growth, chemical composition and resistance to the solvent action of acids became manifest in four weeks and were found to increase during the following two weeks.

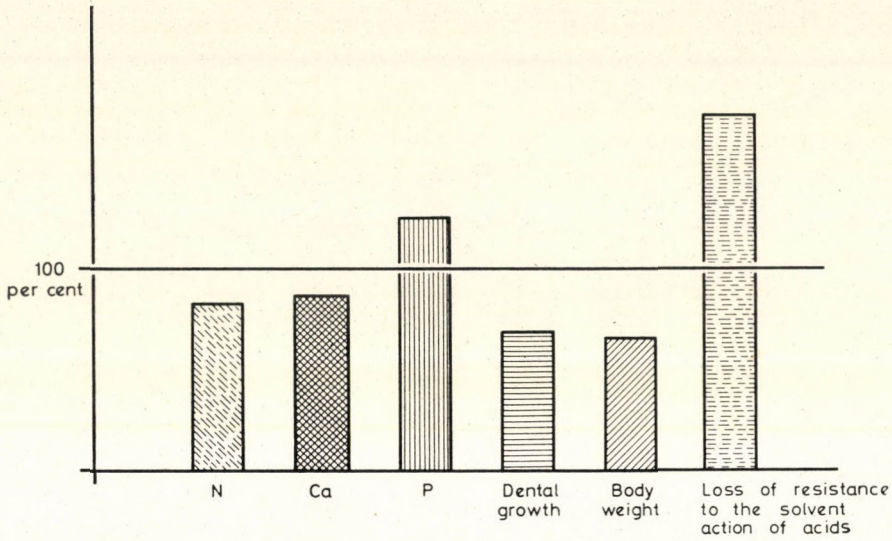

Fig. 7. **Changes in the parameters studied, in per cents (Reference value, 100%)**

Discussion

As a result of the low-protein diet, development in the animals gave way to a loss of weight in one or two weeks. Some two or three weeks later the rate of growth of the incisors also slowed down. The high-protein diet in itself had no influence on the growth of the incisors. However, it helps the restoration of body weight and the growth of the incisors more rapidly than did the normal protein diet.

In the animals fed a protein-deficient diet the chemical structure of the hard dental substance underwent profound alterations which became measurable four weeks after the start of the diet when the rate of dental growth also showed a sharp decline. The changes in the parameters under study were of opposite direction. While the dental substance lost calcium, it retained phosphorus. As a result, the Ca/P-ratio shifted to the advantage of phosphorus and the dental substance became less resistant to acids.

The adverse influence of protein lack on the teeth was more closely reflected by the loss of resistance of dental tissue to the solvent action of acids than by the changes noted in the other parameters (Fig. 7).

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RENAL VENOUS PLASMA RENIN ACTIVITY AFTER UNILATERAL SPLANCHNICOTOMY IN DOGS WITH INDUCED HYPERTENSION

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Development and persistence of arterial hypertension have been observed in dogs after complete ligation of one renal artery, whether the opposite kidney had been left innervated or denervated by splanchnicotomy. In the early phase of hypertension, renin activity in the renal venous blood of the ischaemic kidney was high, in contrast to the denervated kidney where it remained in the range of the control values. A gradual increase in renin activity occurred in the later stage of hypertension after removal of the ischaemic kidney. The results are indicative of a role of the renin-angiotensin system in the stabilization of hypertension.

In earlier studies it has been found that ligation of one renal artery in dogs is followed within a few days by arterial hypertension and later by its stabilization in the presence of the other, unmanipulated, kidney (FEKETE 1970). This induced hypertensive state may be regarded morphologically and clinically as an experimental model of human renovascular hypertension (FEKETE 1970, FEKETE et al. 1970). Both the ischaemic and the nonoperated kidney revealed an enhanced venous renin activity, and removal of the ischaemic kidney failed to normalize the renin activity in the venous blood of the unoperated kidney (FEKETE et al. 1971). In view of the involvement of the sympathetic system in the control of renin secretion, the model was reproduced in the present study by combining it with splanchnicotomy of the unoperated kidney with the purpose of its denervation. The aim of the experiments was to throw light on the mechanism and stabilization of renovascular hypertension.

Material and methods

A total of 20 adult mongrel dogs of either sex was used. The operation was performed under pentobarbital anaesthesia. From midline approach the right renal artery was ligated close to its origin, and the major splanchnic nerve supplying the left kidney was surgically divided. Arterial pressure was measured percutaneously in the femoral artery with a mercury manometer preoperatively and at regular intervals postoperatively in conscious state of the animals which had been used to the manipulation. Blood samples for determination of the renin activity were obtained preoperatively (day 0) and postoperatively at determined intervals from both renal veins and from peripheral arterial blood. The procedure was as follows. Between the 20th and 70th days of hypertension, pentobarbital anaesthesia was induced, blood samples were drawn from the femoral artery by needle puncture, subsequently the abdominal cavity was opened under aseptic conditions, blood samples were taken from the vein of the ischaemic kidney which was then removed and weighed. Then the vein of the

denervated kidney was also punctured for blood samples, care being taken that all venous blood samples should be strictly of renal origin. After these manipulations the abdominal wound was closed.

In the further course of the study, blood pressure was measured at 20-day intervals. Renin activity was repeatedly estimated in the venous blood of the denervated kidney as well as in peripheral arterial blood. At some later stage of hypertension the animals were sacrificed and at the same time the denervated kidney was also removed.

Plasma renin activity was estimated by the method of KANEKO et al. (1967) with slight modifications. The procedure has been described in detail earlier (FEKETE et al. 1971).

Results

1) *Arterial blood pressure* (Fig. 1). Compared with the control value of 130 ± 2 mm Hg, an approximately 30 to 40 mm Hg increase was demonstrable in the first 10 days after ligation of one renal artery. Blood pressure was significantly increased all throughout the experimental period ($p < 0.02$

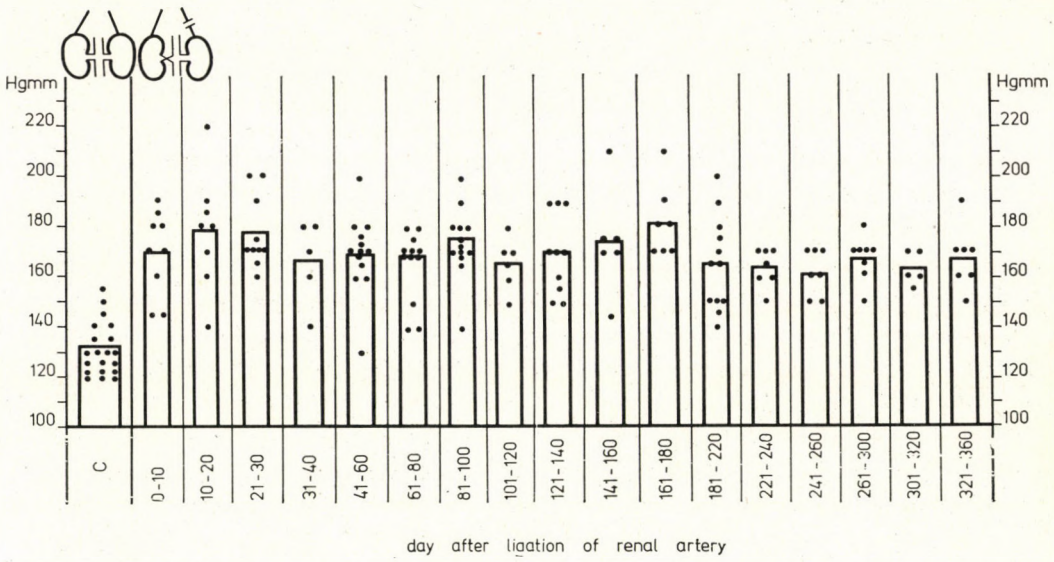

Fig. 1. Arterial blood pressure in dogs before ligation of the right renal artery (K) and after production of renal ischaemia, in the presence of a denervated left kidney. Removal of the ischaemic kidney between the 20th and 70th days of hypertension

and < 0.01, respectively). Removal of the ischaemic kidney between the 20th and 70th day had no influence on high blood pressure.

2) *Renin activity*. a) Values for renin activity in renal venous and peripheral arterial blood are summarized in Table I ($\bar{x} \pm$ S. E. M.). The first column represents the data for the normotensive controls, the other columns show those for the hypertensive animals. The plasma sodium level estimated simultaneously is also shown.

Table I

Renin activity in the normotensive state and in hypertension induced by unilateral ligation of the renal artery ($\bar{x} \pm$ S.E.M.). Renin activity in venous blood of the ischaemic and of the denervated kidney, and peripheral arterial blood, ng/ml plasma; plasma sodium concentration, mEq/l

Time of assay (day)	Normotensive		Hypertensive									
	renal venous blood	peripheral arterial blood	ischaemic renal venous blood	peripheral arterial blood	denervated renal venous blood	renal venous blood	peripheral arterial blood	renal venous blood	peripheral arterial blood	renal venous blood	peripheral arterial blood
0	47.7±7.2	35.2±6.2									
	n = 15										
	x = 153±1										
20—70			85.5±11.4	31.3±4.0	41.6±4.5						
				n = 12							
				x = 152±1							
71—160						78.0±11.1	40.1±6.9				
						n = 15					
						x = 148 ±2					
161—300								74.9±9.1	34.7±5.1		
								n = 12			
								x = 152±2			
301—540										84.9±8.9	36.7±4.2
										n = 12	
										x = 152±1	

b) The renin activity ratio (RAR: the renin activity of the renal venous blood divided by the renin activity of the peripheral blood) in the normotensive state and in the successive stages of experimental hypertension is presented in Fig. 2. The RAR values of the ischaemic kidney (column 2) as compared with the normotensive controls (column 1) was significantly increased. On

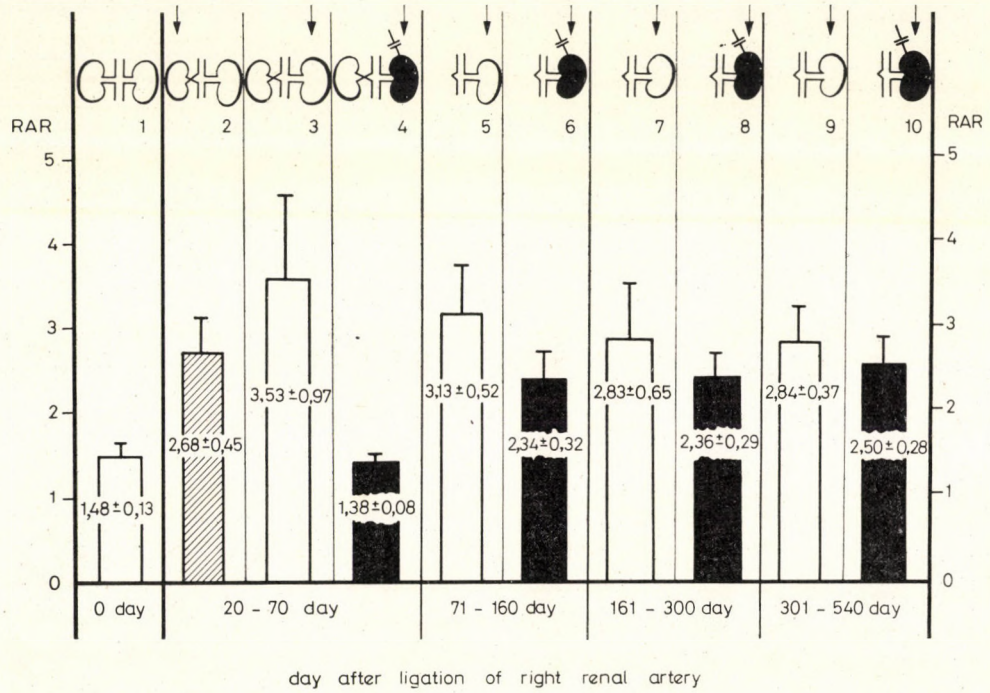

Fig. 2. Renin activity ratio ($\bar{x} \pm$ S.E.M.) in the normotensive state (column 1) and in the successive stages of hypertension after unilateral ligation of renal artery in the ischaemic kidney (column 2), in the opposite unmanipulated kidney (columns 3, 5, 7, 9), in the opposite denervated kidney (columns 4, 6, 8, 10). The ischaemic kidney was removed between the 20th and 70th days of the hypertensive state. All mean RAR-values differ significantly from those of columns 1 and 4

the contrary, the RAR values of the splanchnicotomized kidney determined simultaneously (column 4) was in the control range. After removal of the ischaemic kidney repeated measurements of renin activity in blood samples obtained from the denervated kidney revealed a significant increase in the RAR values between the 20th and 70th days, whether referred to the controls or to the former values for the same kidney (columns 6, 8 and 10).

c) The RAR values of the unmanipulated innervated kidney is shown in Fig. 2 (columns 3, 5, 7 and 9), on the same basis as above. These data have been published earlier (FEKETE et al. 1971) but are presented here for comparative purposes.

Table II

Renin activity ratio ($\bar{x} \pm$ S.E.M.) in the normotensive state and in the successive stages of induced hypertension

Time of assay (day)	Normotensive controls (1)	Hypertensive								
		ischaemic (2)	unmanipulated		unmanipulated		unmanipulated		unmanipulated	
			innervated (3)	denervated (4)	innervated (5)	denervated (6)	innervated (7)	denervated (8)	innervated (9)	denervated (10)
0	1.48 ± 0.13 n = 15									
20—70		□ c +d 2.68 ± 0.45 n = 19	□ c +c 3.53 ± 0.97 n = 6	1.38 ± 0.08 n = 12						
71—160					□ c +c 3.13 ± 0.52 n = 16	□ b +b 2.34 ± 0.32 n = 15				
161—300							□ a +a 2.83 ± 0.65 n = 11	□ c +c 2.36 ± 0.29 n = 12		
301—540									□ d +d 2.84 ± 0.37 n = 6	□ c +d 2.50 ± 0.28 n = 12

□ significance in comparison with the controls
\+ significance in comparison with the denervated kidney (column 4)
a: $p < 0.05$
b: $p < 0.02$
c: $p < 0.01$
d: $p < 0.001$

d) All data relative to RAR (mean values with standard deviations, results of statistical evaluation based on the *t* test) have been summarized in Table II. The RAR values for the normotensive controls are shown together with those for the groups with ischaemic kidney in the presence of innervated and denervated opposite kidney, respectively, together with the distinctive differences.

3) *Kidney weight.* a) The weight of the ischaemic kidney removed 20 to 70 days after ligation of the renal artery decreased to 38% of the original, as a sign of atrophy (Table III, column 6). Calculation of the normal renal weight was based on the equation $y = 12 \pm 4.33$ body weight, where y represents total kidney weight, i.e. the weight of both kidneys of a dog of a given body weight (BÁLINT 1969).

Table III

Renal weight in hypertension induced by unilateral ligation of renal artery

	Number of animals (1)	Body weight, kg (2)	Weight of ischaemic kidney in g removed between 20—70 days (3)	Weight of unmanipulated kidney g (4)	Weight calculated for one kidney of normal animal (5)	3/5 (6)	4/5 (7)
Unilateral renal artery ligation, other kidney unmanipulated	14	17.2±0.7	14.6±1.3	42.7±1.8	43.3±1.4	34±3	99± 8
Unilateral renal artery ligation, other kidney splanchnicotomized	17	13.8±1.1	13.7±1.9	37.8±2.6	36.0±2.4	38±4	105± 7
Normotensive controls after unilateral nephrectomy	20	14.6±0.6	36.5±1.3	50.2±1.8	37.6±1.4	97±3	138±13

b) The opposite, denervated but otherwise unmanipulated, kidney removed after 100 to 400 days of hypertension was found macroscopically as normal and its weight agreed with the predicted value calculated from the total kidney weight of normal animals (Table III, column 7). In contrast, unilateral nephrectomy in normal dogs resulted in a marked hypertrophy of the other kidney in 20 days.

Discussion

The mechanism responsible for the development and persistence of renal hypertension has been widely discussed in the literature but no convincing explanation has been offered. PAGE (1936), HOUSSAY and BRAUN-MENENDEZ (1942), HAAS and GOLDBLATT (1959), PICKERING (1945, 1968), FLOYER (1957), FRANK (1963), GENEST et al. (1964), MCPHAUL et al. (1964), BORKOWSKY

et al. (1965), HAYDUK et al. (1970) found a high renin activity in the venous blood, or in the urine of the ischaemic kidney of laboratory animals and ANICHINI and GROSS (1965) regard it as a primary causal factor of the increase in peripheral resistance. Other authors including MCDONALD et al. (1970) and FUNDER et al. (1970) disclaim any participation of renin-angiotensin system in the production of renal hypertension.

There is still less experimental evidence to support the various theories offered for the explanation of the mechanism responsible for the maintenance of permanent hypertension. As GOLDBLATT (1937, 1958), SKEGGS and KAHN (1958), HUBER (1960), HELMER (1964), MASSON et al. (1962, 1964), SCORNIK and PALLADINI (1964) believe, there is a slight but continuous production of renin in the ischaemic kidney, and the increase in vascular resistance produced by renin-angiotensin is responsible for the stabilization of the hypertension; no simultaneous enhanced renin-activity having been found in the unaffected kidney. This interpretation agreed with the results of KOLETSKY et al. (1965) and of BERMAN (1971) who could induce an acute hypertensive state associated with vascular changes, a condition indistinguishable from experimental renal hypertension.

In our earlier studies in dogs (FEKETE et al. 1971), after unilateral ligation of the renal artery an increased renin activity was found in the venous blood of the ischaemic kidney and, after its removal, in that of the unmanipulated kidney, over long periods of observation. It seemed obvious to connect the maintenance of the hypertension with the production of renin by the intact kidney itself, marked by profound changes of the vascular walls. Studies showing that antirenin treatment in dogs and monkeys was followed by a reduction in blood pressure are also consistent with the role of renin in the maintenance of permanent hypertension (GOLDBLATT 1958, HELMER 1958, WAKERLIN 1958, DEODHAR et al. 1964, FRANK 1963).

MCCUBBIN and PAGE (1963) reconcile the "humoral" and "neural" theories by assigning to renin, more accurately to angiotensin, merely an additional stimulating influence on the sympathetic system. PRITCHARD et al. (1964) attributed the increase in the tone of the vascular smooth musculature to a neuropressor mechanism elicited by the humoral factor. The results by GUYTON and COLEMAN (1969), FUNDER et al. (1970), MCDONALD et al. (1970) are at variance with intermediate theories outlined above, the authors having been able to induce a Goldblatt type hypertension in rabbits immunized against angiotensin-II, an observation which seems to disprove the existence of any relationship between blood pressure and the renin activity in renal venous blood, thus connecting the maintenance of permanent hypertension with extrarenal factors instead of a renal mechanism.

The current theories attribute the release of renin, besides the well-established intrarenal mechanisms, primarily to sympathetic innervation

(GENEST 1971), as confirmed by ultramicroscopic studies of the JGS (BARAJAS 1964) as well as by histochemical immunofluorescence studies (WAGERMARK et al. 1968, GOMBA et al. 1969), furthermore by experimental evidence showing that denervation of the kidney had a depressive effect on renin secretion (TAQUINI et al. 1964, HOLLANDER et al. 1964, TOBIAN et al. 1965, UEDA et al. 1967) and that ganglion (BUNAG et al. 1966) and alpha receptor blocking agents (FORGÁCS et al. 1972, BIRBARI 1971) inhibit the release of renin, whereas sympathetic stimulation or infusion of catecholamine are enhancing its secretion (VANDER 1965, GORDON et al. 1967, NIIJIMA 1971, RIEU et al. 1971). The role of the renal nerves, as far as the JGA is concerned, may well be that of a signalling mechanism rendering the renal vasculature abnormally sensitive to changes in pressure and volume (TOBIAN 1967).

The aim of the present study has been to obtain convincing proof of the adrenergic control of renin secretion in experimental hypertension. Under these aspects the ratio renal vein renin to peripheral arterial renin of the denervated kidney was compared with the same values for the unoperated, innervated kidneys of hypertensive dogs (FEKETE et al. 1971). No increased secretion of pressor agents by the unoperated kidney subjected to splanchnicotomy simultaneously with the production of ischaemia of the other kidney was demonstrable, whereas in the case of a preserved sympathetic nerve supply its pressor activity was significantly increased. The effect of denervation may be either a direct mechanism resulting from the cessation of the direct efferent effect on the JGA, or an indirect mechanism owing to an interruption of the connection between the sympathetic pathways and the afferent arteriole, the dilatation of which is unfavourable to the release of pressor agents (TOBIAN 1964). It is moreover possible that the denervated tissue fails to store catecholamines (HOLLANDER et al. 1964) or to release renin.

These interpretations are, however, not valid in the advanced stages of hypertension. A follow-up over longer periods revealed that the hypertension remains unaffected after removal of the ischaemic kidney and the blood samples obtained at regular intervals showed a gradual rise in RAR as compared with the normotensive control values as well as with the low figures exhibited by the same animal during the early stage of hypertension. The RAR and the weight of the denervated kidneys no longer differ from those of innervated kidneys.

According to GRIMSON (1949), regeneration of nerve fibres is a fairly rapid process. Therefore, a second splanchnicotomy may again induce a hypotensive response. The surgical dividing of the major splanchnic nerve, though being a generally adopted procedure, is certainly inadequate for radical denervation. In the case under study, hyperactivity of the residual fibres may have reactivated renin secretion which then resulted in a further constriction of

the vessels and a further release of renin. Moreover, in view of the excessive reactivity of denervated tissue, the loss of nerve supply may well lead to an undue responsiveness to catecholamines of the JGA and of the vascular structures.

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DIE KREATININAUSSCHEIDUNG BEI FRAUEN WÄHREND DER FASTENKUR

Von

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FORSCHUNGSSTELLE FÜR MEDIZINISCHE ERNÄHRUNGSLEHRE AN DER HAUTKLINIK DER ERNST-MORITZ-ARNDT-UNIVERSITÄT GREIFSWALD (D.D.R.)

(Eingegangen am 5. Juli, 1972)

Urinary creatinine excretion has been studied in 24 fasting adipose female patients. The 24-hour excretion of creatinine as well as the creatinine coefficient decreased during fasting. The results are interpreted as due to an influence of food on creatinine excretion. If urinary creatinine is used as a standard parameter of other excreted metabolic substances, the changes caused by fasting should be taken into consideration.

Einleitung

In der Diskussion über die Variabilität der Kreatininausscheidung nimmt die Frage der Beeinflussung durch die Nahrungszufuhr größeren Raum ein. Neuere Arbeiten (Übersicht bei [13]) stellen das klassische Konzept von der Konstanz der Kreatininausscheidung [8, 18, 22, 23] in Frage, wobei dieses Problem auch das Interesse des Ernährungswissenschaftlers findet [33]. Die unveränderte Kreatininausscheidung während einer Fastenkur dient als Kontrollparameter, inwieweit während des Fastens nur Fettgewebe unter Schonung des Proteinbestandes des Körpers eingeschmolzen wird [24]. ALLEYNE et al. [2] benutzen einen Kreatinin-Körperhöhe-Index (c. h. i.), um bei unterernährten Kindern das Defizit an Muskelmasse einfach und schnell charakterisieren zu können. Ein für die Praxis brauchbares Nomogramm wurde von MENDEZ und BUSKIRK [26] publiziert. Wir haben bei der Bearbeitung klinikseigener Normalwerte für die Kreatininausscheidung bei Kindern und Erwachsenen [30] dem Problem der individuellen Variabilität dieser Ausscheidungsgröße besondere Aufmerksamkeit gewidmet undzur Brauchbarkeit des Kreatinins als Standardisierungsgröße Stellung genommen [31].

Über die Kreatininausscheidung bei Fettsüchtigen findet man keineswegs einheitliche Angaben (Übersicht bei [38]). In der vorliegenden Arbeit soll über die Kreatininausscheidung bei weiblichen adipösen Patienten während einer Fastenkur berichtet werden, wobei wir auftretende Veränderungen im Zusammenhang mit dem Nahrungsentzug betrachten wollen.

Methodik und Untersuchungsgut

Wir bestimmten die Kreatininausscheidung im 24-Stunden-Urin nach der Methode des DAB 7 [14] bei obesen Patienten im Verlaufe eines mehrwöchigen Saftfastens. Zur Aus-

Tabelle I

Übersicht über die Patientengruppe

Nr.	Name	Alter	*n*	Dauer des Fastens	Aufnahme-gewicht kg	Broca-Index	Entlassungs-gewicht kg	Gewichts-abnahme kg	Gewichts-abnahme pro Tag kg/Tag
1	E. V.	27	2	25	87,5	1,37	78,5	9,0	0,36
2	E. A.	65	3	32	111,0	1,68	104,2	6,8	0,21
3	E. B.	47	3	32	98,5	1,76	84,9	13,6	0,43
4	R. F.	49	3	32	101,3	1,51	89,9	11,4	0,36
5	E. G.	39	3	35	91,4	1,83	82,2	9,2	0,26
6	M. S.	33	3	32	83,8	1,44	74,5	9,3	0,29
7	B. P.	56	2	26	86,5	1,29	81,0	5,5	0,21
8	K. R.	18	3	36	86,0	1,30	75,2	9,8	0,27
9	I. L.	42	3	22	90,1	1,24	83,2	6,9	0,31
10	E. M.	26	3	30	83,8	1,21	73,6	10,2	0,34
11	R. E.	17	3	31	75,6	1,24	66,0	9,6	0,31
12	S. H.	19	3	29	74,0	1,21	67,2	6,8	0,23
13	I. P.	39	2	30	84,1	1,40	78,8	5,3	0,18
14	H. K.	41	3	27	85,6	1,36	78,0	7,6	0,28
15	G. H.	44	3	27	91,8	1,35	86,0	5,8	0,22
16	B. T.	38	2	20	110,0	1,41	101,5	8,5	0,43
17	K. Z.	42	3	32	108,5	1,62	97,6	10,9	0,34
18	T. P.	49	3	32	84,4	1,36	75,9	8,5	0,27
19	G. B.	48	3	29	91,0	1,36	82,5	8,5	0,29
20	E. B.	44	2	25	80,5	1,46	74,4	6,1	0,24
21	F. E.	46	3	32	84,0	1,50	78,9	5,1	0,16
22	C. M.	32	2	19	77,2	1,15	70,8	6,4	0,34
23	O. M.	35	3	22	68,2	1,12	65,0	3,2	0,15
24	A. S.	55	3	51	101,0	1,74	91,8	9,2	0,18

wertung gelangten 24 weibliche Patienten, die im Jahre 1970 stationär behandelt wurden. Tabelle I gibt eine Übersicht über die Patientengruppe. Es handelt sich um Patienten mit essentieller Fettsucht [38]. Metabolische Störungen sowie andere Krankheiten konnten durch ein umfangreiches Routine-Untersuchungsprogramm ausgeschlossen werden. Das Alter der Stichprobe betrug 40 Jahre (Zentralwert 41,5 J.). Aufnahme- und Entlassungsgewicht sowie Gewichtsabnahme sind der Tabelle I zu entnehmen. Durchschnittlich wurden Gewichtsabnahmen von 8,1 $\pm$ 2,1 kg (VK = 26%) erzielt, wobei unsere täglichen Durchschnittswerte von 0,28 $\pm$ 0,08 kg (VK = 29%) unter den bei Null-Diät zu erreichenden täglichen Gewichtsabnahmen lagen [15, 19]. Wir führten die Kreatininbestimmung 1—2 Tage vor Fastenbeginn, in der Fastenmitte (10.—16. Tag) und am Fastenende (19.—30. Tag) durch. Als Vergleichsgruppe wurden 14 Frauen aus vorangehenden Untersuchungen ausgewählt, deren einfacher Broca-Index nicht über 1,10 lag.

Die statistischen Maßzahlen berechneten wir nach dem bei RICHTERICH [36] angegebenen Verfahren. Das Rechenzentrum der Ernst-Moritz-Arndt-Universität Greifswald hatte die Korrelations- und Regressionsanalysen übernommen.*

* Herrn Dipl.-Math. H. Poser sei für die Ausführung der Berechnungen an dieser Stelle gedankt.

Ergebnisse

In Tabelle II sind die in unseren Untersuchungen ermittelten Werte für die Kreatininausscheidung angegeben. Trotz höherer Alterszusammensetzung der Adipösen betrug die als Normalwert anzusehende Kreatininausscheidung bei stationärer Aufnahme 1,30 ± 0,24 g und liegt damit signifikant über den Werten der Vergleichsgruppe. Die größere Varianz der Normalgruppe erweist

Tabelle II

Kreatininausscheidung und -koeffizient bei adipösen Frauen gegenüber Normalpersonen

		Kreatininausscheidung mg/24 Std.	Kreatininkoeffizient mg/kg	Körpergewicht kg
Vergleichsgruppe n = 14	$\bar{x} \pm s$	1033±393	18,9±6,1	54,4±8,4
	$s_{\bar{x}}$	105	1,6	2,2
	VK	38%	32%	15%
Fastenpatienten n = 23	$\bar{x} \pm s$	1302±241	14,8±3,4	88,9±11,3
	$s_{\bar{x}}$	50	0,7	2,3
	VK	19%	23%	13%
Irrtumswahrscheinlichkeit		$P \leq 2\%$	$P \leq 2\%$	$P \leq 0{,}1\%$

sich im Streuungsvergleich (F-Test) als signifikant (P = 5%). Der Kreatininkoeffizient der Fettsüchtigen ist mit 14,8 ± 3,2 mg/kg signifikant vermindert.

Während der Fastenkur (Saftfasten) sinkt die Kreatininausscheidung ab (P = 1%, Abb. 1). Der Korrelationskoffizient beträgt $r = -0{,}387$, wobei die dazugehörige Regressionsgerade durch die Funktion

$$y = 1321 - 11{,}2\,x$$

bechrieben wird.

Das abweichende Verhalten einiger Probanden kann durch Urinverluste bedingt sein. Da der Sammelfehler im Gesamtmaterial zufallsmäßig verteilt ist, wurden keine Einzelwerte eliminiert.

Der Kreatininkoeffizient (Abb. 2) fällt in gleichsinniger Weise wie die Gesamtausscheidung ab. Die berechneten statistischen Prüfgrößen überschreiten hier jedoch nicht das geforderte Signifikanzniveau für ein P = 5%.

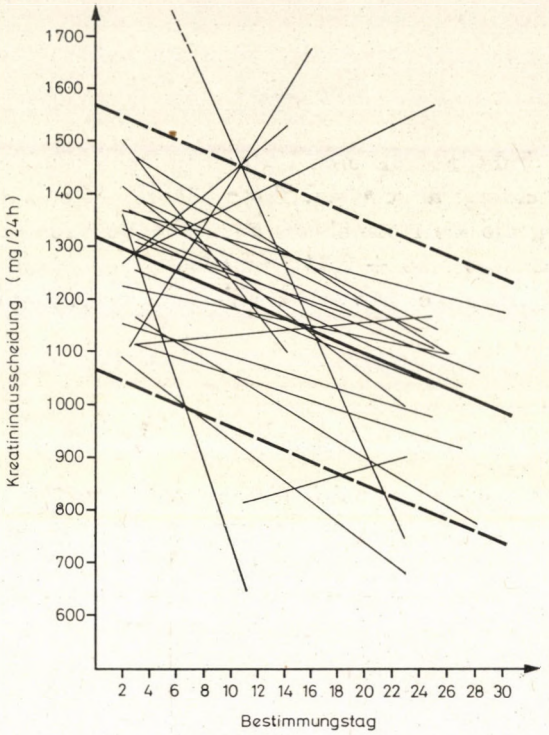

Abb. 1. Kreatininausscheidung (mg/24-Std.) bei obesen Frauen während einer Fastenkur $y = 1321 - 11{,}2x$; $s = \pm 250$ ($r = -0{,}387$)

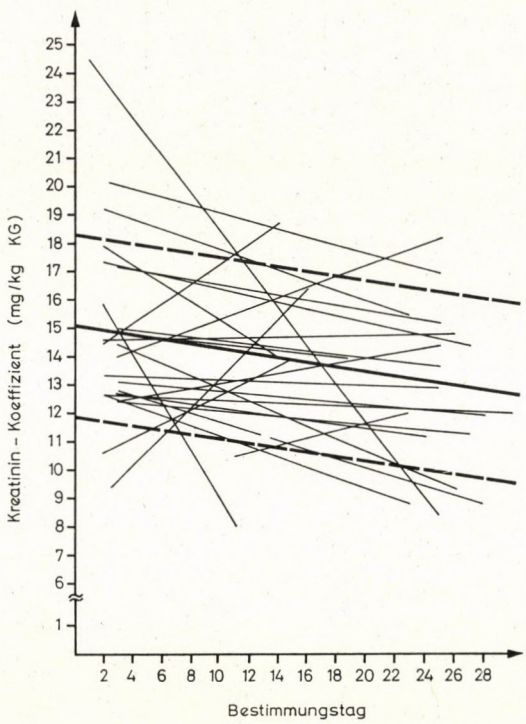

Abb. 2. Kreatininkoeffizient (mg/kg Körp.-Gew.) bei obesen Frauen während der Fastenkur $y = 15{,}1 - 0{,}08x$; $s = \pm 3{,}2$ ($r = -0{,}235$)

Diskussion

Die Höhe der Kreatininausscheidung wird durch den Körperbestand an Muskelmasse bestimmt. Isotopenversuche an Ratten und beim Menschen ergaben, daß täglich etwa 1,6%—2% des zu 95% in der Muskulatur lokalisierten Kreatinbestandes des Organismus als Kreatinin renal eliminiert werden [6, 21]. Untersuchungen über die normale Kreatininausscheidung bei Kindern und Erwachsenen widerspiegeln deren altersparallelen Verlauf zur Entwicklung und Rückbildung der Muskelmasse [1, 12, 30, 39, 41]. Mit 12 Jahren tritt ein signifikanter Geschlechtsunterschied auf [12]. Bei Adipösen ist die Kreatininausscheidung im 24-Std.-Harn vermehrt. Andererseits ist die auf das Körpergewicht bezogene Ausscheidungsmenge (Kreatininkoeffizient) gegenüber Normalgewichtigen deutlich vermindert (Tab II). Aufgrund des höheren Körperfettanteils ist der niedrigere Kreatininkoeffizient der Adipösen erklärbar [8a, 18, 37].

Die Untersuchung der Kreatininausscheidung während des Fastens erfolgte vor allem deshalb, um Aufschlüsse über nahrungsbedingte Veränderungen dieser Ausscheidungsgröße zu erhalten. In einem vorangehenden Vergleich fanden wir bei 11 Kindern unter 24-stündiger Nahrungskarenz keine unterschiedlichen Werte zu einer Vergleichsgruppe [30]. In der Literatur wird der Einfluß der Nahrung auf die Kreatininausscheidung nicht einheitlich beurteilt. Neuere Arbeiten machen im Hinblick auf die erhebliche individuelle

Tabelle III

Regressionsgleichung ($y = a + bx$), Korrelationskoeffizient (r) sowie Prüfgröße (t) und Streuung der y-Werte (s) bei den untersuchten Beziehungen (y zu x)

y	x	$y = a + bx$	r	t	$s = \pm$
Kreatininausscheidung mg/24 Std.	Bestimmungstag	$y = 1321 - 11{,}2\ x$	−0,387	−3,36**	249,6
Kreatininkoeffizient mg/kg Körp. Gew.	Bestimmungstag	$y = 15{,}1 - 0{,}08\ x$	−0,235	−1,94	3,2
Körpergewicht kg	Bestimmungstag	$y = 88{,}9 - 0{,}33\ x$	−0,273	−2,27*	10,8

($n - 2 = 64$, * $p \leq 5\%$, ** $p \leq 1\%$)

Variabilität der Kreatininausscheidung auf den Faktor Nahrungszufuhr aufmerksam [4, 5, 10, 25, 32, 35]. Während der Fastenkur sinkt die Kreatininausscheidung bei unseren Patienten täglich um etwa 11 mg. Der Kreatininkoeffizient bleibt ebenfalls nicht konstant, sondern fällt im Gesamtmaterial durchschnittlich um 2 mg/kg ab. JOHNSON et al. [24] sahen unter 10tägiger

Diättherapie (500 kcal, Eiweiß- und Kohlenhydratkost) bei Männern keine Veränderungen. Daß unter vollständiger Nahrungskarenz die Kreatininausscheidung im Urin abfällt, ist bereits früher publiziert worden [22, 23, 27, 28]. BLEILER u. SCHEDL [5] untersuchten über 2—3 Wochen unter standardisierten Bedingungen nahrungsabhängige Veränderungen der Kreatininwerte in Blut und Urin. Wurde Casein als Nahrungseiweiß verabfolgt, fielen sowohl das Serumkreatinin als auch die Urinausscheidung bei konstanter Kreatininclearance ab. Auf unveränderte Clearancewerte während des Fastens wies auch CHEIFETZ [11] hin. Ein Einfluß der Nahrungszufuhr auf das Serumkreatinin wird allgemein negiert [4, 16, 17, 20, 34, 40], obwohl die Befunde von CAMARA et al. [9] und BLEILER und SCHEDL [5] eine Bestätigung sind. Der diurnale Rhythmus von Serumkreatininkonzentration und Harnausscheidung wird zwar durch eine Nahrungskarenz modifiziert, aber nicht aufgehoben [29]. BROWN et al. [7] berichten über einen ansteigenden Hydroxyprolin-Kreatinin-Index im Urin bei fastenden adipösen Patienten, ohne auf kreatininbedingte Änderungen dieses Quotienten einzugehen. KORTE et al. [25] machen darauf aufmerksam, daß bei unterernährten Kindern durch die herabgesetzte Kreatininausscheidung höhere Indexwerte erhalten werden, wenn die Ausscheidung anderer Stoffwechselmetaboliten auf die Kreatininmenge bezogen wird. Stoffwechseluntersuchungen bei adipösen Kindern zeigen, daß diese Kinder neben anderen Stoffwechselparametern weniger Kreatinin ausscheiden [3]. Während bei Normalkindern unter Eiweißbelastung eine Erhöhung der Kreatininausscheidung um 22,5% eintrat, war bei den Adipösen als Ausdruck der größeren N-Retention lediglich ein Anstieg um 12,9% zu beobachten. Im Hinblick auf unsere Fragestellung entnehmen wir diesen Ergebnissen noch, daß bereits kurzfristige Änderungen der Nahrungszusammensetzung die 24-Std.-Menge des Kreatinins beeinflussen. Unsere Ergebnisse bestätigen ebenfalls, daß die Kreatininausscheidung bei längerer Nahrungskarenz kontinuierlich abfällt. Wir werten diese Verminderung im Sinne eines nahrungsbedingten Einflusses und nicht eindeutig als Indikator einer abnehmenden Muskelmasse unter der Fastenkur. Um Aufschlüsse über Veränderungen des Proteinbestandes des Organismus während des Fastens zu erhalten, sind umfangreichere Untersuchungen der N-Bilanz erforderlich.

Zusammenfassung

Es wird über das Verhalten der Kreatininausscheidung im Urin bei 24 adipösen Frauen während einer Fastenkur berichtet. Sowohl die 24-Stunden-Ausscheidung als auch der Kreatininkoeffizient fielen im Verlauf der Fastenkur ab. Die Ergebnisse werden als Ausdruck des Nahrungseinflusses auf die

Kreatininausscheidung gedeutet. Soll das Harnkreatinin als Standardisierungsgröße anderer Stoffwechselparameter herangezogen werden, sind die durch den Nahrungsentzug bedingten Veränderungen zu berücksichtigen.

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РЕЗЮМЕ

О СУБКЛИНИЧЕСКОМ ИНФАРКТЕ СЕРДЦА

И. КЕНЕДИ, Ю. ДЕВАИ и Ф. ГОНДА

У 36 больных, в анамнезе которых не было данных об остром приступе инфаркта, было обнаружено состояние после инфаркта сердца по. **Minnesota Code.**

На ЭКГ картину постинфарктного рубца без острого, длительного болевого приступа могут вызвать четыре различных патомеханизма: 1. истинный, немой, безболевой инфаркт, 2. преходящая, однократная сердечная боль, подражающая грудную жабу, 3. острые симптомы со стороны сердца (недостаточность кровообращения или ритм галопа) без болей, 4. симптомы другого заболевания маскируют признаки инфаркта сердца.

У больных старше 55 лет симтомокомплекс, названный подклиническим инфарктом, был выявлен чаще, причем при инфаркте задней стенки чаще, чем при инфаркте передней стенки.

СОСТАВ ФОСФОРЛИПИДОВ В КРОВИ ПРИ РАССЕЧЕНОМ СКЛЕРОЗЕ

И. ХУСАК, А. РИМАНОЦИ, Л. ЛАЦКОВИЧ и Л. ЛЕХОТАИ

Уровень и состав фосфорлипидов в плазме и в форманных элементах крови больных рассеянным склерозом и контрольных лиц былы определены при помощи метода хроматографии на тонком слое и на столбе.

Ни общее, ни частное содержание фосфорлипидов не показало никаких достоверных отклонений. Однако следует отметить, что уровень частных фосфолипидов в плазме и в кровяных клетках колебается как у контрольных лиц, так и у больных рассеянных склерозом, причем колебания, быть может, более значительны у больных рассеянным склерозом. Это наблюдение может быть объяснением того факта, что некоторые авторы в связи с содержанием фосфорлипидов в крови больных рассеянным склерозом публиковали противоречивые данные.

СЫВОРОТОЧНЫЕ ИММУНОГЛОБУЛИНЫ У ДЕТЕЙ С БИЛГАРЦИОЗОМ

М. Х. СОЛИМАН, А. ЭЛ-ВАСЕЕФ, А. А. КОМБОР и К. М. ЭЛ-КАШЛАН

1. Исследования были проведены у 23 детей, зараженных S. **haematobium** и 15 детей, зараженных S. **mansoni** и у 5 контрольных детей.

2. В обоих типах шизостомоза было выявлено достоверное понижение концентрации альбумина в крови, вызванное либо фактором питания (при заражении **S. haematobium**), либо снижением синтеза альбумина вследствие поражения печени (при шитозомозе Мансона).

3. Гипо-альбуминемия была сопровождена достоверным повышением содержания γ-глобулинов, вызванного значительным повышением только фракции **IgG**. Как содержание общих γ-глобулинов, так и компонента **IgG** оказались гораздо более повышенными у больных кишечным шистозомозом, осложненной гепатоспленомегалией, чем при шистозомозе мочевых органов. Это явление приписывается более значительной активности ретикулоэндоталиальных клеток при инфекциях S. mansoni.

4. Рекомендается обратить внимание на факторы питания, так как они могут быть отчасти ответственными за некоторое снижение концентрации альбуминов и за компенсаторное повышение содержани γ-глобулинов в крови.

5. Следует отметить, что увеличение фракции было обнаружено шесть месяцев после инфекции билгарциозом.

ОПРЕДЕЛЕНИЕ ИММУНОРЕАКТИВНОГО ИНСУЛИНА В СЫВОРОТКЕ И В ГЕПАРИНОВОЙ И ЭДТА-ПЛАЗМЕ

И. БАЛАЖИ и М. ШТЮЦЕЛЬ

В сыворотке, в гепариновой и ЭДТА-плазмах, полученных из тождественных образцов человеческой крови, авторами были проведены параллельные определения иммунореактивного инсулина. Было установлено, что добавление гепарина к крови в целях получения плазмы (в концентрации 12,5 и 50 МЕ на мл крови) или ЭДТА (в концентрации 5 мг на мл крови) не вызывает достоверного изменения количества иммунореактивного инсулина, определенного в этих плазмах, по сравнению с величинами иммунореактивного инсулина в тождественных сыворотках. Следовательно плазмы, полученные после добавления гепарина или ЭДТА в указанных концентрациях, и сыворотки равным образом пригодны для определения иммунореактивного инсулина. Ввиду того, что между величинами иммунореактивного инсулина в сыворотке и в плазме были получены недостоверные различия, рекомендуется при проведении экспериментальных серий подготовлять образцы крови для определения в одинаковых условиях.

В случае применения гепарина, растворенного в буферном растворе в концентрации, не применямой для получения плазмы (500 МЕ на мл), были получены более высокие величины иммунореактивного инсулина. При определении в человеческой плазме в присутствии ЭДТА в более высокой концентрации (25 мг на мл крови), величины иммунореактивного инсулина оказались достоверно более низкими. С другой стороны прибавление ЭДТА не влияло на рекуперацию инсулина, растворенного в буферном растворе, содержащем альбумин.

На основе этих результатов авторы считают вероятным, что гепарин вызывает сни жение реакции инсулина с противотелами, связывающими инсулин, что происходит и независимо от наличия плазмы, в то время как действие ЭДТА, повышающее реакцию между инсулином и связывающим инсулин антителами, зависит от какого-то фактора, присутствующего в плазме.

ДЕЙСТВИЕ КЛОФИБРАТА НА АОРТАЛЬНЫЕ ГЛЮКОЗАМИНОГЛИКАНЫ, БЕЛКИ И НА УРОВНЬ СЫВОРОТОЧНЫХ ЛИПИДОВ ПРИ ЭКСПЕРИМЕНТАЛЬНОМ АТЕРОСКЛЕРОЗЕ

М. БИХАРИ-ВАРГА, Я. ФЕХЕР, М. ВАРШАНЬИ и Ш. ГЕРЕ

Авторами было изучено действие клофибрата на кроликов, получавших холестерол.

1. Математически очень достоверное уменьшение уровня сывороточных липидов было установлено уже спустя две недели после введения клофибрата.

2. В аортах кроликов, кормленных холестеролом, при гистологическом, гистохимическом и электронномикроскопическом исследованиях было выявлено незначительное повышение концентрации глюкозаминогликана и интенсивное отложение липивод. При помощи термального анализа в интиме животных удалось доказать наличие комплексов аортальных глюкозаминогликанов — сывороточных бета-липопротеинов и аккумуляцию «патологических» перекрестных соединений в волокнистых тканевых протеинах.

3. В результате введения клофибрата в аортах кроликов с экспериментальным атеросклерозом было найдено достоверное повышение содержания глюкозаминогликана. В то же тремя было установлено уменьшение количества аортальных глюкозаминогликан-бета-липопротеиновых комплексов. Противо-атерогенная активность клофибрата влияла также на патологически измененную протеиновую структуру: вызванные кормлением холестерином изменения отчасти оказались обратимыми.

ТАК НАЗ. "WASTING DISEASE" И ТЕТАНИЯ ПОСЛЕ ПОСЛЕРОДОВОГО УДАЛЕНИЯ ШИШКОВИДНОГО ТЕЛА

Д. ЧАБА, И. РАДОШ, Э. ВОЛМУТ

Авторы исследовали после удаления шишковидного тела у цыплят (Gallus domesticus) развитие, физиологические параметры и гистологическую картину щитовидной, околощитовидных желез, ультимобранхиального тела и Фабрициевой сумки. Они установили,

что эпифизектомированные цыплята отстают в развитии, они теряют перья, что объясняется болезнью «wasing disease», возникающей в результате атрофии зобной железы и Фабрициевой сумки. У 50% цыплят наступила тетания и 35% погибли от нее. При этом околощитовидные железы были интактными. Авторы рассматривают возможный механизм того заболевания и предполагают существование пинеального регулирования эндокринных желез энтодермального происхождения.

ЦЕРЕБРАЛЬНЫЕ ГЕМОДИНАМИЧЕСКИЕ ПРОЦЕССЫ И МЕТАБОЛИЗМ ПРИ ИШЕМИЧЕСКИХ ПОРАЖЕНИЯХ КРОВООБРАЩЕНИЯ ГОЛОВНОГО МОЗГА

Д. ХАДЖИЕВ, Б. ЙОРДАНОВ, ШТ. ЯНЧЕВА и Б. БАЙКУШЕВ

Определенные параметры церебральных гемодинамических процессов (кровообращение, сосудистое сопротивление, перфузионное давление гоовтного мозга) и церебральный метаболизм (потребление кислорода, поглощение глюкозы, образование двуокиси углерода, дыхательный коэффициент, соотношение лгюкоза: кислород) были изучены у 30 больных с ишемическим поражениями церебрального кровообращения. Величина церебрального кровотока была определена методом венной дилюции изотопа, а дыхательные газы — при помощи микрогазометрической техники. В полушарии, пораженном патологическим процессом, было выявлено понижение кровотока (268,76 мл/мин), потребления кислорода (35,10 мл/мин), поглощения глюкозы (28,90 мг/мин), образования двуокиси кислорода (18,72 мл/мин), дыхательного коэффициента (0,58) и соотношения глюкоза: кислород (0,89). Церебральное перфузионное давление оказалось повышенным (106,33 мм рт. ст). Были анализированы корреляции между параметрами церебральной гемодинамики и церебральным метаболизмом. Корреляционные коэффициенты были сопоставлены с величинами, исчисленными на основе данных, полученных методом ^{131}У-иоантипирина О. М. Рейнмута и сотрудников у здоровых лиц и у больных аналогичной группы. У больных были выявлены расстройства в гемодинамико-метаболических корреляциях, за исключением тесной корреляции между церебральным кровотоком и кислородным потреблением мозга. При церебральной ишемии возникают новые гемодинамико-метаболические взаимоотношения, объясняющие некоторые патогенетические механизмы, и таким образом способствующие более рациональному терапевтическому воздействию на поражение церебрального кровообращения.

ИЗМЕНЕНИЯ, НАСТУПАЮЩИЕ ВСЛЕД ЗА ХРОНИЧЕСКОЙ ЗАТРАВКОЙ КРЫС ПРЕПАРАТОМ САЦЕРНО

Д. СЕГЕДИ и Г. КПАСНАИ

Авторы проводили после хронической дачи крысам препарата сацерно иммуносерологические и гистологические исследования. Параллельно с увеличением продолжительности воздействия повышался процент иммуносерологических изменений: появления антинуклеопротеиновых антител, анти-гаммаглобулина. Параллельно с этим наблюдались гиперпротеинамия и гипергаммаглобулинемия. В лимфатических узлах крыс, длительно получавших препарат, наблюдались различные гистологические отклонения. Из них наибольшее внимание заслуживает плазмаклеточная плеоморфно-клеточная опухолевидная пролиферация. Возникновения истинной опухоли авторы не наблюдали. Авторы рассматривают механизм свойства гидантионовых препаратов провоцировать аутоиммунные заболевания. По мнению авторов, морфологические изменения, возникающие в лимфатических узлах под прямым воздействием медикамента, представляют основу различных иммуносерологических изменений.

МОДЕЛЬНЫЙ ОПЫТ ДЛЯ ИЗУЧЕНИЯ КЛИНИКОПАТОЛОГИИ МИОКАРДИТА

П. ШВАРЦМАН, Й. ДЕМЕТЕР, Е. МАДЯР

Авторы прибавили к обычной диете крыс этиллауреат. При этой диете в миокарде возникали такие диффузные изменения, которые соответствуют критериям миокардита. Авторы исследовали параллельность между тяжестью гистологической картины и изменениями ЭЭГ. Они сообщают о выводах, сделанных на основании 242 электрокардиографических исследований.

ВЫЗВАННАЯ ОБЛУЧЕНИЕМ УЛЬТРАЗВУКОМ МЕСТНАЯ РЕАКЦИЯ РЕАКЦИЯ ШВАРЦМАНА

Ш. ТОТ, Т. СИЛАДИ, Г. КАРОИ, Г. ЛЭВАИ

В коже, предварительно подготовленной внутри кожным введением эндотоксина кишечной палочки 0111, местным облучением ультразвуком (2 ватт/см²) на протяжении 10 минут мы вызвали на кроликах тромбогеморрагическое, некротическое поражение кожи. Кинетика возникновения реакции, а также макроскопическая и микроскопическая ее формы проявления, по сути дела, были тождественными с таковыми местной реакции Шварцмана, вызываемой внутрикожным и внутривенным введением эндотоксина.

Провоцирующий местный феномен Шварцмана эффект облучения объясняется комплексным действием ультразвука, в котором роль играют поражение местной ткани и лейкоцитов, микроциркуляторные изменения, а также — вероятно — активация факторов свертывания крови.

ЗАЩИТНОЕ ДЕЙСТВИЕ ОБРАЩЕННОГО ПРОТИВ МЫШИНОГО КОЖНОГО ЭПИТЕЛИЯ КРОЛИЧЬЕГО IgG НА КОЖНЫЙ ГРАФТ МЫШЕЙ

Б. ФЕКЕТЕ, Д. СЕГЕДИ, Г. ГАСТОНИ, Г. САБО, Д. ПЕТРАНИ

Авторы констатируют, что кроличьи IgG, обращенный против Бальб/с мышиного эпителия кожи увеличивает время выживания Баль/с кожного графта в мышах СВА, В действии IgG обращенного против эпителия кожи и такового, обращенного против типоцитов, много сходных черт. В защищающем кожный графт действии обращенного против эпителия кожи кроличьего IgG авторы предполагают наличие трех связанных между собой факторов:

1. антимоцитарное действие,
2. защитное, покрывающее поверхность действие и
3. проявляющуюся на макрофагах супрессию антител.

ИССЛЕДОВАНИЕ ЧУВСТВИТЕЛЬНОСТИ В ОТНОШЕНИИ ЦИТОСТАТИЧЕСКИХ ПРЕПАРАТОВ IN BITRO ПРИ ХРОНИЧЕСКОЙ МИЕЛОИДНОЙ ЛЕЙКЕМИИ

П. ГЕРГЕЙ, Г. САБО, Д. СЕГЕДИ, Б. ФЕКЕТЕ, Д. ПЕТРАНИ

Авторы исследовали при хронической миелоидной лейкемии чувствительность культуры периферических лейкоцитов в отношении цитостатических препаратов. Из 8 больных, у которых проводилось исследование, в 7-ми случаях медикамент, который на основании теста in vitro оказался эффективным, вызавал клиническую ремиссию.

СВЯЗЬ МЕЖДУ ГИСТОЛОГИЧЕСКОЙ КАРТИНОЙ СЛИЗИСТОЙ ЖЕЛУДКА И ЕЕ СЕКРЕТОРНОЙ АКТИВНОСТЬЮ У ЧЕЛОВЕКА

В. ВАРРО, Г. КАРАЧОНЬ и Э. ПАХ

Авторы проводили на неподобранцом больничнном материале клинического отделения сравнение гистологических данных слизистой дна желудка, полученной путем слепой биопсии, и данных секреции железами соляной кислоты.

Было установлено, что у лиц с нормальной слизистой, после дачи гистамина, образование соляной кислоты достоверно превышает секрецию у больных гастритом. У последних по мере тяжести гастрита уменьшается количество соляной кислоты:

Выявлено также, что толщина активной паренхимы более верно отражает степень морфологического и функционального поражения слизистой, чем величины толщины всей слизистой.

Число клеток, образующих активную паренхиму, снижается параллельно степени выраженности гастритического поражения.

Авторы считают интересным, что среди железистых клеток главные клетки, по-видимому, более чувствительны в отношении прогрессирующих гастритических процессов.

КЛИНИЧЕСКИЕ И ГИСТОПАТОЛОГИЧЕСКИЕ ИССЛЕДОВАНИЯ ПРИ ТЕРАПЕВТИЧЕСКИХ ПОЧЕЧНЫХ БОЛЕЗНЯХ

И. ВАРГА, Э. БЕРЕГИ, Б. КЕНЕЗ

На основании изучения клинических и гистопатологических данных 41-го взрослого больного и по литературным данным мы деламем выводы относительно диагностической ценности гематурии и клинического значения очагового нефрита:

1. Повторно возникающая в связи с катарром верхних дыхательных путей, с физической нагрузкой гематурия или постоянная микроскопическая гематурия является во взрослом возрасте очень редким симптомом очагового нефрита и в большинстве случаев этот симптом означает диффузный гломерулонефрит. Синдром гломерулонефрита, проявляющегося с очаговой структурой не является единым и поэтому его клиническое распознавание невозможное. Очаговый нефрит — это гистологический диагноз.

2. Очаговый нефрит — как и диффузный гломерулонефрит — является сопровождающейся с иммунопатологическим процессом, воспалительной клубочковой реакцией с очаговой структурой. В клубочковых петлях отложение иммуноглобулинов более распространенное, чем этого следовало бы ожидать на основании очаговой воспалительной реакции. Роль стрептококковой инфекции в патогенезе не доказана; в отдельных случаях нефритогенной вредностью может быть вирусная инфекция или другая бактериальная инфекция.

3. Очаговый нефрит является типом гломерулонефрита со сравнительно доброкачественным течением, но он не может рассматриваться невинным явлением. У взрослых это обычно хронически протекающая болезнь, обострения которой могут, однако, сопровождаться ухудшением почечной функции вплоть до уремии. Вероятно отдельные случаи представзяют начазьнуи фазу диффузного гломерулонефрита, хотя в настояще время это предположение еще не доказано. В терапии, наряду с защитой от инфекции и с избежаним нагрузки можно применять также и иммуносупрессию.

О ПАТОГЕНЕЗЕ ПЕРНИЦИОЗНОЙ АНЕМИИ

М. ХОРАНИ, Э. Х. ШАРФИ

Для идентификации вещества, встречающегося в дуоденальном соке, описанного авторами в своем предыдущем сообщении, во многих отношениях похожего на внутренний (intrinsic) фактор, авторы проводили иммунобиологическое исследование. Они выявили, что прибавление сыворотки больного пернициозной анемией ингибирует действие этого дуоденального фактора за счет содержащегося в этой сыворотке протвотела против внутреннего фактора. Это наблюдение также подтверждает старое предположение авторов, что дуоденальный фактор действительно во многом похожий на внутренний фактор. Исследования предоставили новые данные к патогенезу пернициозной анемии.

ИССЛЕДОВАНИЕ ОТДЕЛЬНЫХ ПАРАМЕТРОВ ПОСТИШЕМИЧЕСКОЙ ПОЧКИ В ХРОНИЧЕСКОМ ОПЫТЕ

И. ТАРАБА, А. ФЕКЕТЕ, Л. МОЛНАР

Авторы исследовали в хронических опытах на собаках изменение выделения креатинина и ПАГК после 2-часовой почечной ишемии. У тех животных, у которых ишемизация проводилась под наркозом, вышеуказанные величины улучшались, в то время как у бодрствующих животных они ухудшались. Разницу авторы объясняют внутрипочечным распределением кровообращения.

ИСПОЛЬЗОВАНИЕ ТОРМОЖЕНИЯ ОБРАЗОБАНИЯ ИММУНОСПЕЦИЛИЧЕСКОЙ РОЗЕТКИ ДЛЯ СРАВНИТЕЛЬНОГО ИССЛЕДОВАНИЯ ДВУХ ПРОИЗВОДНЫХ ПРЕДНИЗОЛОНА

Б. ФЕКЕТЕ, Д. СЕГЕДИ, П. ГЕРГЕЙ, Г. САБО, Д. ПЕТРАНИ

Методом торможения образования иммуноспецифической розетки авторы исследовали иммуносупрессивное действие деперсолона и диадркзон Ф-аквозум. Они установили, что торможение образования иммуноспецифической розетки in vivo является для этого

более пригодным методом, чем in vitro. В большой дозе диадрезон Ф-аквозум задерживает образование розетки значительно сильнее, чем деперсолон, но с понижением дозы разница в ингибирующем образование розетки действии двух производных преднизолона уменьшается.

МЕТАБОЛИЗМ И АККУМУЛЯЦИЯ ЖЕЛЕЗА ПРИ БОЛЕЗНИ ЖИЛЬБЕРТА И СИНДРОМАХ РОТОРА И ДУБИН-ДЖОНСОНА

Т. БУРГЕР, А. ГОГЛ, Т. ЯВОР и А. ПАТАКФАЛЬВИ

Кинетика железа была изучена у 3 пациентов с синдромом Дубин-Джонсона, у двух больных с синдромом Ротора и у 7 больных с болезнью Жильберта.

Обнаружены следующие аномалии:

1. 4 из 12 пациентов с функциональной билирубинемией оказались анемическими, и один из них страдал анемией от недостатка железа.

2. Исчезновение радиожелеза из плазмы было у трех больных ускоренным и у остальных нормальным.

3. У 7 нациентов обмен железа в плазме был повышенным, у остальных больных он был нормальным.

4. Утилизация радиожлеза эритроцитами была значительно пониженной у 4 пациентов, и умеренно пониженной у шести больных.

5. Выживание тромбоцитов было — за исключением одного пациента — у всех обследованных больных нормальным.

6. Измерениями радиоактивности in vivo было выявлено повышение аккумуляции железа в печени.

ДЕЙСТВИЕ ДИЕТ С РАЗЛИЧНЫМ СОДЕРЖАНИЕМ БЕЛКОВ НА РЕЗЦЫ БЕЛЫХ КРЫС

Т. ФАБИАН, И. СЕЛЕНИ, Т. ЦЕЛЛЕШ, П. ФЕЕРДИ

В ходе своих опытов авторы установили, что бедная белками диета вызывает общее нарушение развития зубов. Рост зубов замедляется, меняется их химический состав, уменьшается их кислотоустойчивость. Из исследованных критериев наибольшее изменение показывает растворимость в кислотах. Результаты исследований позволяют делать вывод, что в результате недостатка в белках в пище в период развития зубов возникают менее устойчивые в отношении действия внешних факторов зубы.

ДЕЙСТВИЕ ОДНОСТОРОННЕЙ ПЕРЕРЕЗКИ ЧРЕВНОГО НЕРВА НА АКТИВНОСТЬ РЕНИНА В ПОЧЕЧНОЙ ВЕННОЙ КРОВИ СОБАК С РЕНОВАСКУЛЯРНОЙ ГИПЕРТОНИЕЙ

А. ФЕКЕТЕ, А. ФАЗЕКАШ, И. ФОРГАЧ, К. ГАЛ

Полной перевязкой почечной артерии на одной стороне, авторы наблюдали у собак при существовании как иннервированной, так и «денервированной» спланхникотомией другой почки, возникновение и прочное существование гипертонии. В начальной стадии гипертонии активность ренина в венной крови ишемической почки высокая, в то время как в «денервированной» почке она не отличается от контрольных величин. После удаления ишемической почки новые определения, проведенные в более поздней стадии гипертонии, показывали повышающуюся активность ренина. Результаты опытов указывают на роль ренин-ангиотензиновой системы в деле поддержания хронической гипертонии.

ВЫДЕЛЕНИЕ КРЕАТИНИНА У ЖЕНЩИН ВО ВРЕМЯ КУРСА ЛЕЧЕНИЯ ГОЛОДАНИЕМ

В. Х. ПЕТЕРС, В. ГРОСЕР и А. КНАПП

Обсуждается динамика выделения креатинина в моче у 24 женщин с ожирением во время курса лечения голоданием. Как суточное выделение, так и коэффициент креатинина понизились во время курса. Авторы рассматривают результаты как выражение влияния питания на выделение креатинина. При использовании содержания креатинина в моче в качестве величины для стандартизации других параметров обмена веществ необходимо учитывать также изменения, вызванные лишением пищи.

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