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Inhibition by cysteamine of steroid 11 β-hydroxylation

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RECENTLY it has been found in rats that the increase in the corticosterone content of the adrenals and the peripheral blood following pharmacological stress (X-rays, salicylate, histamine) and ACTH was inhibited by cysteamine.[1–5] This effect is of interest because cysteamine (β-mercaptoethylamine) is physiologically important as a component of co-enzyme A, and it has also been clinically used. Moreover, it is a simple aliphatic substance compared to the ring-shaped aromatic inhibitors of the adrenal cortex hitherto known. Therefore, it seemed worthwhile to ascertain the effect of cysteamine upon the adrenal cortex in more detail.

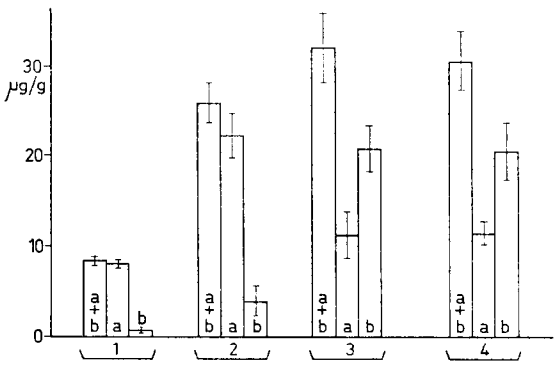

FIG. 1. Effect of cysteamine on the corticosterone and DOC content of the adrenals. Four male rats/group, 4 groups: 1, saline controls; 2, histamine only; 3, cysteamine, 120 min later histamine; 4, cysteamine only. a, corticosterone; b, DOC; a + b, corticosterone plus DOC. Mean ± S.D. All injections were made intraperitoneally with a volume of 0·8 ml 30 min after the injection of physiological saline or histamine (7·5 mg/kg) and 150 min after the injection of cysteamine (100 mg/kg), respectively, the animals were sacrificed. Chloroform extracts from adrenal homogenates and plasma of peripheral blood were used for thin layer chromatography. Solvent mixture: benzene–ethyl acetate–acetone = 60:10:10. Tetrazolium blue reaction, ethanol extraction and photometry were used for the quantitative determination of corticosteroids.[6,7]

In rats, corticosterone is the end-product of the corticosteroid biosynthesis. With regard to quantity, it predominates the other corticosteroids found in the adrenal cortex. It is formed by the β-hydroxylation of deoxycorticosterone (DOC) at the C 11 position. If cysteamine affects this step in the corticosterone biosynthesis, the decrease in the corticosterone content of adrenals and blood would be accompanied by an increase of DOC. In order to examine this hypothesis, 16 male albino rats of an inbred strain were divided into 4 groups and treated with saline, histamine, cysteamine or histamine plus cysteamine.* The experimental set-up and the methods for corticosteroid assay were as described in the legend of Fig. 1.

The experimental results are shown in Fig. 1. The sum of the values for corticosterone plus DOC (a + b) as well as the values for corticosterone only (a) and for DOC only (b) are delineated for the various groups. Compared to the controls (group 1), a sharp increase occurred in the sum of corticosterone and DOC in all experimental groups. No significant difference was found for the (a + b)-values between the groups 2–4. Regarding the single values for corticosterone (a) and DOC (b), however, a remarkable difference was found. Following histamine injection only (group 2), both corticosterone and DOC rose steeply, but the ratio of corticosterone–DOC had not essentially changed when compared to the controls. However, following cysteamine + histamine treatment

* Cysteamin-hydrochlorid purum, (Fluka, reagent grade No. 30 080); histamin-dihydrochlorid puriss (Fluka, reagent grade No. 53 300).

(group 3) as well as following cysteamine treatment only (group 4), the change in the corticosterone level was small (from 8·0 μg for the controls to 10·0 μg or 11·0 μg, respectively for groups 3 and 4), whereas the DOC level increased immensely (from 0·4 μg for the controls to 20·9 μg or 20·2 μg, respectively for groups 3 and 4).

These results indicate that cysteamine has two different effects on the adrenals. Firstly it stimulates the corticosteroid biosynthesis indirectly by releasing ACTH from the pituitary, similarly to histamine and many other drugs. This is in agreement with statements in the literature that, after treatment of rats with cysteamine, a drop in the ascorbic acid content of the adrenals[8,9] was found and an increase in the 17-hydroxysteroid level of the peripheral blood.[10] Secondly cysteamine has a direct effect on the adrenal cortex itself causing a shift in the ratio of corticosterone–DOC in favour of DOC. It appears that this direct effect on the adrenals is responsible for the fact that, after treatment with cysteamine, histamine and presumably all other agents causing a release of ACTH produce the same corticosteroid shift in favour of DOC as cysteamine.

The indirect stimulation by cysteamine of the adrenal cortex via a release of ACTH from the pituitary occurs within a few minutes. One might assume, however, that the direct effect of cysteamine causing a shift in corticosteroid synthesis does not take place before a certain period of time has elapsed, which is required for the transport of the drug to the adrenals and the uptake into the adrenal cortex. If this assumption applies, a time lapse should be ascertainable between the indirect and the direct effect of cysteamine.

TABLE 1. CORTICOSTEROID LEVEL IN THE ADRENALS FOLLOWING INJECTION OF CYSTEAMINE (100 mg/kg BODY WEIGHT)

	Saline controls	Cysteamine			
		After injection (min)			
		15	30	60	150
Corticosterone	7·9 ± 2·3	25·3 ± 1·9‡	24·7 ± 0·3§	18·0 ± 2·1†	6·7 ± 1·7
DOC	1·0 ± 1·1	6·5 ± 2·3	4·3 ± 2·4	13·1 ± 2·5‡	21·8 ± 2·2§
Corticosterone + DOC	8·9 ± 2·3	31·8 ± 1·6§	29·0 ± 2·0§	31·1 ± 4·4‡	28·5 ± 2·9‡

Shift from corticosterone to DOC. Corticosteroid content = μg/g adrenal. For details of corticosteroid content = μg/g adrenal. For details of corticosteroid assay see legend of Fig. 1. Four male rats/group. Mean ± S.D. Significance compared to controls: † = $P < 0{\cdot}05$; ‡ = $P < 0{\cdot}01$; § = $P < 0{\cdot}001$.

The corticosterone and DOC content was established repeatedly between 15 and 150 min after the injection of 100 mg cysteamine per kilogram body weight in order to test the course of the adrenocortical reaction. Regarding quantity, the results show that the adrenocortical stimulation (as indicated by the sum of corticosterone + DOC) reached its maximum as early as 15 min after the injection and remained undiminished up to 150 min afterwards (Table 1). The comparison between the single values for corticosterone and DOC shows that up to 30 min alone the corticosterone content increased. The direct qualitative effect of cysteamine was observed only after 60 min by a significant rise in the DOC content, which continued to increase still further up to 150 min. It is remarkable that, from 30 min onwards, there was a concurrence between the corticosterone decrease and the DOC increase. Thus, the aforementioned time lapse between the indirect and direct action of cysteamine on the adrenal cortex was established.

The corticosteroid changes in the adrenals are largely reflected in the blood.[11,12] Therefore, the shift in corticosterone biosynthesis from the end-product corticosterone to the precursor DOC due to cysteamine should be detectable also in the peripheral blood. This was confirmed in preliminary experiments. The chromatographic analysis of extracts from the peripheral blood of rats showed a marked increase in the content of DOC 150 min after treatment with cysteamine.

With respect to generalizing the described results obtained with rats, preliminary findings with mice and guinea pigs should be mentioned. In mice pre-treated with cysteamine, it was found that 60 min after the injection of histamine, the adrenals contained more DOC and less corticosterone than the controls. In the case of guinea-pigs the physiologically predominant end-product of corticosteroidogenesis is cortisol. However, compared with untreated control animals, in the adrenals of cysteamine-treated guinea-pigs large amounts of 11-deoxycortisol were found.

It is well known that corticosterone or cortisol are synthetized by hydroxylation of the precursors DOC and 11-deoxycortisol, respectively. It is then reasonable to ask whether cysteamine is able to inhibit the enzyme concerned, i.e. the 11 β-hydroxylase. In order to answer this question experimentally, the effect of cysteamine on the activity of the steroid 11 β-hydroxylase was investigated *in vitro* and *in vivo*.

In the *in vitro* experiments, using homogenates of adrenals from untreated rats, cysteamine was added to the incubation vessels before incubation. The enzyme activity was strongly inhibited. The effect of cysteamine was proportional to the concentration as demonstrated in Table 2a.

In the *in vivo* experiments, rats were injected intraperitoneally with cysteamine and sacrificed 2 or 4 hr later. The assay of adrenal homogenates demonstrated an inhibited 11 β-hydroxylase activity when compared to the controls (Table 2b).

TABLE 2. CYSTEAMINE INHIBITION OF STEROID 11 β-HYDROXYLASE ACTIVITY

			Cysteamine		
		Controls	Final concn		
(a)	Exp. *in vitro*		$0{\cdot}4 \times 10^{-4}$ M	1×10^{-4} M	4×10^{-4} M
	Enzyme activity (%)	100 ± 15	66 ± 9†	53 ± 5‡	22 ± 5§
			Cysteamine		
		Controls	Time of removal of adrenals		
(b)	Exp. *in vivo*		(2 hr)		(4 hr)
	Enzyme activity (%)	100 ± 8	20 ± 5§		18 ± 6§

Controls 100 per cent. Mean ± S.D. ($n = 4$), † $P < 0{\cdot}05$; ‡ $P < 0{\cdot}01$; § $P < 0{\cdot}001$.

(a) *In vitro*: Homogenates of rat adrenals, incubation. Cysteamine was added to the incubation medium in a final concentration ranging from $0{\cdot}4 \times 10^{-4}$ M to 4×10^{-4} M. (b) *In vivo*: The experimental rats were injected intraperitoneally with cysteamine, 100 mg/kg body wt. The animals were killed 2 or 4 hr after the injection and the adrenals removed for preparation of homogenates. No cysteamine was added to the incubation medium. The determination of enzyme activity was carried out according to Rosenthal and Narasimhulu[13] and Netter *et al.*[14] Adrenal homogenates in isotonic sucrose solution (0·25 M sucrose; 0·02 M Tris; 0·005 M EDTA). Incubation: An aliquote of the homogenate containing 2 mg protein was incubated in 1·35 ml phosphate buffer (0·004 M; pH 7·4) in the presence of the NADPH regenerating system and after addition of 1×10^{-4} M DOC as substrate for 60 min at 37° with shaking. NADPH regenerating system: $0{\cdot}74 \times 10^{-2}$ M potassium fumarate; $0{\cdot}74 \times 10^{-2}$ M nicotinamide; $1{\cdot}85 \times 10^{-3}$ M glucose-6-phosphate; 3×10^{-3} M $MgCl_2$; 9×10^{-4} M NADP. Chloroform extraction of the incubates. The corticosterone content was determined fluorometrically.[15]

These experiments show conclusively that cysteamine has an effect on corticosteroid biosynthesis by producing a shift from the C 11 hydroxylated steps to the non-hydroxylated precursors. This effect is caused by an inhibition of the 11 β-hydroxylase system. This system consists of adrenodoxin, adrenodoxin reductase and cytochrome P-450.[16,17] It was suggested that cysteamine operates as a competitive inhibitor of adrenodoxin.[18] Experiments are scheduled to check this hypothesis and to find out whether an effect of cysteamine on the cytochrome P-450 is also involved.

The 11 β-hydroxylase also controls other steps of the corticosteroid biosynthesis, for example the formation of 11 β-hydroxyprogesterone from progesterone and the formation of aldosterone from DOC. It is assumed that these steps of the corticosteroid biosynthesis are also inhibited by cysteamine. Experiments to check this assumption will be made in the near future. Further investigations are planned as to whether cysteamine also exercises an influence on other hydroxylation reactions in corticosteroid biosynthesis, for instance a hydroxylation in the C 18 or in the C 21 position.

In conclusion one should mention that the mechanism of cysteamine inhibition on the 11 β-hydroxylation remains to be clarified. In this respect, one must consider that cysteamine is oxidized to cystamine in aqueous solutions. An equilibrium is achieved between these forms which, *in vivo*[19,20]

and, in the presence of living tissue, also *in vitro*,[21] is influenced by the reverse reaction, i.e. the reduction of cystamine to cysteamine. Further, *in vivo* both substances are partially metabolized and excreted in the urine.[22–24] As a result, the blood concentration of cysteamine decreases very rapidly after injection. From this and other evidence in the literature,[25] one may assume that only a small part of the injected dose is still present at time of maximum inhibition seen in our experiments (150 min post-injection).

The above data suggest that the native cysteamine itself is not responsible for the hydroxylase inhibition. This view is supported by our preliminary findings *in vitro*. They showed that cysteine* (free SH group; reducing capacity) like cysteamine does not affect the 11 β-hydroxylation while cystamine† (disulfide, no reducing capacity) inhibits the reaction similar to cysteamine. Therefore, it remains to be investigated whether the cysteamine effect is due to a metabolite or whether the formation of mixed disulfides between cysteamine and sulfur containing components of the 11 β-hydroxylase system is responsible for the inhibition as has been assumed recently.[18] Eldjarn[26] has emphasized that mixed disulphide formation[27] with cellular constituents would mean a temporary interference with enzyme activity and cellular metabolism.

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Effect of administration of clofibrate and clofenapate on kidney mitochondria of the rat

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THIS LABORATORY has previously reported[1] that the administration of the hypolipidaemic compound, clofenapate (methyl-2-[4-(*p*-chlorophenyl) phenoxy]-2-methylpropionate) to the rat increased the content of mitochondrial protein and the activities of catalase (hydrogen peroxide–hydrogen peroxide oxidoreductase, EC 1.11.1.6) and glycerolphosphate dehydrogenase (L-glycerolphosphate–acceptor oxidoreductase, EC 1.1.99.5) in the liver. In this respect it resembled the analogous compound clofibrate (ethyl-α-*p*-chlorophenoxyisobutyrate) which is widely used as an antihypercholesterolaemic drug. These compounds and their metabolites are excreted in the urine of man.[2] However, little information is available about the changes, if any, brought about by these drugs in the kidney. The results presented in this communication indicate a significant increase in kidney mitochondrial protein on administration of clofibrate or clofenapate to the rat. Kidney mitochondria from drug-fed animals showed better oxidation of NAD^+-linked substrates.

Male albino rats, weighing 140–160 g, drawn from the stock colony of this Institute were used. The composition of the normal diet and feeding schedule were the same as previously described.[3] After 7–10 days feeding on the normal diet, the experimental animals were given 0·5% (w/w) clofibrate or 0·005% (w/w) clofenapate mixed with the diet for the period indicated in the Tables. In the withdrawal experiments, the animals were maintained on a diet containing the drugs at the above concentrations for the time period indicated and were then replaced on the stock diet and kept on it for an equal period of time. The control animals were supplied with the normal diet. An equal number of control and experimental animals were killed and analysed at any one time with a view to keeping variations to a minimum.

The animals were stunned and killed by decapitation. The kidneys were removed and freed of capsule and medulla.[4] The cortex was homogenized in 0·25 M sucrose and the subcellular fractions sedimented as described earlier.[1,3] The nuclear and mitochondrial fractions were washed once with 0·25 M sucrose. The rate of oxygen uptake was measured polarographically with a Gilson KM oxygraph. The reaction medium contained 0·4 M mannitol, 50 mM Tris–HCl buffer, pH 7·4, 25 mM potassium phosphate buffer, pH 7·4, 25 mM KCl, 5 mM $MgCl_2$, 20 mM EDTA, 3·4 mg bovine serum albumin and 2–3 mg (protein) of freshly isolated mitochondria in a total reaction volume of 2 ml. Respiratory control and ADP/O[5] (ratio of ADP phosphoxylated to oxygen consumed) were determined as has already been described.[3] All other determinations and conditions of assay were the same as was earlier reported.[1,3]

Administration of clofibrate or clofenapate to the rat has been shown to increase the mitochondrial protein content of the liver 50–100 per cent, without appreciably affecting the protein content of the other subcellular components. The results presented in Table 1 show that the mitochondrial fraction of the kidney also responded in a similar manner to these drugs. Dietary administration of clofibrate increased the content of kidney mitochondrial protein by 38 per cent. Clofenapate also produced the same effect, but at one-hundredth the concentration of clofibrate. In control animals the mitochondrial protein accounted for 21 per cent of the total cellular protein, while in the drug-administered animals the mitochondrial fraction increased to 26–27 per cent of the total protein.

When the animals were returned to the normal diet, after the indicated period of drug administration, the protein in the kidney mitochondrial fraction returned to its normal value (Table 1). This indicates that the effect produced by these drugs on the kidney is reversible as observed previously[1] in the case of liver.

Administration of these drugs did not adversely affect the oxidative or phosphorylative activity of liver mitochondria.[1,3] The results obtained in Table 2 indicate that respiratory control and ADP/O of kidney mitochondria were not affected by the administration of these drugs. However, active