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A COMPARATIVE STUDY ON THE INFLUENCE OF CYSTEAMINE AND METYRAPONE ON MIXED-FUNCTION OXYGENASE ACTIVITIES IN VARIOUSLY PRETREATED LIVER MICROSOMES FROM RATS AND MICE

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Summary

It has been found that metyrapone can inhibit both type I and type II mixed-function oxygenase reactions, while cysteamine inhibits only type I activity in this mammalian system. Following pretreatment with phenobarbital and 3-methylcholanthrene the half-maximal inhibiting concentrations for the O-demethylation of paranitranisol are increased for cysteamine and decreased for metyrapone. Both cysteamine and metyrapone give type II binding spectra with oxidized cytochrome *P*-450. The negative and positive peaks are at 393 and 426 nm respectively for metyrapone, and 410 and 434 nm for cysteamine. Cysteamine showed no binding comparable to that of metyrapone for reduced cytochrome *P*-450. Metyrapone showed little or no inhibition of the NADH cytochrome-*c* reductase (EC 1.6.1.1) or NADPH (EC 1.6.2.3) cytochrome-*c* reductase while cysteamine had a more or less strong inhibiting effect depending on the pretreatment of animals.

Neither the binding to *P*-450 heme nor the inhibition of NADH and NADPH cytochrome-*c* reductase correlates well with cysteamine inhibition of total activity. It is therefore suggested that cysteamine reacts with an intermediate electron carrier of non-heme iron or glycoprotein character thus inhibiting mixed-function oxygenase activity.

I. Introduction

Cytochrome *P*-450 is the CO-binding enzyme [1] responsible for drug and steroid metabolism in liver microsomes [2,3], and cortocosteroid biosynthesis in adrenal glands [4]. The various substrates for this enzyme have been classified largely as polycyclic hydrocarbons, type I, and aliphatic amines, type II [5]. This system is complicated by an array of inhibitors also giving type I and II binding spectra [6], and the apparent competition between type I and type

II substrates for cytochrome *P*-450 [7]. Further complications enter the picture with the use of *P*-450-inducing drugs. They also can be divided into two groups, e.g. phenobarbital-like, inducing more type I substrate activity, and 3-methylcholanthrene-like, inducing more type II substrate activity [8]. The so-called cytochrome *P*-420 [1] does not appear to have any functional importance in this system, and could be an artefact of non-specific heme binding to liver microsomes [9].

In this laboratory, it was recently found that the 11 β-hydroxylation of desoxycorticosterone in adrenal mitochondria was inhibited in vivo and in vitro [10,11] by cysteamine. The fact that the order of magnitude for this effect was comparable to that of metyrapone, led us to ask if cysteamine could also be a specific inhibitor of the mixed-function oxygenase system in the liver and by what mechanism this might proceed.

At least four groups have tested cysteamine or its disulfide cystamine, on liver mixed-function oxygenase reactions [12—15] but none have, to the best of our knowledge, made parallel experiments with a known inhibitor of "similar type", or chosen a group of reactions and treatments large enough to answer the above questions *.

II. Methods

(1) Animals

All animals were fed an Altromin diet and water ad lib. Rats were of the SIV-50 strain obtained from S. Ivanovas, Kisslegg/Allgäu, West Germany, when 7 weeks old, with an average weight of 200 g (untreated). Phenobarbital-treated animals were injected twice daily for two days with 70 mg/kg phenobarbital in physiological NaCl and killed by decapitation 48 h after the last injection; 3-methylcholanthrene was injected once daily for 4 days and the animals were killed 24 h after the last injection.

Mice were of an inbred strain from our institute. They were also treated when 7 weeks old, having an average weight of 24—30 g. Phenobarbital and 3-methylcholanthrene doses were the same as for rats. Mice were killed by breaking the neck.

(2) Tissue preparation

All procedures were done a 4°C after removal from the animal, unless otherwise stated. Animals were fasted for 15 h and killed as described above.

Livers of rats and mice were perfused in situ by forcing 20 and 10 ml respectively of chilled sucrose/Tris solution (0.25 M sucrose, 0.02 M Tris, pH 7.4) through the tissue with an automatically refilled syringe and needle (2A or 16) via the portal artery. This removed hemoglobin (which interfered with spectral measurements) and it slowed degenerative processes generally. When all livers were collected and weighed the first volume of sucrose/Tris was decanted and the tissue diluted with 1 g/2—3 ml of sucrose/Tris solution. Liver homogenate was centrifuged according to the scheme of Dallner and Fleischer [16,17] with

* A preliminary report of this work was made at the Third International Symposium on Microsomes and Drug Oxidations, Berlin 1976.

an extra step added for removing light mitochondria from microsomes.

The washed microsomal sediment was then resuspended in 0.25 M sucrose solution to obtain a protein concentration of approximately 20 mg/ml (1 ml 0.25 M sucrose/4 g liver). The preparation was divided into appropriate aliquots and immediately stored in liquid N_2 (−196°C). Microsomes so prepared and stored showed no significant loss of activity over 3 weeks. Microsomes used in inhibition experiments were not stored for more than five days as other groups have reported changes in activity during storage.

(3) Photometric determinations

Protein was determined by the Lowry method with commercial Folin reagent from Merck [18].

All coenzymes were tested in each system as used by this laboratory for the concentration which gave the highest activity, thus the values vary according to the activity being measured and often differ significantly from the cited source of the method.

The use of $MgCl_2$ and nicotinamide present two points of controversy here:

(1) Nicotinamide is well known for its inhibition of NADP dephosphorylation in microsomes but has also been reported to given a type II binding spectrum with cutochrome *P*-450 and to inhibit related hydroxylation reactions [19]. We therefore ran a series of experiments to test the concentration dependence of these effects. These experiments showed a 60% inhibition of O-demethylase activity without nicotinamide, which was larger than the inhibition with this component (40% at $2 \cdot 10^{-1}$ M). A plateau also exists between $2 \cdot 10^{-2}$ M and $5 \cdot 10^{-2}$ M where no further stimulation and no apparent inhibition occur, thus all our methods were tested for the lowest and most effective nicotinamide concentration. This was seen as a lesser evil than having a significantly reduced NADP concentration during the latter part of the incubation.

(2) $MgCl_2$ is a recognized stabilizer of mitochondrial membranes but generally not considered necessary for microsomal integrity [20,21]; however, we had already used it in our 11 β-hydroxylation experiments on adrenal mitochondria and decided to test its effects on our microsomal activities. It was found that approximately 15 mM $MgCl_2$ stimulated O-demethylation 20—40% depending on the source of microsomes. From other experiments in this lab it has been shown that Mg^{2+} is an excellent inhibitor of the NADPH-dependent lipid peroxidase (Mull, R., Gertz, J. and Flemming, K., unpublished) and may thereby protect the hydroxylase activity of cytochrome *P*-450. A standard experiment consisted of the following groups: (1) Blank (buffer + any addition after incubation); (2) Standard (buffer + 10 μg end product to be measured); (3) Control (buffer + coenzymes, substrate and microsomes); (4) Null control (like Control but reaction mixture was stopped prior to incubation); (5) Sample (Control + substance of unknown effect on system). Blank was always substracted from Standard, and Null control from Control and Sample values in order to correct for extraneous absorption not attributable to incubation and/or end product; thus addition of tested substances to Blank or Null control could be used to correct for non-enzymatic effects on the system.

All tests were carried out at 37°C in a Köttermann shaking water bath. Erlenmeyer flasks, 25-ml volume, equipped with ground glass stoppers were

used for incubation and extraction procedures. All solutions of cysteamine were prepared fresh because of uncertainty about auto-oxidation to cystamine in solution.

Protein content for all methods was chosen to give not less than 0.100 absorbance upon maximum inhibition and not more than 0.800 upon maximum stimulation. The absolute value was between 1 mg and 3 mg/assay depending on the reaction and source of microsomes.

O-demethylation was determined by a method modified from that of Netter [5]. The reaction was incubated for 15 min and contained: Glc-6-*P*, 5 μmol; nicotinamide 0.05 μmol; NADP 0.1 μmol; $MgCl_2$ 1.0 μmol; *p*-nitroanisol 1.0 μmol; Glc-6-*P* dehydrogenase 0.42 units; microsomes, 1—3 mg/assay and buffer 0.067 M potassium phosphate (pH 7.9) added to given a final volume of 2.0 ml.

The incubation of the above-described mixture was stopped with 0.5 ml 20% trichloroacetic acid and the pH brought back above 7.9, where the end product, *p*-nitrophenol, has an absorbance maximum at 420 nm. This mixture was then centrifuged at 27 300 × *g* for 20 min in a Sorvall GSA rotor to sediment the protein. The supernatant was measured at 420 nm on a Beckman Acta II photometer.

Acetanilide hydroxylation was determined by the method of Kirsch and Staudinger [22]. The reaction mixture was incubated for 20 min and contained: glucose-6-*P*, 5 μmol; nicotinamide, 0.1 μmol; NADP, 0.08 μmol; $MgCl_2$ 0.2 μmol; acetanilide 16 μmol; glucose-6-phosphate dehydrogenase 0.42 units, microsomes 1—3 mg/assay and buffer 0.04 M potassium phosphate (pH 7.4) added to give a final volume of 2.0 ml.

Reaction of the above mixture was stopped by addition of 10 ml ether/isoamylalcohol in order to avoid acid artefacts [23]. The assay mixture was then saturated with NaCl and shaken for 5 min with the ether mixture. Then 8 ml of the organic phase were added to 2 ml of 0.1 M NaOH and extracted again for 5 min (insufficient removal of aqueous phase from ether allowed protein to be carried over into the NaOH and gave a false color development). Finally, 1.5 ml of the 0.1 M NaOH solution were used for the color development with Folin reagent. The resulting blue solution was measured at 720 nm on a Beckman Acta II photometer as an index of *p*-hydroxyacetanilide formed.

Cytochrome-*c* reductase determinations were as described by Masters [24], and binding spectra as described by Shoeman [25].

(4) Chemicals

All coenzymes were obtained from Boehringer Laboratories of Mannheim, West Germany. Metyrapone was a gift of the Ciba Geigy Laboratory, Basel, Switzerland, and cysteamine and cystamine were from Merck, synthetic grade. Diethylether was from Merck, Uvasol grade for spectroscopic determination, due to the high blanks experienced with less pure substances. All other chemicals were reagent or analytical grade and obtained commercially.

III. Results and Discussion

Cysteamine and metyrapone effects on total mixed-function oxygenase activity

O-demethylation of p-nitroanisol. Cysteamine showed an inhibiting effect

similar to that of metyrapone in both untreated and induced rats, however, the effect of metyrapone was stronger than that of cysteamine (Table I). Only small quantitative differences were found between rats and mice (data for mice not presented).

If one compares the half-maximal inhibiting concentrations (I_{50}), determined from a semi-log plot presented in a preliminary report [26], to the specific activity of O-demethylation in control and 3-methylcholanthrene or phenobarbital induced microsomes, it immediately becomes apparent that the I_{50} value for metyrapone decreases with induction while that of cysteamine increases (Table II). The increase in cysteamine I_{50} after 3-methylcholanthrene pretreatment is especially large. Another interesting variable is seen when one pretreats animals with phenobarbital, 1 × 70 mg/kg, killed 48 h later, so as to induce O-methylation to about the same specific activity as in 3-methylcholanthrene pretreated microsomes. In the case of metyrapone, one then sees a decrease in I_{50} comparable to that for 3-methylcholanthrene microsomes, and about $\frac{1}{4}$ of that for microsomes pretreated four times with the same doses, but it is not comparable to the large change after 3-methylcholanthrene pretreatment, thus demonstrating that the effects of 3-methylcholanthrene induction on cysteamine inhibition differ quantitatively as well as qualitatively from those of metyrapone.

Acetanilide hydroxylation. Here we found a complicated series of species and induction dependent variations (Table III A + B). In untreated and 3-methylcholanthrene-treated rats (Table III A) our data indicate a two-phase system for metyrapone: in the first phase, from $1 \cdot 10^{-4}$ to $5 \cdot 10^{-4}$ M metyrapone, one sees an inhibition of only 15% for both groups of animals; in the second phase, from $5 \cdot 10^{-4}$ to $1 \cdot 10^{-2}$ M, an average maximum inhibition of 50% for untreated and 70% for 3-methylcholanthrene-treated animals was achieved.

These results are in partial disagreement with those of Leibman [27] insofar that we found no statistically significant stimulation of the acetanilide hydroxylation by metyrapone in control microsomes. In phenobarbital-treated

TABLE I

THE EFFECT OF CYSTEAMINE AND METYRAPONE ON THE O-DEMETHYLATION OF *p*-NITROANISOL IN VARIOUSLY PRETREATED LIVER MICROSOMES FROM RATS

Specific activities for controls (100%) were: 3.0, 3.8, and 9.2 μmoles para-nitrophenol per minute and per milligram protein for untreated, 3-methylcholanthrene and phenobarbital, pretreated microsomes respectively. Each point is the mean ± S.E. of at least 6 experiments, 3 samples per experiment, from 3 microsome preparations.

	[Inhibitor] (M)				
	$1 \cdot 10^{-2}$	$5 \cdot 10^{-3}$	$1 \cdot 10^{-3}$	$5 \cdot 10^{-4}$	$1 \cdot 10^{-4}$
Cysteamine					
Untreated	22 ± 5	32 ± 7	60 ± 8	72 ± 5	88 ± 3
Phenobarbital	65 ± 5	66 ± 3	94 ± 6	94 ± 8	100 ± 6
3-Methylcholantrene	74 ± 5	80 ± 8	96 ± 4	102 ± 8	100 ± 66
Methyrapone					
Untreated		28 ± 7	57 ± 5	72 ± 8	95 ± 10
Phenobarbital		21 ± 2	43 ± 6	54 ± 4	75 ± 5
3-Methylcholantrene		26 ± 4	55 ± 3	66 ± 3	86 ± 5

TABLE II

HALF-MAXIMAL INHIBITING CONCENTRATIONS (I_{50}) FOR CYSTEAMINE AND METYRAPONE IN VARIOUSLY TREATED MICE

Half-maximal inhibiting concentrations were determined from semi-logarithmic plots of rest activity (x-axis, linear scale) and inhibitor concentration (y-axis, log scale). Concentrations of inhibitors used, and the number of experiments were as described in Table I.

Treatment	Cysteamine (mmol/l)	Metyrapone (mmol/l)	O-Demethylation (nmol · p-nitrophenol)/(mg protein · min)
Untreated	2.0	2.0	3.0
Phenobarbital			
1 X 70 mg/kg	4.0	1.2	4.0
4 X 70 mg/kg	20.0	0.5	9.2
3-Methylcholanthrene	50.0	1.0	3.8

rats, however, our results were similar to Leibman's, e.g. from $1 \cdot 10^{-4}$ to $5 \cdot 10^{-4}$ M metyrapone hydroxylase activity increased up to 185% and then decreased so that at a concentration of $1 \cdot 10^{-2}$ M we measured a 20% inhibition.

When we tested cysteamine at the same concentrations in the same experiments, we found no statistically significant effects in treated or untreated animals (Table III A).

As opposed to its effect in rats, metyrapone showed no stimulation of acetanilide hydroxylation for phenobarbital-induced mice, but rather a linear inhibition from $5 \cdot 10^{-4}$ M, the concentration of maximal stimulation in rats. An average maximum inhibition of 40% was achieved with $1 \cdot 10^{-2}$ M metyrapone. Control animals gave similar results, reaching an average maximum inhibition of 20%. For 3-methylcholanthrene-treated microsomes, metyrapone also had an average maximum inhibition of up to 40% by $1 \cdot 10^{-2}$ M.

Cysteamine also had no statistically significant effect on acetanilid hydroxylation in mice (Table III B).

The most important points from these data are: (1) that the metyrapone stimulation in phenobarbital treated rats is unique and thus could be of special importance in studying the mechanism of the effect of metyrapone on the mixed-function oxygenase system; (2) that cysteamine-inhibition appears restricted to type I substrate hydroxylation indicating a very specific mechanism of action. From the above data our first question can be answered positively. Experiments on total activity indicate that cysteamine inhibition occurs by way of some component specific for type I activity, but offer no further indication of a molecular mechanism. We thus turned to experiments on individual components of the mixed-function oxygenase system to localize better the effect of cysteamine.

Effects of cysteamine and metyrapone on individual components of the mixed-function oxygenase system

(1) Hemoproteins. We and others have been able to demonstrate the presence of two CO-binding cytochromes in liver microsomes from mice [28] and rats [29].

TABLE III

THE EFFECT OF CYSTEAMINE AND METYRAPONE ON THE HYDROXYLATION OF ACETANILIDE IN VARIOUSLY PRETREATED LIVER MICROSOMES FROM RATS (A) AND MICE (B)

Specific activities for controls (100%) were: 1.2, 2.5, and 1.9 nmol *p*-hydroxyacetanilide per min and per mg protein for untreated 3-methylcholanthrene- and phenobarbital-pretreated microsomes respectively. Each point is the mean ± S.E. of at least 6 experiments, 3 samples per experiment, from 3 microsome preparations.

	(A) Rats: [Inhibitor] (M):					(B) Mice; [Inhibitor] (M):				
	$1 \cdot 10^{-2}$	$5 \cdot 10^{-3}$	$1 \cdot 10^{-3}$	$5 \cdot 10^{-4}$	$1 \cdot 10^{-4}$	$1 \cdot 10^{-2}$	$5 \cdot 10^{-3}$	$1 \cdot 10^{-3}$	$5 \cdot 10^{-4}$	$1 \cdot 10^{-4}$
Cysteamine										
Untreated	80 ± 11	90 ± 6	91 ± 7	92 ± 8	90 ± 7	116 ± 28	94 ± 22	106 ± 16	100 ± 8	94 ± 6
Phenobarbital	120 ± 18	117 ± 9	115 ± 7	105 ± 9	100 ± 6	104 ± 18	106 ± 8	110 ± 10	105 ± 9	90 ± 14
3-Methyl-cholantrene	116 ± 7	123 ± 32	112 ± 8	114 ± 5	102 ± 6	88 ± 16	94 ± 6	106 ± 4	104 ± 12	104 ± 8
Methyrapone										
Untreated	49 ± 11	63 ± 16	81 ± 36	84 ± 24	86 ± 7	78 ± 14	84 ± 16	88 ± 18	89 ± 8	90 ± 15
Phenobarbital	73 ± 13	118 ± 10	182 ± 16	185 ± 10	130 ± 10	58 ± 14	68 ± 12	86 ± 8	89 ± 10	96 ± 10
3-Methyl-cholantrene	30 ± 9	38 ± 9	66 ± 14	80 ± 10	82 ± 10	62 ± 26	71 ± 24	93 ± 11	98 ± 9	86 ± 12

As these two cytochromes were differentially induced by phenobarbital and 3-methylcholanthrene, differences in these components could explain the variations in inhibition after induction and thus reveal the locus of action for cysteamine and metyrapone. To examine this possibility, we studied the binding spectra of cysteamine, ethandithiol, a sulfur containing derivative of cysteamine, and metyrapone in microsomes from variously treated mice (Fig. 1).

As has been previously reported [14,15], cysteamine gives a so-called type II binding spectrum similar to that of metyrapone indicating an amine-heme iron ligand [15] similar to the pyridine-heme iron ligand of metyrapone. Ethandithiol, however, gave a totally different oxidized spectrum with a broad maximum at 468 nm which is similar to the carbene ligands described by Ullrich [32]. This ethandithiol spectrum is probably some type of S-Fe binding. It is important to note that the replacement of an amine with another sulfhydryl, as

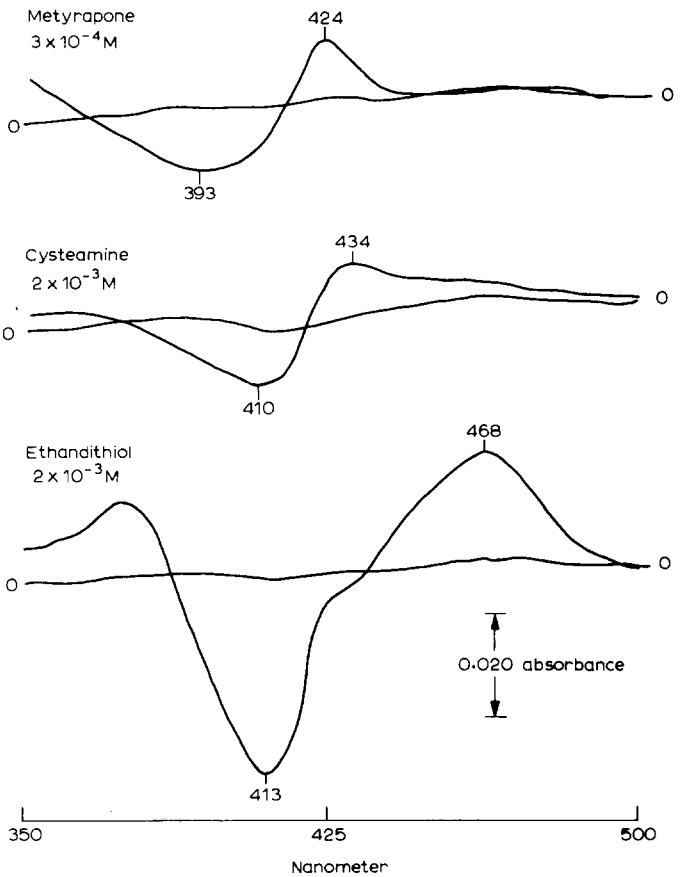

Fig. 1. Difference spectra of Metyrapone, Cysteamine and Ethandithiol on Oxidized Cytochrome *P*-450 from Untreated Mouse Liver Microsomes. Spectra were determined in solubilized and partially purified cytochrome *P*-450 preparations as described in Table IV, in a Beckman Acta II photometer. All solutions of inhibitors were prepared immediately before use for the sulfhydryl compounds. The protein concentration was 1 mg/ml, and samples were stirred 30 s after addition of test substance to allow for good binding and to minimize sedimentation effects. The latter was also controlled by stirring and scanning without adding substance (null). Scans were made from higher to lower wavelengths at a rate of 100 nm per min.

in the case between cysteamine and ethandithiol, causes at least a 10-fold increase in the reducing capacity of the SH groups. This in turn must have a dramatic effect on the sulfhydryl-heme binding. However, neither cysteamine nor ethandithiol gave binding spectra with reduced cytochrome *P*-450 preparations, although this form of cytochrome *P*-450 is thought to be the active state of hydroxylation.

Spectral binding constants, K_s, were also determined for these compounds (Table IV). The metyrapone K_s values were strongly influenced by pretreatment and agree well with previously reported values [33] and partially with the changes in I_{50} for O-demethylation (Table II), making hemoprotein binding the most probable explanation for metyrapone inhibition.

The K_s values for cysteamine and ethandithiol were unchanged after induction. This and the absence of a reduced binding spectrum would indicate that cysteamine binding to cytochrome *P*-450 heme cannot form the basis of any inhibition mechanism. This contention is further supported by the fact that cysteamine inhibits only type I activity, a very specific effect, while both *P*-450 hemoproteins bind CO and give similar ESR spectra. This similarity leaves little possibility for such a specific effect of cysteamine on one or the other hemoprotein, although the relationship of cytochromes *P*-450 and P_1-450 to type I and type II activities remains unclear [28]. From the above we therefore propose that cysteamine acts on some intermediate electron carrier specific for type I activity.

(2) Reductase. Since type I and II activities have differing synergetic relationships to NADH as an electron donor in NADPH-dependent hydroxylation [34], we decided to study the effect of inhibitors on the NADH- and NADPH-dependent cytochrome-*c* reductases. Unfortunately, ethandithiol could not be tested in this system as cystochrome-*c* was reduced by ethandithiol alone, even at very low concentrations.

TABLE IV

MICROSOMAL BINDING CONSTANT (K_s) * VALUES FOR CYSTEAMINE AND METYRAPONE IN VARIOUSLY PRETREATED MICE FOR OXIDIZED CYTOCHROME *P*-450

K_s values were determined in a solubilized and partially purified cytochrome *P*-450 preparation. As the values for metyrapone were comparable to those for whole microsomes [33], it was assumed that binding characteristics were unchanged by this modification, while the problems of turbidity and sedimentation seen when using whole microsomes were eliminated. Values are the mean of the number of experiments given in parentheses.

Inhibitor (M)	Untreated	Phenobarbital-treated	3-Methylcholanthrene-treated
Methyrapone			
Preparation 1	$1.0 \cdot 10^{-5}$ (3)	$2.2 \cdot 10^{-6}$ (3)	$1.0 \cdot 10^{-4}$ (3)
Preparation 2	$2.0 \cdot 10^{-5}$ (3)	$1.5 \cdot 10^{-6}$ (3)	$1.8 \cdot 10^{-4}$ (3)
Cysteamine			
Preparation 1	$3.0 \cdot 10^{-3}$ (3)	$2.5 \cdot 10^{-3}$ (3)	$3.5 \cdot 10^{-3}$ (3)
Preparation 2	$1.3 \cdot 10^{-3}$ (3)	$5.5 \cdot 10^{-3}$ (3)	$2.7 \cdot 10^{-3}$ (3)
Ethandithiol			
Preparation 1	$2.3 \cdot 10^{-4}$ (2)	$1.2 \cdot 10^{-4}$ (2)	$1.2 \cdot 10^{-4}$ (2)
Preparation 2	$1.4 \cdot 10^{-4}$ (3)	$2.3 \cdot 10^{-4}$ (3)	$3.0 \cdot 10^{-4}$ (3)

* K_s is defined as the concentration which gives a half-maximal spectral development.

In agreement with our previous studies [28], the specific activity of the NADH-dependent reductases appeared to be unchanged after pretreatment with phenobarbital and 3-methylcholanthrene while the NADPH-dependent activity was strongly induced by phenobarbital and unchanged by 3-methylcholanthrene pretreatment (Table V).

Inhibition studies with cysteamine and metyrapone from the same preparations revealed several interesting effects, the largest of these being an inhibition of both NADPH- and NADH-dependent reductases by cysteamine in microsomes from untreated animals. Only 65% of control activity was obtained by $1 \cdot 10^{-2}$ M cysteamine. Metyrapone, however, had little or no effect on either the NADH- or NADPH-dependent reactions.

Cysteamine's inhibiting effect was slightly decreased after phenobarbital and more so after 3-methylcholanthrene pretreatment. The decrease in inhibition was most prominent for the NADH-dependent reductase. This reaction still demonstrated 88% of control activity by $1 \cdot 10^{-2}$ M cysteamine in microsomes for 3-methylcholanthrene pretreated animals. As can be seen from the above data, the flavoprotein components of microsomal electron transport can be inhibited by cysteamine but not metyrapone. The inhibition of the cytochrome-*c* reductase is also changed in the same manner as the inhibition of the O-demethylase after induction with 3-methylcholanthrene and phenobarbital, e.g. induction decreases cysteamine inhibition, but the magnitude of the effects do not correlate well with the inhibition seen in the total reaction.

This lack of correlation between the influence of cysteamine on individual components and inhibition of total hydroxylase activity before and after induction could be explained by at least two hypotheses:

(1) Due to the existing stoichiometry between flavoprotein(s) and hemoprotein(s) (1 : 10 as reported by Estabrook [38]) and changes therein after induction, an amplification of the inhibition on any single component could occur; (2) The often suggested presence of unknown intermediate electron carriers [35] where cysteamine may react, could also explain the weak effects on known components as compared to the strong inhibition of total activity.

Our experiments characterizing the protein components of microsomal electron transport before and after induction [28,36] tend to support the latter.

On the one hand, we found a ratio of NADPH dependent flavoprotein to CO binding between 1 : 1.5 in controls and 1 : 3 after phenobarbital induction. These values do not agree with previous reports [38], and are thus not of a magnitude which could directly explain cysteamine inhibition of *P*-450 activities. One must, however, also consider the role of the NADH-dependent flavoprotein.

We have shown that the NADH cytochrome-*c* reductase can be made more resistant to cysteamine than the NADPH-dependent reductase and one could suggest that 3-methylcholanthrene induction switches the flow of more electrons from the NADPH- to the NADH-dependent reductase. Assuming the above to be correct, a partial dependence of type I activity, varying with pretreatment, and a total dependence of type II activity on the cysteamine-resistant pathway could be the basis of the inhibition mechanism of cysteamine. However, our studies on the NADH cytochrome-*c* reductase are limited in scope, thus such interpretations are very speculative and more detailed studies

TABLE V

INHIBITION OF MICROSOMAL CYTOCHROME-*c* REDUCTASES BY CYSTEAMINE AND METYRAPONE IN VARIOUSLY PRETREATED MICE

Experimental methods were as cited. The data represent the mean ± S.E. of five experiments, three samples per experiment, for two preparations in each group of animals. Enzyme activity is expressed as nmol cytochrome-*c* reduced per min and per mg protein.

	Control	Cysteamine concn. (M)			Metyrapone concn. (M)	
		$1 . 10^{-2}$	$1 \cdot 10^{-3}$	$1 \cdot 10^{-4}$	$1 \cdot 10^{-2}$	$5 \cdot 10^{-3}$
Untreated						
NADPH						
Enzyme Activity	26.1 ± 3.5	17.0 ± 3.2	23.7 ± 3.5	25.6 ± 3.9	22.8 ± 3.0	25.1 ± 3.0
per cent of Control	100.0 ± 5.9	64.5 ± 5.3	90.4 ± 2.1	97.8 ± 3.5	86.9 ± 4.0	96.3 ± 2.4
NADH						±
Enzyme Activity	325.6 ± 15.5	213.1 ± 22.2	276.7 ± 8.9	301.2 ± 12.3	341.6 ± 10.5	306.0 ± 26.7
per cent of Control	100.0 ± 5.3	65.5 ± 7.5	85.7 ± 3.4	93.3 ± 4.8	105.2 ± 7.4	93.9 ± 4.6
Phenobarbital						
NADPH						
Enzyme Activity	79.1 ± 7.6	50.2 ± 5.2	68.2 ± 7.3	77.6 ± 11.3	67.1 ± 8.3	70.6 ± 7.0
per cent of Control	100.0 ± 4.5	63.4 ± 1.9	86.7 ± 1.0	97.8 ± 5.6	85.7 ± 2.8	89.4 ± 5.6
NADH						
Enzyme Activity	301.5 ± 27.3	215.4 ± 23.3	276.0 ± 29.7	311.9 ± 41.2	306.6 ± 27.5	299.3 ± 24.7
per cent of Control	100.0 ± 4.7	71.4 ± 2.3	91.6 ± 5.9	103.3 ± 6.8	101.7 ± 2.6	99.4 ± 5.1
3-Methylcholanthrene						
NADPH						
Enzyme Activity	38.7 ± 2.2	30.2 ± 3.0	38.7 ± 2.0	39.9 ± 4.8	41.4 ± 7.8	40.1 ± 5.24
per cent of Control	100.0 ± 4.4	77.8 ± 4.1	100.1 ± 2.5	102.8 ± 8.1	106.5 ± 16.0	103.2 ± 8.5
NADH						
Enzyme Activity	334.6 ± 9.9	293.6 ± 18.8	314.6 ± 5.0	341.6 ± 14.7	351.8 ± 16.1	344.0 ± 11.0
per cent of Control	100.0 ± 6.5	87.7 ± 4.1	94.1 ± 1.7	102.1 ± 2.1	105.2 ± 4.9	103.0 ± 6.1

will be necessary before a final answer to this question can be attempted.

On the other hand, we have found only two types of cytochrome *P*-450. The recent reports of three or seven cytochrome *P*-450 [30,31] types are not thought seriously to contest our results. Even if seven hemoprotein forms can be demonstrated, no relationship to CO binding has been seen. Further, two facts argue strongly against this interpretation of these experiments: (1) the hemoprotein staining on SDS gels used by the cited groups is well known for its lack of specificity largely because of the separation and non-specific rebinding of heme in the presence of SDS. (2) It has also been shown by McLean [9] that native microsomes can bind large quantities of exogenous heme. Furthermore, from our data these two hemoproteins do not appear to differ enough to account for specific inhibition by cysteamine of type I activity.

Additional components, however, do appear to be active in the mixed-function oxygenase system [28,37] and therefore could help explain the complex inhibition patterns described in the first part of this paper, i.e. could represent the intermediate electron carrier with which cysteamine reacts.

One of these components has an apparent molecular weight of 50 000 [28]. Its relative content, 15% of total microsomal protein, is decreased to half of the original value in control animals after pretreatment with phenobarbital and it could be the often proposed [39,40] and recently demonstrated [41,42] iron-sulfur protein component of liver microsomes. As iron-sulfur proteins are very sensitive to other sulfur-containing compounds such as cysteamine, this component is an excellent candidate for the site of cysteamine inhibition. This, however, is not easily compatible with the effect of induction upon inhibition effects of cysteamine, i.e. this component is decreased after phenobarbital and unchanged after 3-methylcholanthrene induction [28], indicating a closed relationship to type II activity while cysteamine inhibition was very selective for type I activity.

Our second component has an apparent molecular weight of 68 000 [2]. It is a glycoprotein and was increased after phenobarbital and unchanged after 3-methylcholanthrene induction. This component appears to be related to the increase in NADPH cytochrome-*c* reductase and type I hydroxylase activity after phenobarbital induction; therefore, this 68 000 dalton component offers a specific site for cysteamine action on type I hydroxylation.

As can be seen from the preceding data and discussion, the mixed-function oxygenase system is very complex, thus any single explanation for a given effect will probably be insufficient if not completely incorrect. However, the fact that we have been able not only to characterize the changes in microsomal protein composition after various pretreatments [28,35,37] but also to demonstrate very specific inhibition patterns for various substances as presented in this paper, leads us to hope that one may soon be able to say which part of the above can be of use in understanding the mechanism of any given effect.

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