BBA 12257

THE INHIBITION OF CEREBRAL NAD(P)H:(ACCEPTOR) OXIDOREDUCTASE* BY BARBITURATES AND NERVOUS DEPRESSANTS

RELATIONSHIP WITH CHEMICAL STRUCTURE

ANTONIO GIUDITTA AND GUIDO DI PRISCO

The International Laboratory of Genetics and Biophysics, Naples (Italy)

(Received February 15th, 1963)

SUMMARY

Cerebral NAD(P)H:(acceptor) oxidoreductase I (diaphorase I) is inhibited by barbiturates and nervous depressants which compete with the substrate. Determination of K_i values for each compound provides a quantitative measure of their inhibitory effect. An investigation has been made on the chemical structures required for inhibition. The importance of the –CO–NR– group and of the size of substituent groups is discussed. Other groups of atoms can, however, substitute for –CO–NR–. Cyclic structures are not required for inhibition. A parallel investigation on the structural requirements for inhibition of NADH oxidase of heart mitochondria has revealed a general similarity in the chemical structures required. The possible nature of inhibitory groups involved in binding with the enzyme is discussed.

INTRODUCTION

The relationship between chemical structure and biological activity of barbiturates and other nervous depressants of similar formula has stimulated the interest of investigators since the effect of these drugs was first studied with pharmacological and biochemical techniques. The first investigations were made in the whole animal by comparing the ability of various barbituric acid derivatives to produce hypnosis[1,2]. After the action *in vitro* of these compounds on biological oxidations was recognized[3–9], similar studies were carried out with tissue slices and cell-free preparations[10,11] by following the degree of inhibition produced by different types of compounds. As a result of this work, biological activity has been found to depend among other things on the length and branching of the side chains present at C-5 of barbituric acid and on the presence of the –CO–NR– group that occurs in the majority of active compounds.

More recent work has further localized the point of action of these drugs *in vitro*

* This enzyme, previously referred to as cerebral diaphorase I, is listed with the systematic name of NAD(P)H_2: 2-methyl-1,4-naphthoquinone oxidoreductase and the trivial name of menadione reductase (EC 1.6.5.2) in the Report of the Commission on Enzymes of the I.U.B. (Pergamon Press, 1961). In this paper the names NAD(P)H:(acceptor) oxidoreductase and NAD(P)H dehydrogenase (trivial name) will be used, since they give no emphasis to the nature of the electron acceptor, which, as it occurs *in vivo*, is as yet unknown.

in the flavoprotein region of the mitochondrial NADH-oxidase system[12,13]. Some uncertainty remains, however, as to the exact site of barbiturate inhibition[14,15].

During the last few years, two nicotinamide nucleotide dehydrogenases, NAD(P)H dehydrogenases I and II (diaphorases I and II), have been identified and purified from brain[16–19] and shown to be inhibited by amytal. The inhibition has been found to be of the competitive type[20,21], and to be produced in the case of NAD(P)H dehydrogenase I by several other barbiturates and nervous depressants[22]. It therefore seemed of interest to examine the barbiturate inhibition of this enzyme from the point of view of its structural requirements, and to compare it with that of other enzymic systems such as mitochondria. This comparison might provide information on the possible similarity of the two inhibitory sites. Structural studies with barbiturate-like compounds are carried out more suitably with NAD(P)H dehydrogenase I in view of the relative simplicity of this system when compared with particulate preparations, and of the accuracy with which inhibitory power can be determined from the values of the inhibitor constant. This work has been presented to the 8th Meeting of the Italian Society of Biochemistry[23].

EXPERIMENTAL

Chemicals

NADH was obtained from the Sigma Chemical Co., St. Louis, Mo; FAD from the California Corporation for Biochemical Research, Los Angeles; potassium ferricyanide from E. Merck, A.G., Darmstadt (Germany). Barbiturates and other nervous depressants were gifts from various firms; azetidinones[24], oxazindiones and *N*-methyl-oxazindiones [25] were kindly supplied by Professor E. TESTA, Lepetit Laboratories, Milano (Italy). The sodium salts of the barbiturates were dissolved in water; the acid forms in dilute sodium hydroxide. Addition of these solutions in the quantities used for experimentation did not modify the pH of the assay mixture. Non-barbiturate drugs which were not readily soluble in water were dissolved in 95% ethanol and added in quantities not exceeding 0.1 ml. The same amount of 95% ethanol did not effect diaphorase activity. Glass-distilled water was used throughout.

Enzymic preparations

NAD(P)H dehydrogenase I (diaphorase I) was extracted from fresh ox brain obtained from the local slaughterhouse and purified by methods already described[18,19]. The enzyme had a minimal specific activity of 400 μmoles NADH oxidized per min per mg of protein under the standard conditions of assay, using vitamin K_3 as electron acceptor. Purified preparations of the enzyme were stored at $-15°$ in 0.05 M potassium phosphate buffer (pH 7.3) containing 10^{-5} M FAD and 10^{-5} M NADH. Very little loss of activity occurred under these conditions in a period of several months.

Beef-heart mitochondria were prepared as described by CRANE *et al.*[26] for the so-called "sucrose mitochondria" and stored at $-15°$ before use.

Methods

NAD(P)H dehydrogenase activity was measured at 25° by following at 420 mμ

the disappearance of the band of ferricyanide in a Beckman-DU spectrophotometer equipped with an absorbancy log converter connected to a Texas Servoriter recorder. The reaction mixture contained 0.05 M potassium phosphate buffer (pH 7.3), $0.74 \cdot 10^{-6}$ M FAD, $5.0 \cdot 10^{-4}$ M potassium ferricyanide and varying amounts of NADH ranging from $3.0 \cdot 10^{-5}$ M to $2.0 \cdot 10^{-4}$ M in a total volume of 3.0 ml. Enzyme activity was corrected for the small non-enzymic ferricyanide reduction obtained with NADH alone. The reaction was started by addition of the enzyme in volumes not exceeding 20 μl. Potassium ferricyanide was used instead of menadione as electron acceptor in order to avoid the possible interference resulting from the competitive inhibition taking place with the quinone[18].

Oxidation of NADH by beef-heart mitochondria was measured by following the decrease in absorbancy at 340 mμ in a reaction medium containing 0.05 M potassium phosphate buffer (pH 7.3), $1.0 \cdot 10^{-4}$ M NADH and suitable amounts of mitochondrial suspension in a total volume of 3.0 ml. The reaction was started by addition of the mitochondria in volumes not exceeding 50 μl.

K_i values for each inhibitor were calculated from double reciprocal plots[27] of enzyme activity *versus* substrate concentration in presence and absence of a suitable amount of inhibitor. The formula $K_i = \frac{I}{K_p/K_m - 1}$ was used[22]. Values of K_p and K_m were obtained graphically.

With mitochondria, the concentration of inhibitor giving 50% inhibition was calculated from plots of reciprocal activity *versus* inhibitor concentration and is indicated in the tables as $[I]_{50}$. Values of K_i and $[I]_{50}$ reported in the tables represent the average of experiments whose number is given in parentheses.

RESULTS

Experiments with NAD(P)H dehydrogenase I

Barbiturates: Previous work on the inhibition of oxidations in tissue slices and cell-free preparations[10,11] has shown the importance of the length and branching of the C-5 side chains of barbituric acid in determining the degree of inhibition. To investigate the influence of the same structural parameters in the case of NAD(P)H dehydrogenase I, the inhibitor constants of several barbiturates were measured as described in the methods. Barbiturates were selected so that only one of the two side chains in C-5 increased in size, while the other one was kept the same in each compound. Variation in only one substituent group was desirable to facilitate interpretation of the results. Similar considerations directed the choice of the other cyclic inhibitors that will be considered later. Inhibitory power was measured by the value of the inhibitor constant, K_i, and decreased when K_i increased.

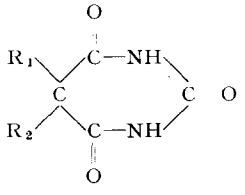
The data presented in Table I indicate the occurence of a direct relationship between the size of the substituent group in C-5 and the inhibitory effect. An apparent exception to this rule is given by the phenyl and cyclohexenyl derivatives whose values for inhibitor constant differ although both compounds have similar size. Possible explanations for this behaviour will be considered in the discussion.

Other cyclic compounds: To confirm and extend the results obtained with the barbiturates, other cyclic structures recently shown[24,25] to be endowed with hyp-

TABLE I

EFFECT OF SUBSTITUENT GROUPS OF BARBITURIC ACID ON THE INHIBITION OF NAD(P)H DEHYDROGENASE, K_i, AND NADH OXIDASE, $[I]_{50}$. BARBITURATES

R_1, ethyl-. The values of K_i and $[I]_{50}$ are the average of the number of experiments given in parentheses

Formula	R_2	Compound	K_i (M)	Standard error (%)	$[I]_{50}$ (M)	Standard error (%)
	phenyl-	Phenobarbital	$1.7 \cdot 10^{-3}$ (3)	10.7	$1.1 \cdot 10^{-3}$ (2)	4.3
	isoamyl-	Amylobarbital	$2.2 \cdot 10^{-3}$ (3)	12.0	$3.0 \cdot 10^{-4}$ (2)	3.3
	methylbutyl-	Pentobarbital	$3.7 \cdot 10^{-3}$ (3)	3.0	$3.1 \cdot 10^{-4}$ (2)	4.8
	cyclohexenyl-	Cyclobarbital	$4.0 \cdot 10^{-3}$ (3)	12.5	$2.3 \cdot 10^{-3}$ (2)	6.5
	butyl-	Butobarbital	$4.7 \cdot 10^{-3}$ (3)	4.6	$2.5 \cdot 10^{-3}$ (2)	4.0
	ethyl-	Barbital	$1.7 \cdot 10^{-2}$ (3)	7.2	$1.1 \cdot 10^{-2}$ (2)	9.0

notic properties were also used. Preliminary experiments indicated that azetidinones, oxazindiones and *N*-methyl-oxazindiones were inhibitory to NAD(P)H dehydrogenase I, and that their inhibition was similarly competitive with the substrate, thereby increasing the number of depressant drugs having the same mechanism of action on this enzyme[22]. K_i values could therefore be calculated from competitive types of plots and the inhibitory effect of the compounds compared to each other and to the other drugs.

TABLE II

EFFECT OF SUBSTITUENT GROUPS OF AZETIDINONE, OXAZINDIONE AND *N*-METHYLOXAZINDIONE ON THE INHIBITION OF NAD(P)H DEHYDROGENASE

A, azetidinones; B, oxazindiones; C, *N*-methyloxazindiones. R_1, phenyl-. For further details see Table I.

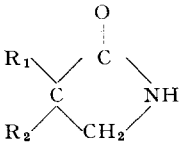
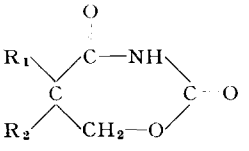
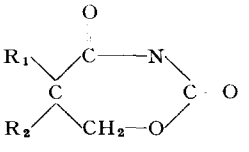
	Formula	R_2	K_i (M)	Standard error (%)
A		phenyl-	$1.5 \cdot 10^{-4}$ (3)	3.3
		butyl-	$2.9 \cdot 10^{-4}$ (3)	7.9
		isobutyl-	$3.8 \cdot 10^{-4}$ (3)	9.7
		ethyl-	$7.9 \cdot 10^{-4}$ (3)	11.8
		methyl-	$9.3 \cdot 10^{-4}$ (3)	4.4
B		phenyl-	$8.0 \cdot 10^{-5}$ (3)	9.6
		benzyl-	$8.2 \cdot 10^{-5}$ (3)	4.0
		isopropyl-	$2.4 \cdot 10^{-4}$ (3)	4.9
		propyl-	$3.6 \cdot 10^{-4}$ (3)	7.5
		methyl-	$4.0 \cdot 10^{-4}$ (3)	8.0
C		phenyl-	$4.0 \cdot 10^{-5}$ (3)	4.0
		benzyl-	$5.2 \cdot 10^{-5}$ (3)	5.4
		butyl-	$9.3 \cdot 10^{-5}$ (3)	1.8
		ethyl-	$1.2 \cdot 10^{-4}$ (3)	8.9

TABLE III

EFFECT OF CERTAIN RING STRUCTURES ON THE INHIBITION OF NAD(P)H DEHYDROGENASE AND NADH OXIDASE

R_1, ethyl-; R_2 phenyl-. For further details see Table I.

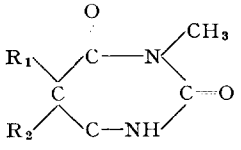
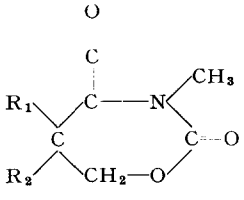
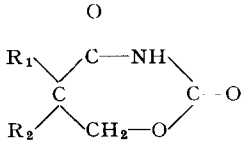
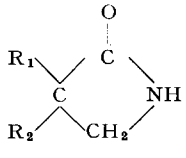
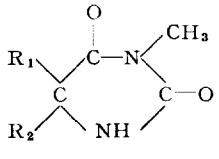
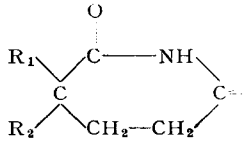
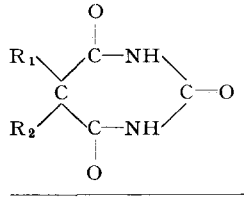
Formula	*Compound*	K_i *(M)*	*Standard error (%)*	$[I]_{50}$ *(M)*	*Standard error (%)*
	Methylphenobarbital	$7.0 \cdot 10^{-5}$ (2)	2.1	$4.3 \cdot 10^{-4}$ (2)	4.7
	*N*-Methyloxazindione	$1.2 \cdot 10^{-4}$ (3)	8.9	$2.8 \cdot 10^{-4}$ (2)	3.6
	Oxazindione	$3.8 \cdot 10^{-4}$ (3)	7.7	$3.9 \cdot 10^{-4}$ (2)	5.1
	Azetidinone	$7.9 \cdot 10^{-4}$ (3)	11.8	$4.3 \cdot 10^{-4}$ (2)	3.5
	Methoin	$1.2 \cdot 10^{-3}$ (2)	7.7	—	—
	Glutethimide	$1.6 \cdot 10^{-3}$ (2)	11.4	—	—
	Phenobarbital	$1.7 \cdot 10^{-3}$ (3)	10.7	$1.1 \cdot 10^{-3}$ (2)	4.3

Table II presents data obtained with several azetidinones selected with the same criteria outlined for the barbiturates. With this class of compounds an increase in size of the side chain also produced an increase of inhibitory power.

In the same table are shown similar results obtained with oxazindiones and *N*-methyl-oxazindiones in which essentially the same relationship of structure to inhibition was found, namely an increase in inhibitory effect brought about by an increase in the size of the substituent group.

The slightly higher K_i values obtained with the benzyl derivative as compared to the phenyl derivative are within the limits of experimental error. However, the same results have been obtained with both series of compounds. Since a similar larger effect of the phenyl group has been found in the case of barbiturates it seems possible that also in the case of oxazindiones the phenyl group may exert an effect larger than its size would account for.

Ring type and N-substituent groups: Other structural features examined for their possible influence on inhibition concern the type of ring and the effect of methyl substitution on the ring nitrogen. Table III presents the formulas of the compounds studied and the values of K_i obtained with them. The side chains in R_1 and R_2 are the same for each compound. *N*-Methyl substitution greatly enhances inhibitory power, as shown by comparing methylphenobarbital and phenobarbital and by comparing *N*-methyl-oxazindione and oxazindione. No ready explanation however can be given for the different extent of increase in inhibitory effect in the case of barbiturates as compared to that of oxazindiones.

The effect of various cyclic structures is also shown in the same table. A common feature of the compounds listed seems to be the occurrence of the –CO–NR– group. A similar observation has been made by other authors in studies involving tissue slices and particulate preparations[10,11]. No other relationship between ring structure and inhibitory power is immediately apparent, although the significantly different values of K_i suggest that some as yet undefined property of the rings is concerned. At variance with what has been found for the side chains, ring size does not seem to be directly related to inhibitory power, as shown by the K_i value for the four-membered ring azetinone, which are intermediate between those of compounds with larger rings.

Acyclic structures: It has been shown previously[22] that non-cyclic compounds endowed with hypnotic properties, such as open-chain ureids, urethans and sulphonals, inhibit NAD(P)H dehydrogenase I with the same competitive mechanism ascertained for cyclic drugs. Data shown in Tables IV, V and VI confirm these findings and allow a ready comparison of the inhibitory effect of these compounds. Open-chain ureids, sulphonals and Nirvotin (R) had an inhibitory power similar to or greater than that of typical cyclic structures such as barbiturates, as indicated by their K_i values (Tables IV and V).

With some of these compounds, the possibility of formation of internal chelates has to be considered and can actually be shown to take place using molecular models. Nirvotin (R), sulphonals, chloral hydrate and chlorbutol however cannot be expected to form cyclic structures. The results with these compounds therefore conconclusively prove that the existence of a ring is not a necessary requirement for inhibition.

A similar direct relationship between size of the substituent group and inhibitory

TABLE IV

EFFECT OF CERTAIN OPEN-CHAIN UREIDS AND URETHANES ON THE INHIBITION OF NAD(P)H DEHYDROGENASE AND NADH OXIDASE

A, open-chain ureids; B, urethanes. For further details see Table I.

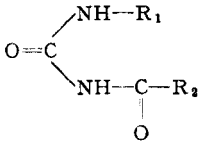
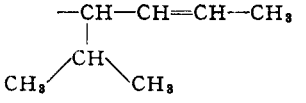
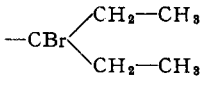
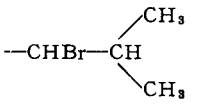
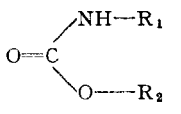
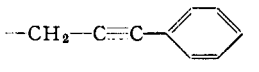
Formula	R_1	R_2	*Compound*	K_i (*M*)	*Standard error* (%)	$[I]_{50}$ (*M*)	*Standard error* (%)
A 	H		Apronal	$1.1 \cdot 10^{-3}$ (2)	0.9	$2.9 \cdot 10^{-4}$ (2)	5.2
	H		Carbromal	$1.3 \cdot 10^{-3}$ (2)	2.7	$4.0 \cdot 10^{-4}$ (2)	5.0
	H		Bromvaletone	$1.7 \cdot 10^{-3}$ (2)	1.5	$5.8 \cdot 10^{-4}$ (2)	5.8
B 	H		Nirvotin (R)	$1.3 \cdot 10^{-3}$ (2)	6.7	$1.8 \cdot 10^{-4}$ (2)	7.1
	$-CH_2-CH_3$	$-CH_2-CH_3$	Ethyl urethane	$1.5 \cdot 10^{-2}$ (2)	0.0	$6.7 \cdot 10^{-2}$ (2)	6.0
	H	$-CH_2-CH_3$	Urethane	$1.1 \cdot 10^{-1}$ (2)	10.3	$1.5 \cdot 10^{-1}$ (2)	11.8

TABLE V

EFFECT OF CERTAIN SULPHONALS ON THE INHIBITION OF NAD(P)H DEHYDROGENASE AND NADH OXIDASE.

SULPHONALS

For details see Table I

Formula	R_1	R_2	*Compound*	K_i (*M*)	*Standard error* (%)	$[I]_{50}$ (*M*)	*Standard error* (%)
	ethyl-	ethyl-	Dimethylsulphonal	$3.8 \cdot 10^{-4}$ (3)	8.6	$5.3 \cdot 10^{-4}$ (2)	4.7
	ethyl-	methyl-	Methylsulphonal	$6.0 \cdot 10^{-4}$ (3)	8.7	$5.7 \cdot 10^{-4}$ (2)	3.5
	methyl-	methyl-	Sulphonal	$8.0 \cdot 10^{-4}$ (3)	11.6	$5.8 \cdot 10^{-4}$ (2)	5.2

TABLE VI

EFFECT OF CERTAIN MISCELLANEOUS COMPOUNDS ON THE INHIBITION OF NAD(P)H DEHYDROGENASE

MISCELLANEOUS COMPOUNDS

For details see Table I.

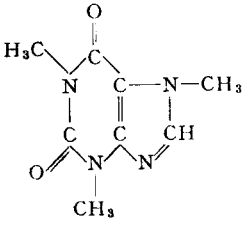
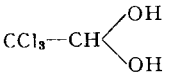
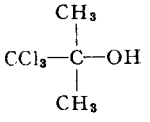
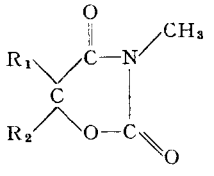
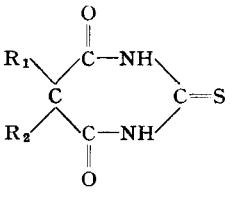
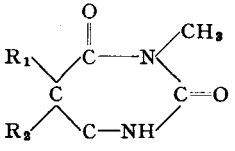
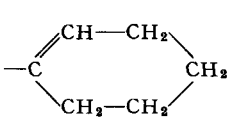
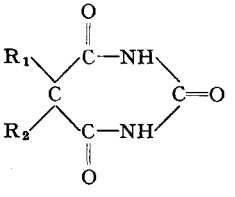
Formula	R_1	R_2	*Compound*	K_i (*M*)	*Standard error* (%)
	—	—	Caffeine	$6.4 \cdot 10^{-4}$ (2)	3.1
	—	—	Chloral hydrate	$1.5 \cdot 10^{-3}$ (2)	8.4
	—	—	Chlorbutol	$3.6 \cdot 10^{-3}$ (2)	6.7
	$-CH_3$	$-CH_3$	Trimethadione	$6.8 \cdot 10^{-3}$ (2)	1.5

TABLE VI (continued)

Formula	R_1	R_2	*Compound*	K_i (*M*)	*Standard error* (%)
	$-CH_2-CH_3$	$-CH(CH_3)-(CH_2)_2-CH_3$	Thiopentone	$6.4 \cdot 10^{-4}$ (2)	5.4
	$-CH_2-CH=CH_2$	$-CH(CH_3)-(CH_2)_2-CH_3$	Thiamylal	$4.0 \cdot 10^{-4}$ (2)	0.3
			Hexobarbital	$1.1 \cdot 10^{-3}$ (2)	2.7
	$-CH_2-CBr=CH_2$	$-CH(CH_3)-CH_2-CH_3$	—	$5.3 \cdot 10^{-3}$ (2)	1.7
	$-CH_2-CBr=CH_2$	$-CH(CH_3)_2$	—	$2.5 \cdot 10^{-3}$ (2)	5.0
	$-CH_2-CH=CH_2$	$-CH_2-CH_2=CH_2$	Allobarbital	$5.4 \cdot 10^{-3}$ (2)	4.2

power was also shown by these compounds. In the case of urethanes, the decreasing inhibition of Nirvotin (R), ethyl urethane and urethane was paralleled by a decrease in size of their side chains. In the case of open-chain ureids similar values of K_i were obtained for compounds with only slight differences in the size of their substituent groups. In the case of sulphonals small increases in the size of R_1 and R_2 produced equally small decreases in K_i. Furthermore sulphonals are among the few compounds capable of this type of inhibition in which the –CO–NR– group is not present in the molecule (Table V). Other examples are chloral hydrate and chlorbutol (Table VI)[22].

Experiments with heart mitochondria

To determine the structural requirements for the inhibition of NADH oxidation in mitochondria, beef heart was used as source of these particles since beef-heart mitochoncria can be prepared free from the NADH oxidation system present in microsomes. Complete inhibition by antimycin A and barbiturates was observed in our preparation. As shown in Table I, the size of the side chains in C-5 of barbiturates is important for mitochondrial inhibition. A significant parallelism between the values of K_i and $[I]_{50}$ was obtained for barbital, butobarbital, cyclobarbital and phenobarbital. The $[I]_{50}$ values for the last two compounds show that the phenyl derivative has a greater effect than the cyclohexenyl derivative also in mitochondria. Amylobarbital and pentobarbital are however considerably more effective in the mitochondrial system. It is possible that in the case of mitochondrial inhibition additional properties of these compounds, such as lipid solubility, become of importance.

The values of $[I]_{50}$ listed in Table III for various ring systems clearly show that similar structures are required for the inhibition of NADH oxidation in mitochondrial as well as in NAD(P)H dehydrogenase I preparations. *N*-Methyl substitution of barbiturates and oxazindiones produced an increase in inhibitory power also in mitochondria, although the inhibition produced by methylphenobarbital is appreciably lower. The same lack of relationship between ring size and inhibition exists for mitochondria as is shown by comparing the $[I]_{50}$ value for azetidinone with those of phenobarbital and oxazindione.

The inhibition of mitochondrial oxidations by non-cyclic compounds is shown in Tables IV and V. The relative sequence of inhibitors found for NAD(P)H dehydrogenase I applies also to this system, although differences in absolute values exist for open-chain ureids as well as for some of the urethanes. The same relationship exists for sulphonals (Table V) and in this instance inhibition of the mitochondrial system was produced at concentrations closely approaching those effective with NAD(P)H dehydrogenase I. As shown by the $[I]_{50}$ values of Tables IV and V, cyclic structures are not required for inhibition and, as for NAD(P)H dehydrogenase I, the –CO–NR– group can be substitued by other groups of atoms, as shown by the inhibition produced by sulphonals.

Comparable data have been obtained by other authors with tissue slices[10] and an NADH oxidase preparation from heart[11] using similar types of compounds. Sulphonal at concentrations of $2 \cdot 10^{-3}$ M has however been reported not to produce any effect on a mitochondrial NADH oxidase preparation from heart[11]. No ready explanation can be given for this apparent difference.

DISCUSSION

The large majority of compounds, both cyclic and non-cyclic, found to be competitive inhibitors of NAD(P)H dehydrogenase I are characterized by the presence of the group –CO–NR– in their molecules. It therefore seems reasonable to assume that the –CO–NR– group is involved in the inhibition mechanism, possibly by combining with some group on the enzyme. However, in sulphonals and in a few other compounds (chloral hydrate, chlorbutol) the –CO–NH– group is not present, showing that its presence is not indispensable for inhibition. It may be that in these compounds other chemical groups, *e.g.* $-SO_2-$ in the sulphonals, halogen in chloral hydrate and chlorbutol, are able to simulate its properties. Furthermore, the –CO–NR– group is not by itself sufficient to produce inhibition. A side chain of large enough size is also required, at least in the barbiturates, as shown by the lack of inhibition of barbituric acid at a concentration of $5.0 \cdot 10^{-2}$ M. Increase in size of the substituent groups of the carbon α to the carbonyl group is usually accompanied by increase in inhibitory power, as shown by the behaviour of barbiturates, oxazindiones, *N*-methyloxazindiones, azetidiones, open-chain ureids, urethanes and sulphonals. This effect seems therefore to be of general significance. These side chains might influence the inhibition of NAD(P)H dehydrogenase I by at least three mechanisms, namely (a) steric interference with the binding of substrate, (b) binding of the inhibitor to hydrophobic areas on the enzyme surface, and (c) resonance and inductive effects on C-5 and nearby atoms.

If only the first mechanism were operative, some exceptions would immediately be apparent. The phenyl group in fact produced a larger effect than its size would account for when compared to the cyclohexenyl group (Table I) and to the benzyl group (Table II, B and C). However, these apparent exceptions would readily be explained by an additional effect of the aromatic structure according to the third mechanism. As to the second possibility, it would be the only manner in which the OVERTON–MEYER theory of hypnotic action[28,29], as explained by lipid solubility, could be formulated for a soluble and highly purified system such as NAD(P)H dehydrogenase I. Experimentation with suitable compounds might be used to discriminate between these hypotheses or to attribute to each one its relative significance.

The cyclic structure in barbiturates and in a number of depressant drugs (hydantoins, glutethimide, oxazindiones, azetidinones *etc.*) is not a necessary requirement for inhibition. Open-chain ureids, urethanes, sulphonals, chloral hydrate and chlorbutol are all inhibitory, and for most of them K_i values of the same order of magnitude as those found for cyclic compounds have been obtained. For some of the acyclic inhibitors the possibility of internal chelates has been considered, and molecular models show that such possibility exists for some of these compounds. However, no internal chelates can be formed with sulphonals, chloral hydrate, chlorbutol, Nirvotin (R) and other urethanes, showing that ring structure is not necessary for inhibition. This accords with the results presented in Table III which indicate that ring size has little or no importance in determining inhibitory power. At variance with this observation is the striking influence of the size of the side chains present in the α-C to the carbonyl group.

The inhibitory action of caffeine on NAD(P)H dehydrogenase I (Table VI) is relevant to the form (enolic or ketonic) in which the –CO–NH– group might partici-

pate in the inhibition. No enolization is in fact possible for caffeine since all nitrogen atoms are substituted with methyl groups. Evidently the enol form is not involved in the inhibition mechanism. Since it seems reasonable to assume that caffeine has a similar mechanism of competition as the other drugs, it may be concluded that the enolic form is not involved in the inhibition by these inhibitors.

No conclusion can yet be reached as to which property of the –CO–NR– group produces the inhibition. The unspecificity of the inhibition produced by such widely different structures as barbiturates, sulphonals, chloral hydrate *etc.* points to a parameter of general occurrence. The relative negativity of the carbonyl oxygen in the –CO–NR– containing compounds, of the $-SO_2-$ oxygen in the sulphonals and of the halogen atoms in chloral hydrate and chlorbutol might be important. Another possible explanation, somewhat related to the first one, might be the capacity of these compounds to form hydrogen bonds with specific groups on the enzyme surface. Further work is necessary to investigate this point.

The results presented in this paper, in which several series of compounds have been examined with NAD(P)H dehydrogenase I and mitochondrial NADH oxidase, show that substantially similar chemical structures produce inhibition in both systems. Results obtained by other authors with tissue slices from brain[10] and submitochondrial particles from heart[11] provide support for this view. The differences in the extent of inhibition of NAD(P)H dehydrogenase I as compared to mitochondrial NADH oxidation may be accounted for by the different nature of the two enzymic systems. The presence of membranes and lipids in mitochondria must be important in determining inhibition, at least for some of these compounds. In view of the general similarity of chemical structures required to produce inhibition in both systems, it seems reasonable to expect that further studies on the nature of the barbiturate site of action in NAD(P)H dehydrogenase I will also shed some light on the barbiturate site in mitochondria. As already pointed out[22], studies with NAD(P)H dehydrogenase I are conveniently carried out in view of the soluble and purified nature of the enzyme.

REFERENCES

1 F. F. BLICKE AND R. H. COX, *Medicinal Chemistry*, Vol. 4. J. Wiley and Sons, New York, 1959.
2 L. DONATELLI, *Narcosi e Narcotici Barbiturici*, Edizioni Scientifiche Italiane, Napoli, 1953.
3 J. H. QUASTEL AND A. H. M. WHEATLEY, *Proc. Roy. Soc. London Ser. B.*, 112 (1932) 60.
4 D. R. DAVIES AND J. H. QUASTEL, *Biochem. J.*, 26 (1932) 1672.
5 M. F. GREIG, *J. Pharmacol. Exptl. Therap.*, 87 (1946) 185.
6 J. J. EILER AND W. K. MC EWEN, *Arch. Biochem.*, 20 (1949) 163.
7 T. M. BRODY AND J. A. BAIN, *Proc. Soc. Exptl. Biol. Med.*, 77 (1951) 50.
8 J. L. WEBB AND K. A. C. ELLIOT, *J. Pharmacol. Exptl. Therap.*, 103 (1951) 24.
9 W. J. JOHNSON AND J. H. QUASTEL, *J. Biol. Chem.*, 205 (1953) 163.
10 F. A. FUHRMAN AND J. FIELD, *J. Pharmacol. Exptl. Therap.*, 77 (1943) 392.
11 M. L. COWGER, R. F. LABBE AND B. MACKLER, *Arch. Biochem. Biophys.*, 96 (1962) 583.
12 L. ERNSTER, O. JALLING, H. LOW AND O. LINDBERG, *Exptl. Cell Res., Suppl.* 3, (1955) 124.
13 B. CHANCE, in O. GAEBLER, *Enzymes: Units of Biological Structure and Function*, Academic Press, New York, 1956, p. 447.
14 R. W. ESTABROOK, *J. Biol. Chem.*, 227 (1957) 1093.
15 T. P. SINGER, in T. W. GOODWIN AND O. LINDBERG, *Biological Structure and Function*, Vol. II, Academic Press, 1961, p. 103.
16 W. LEVINE, A. GIUDITTA, S. ENGLARD AND H. J. STRECKER, *J. Neurochem.*, 6 (1960) 28.
17 A. GIUDITTA AND H. J. STRECKER, *Biochem. Biophys. Res. Commun.*, 2 (1960) 159.
18 A. GIUDITTA AND H. J. STRECKER, *Biochim. Biophys. Acta*, 48 (1961) 10.
19 E. HARPER AND H. J. STRECKER, *J. Neurochem.*, 9 (1962) 125.

[20] A. GIUDITTA, H. J. STRECKER AND L. TRINCAS, *Boll. Soc. Ital. Biol. Sper.*, 36 (1960) 1975.
[21] A. GIUDITTA AND H. J. STRECKER, *Nature*, 193 (1962) 979.
[22] A. GIUDITTA, *J. Neurochem.*, 9 (1962) 329.
[23] G. DI PRISCO AND A. GIUDITTA, *8th Meeting of the Ital. Soc. Biochem.*, Padova, October 1962, *Boll. Soc. Ital. Biol. Sper.*, in the press.
[24] E. TESTA, L. FONTANELLA AND F. FAVA, *Il Farmaco*, 13 (1958) 152.
[25] E. TESTA, L. FONTANELLA, G. CRISTIANI AND G. GALLO, *J. Org. Chem.*, 24 (1959) 1928.
[26] F. L. CRANE, J. L. GLENN AND D. E. GREEN, *Biochim. Biophys. Acta*, 22 (1956) 475.
[27] H. LINEWEAVER AND D. BURK, *J. Am. Chem. Soc.*, 56 (1934) 658.
[28] H. H. MEYER, *Arch. Exptl. Pathol. Pharmakol.*, 42 (1899) 109.
[29] E. OVERTON, *Studien über Narcose*, Jena, 1901.