Biochemical Pharmacology, Vol. 37, No. 13, pp. 2551–2558, 1988.
Printed in Great Britain.

0006-2952/88 $3.00 + 0.00

PETHIDINE ANALOGUES, A NOVEL CLASS OF POTENT INHIBITORS OF MITOCHONDRIAL NADH:UBIQUINONE REDUCTASE

MECHTHILD FILSER and SIGURD WERNER*

Institut für Physiologische Chemie, Physikalische Biochemie und Zellbiologie der Universität München, Goethestrasse 33, D-8000 München 2, Federal Republic of Germany

(*Received* 18 *December* 1987; *accepted* 22 *December* 1987)

Abstract—Analogues of the analgetic drug pethidine were synthesized. Two N-aralkylen derivatives displayed a superior inhibitory effect on the activity of NADH:ubiquinone reductase in beef heart mitochondrial membranes. Dose–response curves revealed that the potency of these compounds is very comparable to that of the standard probe rotenone. The inhibitors were characterized by (a) their action on the reductase activity in various (eukaryotic and prokaryotic) organisms, (b) their influence on the enzyme kinetics, (c) their effects on the NADH dependent reduction of different electron acceptors, (d) their interference with the activities of other mitochondrial oxido-reductases. With regard to many of these aspects a close analogy between pethidine derivatives and rotenone was observed. A computer simulation of the steric structures of these molecules indicates that both classes of the chemically rather unrelated inhibitors may imitate very similar conformations. The potential advantages of the pethidine derivatives for the investigation of structure – function relationships within complex I of the respiratory chain is discussed.

Complex I is the segment of the respiratory chain, which catalyzes the electron transfer from NADH to ubiquinone. Despite a large body of literature, our knowledge on the mechanism of action of this membrane unit remains rather limited. This is mainly due to the exorbitant complexity of both protein composition (about 25 individual polypeptide subunits) and redox group content (for recent review see Ref. 1).

Among the diverse experimental approaches to establish both function and structure–function relationship within an oligomeric protein complex, specific compounds, which interfere at defined catalytic sites (e.g. inhibitors) have been used as potent tools. The reduction of ubiquinone by NADH in complex I is inhibited by a variety of chemically rather unrelated substances, reviewed by Storey [2]. The most frequently applied compounds have been barbiturates (e.g. amytal), isoflavinoids (e.g. rotenone) and the antibiotic piericidin A. Although the latter two species have turned out to be highly potent inhibitors of the NADH:ubiquinone reductase activity [1, 3], they suffer from two features: (1) the chemical structures of these molecules are rather complicated and do not allow an extensive derivatization and modification; (2) the highly hydrophobic character of the compounds may cause strong unspecific interactions with other membrane proteins, leading to difficult interpretations of binding studies; compare for instance Refs 4 and 5.

We recently investigated the chemical class of 4,4′-disubstituted piperidine derivatives and found several very effective inhibitors of the reductase. Out of the large number of compounds which we have synthesized subsequently, we report on two derivatives of rather simple structures, as a matter of fact, analogues of the analgetic drug pethidine. The inhibitors, nearly equipotent to rotenone, are characterized by their effects on enzymatic reactions and a rationale for their inhibitory activities with respect to structural similarities of the molecules is presented.

MATERIALS AND METHODS

Rotenone and amytal (sodium salt of 5-ethyl-5-isopentylbarbituric acid) were obtained from Sigma and Serva (Heidelberg), respectively.

Synthesis of compounds No. 29 and No. 49

Intermediates. 4-Phenyl-4-piperidinecarboxylic acid ethyl ester: hydrolysis of 4-phenyl-4-cyanopiperidine (Aldrich) and subsequent esterification was performed according to the procedure described by Eisleb [6].

(E)-1-(4-Chloro-2,3-dimethyl-2-butenyl)-4-nitrobenzene: Meerwein arylation reaction of butadien was employed [7]. The diazonium salt obtained from 4-nitroaniline was added to 2,3-dimethylbutadien, copper (II)-chloride and calcium oxide in aqueous acetone [8]. Predominantly the 1,4-addition product was obtained. The crude halide was chromatographed on an Al_2O_3-column in the presence of acetone and the eluent was used without further purification.

N-*Substitution of piperidine derivatives.* 4-Phenyl-4-piperidinecarboxylic acid ethyl ester (2.15 mmol) and the appropriate halide (cinnamylbromide or (E)-

* To whom correspondence should be addressed.

1-(4-chloro-2,3-dimethyl-2-butenyl)-4-nitrobenzene) (2.15 mmol) were dissolved in 5 ml of dry butanol-(1); 0.8 g of powdered anhydrous sodium carbonate and a catalytic amount of KI were added. The stirred mixture was heated at reflux for 24 hr. The suspension was filtered and the residual salts were washed with hot ethanol. The filtrate was subsequently treated with a chip of dry ice and decolorizing charcoal and, after a further filtration step, evaporated to dryness. The oily material was taken up in diethyl ether, filtered, and the solution was bubbled with hydrogen chloride. The resulting precipitate was crystallized first from ethanol–ether and a second time from acetone–petroleum benzine. Yields were in the range of 35–50%.

Analytical data. (E)-1-Cinnamyl-4-phenyl-4-piperidinecarboxylic acid ethyl ester (compd. No. 29), white needles (hydrochloride), mp 208° uncorr., (lit. m.p. 211–213° corr. [9]; NMR 200 MHz ($CDCl_3$), d 1.2 (t, 3 H, CO_2—CH_2—CH_3), 2.8 (m, 6 H, pip., CH_2—CH=CH), 3.7 (m, 4 H, pip.), 4.2 (q, 2 H, CO_2—CH_2—CH_3), 6.5 (m, 1 H, CH_2—CH=CH—Ar), 6.8 (d, 1H, CH_2—CH=CH-Ar, J=17 Hz), 7.4 (m, 10 H, Ph), 12.4 (s, (br), 1 H, N^+H). 1-(4-Nitrophenyl-2,3-dimethyl-2-butenyl)-4-phenyl-4-piperidinecarboxylic acid ethyl ester (compd. No. 49), light yellow needles (hydrochloride), m.p. 197° (uncorr.); NMR 200 MHz ($CDCl_3$), d 1.15 (t, 3 H, CO_2—CH_2—CH_3), 1.65 (s, 3 H, CH_3—C=C(Ar)—CH_3), 1.8 (s, 3 H, CH_3—C=C(Ar)—CH_3), 2.1 (m. 4 H, pip.), 2.7 (m, 4 H, pip.), 3.0 (s, 2 H, C=C—CH_2—Ar), 3.5 (s, 2 H, C=C—CH_2—pip.), 4.15 (q, 2 H, CO_2—CH_2—CH_3), 7.3 (m, 7 H, Ph), 8.15 (d, 2 H, Ar—NO_2); MS, *m/e* 436 (M^+), 363, 233, 57.

Preparation of membrane fractions

Mitochondria from beef heart [10], human heart (analog to the beef heart preparation), and *Neurospora crassa* [11] were isolated. Mitochondria from *Locusta migratoria* were obtained from W. Kleinow (University of Cologne). Mitochondrial membranes were prepared by sonication in phosphate buffer [12]. *E. coli* (strain K 12, grown on Lurea broth) was suspended in Tris–buffer (0.1 M, pH 7.5), deeply frozen and forced through a French press. After a brief low speed centrifugation step, membranes were spun down at 100,000 *g* (30 min).

Complex I from Neurospora crassa

The isolation was carried out essentially as described by Ise *et al.* [13]. For chromatography on DEAE-Sepharose, salt step gradients were applied rather than a continuous gradient. The material eluted with 0.2 M NaCl was collected, and an amount of Amberlite XAD-2 (20–50 mesh), sufficient to remove 75% of Triton X-100 was added. After an incubation period the mixture was filtered through a cheese cloth and centrifuged at 100,000 *g* for 1 hr. In most cases the pelleted complex I was found to be fairly homogeneous. Further chromatographic steps (like gel filtration) were dispensable, since they did not improve the "purity" of the preparation, as judged by SDS gel electrophoresis.†

Enzyme analyses

NADH:quinone reductase activity was tested at 25° by following the absorbance at 340 nm. The standard assay contained: 50 mM Tris, pH 7.5, 75 μM NADH, 2 mM NaN_3 and 76 μM either 2,3-dimethoxy- 5-methyl- 6-decyl- 1,4-benzoquinone (DB) (synthesized according to Wan *et al.* [14]), or 2,3-dimethoxy-5-methyl-1,4-benzoquinone, or 5-hydroxy-1,4-naphthoquinone (juglone), or 2-methyl-1,4-naphthoquinone (menadione). In most cases the reaction was started by addition of the enzyme.

NADH-ferricyanide reductase activity was measured at 25° according to Hatefi [15].

3-Hydroxybutyrate dehydrogenase, malate dehydrogenase, succinate-, glycerol-3-phosphate-, and NADH-cytochrome *c* reductase activities were assayed as described previously [16].

All enzyme activities are expressed as μmoles of substrate reacting per min (U). IC_{50} values (inhibitor concentrations resulting in a 50% loss of enzyme activity) were extrapolated from series of experiments varying the concentrations of the inhibitor only. Compounds No. 29 and No. 49 were dissolved in buffer, rotenone in methanol (final conc. of the solvent in the cuvette did not exceed 1%), and added to the complete assay mixture.

Computer calculations

The Parafit Iterative Regression program (Dynacomp Inc.) modified by J. G. Filser was used for all kinetic analyses. Molecular imaging of rotenone and pethidine analogues was achieved by means of the SYBYL graphics software.

RESULTS AND DISCUSSION

Inhibition of NADH: ubiquinone reductase activity by pethidine analogues

Figure 1 shows the structures of pethidine and of two synthesized *N*-aralkenyl derivatives (compounds No. 29 and No. 49).

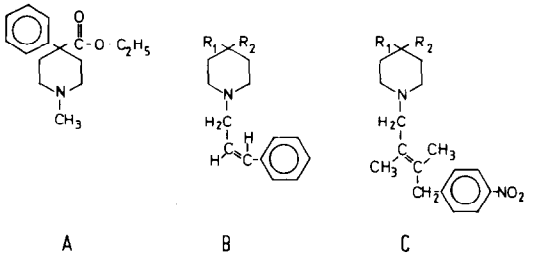

Fig. 1. Structures of pethidine and of two N-substituted analogues. (A) Pethidine; (B) compound No. 29; (C) compound No. 49. Ligands R_1 and R_2 are the same as in pethidine.

† Abbreviations used: DB, 2,3-dimethoxy-5-methyl-6-decyl-1,4-benzoquinone; Q_0, 2,3-dimethoxy-5-methyl-1,4-benzoquinone; Q_1, 2,3-dimethoxy-5-methyl-6-(3 methyl-2-butenyl)-1,4-benzoquinone; SDS, sodium dodecyl sulfate.

Enzymes: NADH: quinone reductase (EC 1.6.99.5); succinate dehydrogenase (EC 1.3.99.1); 3-hydroxybutyrate dehydrogenase (EC 1.1.1.30); malate dehydrogenase (EC 1.1.1.37); NADH dehydrogenase (EC 1.6.99.3).

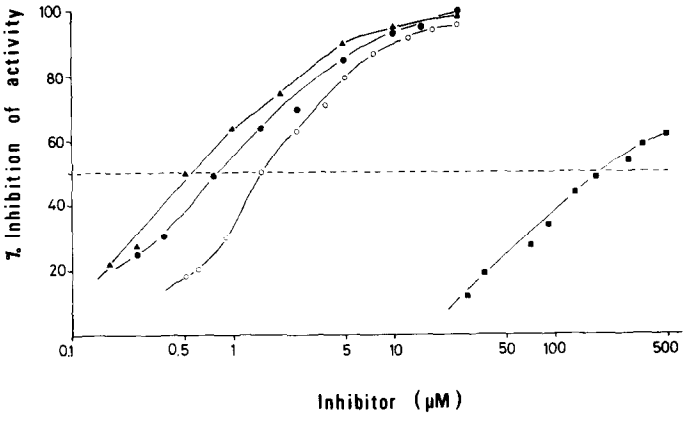

Fig. 2. Semi-logarithmic dose–response curves for the inhibition of NADH-ubiquinone reductase activity by various compounds. Pethidine (■), compound No. 29 (○) compound No. 49 (●), and rotenone (▲) were added in different amounts to the standard assay (see Materials and Methods), containing the decylbenzoquinone derivative (DB) and 100 μg of beef heart mitochondrial membranes (specific activity: 0.30 U/mg protein).

A "rotenone-like" action of pethidine (synonyms are meperidine and demerol) on site I of the respiratory chain has already been mentioned in several review articles (e.g. see Refs 1 and 17). However, no data are available. The dose–response curve (see Fig. 2) for this analgetic substance reveals that its inhibitory effect on the NADH: ubiquinone reductase activity is rather weak (IC_{50} value about 200 μM). Moreover, maximal inhibition levels did not exceed 70–75%, even when extreme concentrations of the drug were applied. However, substitution of the methyl group at the piperidine nitrogen by a aralkyl moiety led to a considerable improvement of the inhibitory potency. The optimal chain length was found to be 3 to 4 carbon atoms. Most remarkably, analogues with unsaturation between the 2, 3 positions of this moiety affected strongly the activity of the compounds. For example, derivatives including phenylpropyl or phenylbutyl groups were found to be less inhibitory by a factor of ten, compared to their 2,3-unsaturated counterparts. (Details on the systematic derivatization including the substitution pattern at the aryl group will be given elsewhere.)

Figure 2 compares the inhibition curves of compounds No. 29 and No. 49 to those of pethidine and rotenone. With the aralkenyl derivatives nearly complete (95%) inhibition of the beef heart NADH: ubiquinone reductase activity has been obtained. On an IC_{50} basis (see also Table 2) compounds No. 29 and No. 49 exhibit a 130- and 250-fold increased potency, respectively, when compared to the parent substance. Thus, the inhibitory effect of compound No. 49 and of rotenone on the enzymatic activity is almost equivalent.

Sensitivity of the reductase from various organisms

In order to get a more general idea of the action of piperidine derivatives on the reductase activity, a number of widely different species (eukaryotic and prokaryotic) were employed as an enzyme source. Table 1 lists the IC_{50} values for compounds No. 29 and No. 49; for comparison the data obtained with the established inhibitors rotenone and amytal are

Table 1. Determination of inhibitor concentrations giving rise to a 50% loss of NADH-ubiquinone reductase activity in various organisms

Source of enzyme	Specific activity (U/mg protein)	IC_{50} values (μM)			
		Compd No. 29	Compd No. 49	Rotenone	Amytal
Beef heart mt. membranes	0.32	1.5	0.8	0.55	170
Human heart mt. membranes	0.23	1.6	1	0.60	120
Locust muscle mt. membranes	0.05	2	n.d	0.06	130
Neurospora crassa mt. membranes	0.19	30	10	5	600
E. coli membranes	0.10	7	n.d.	20	>1000
Neurospora crassa isol. complex I	0.25	15	7	2	800

The activities were recorded under standard assay conditions (see Material and Methods) with DB as electron acceptor, the amounts of protein applied were 100 μg both for mitochondrial membranes and *E. coli* plasma membranes, and 10 μg for isolated complex I of *Neurospora crassa*.

n.d., not determined.

included. The mammalian and the locust enzyme activities were affected by similar concentrations of the pethidine analogues. However, for the inhibition of the *Neurospora crassa* and *E. coli* enzymes, at least 10-fold higher concentrations were required. Rotenone produced nearly identical behaviour among the different species, except with locusts. The insect reductase was affected by one-tenth of the concentrations compared to those necessary for the mammalian enzymes. The extremely low IC_{50} value of about 60 nM may explain the successful application of the drug as an insecticide.

The low sensitivity of the enzyme activity in the fungus *Neurospora* to both pethidine analogues and rotenone most probably reflects the presence of a "second" enzyme, which oxidizes exogenous NADH without proton translocation [18]. This particular reductase is considered to be insensitive to rotenone at concentrations up to 1 μM [13], whereas higher concentrations may affect the activity of this enzyme as well. This assumption was tested with the isolated complex I of *N. crassa*, containing only the "rotenone sensitive" species. We found that the relevant IC_{50} values for the inhibitors dropped only by a factor of two when the purified complex I was employed. We have to state, however, that our enzyme preparation still contained some Triton X-100, and it has been demonstrated that the replacement of the detergent by phospholipids is able to restore the "rotenone sensitivity" to a certain degree [13, 19, 20].

On the other hand, the situation in *E. coli* seems to be even more complex: the presence of multiple NADH-reductases has been discussed [21]. Therefore, the poor effect of the inhibitors on the overall enzyme activity(ies) cannot be interpreted without clear structural discriminations. Finally the frequently cited "inhibitor" amytal produced only incomplete enzyme inhibitions in all investigated organisms. Moreover, the high IC_{50} values observed throughout raise some doubt about its use as a specific diagnostic tool for site I of the respiratory chain.

Effect on enzyme kinetics

Figure 3 summarizes the kinetic measurements with the membrane bound NADH:ubiquinone reductase, using beef heart mitochondrial membranes. In the absence of inhibitor apparent K_m values of 58 μM and 13 μM (V_{max} app 250 and 88 μmol min^{-1} per mg of protein) were obtained for the decylbenzoquinone derivative (DB) and NADH, respectively. This is in good agreement with previously published data [17, 22], although these assays were performed with a different electron acceptor (Q_1) and recorded at a considerable higher temperature (38°). Nevertheless, we want to stress that the given numbers should be regarded as assessments only, due to the standard deviations obtained in the individual measurements (see Fig. 3). Moreover, the evaluations of the enzyme assays did not enable us to decide clearly whether we are dealing with "pure" hyperbolic Michaelis–Menten kinetics (as adapted by the computer program), or possibly with sigmoidal responses. This is especially true for the measurements employing low quinone concentrations, in which the endogenous ubiquinone present in the membranes affects considerably the enzyme activity.

In the presence of compound No. 29 apparent K_i values of 73 μM and 16 μM were calculated for the quinone derivative and NADH, respectively. No attempt was made to determine the actual K_i values by a secondary plot procedure, due to the limited accuracy of the apparent K_i measurements. Nevertheless, these figures do not differ much from those obtained with the uninhibited assays; on the other hand, the apparent V_{max} values drop markedly (see double-reciprocal plots). This behaviour suggests a non-competitive type of interaction of the inhibitor with both substrates. In contrast to rotenone [20], inhibition by pethidine analogues does not depend on the ubiquinone concentration. The effect of compound No. 29 on the activity was unchanged at ubiquinone concentrations up to 300 μM, indicating that the binding of the inhibitor is not easily affected by this substrate.

After addition of compound No. 29 to the complete enzyme assay a rapid onset of the inhibitory action with a lag period of a few seconds took place. Preincubation studies of the enzyme with both pethidine analogues and substrates showed that the inhibition was not reversible. Furthermore, the extent of inhibition was not altered, when the mitochondrial membranes were washed with buffer (without substrates or containing substrates), after pretreatment with compound No. 29. The strong affinity of the inhibitor indicated by these experiments could be confirmed by direct binding assays using a radioactively labelled pethidine derivative (S. Werner *et* al., *Eur. J. Biochem.*, submitted).

Inhibition of the NADH dependent reduction of different electron acceptors

The enzyme oxidizes NADH with a number of different electron acceptors [17, 22]. We have chosen two benzoquinone and two naphthoquinone derivatives, and in addition ferricyanide, to measure the influence of compound No. 29 on the reactions mediated by the beef heart enzyme (see Table 2). The reduction of ferricyanide was not inhibited by the pethidine analogue. The same behaviour has been reported for rotenone [1, 17]. Furthermore, NADH-juglone reductase activity was not affected by compound No. 29 and not by rotenone (data for rotenone not shown). The reductions achieved with the other electron acceptors were all inhibited by compound No. 29, in a probably non-competitive mode. Since some inconsistency exists in the literature about the sensitivity of the Q_0- and menadione reaction with respect to rotenone [e.g. 17, 23, 24], we repeated these experiments under the conditions applied for compound No. 29. It was found that rotenone (at concentrations of 1–2 μM) also inhibited the reaction with these quinone derivatives, although to a somewhat lesser extent, compared to DB. This may be explained by an altered interference of the enzyme with the very hydrophobic rotenone and the hydrophilic quinone derivatives.

Specificity of enzyme inhibition

In contrast to amytal, which affects the activity of

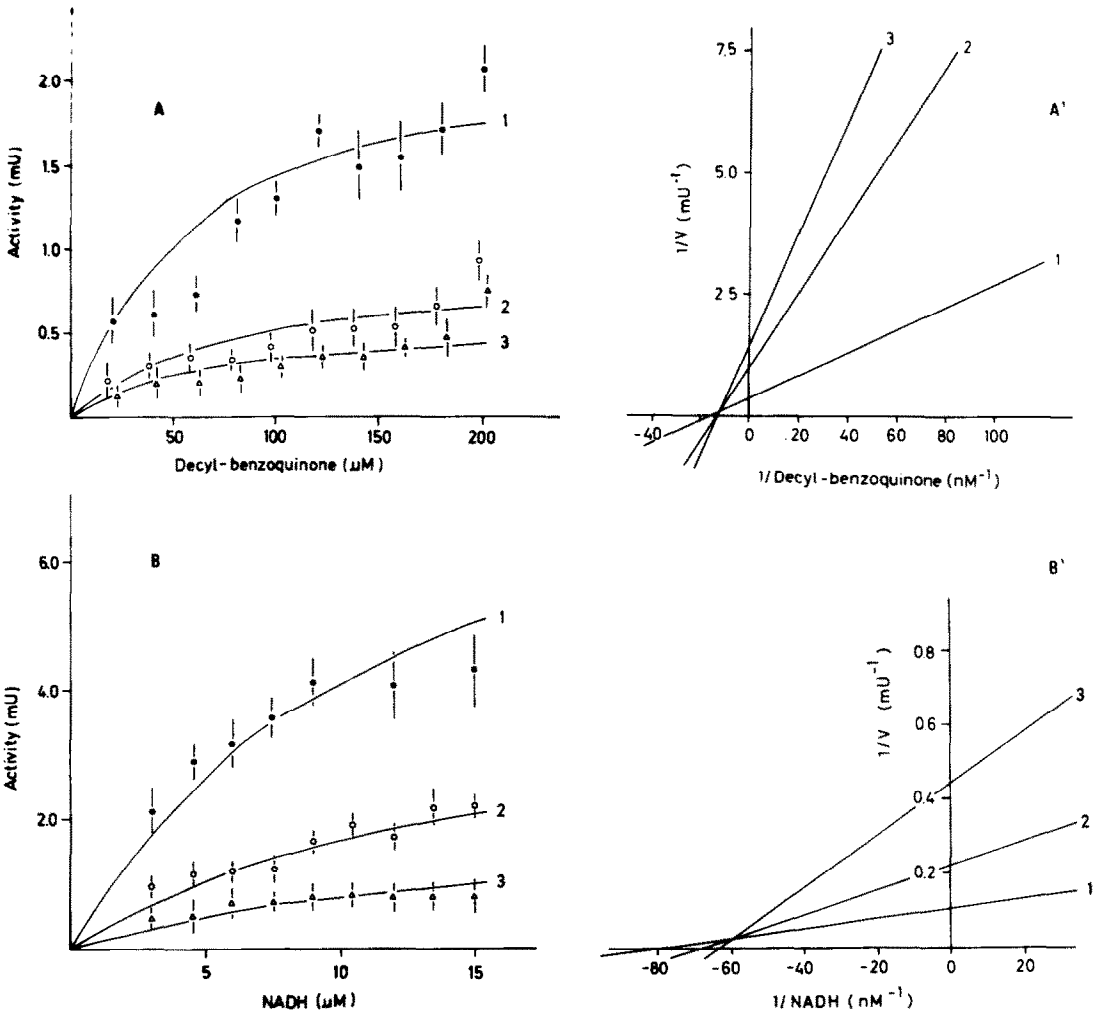

Fig. 3. Effect of substrate concentrations on the activity of NADH: ubiquinone reductase in the absence and presence of compound No. 29. (A) Michaelis–Menten plot, and (A') double-reciprocal plot of velocity versus different DB concentrations; NADH conc. was 75 μM; (1) without, (2) plus 2.5 μM, and (3) plus 4 μM inhibitor. (B) Michaelis–Menten plot and (B') double-reciprocal plot of velocity versus different NADH concentrations; DB conc. was 76 μM; (1) without, (2) plus 1 μM, (3) plus 4 μM inhibitor. Symbols indicate mean values of three to five measurements. Fitted curves were obtained by computer analysis of the experimental data. The reciprocal plots reflect an adequate graphical transposition of the computer fitted Michaelis–Menten curves only, drawn for reasons of a compendious presentation; it is nevertheless subjected to the same range of confidence given by the standard deviations of the measurements depicted in panels A and B. 100 μg of beef heart mitochondrial membranes were employed in each assay.

Table 2. Kinetic properties of beef heart mitochondrial NADH-reductase with different electron acceptors in the absence and presence of inhibitor

Electron acceptor	Without inhibitor		In the presence of compd. No. 29 (2.5 μM)		% Inhibition in the standard assay
	K_m^{app} (mM)	V_{max}^{app} (U/mg)	K_i^{app} (mM)	V_{max}^{app} (U/mg)	
DB	0.06	0.25	0.07	0.09	65
Q_0	0.05	0.34	0.05	0.13	60
Juglone	0.12	0.58	—	—	none
Menadione	0.06	0.16	0.055	0.09	50
$K_3Fe(CN)_6$	0.40	25	—	—	none

100 μg of membranes were applied in the assays containing the quinone derivatives and 10 μg in the test for NADH-$K_3Fe(CN)_6$ reductase. NADH conc. was throughout 75 μM. For further conditions see Methods section.

Table 3. Effects of compound No. 29 and of rotenone on various dehydrogenase activities in beef heart mitochondrial membranes

Enzyme	Amount of protein per assay	Specific activity (U/mg)	% Inhibition by Compd No. 29 (μM) 1.5	30	Rotenone (μM) 0.5	10
Succinate dehydrogenase	10	1.0	n	55	n	50
3-Hydroxybutyrate dehydrogenase	10	0.16	n	n[a]	n	n
Glycerol-3-phosphate dehydrogenase	100	0.01	n	20	n	23
Malate dehydrogenase	1	19	n	n	n	n
NADH-dehydrogenase	10	0.95	98[b]		97	

n: no inhibition (<5%). [a] activation; [b] IC_{50} value for NADH-dehydrogenase, measured via NADH: cytochrome *c* reductase activity, was 0.3 μM.

diverse enzymes and energy conservation reactions, rotenone has been considered to be a fairly specific inhibitor of the site I linked NADH oxidation, at least at low concentrations [2].

Table 3 compares the data obtained with compound No. 29 and rotenone. Three enzyme activities associated with the inner mitochondrial membrane (succinate-,3-hydroxybutyrate-, and glycerol-3-phosphate dehydrogenase), and in addition that of the matrix localized malate dehydrogenase were determined. At inhibitor concentrations in the range of the IC_{50} values for compound No. 29 and rotenone (referring to NADH:quinone reductase) the activities of the tested enzymes were not influenced: at concentrations 20 times higher, the reactions of succinate- and glycerol-3-phosphate dehydrogenase were affected to different extents. Again we note a striking parallelism in the action of both inhibitors.

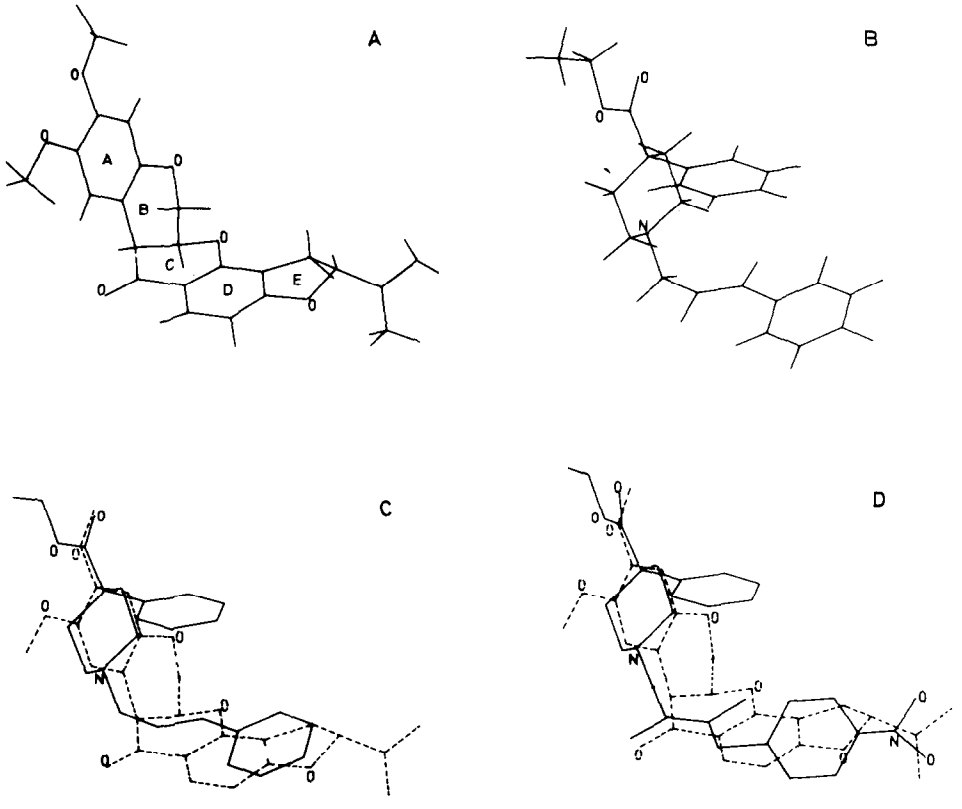

Fig. 4. Computer-generated structures and molecular overlays of rotenone and pethidine analogues. (A) Structure of rotenone according to X-ray analysis [25]; letters A–E indicate the positions of the five condensed rings. (B) Possible structure of compounds No. 29; a phenyl axial conformation for the piperidine 4-substitution was chosen (see discussion). (C) and (D) Superimpositions of compounds No. 29 and No. 49 (solid lines), respectively, and the rotenone molecule (dashed lines); hydrogen atoms are omitted for reasons of clarity.

Since these enzyme activities were measured via the reduction of cytochrome *c*, the NADH dehydrogenase was also assayed with this electron acceptor, to allow a better comparison. We found that the sensitivity of the latter reaction to compound No. 29 was at least two orders of magnitude higher than it was for succinate dehydrogenase.

In order to assess an "unspecific" binding of compound No. 29 to other proteins, enzyme assays were performed in the presence of various concentrations of bovine serum albumin. As a typical consequence, the IC_{50} value for the inhibitor increased by a factor of about 3 when the NADH:quinone reductase activity of mitochondrial membranes (100 μg) was tested in buffer containing 1% albumin, equivalent to an amount of 10 mg. Taking these results together, they indicate that the pethidine analogues indeed represent relative specific inhibitors for the reductase.

Structural considerations

The inhibitory potency of pethidine analogues depends largely on the introduction of conformational restrictions in the aralkyl substitution at the piperidine nitrogen. Compounds possessing a fully saturated three or four carbon chain, which allows the ligand to assume a variety of spatial orientations of the aromatic ring, were less active by an order of magnitude (unpublished results). The unsaturation between the 2,3 positions and a 3-methyl substitution (compound No. 49) of the connecting chain has some resemblance to the isoprenoid moiety of ubiquinone. Possible correlations could be approached by direct binding studies.

One of the persistent findings in this investigation has been the close analogy between the inhibitory effects of pethidine derivatives and rotenone. Although these molecules are rather unrelated in terms of simple chemical categories, we attempted to compare the potential three dimensional structures of these compounds. The task was considerably facilitated by the fact that X-ray diffraction analyses of rotenone [25] and of some pethidine derivatives [e.g. 26, 27] are available.

Figure 4 shows structures and superimpositions of the V-shaped rotenone and of compounds No. 29 and No. 49 respectively. An optimal fit was obtained when the piperidine ring was placed at the A ring of rotenone in a way that its 2-methoxy substituent is correlated with the carboxylic acid ester of the pethidine analogue. The 4-phenyl group at the piperidine then directs into the "cavity" formed by the molecules. This model implies an axial conformation between the phenyl and piperidine groups in compounds No. 29 and No. 49. (Both the axial and equatorial arrangements seem to have equal stabilities in comparable ring systems [26].) Furthermore, the 2,3 carbon double bond in the *N*-substituting aralkylen moiety allows this portion to span the condensed C/D rings and finally to fit on ring E of the rotenone molecule.

These computer simulations of steric structures tempt us to speculate that both the pethidine analogues and rotenone mimic very similar conformations at the protein and therefore bind to the same "receptor" site. This steric model confirms and even predicts the inhibitory activities of the different pethidine derivatives synthesized. Moreover, we asked the question of whether the analgetic potency *per se* of pethidine-like drugs can be correlated with an inhibitory effect on site 1 of the respiratory chain. We found that for example methadone, fentanyl and morphine did not at all affect the enzyme activity, obviously due to their discrete structural deviations. In the case of the very rigid morphine molecule the only "misfit" into the model seems to be the cyclohexenol nucleus. A "removal" of this structural portion leading to the benzomorphan analgetics, results in a considerable inhibitory activity (e.g. pentazocine, IC_{50}:25 μM). Furthermore, compounds of the neuroleptic class—structurally closely related to the pethidine analogues—show the effects already predicted by the model (e.g. haloperidol and benperidol, IC_{50} values:25 μM; details will be given elsewhere).

Conclusions and implications

The presented data indicate that particular substitutions of the pethidine molecule result in highly active and also specific inhibitors of site I of the respiratory chain. The relatively simple structure of the compounds certainly promotes a further derivatization of the molecules, with the aim, to obtain a set of well defined tools for relating discrete catalytic processes to polypeptide conformations. Inhibitors like rotenone are unsuited for this purpose, due to their complicated five rings heterocyclic system and an attempt to modify this inhibitor has remained largely unsuccessful [28]. Moreover, for the design of appropriate photoaffinity analogues, the rotenone molecule appears to be very problematic, because extremely hydrophobic products have been obtained [5]. In contrast, azido-derivatives, synthesized from pethidine analogues, hold an amphipathic character, that permits more specific interactions with membrane proteins. Using such a photoreactive derivative (also labelled radioactively with ^{125}I) five different subunits of complex 1 have been identified as binding sites for the inhibitor molecule (S. Werner, *Eur. J. Biochem.*, submitted).

Acknowledgements—The authors are very much indebted to the following scientists: Dr C. Filser (University of Munich) for supplying autopsied human hearts, Dr W. Kleinow (University of Cologne) for providing locust mitochondria, Dr G. v. Jagow (University of Munich) for advice on the synthesis of quinone derivatives, Dr J. Filser (GSF, Neuherberg) for substituting us with computer software, and Dr C. Hörig (Janssen Pharmaceutica, Beerse, Belgium) for sending a variety of neuroleptic compounds. Especially we want to thank Dr H. Kölbl and Dr F. P. Hoefer (Bayer, A. G., Leverkusen) for very fruitful discussions concerning the molecular modelling. Furthermore, we are grateful to Mrs B. Bürk and Mrs H. Rothe for their expert technical assistance. This research was supported by the Deutsche Forschungsgemeinschaft (project We 706 and through SFB 184/B-5).

REFERENCES

1. Hatefi Y, The mitochondrial electron transport and oxidative phosphorylation system. *Annu Rev Biochem* **54**: 1015–1069, 1985.
2. Storey BT, Inhibitors of energy-coupling site 1 of the

mitochondrial respiratory chain. *Pharmac Ther* **10**: 399–406, 1980.
3. Ragan CI, NADH-ubiquinone oxidoreductase. *Biochim Biophys Acta* **456**: 249–290, 1976.
4. Gutman M and Singer PT, Studies on the respiratory chain-linked reduced nicotinamide adenine dinucleotide dehydrogenase. *J. Biol Chem* **245**: 1992–1997, 1970.
5. Earley FGP and Ragan CI, Photoaffinity labelling of mitochondrial NADH dehydrogenase with arylazidoamorphigenin, an analogue of rotenone. *Biochem J* **224**: 525–534, 1984.
6. Eisleb O, Neue Synthesen mittels Natriumamids. *Chem. Ber.* **74**: 1433–1450, 1941.
7. Müller E, Über die Einwirkung von Diazoniumsalzen auf ungesättigte aliphatische Verbindungen. Ausweitung der Meerwein-Reaktion. *Angew Chem* **61**: 179–183, 1949.
8. Morgan KT, Wohl RA, Lumma WC, Wan CN, Bavey DD, Gomez RP, Marisca AJ, Briggs M, Sullivan ME and Wong SS, Synthesis and class III antiarrhythmic activity of (phenylbut-2-enyl) ammonium salts. Effect of conformation on activity. *J Med Chem* **29**: 1398–1405, 1986.
9. Elpern B, Gardner LN and Grumbach L, Strong analgesics. The preparation of some ethyl 1-aralkyl-4-phenylpiperidine-4-carboxylates *J Am Chem Soc* **79**: 1951–1954, 1957.
10. Smith AL, Preparation, properties, and conditions for assay of mitochondria:Slaughterhouse material, small-scale. *Methods Enzymol* **10**: 81–86, 1967.
11. Werner S, Isolation and characterization of mitochondrially synthesized precursor protein of cytochrome oxidase. *Eur J Biochem* **43**: 39–48, 1974.
12. Werner S, Preparation of polypeptide subunits of cytochrome oxidase from *Neurospora crassa*. *Eur J Biochem* **79**: 103–110, 1977.
13. Ise W, Haiker H and Weiss H, Mitochondrial translation of subunits of the rotenone-sensitive NADH: ubiquinone reductase in *Neurospora crassa*. *EMBO J* **4**: 2075–2080, 1985.
14. Wan YP, Williams RH, Folkers K, Leung KH and Racker E, Low molecular weight analogues of coenzyme Q as hydrogen acceptors and donors in systems of the respiratory chain. *Biochem Biophys Res Commun* **1**: 11–15, 1975.
15. Hatefi Y, Preparation and properties of NADH: ubiquinone oxidoreductase (complex I), EC 1.6.5.3. *Methods Enzymol* **53**: 11–21, 1978.
16. Werner S and Neupert W, Functional and biogenetical heterogeneity of the inner membrane of rat-liver mitochondria. *Eur J Biochem* **25**: 379–396, 1972.
17. Hatefi Y and Striggall DL, Metal-containing flavoprotein dehydrogenases. In: *The Enzymes*, 3rd Edn (Ed. Boyer PD), pp. 175–297, Academic Press, New York, 1976.
18. Weiss H, Jagow v G, Klingenberg M and Bücher T, Characterization of *Neurospora crassa* mitochondria prepared with a grind-mill. *Eur J Biochem* **14**: 75–82, 1970.
19. Ragan CI and Racker E, Resolution and reconstitution of the mitochondrial electron transport system. *J Biol Chem* **248**: 6876–6884, 1973.
20. Ragan CI, The role of phospholipids in the reduction of ubiquinone analogues by the mitochondrial reduced nicotinamide–adenine dinucleotide–ubiquinone oxidoreductase complex. *Biochem J* **172**: 539–547, 1978.
21. Ingledew WJ and Poole RK, The respiratory chains of *Escherichia coli*. *Microbiol Rev* **48**: 222–271, 1984.
22. Hatefi Y and Stempel KE, Isolation and enzymatic properties of the mitochondrial reduced diphosphopyridine nucleotide dehydrogenase. *J Biol Chem* **244**: 2350–2357, 1969.
23. Schatz G and Racker E, Partial resolution of the enzymes catalyzing oxidative phosphorylation. *J Biol Chem* **241**: 1429–1438, 1966.
24. Ragan CI and Bloxham DP, Specific labelling of a constituent polypeptide of bovine heart mitochondrial reduced nicotinamide–adenine dinucleotide–ubiquinone reductase by the inhibitor diphenyleneiodonium. *Biochem J* **163**: 605–615, 1977.
25. Arora SK, Bates RB, Grady RA and Delfel NE, Crystal and molecular structure of the one to one complex of rotenone and carbon tetrachloride. *J Am Chem Soc* **97**: 5752–5755, 1975.
26. Froimowitz M, Conformation-activity study of 4-phenylpiperidine analgetics. *J Med Chem* **25**: 1127–1133, 1982.
27. Tollenaere JP, Moereels H and Raymaekers LA, Structural aspects of the structure–activity relationships of neuroleptics: Principles and methods. In: *Drug Design*, Vol. 10 (Ed. Ariens EJ), pp. 71–118, Academic Press, New York, 1980.
28. Burgos J and Redfearn ER, The inhibition of mitochondrial reduced nicotinamide–adenine dinucleotide oxidation by rotenoids. *Biochim Biophys Acta* **110**: 475–483, 1965.