Biochemical Pharmacology, Vol. 19, pp. 1429–1442. Pergamon Press. 1970. Printed in Great Britain

INHIBITION OF MITOCHONDRIAL OXIDATION AND UNCOUPLING OF PHOSPHORYLATION BY ANTISPERMATOGENIC BIS-DICHLOROACETAMIDES*

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(*Received* 22 *June* 1969; *accepted* 12 *September* 1969)

Abstract—Antispermatogenic bis-dichloroacetamides affect both electron transport and the energy conservation system in heart mitochondria and submitochondrial particles. Oxidation of NADH or pyridine nucleotide-linked substrates is inhibited at a site prior to coenzyme Q but after the primary dehydrogenase, similar to rotenone and amytal inhibition, and as with these latter inhibitors the oxidation of succinate is essentially unaffected at similar concentrations of drug. In this respect the dichloroacetamides also resemble antabuse (tetraethylthiuram disulfide), but unlike the inhibition due to antabuse, the effects of the dichloroacetamides are not prevented by mercaptoethanol. Structure–activity studies suggest the importance of a substantial degree of lipid solubility and an unhindered dichloroacetamide group for activity.

Activity in intact mitochondria appears in both succinate and pyridine nucleotide-linked substrate supported systems. *N,N'*-bis(dichloroacetyl)-1,12-dodecanediamine was synthesized and is the most active member of this group of compounds found thus far. This compound results in a marked decrease in phosphorylation coupled to oxidation, as well as a release from ADP-limited respiration and an increase in the Mg^{2+}-stimulated ATPase activity. Dinitrophenol-stimulated ATPase activity is inhibited at low concentrations of drug, but rises somewhat at higher concentrations. The most dramatic changes in each of these parameters occur at quite similar concentrations of *N,N'*-bis(dichloroacetyl)-1,12-dodecanediamine, i.e. about 5 μM. The implications of these results and their relationship with other pharmacological properties of these drugs are discussed.

THE BIS-DICHLOROACETYLDIAMIDES show diverse effects in man and animals. Among these are an arrest of spermatogenesis,[1, 2] unfortunately accompanied, in at least those cases studied, by an antabuse-like reaction to alcohol[2] and an inhibition of liver aldehyde dehydrogenase.[3] Many of the compounds possess potent amebicidal activity[4] and one of these, the ethoxyethyl derivative of bis-dichloroacetyl phenylenediamine, is marketed as such. In addition to the inhibition of alcohol metabolism, we have found that the more general drug-metabolizing systems in liver microsomes are also inhibited *in vivo* and *in vitro*,[5] perhaps further limiting the usefulness of many members of this group of compounds as therapeutic agents. Fortunately, all of these activities do not always go hand in hand; for example, the ethoxyethyl derivative described as a potent amebicide lacks the antabuse activity and has shown essentially no inhibitory effects on microsomal drug metabolism *in vivo*. Recently we have found

* The investigation was supported by United States Public Health Service Grant AM 11006 from the National Institute of Arthritis and Metabolic Diseases.

evidence suggesting that drug metabolism inhibition may be separable from estrogen-induced testicular and accessory sex organ atrophy* and these findings will be reported separately.

Since a very large number of these compounds have been synthesized and they may be modified without a great deal of difficulty, a very complete structure–activity relationship with respect to the various pharmacological and biochemical activities which obtain is possible. Insight gained in this manner may eventually lead to structure–activity relationships for each of the several activities possessed by these compounds as well as to some clues concerning their biochemical mechanism. To this end, we have been examining the interaction of these compounds in several systems and from several points of view. For example, we are studying the metabolism of derivatives which are active with respect to antispermatogenesis as compared to derivatives inactive in this system. We are also studying the nature of the involvement of these compounds in the microsomal drug-metabolizing systems. These studies will be reported separately. In this paper we would like to report some of our findings on the effects of the bis-dichloroacetamides on the function of intact and fragmented mitochondrial particles.

MATERIALS AND METHODS

Beef heart mitochondria were prepared by the Nagarse procedure as described previously.[6] Electron transfer particles (ETP) were prepared from intact phosphorylating mitochondria by sonication and centrifugation essentially as described by Smith and Hansen,[7] except that ATP was omitted from the original sonicate.

Most of the dichloroacetamides used in this study were kindly supplied by the Sterling-Winthrop Research Institute, Rensselaer, N. Y. Two compounds, *N,N'*-bis(dichloroacetyl)-1,12-dodecanediamine and *N,N'*-bis-(dichloroacetyl)-1,10-decanediamine were synthesized in this laboratory essentially as described by Surrey.[4] The decanediamide had a melting point identical to that reported by Surrey and an R_f in reverse phase chromatography compatible with its structure when compared with other members of its homologous series. The dodecane derivative, for which there was no authentic compound available for comparison purposes, melted at 98–100° (uncorr.) and had a Cl analysis of 32·81 per cent (theoretical, 33·59%) and an N analysis of 6·55 per cent (theoretical, 6·63%). Gas chromatography of the compound on 3% OV-17 showed the compound to be essentially pure, suggesting the presence of a small amount of water in the sample. This slight discrepancy in elemental analysis is still under investigation, along with synthesis of other structural analogs of this compound.

The basic structure of these drugs is shown in Fig. 1. We have used compounds where X is an alkylene chain of varying length or is substituted benzene. The amide nitrogen can be unsubstituted as in the secondary amides or variously substituted with aliphatic groups of varying size. In one case, i.e. WIN 13,146, the potent amebicide described above, Y is the ethoxyethyl derivative.

Assays

Oxygen uptake was measured polarographically using a Yellow Springs Instrument Co. electrode and oxygen monitor fitted to a water-jacketed reaction vessel of 5-ml capacity. Substrates used were reduced nicotinamide adenine dinucleotide (NADH) or

* A. J. Merola, A. L. Beyler, G. O. Potts and J. D. Turnbull, unpublished results.

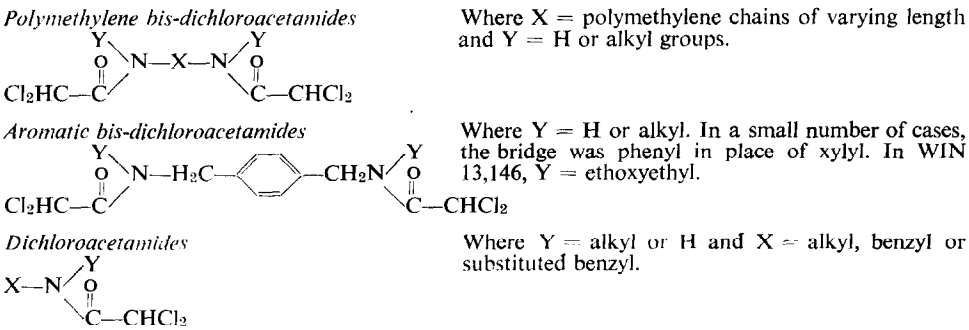


FIG. 1. Bis-dichloroacetamides and some closely allied compounds.

succinate with ETP and pyruvate plus malate or succinate with intact mitochondria. Estimations of oxidative phosphorylation were made by two methods. In the method of Chance and Williams,[8] a medium containing 0·25 M sucrose, 4 mM phosphate (K^+), pH 7·4, 4 mM $MgCl_2$, 2 mM sodium malate, and 2 mM sodium pyruvate or 1 mM sodium succinate in a final volume of 5·0 ml was employed. State 3 respiration was measured by the addition of 1 μmole adenosine diphosphate (ADP). The second method[9] involved the utilization of a hexokinase trap with an excess of hexokinase and glucose, 2 mM phosphate (K^+), pH 7·4, 4 mM $MgCl_2$ and 2 mM ATP in 0·23 M sucrose. The disappearance of inorganic phosphorus was determined by the method of Martin and Doty[10] and compared with oxygen utilization measured polarographically.

Dinitrophenol (DNP) and Mg^{2+} stimulated ATPase activity were determined in intact mitochondria in a medium containing: 12 mM Tris HEPES,* pH 7·0; 2·4 mM $MgCl_2$; 0·24 M sucrose; and 5 mM Tris ATP. DNP-stimulated ATPase activity was obtained using DNP at a concentration of 5°μM.

NADH-coenzyme Q_1 activity and NADH-ferricyanide activity were measured as described by Hatefi *et al.*[11] and Minakami *et al.*,[12] respectively, except that in the ferricyanide assay the electron acceptor was not varied, but was utilized at a concentration of 1 mM in a 3-ml reaction mixture.

In order to determine relative activity of the drugs in the polarographic electron transfer assay, they were added as dimethylsulfoxide solutions to the reaction mixtures containing the ETP and were incubated for exactly 2 min prior to the addition of substrate. In spite of the near water insolubility of these compounds at some of the levels used in these experiments, reproducible dose–response curves obtain when tested under these conditions. These doses are not linear through the entire range of inhibition to 100 per cent, so the concentration needed to inhibit electron transport by 30 per cent of the control value using dimethylsulfoxide alone was determined graphically, and this value was used for comparison of various members of these series.

Evidence for binding was obtained indirectly by adding a dichloroacetamide dissolved in dimethylsulfoxide to a rapidly stirring suspension of ETP followed by two washings with 0·25 M sucrose by centrifugation at 40,000 *g*. After resuspension, the ETP were tested for any evidence of inhibition in the NADH oxidase assay.

Protein was determined by a biuret method according to Gornall *et al.*[13]

* *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid.

RESULTS

Effects on electron transport. When the ability to block NADH-linked electron transport is compared with polymethylene chain length of some secondary and ethyl-substituted tertiary bis-dichloroacetamides, a direct relationship obtains, as seen in Fig. 2. *N,N'*-diethyl-substituted derivatives are, in general, more active than their

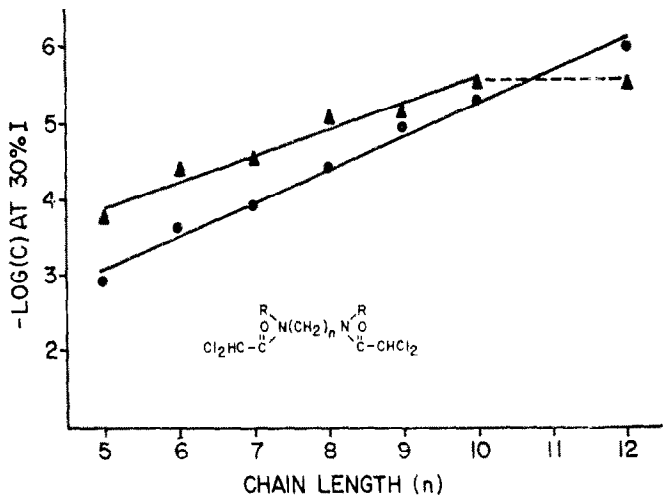


FIG. 2. The effect of varying polymethylene chain length on the inhibitory effects of various dichloroacetamides on the oxidation of NADH in ETP. Secondary amides are represented by the circles and ethyl tertiary amides by the triangles. Activity is plotted as the negative log of the concentration necessary to inhibit electron transport by 30 per cent and was measured as described in the text using NADH as substrate.

corresponding secondary amides, but this difference appears to diminish with increasing chain length. In fact, the secondary dodecanediamide is significantly more active than the corresponding tertiary amide.

If, instead of varying the polymethylene portion of these compounds, the hexamethylene derivative is used and the *N,N'* substituent is varied, a biphasic curve results, as seen in Fig. 3. Included in this figure are some *N,N'*-bis-(dichloroacetyl) heptanediamines. *N,N'* alkyl substitution with short chain alkyl groups increases activity in this case also, but more lengthy alkyl substituents were not available to determine if a biphasic response would obtain with increasing substituent chain length.

Just as an exact polymethylene chain length is not a structural requirement for the antispermatogenic[4] and electron transport inhibitory properties, so can a portion of the alkyl chains be replaced by an aromatic ring (Fig. 4). Within this group of compounds, activity rises when the methyl and ethyl tertiary amides are compared with the secondary amides, but substituting groups with somewhat more bulk, such as ethoxyethyl or isopropyl, appears to render the compounds essentially inactive in inhibiting electron transport. It is interesting to note that both of these compounds possess potent amebicidal activity with no suggestion of antispermatogenic properties.[4] Indeed, none

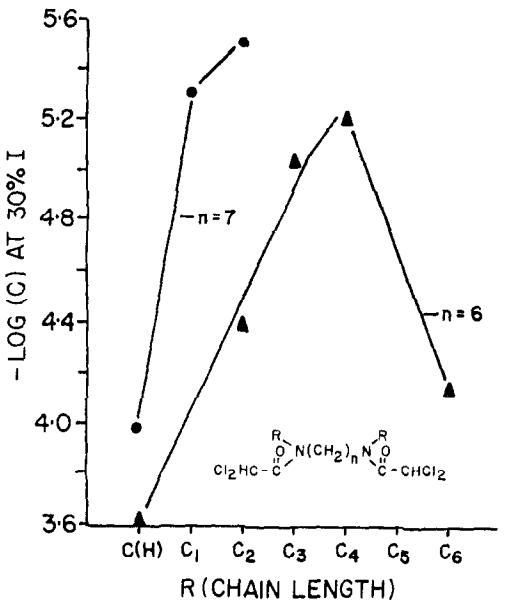


FIG. 3. The effect of varying the amide substituent, R, in hexamethylene and heptamethylene bis-dichloroacetamides on the oxidation of NADH by ETP. The R group in all cases is an alkyl chain. Details of the assay are the same as those for Fig. 2.

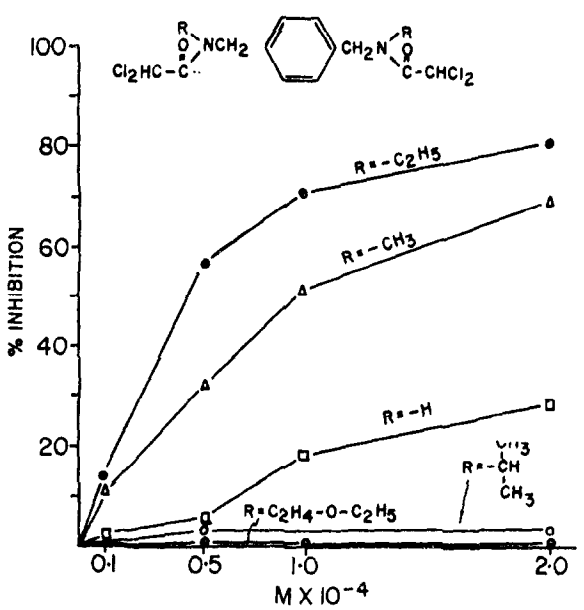


FIG. 4. The effect of various substitutions on the amide nitrogen of aromatic bis-dichloroacetamides on the oxidation of NADH by ETP. Each curve represents a dose-response determination using each drug under conditions as described in the text.

of the tertiary amides which are substituted with groups of somewhat bulky nature, e.g. ethoxyethyl, butyl, hexyl or isopropyl, appears to give any indication of being a potent antispermatogenic agent, so that the inhibition of electron transport appears to correlate better with antispermatogenic activity than with antiamebic activity.

It was of interest to us to determine if the inhibitory properties of these compounds depended on the bis configuration, since to the best of our knowledge this is a necessary structural feature for antispermatogenic activity. In Fig. 5, the titration curves of

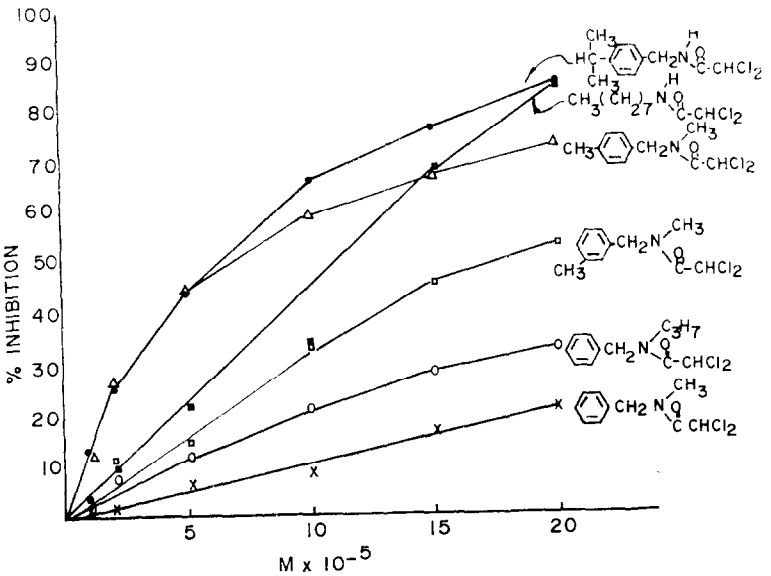


FIG. 5. Inhibition titration curves in the NADH oxidase assay of some dichloroacetamides lacking bis-symmetry. Conditions are the same as for Fig. 4 and the assay conditions are described in the text.

several dichloroacetamides show that this symmetry is not mandatory, although none of these compounds was as active as the most active bis-dichloroacetamide tested. This may simply be due to a less than optimal lipid solubility. In any case, these data suffice to rule out any special importance of the inter-atomic distance between the amide nitrogens, as has been suggested for the inhibition of aldehyde dehydrogenase by Dietrich and Hellerman.[3]

Figure 6 shows that this inhibitory response due to the dichloroacetamides occurs only with pyridine nucleotide-linked electron transport. Like tetraethylthiuram disulfide,[14] these compounds have no effect on succinoxidase activity at the same concentrations which nearly completely block the oxidation of NADH. In this particular experiment, *N,N'*-bis-(dichloroacetyl)-1,12-dodecanediamine, one of the more potent inhibitors, blocks the oxidation of NADH, but when succinate is added, respiration immediately accelerates (experiment 2). If the order of addition is changed such that the dichloroacetamide is added after succinate, there is no change in the slope of the oxygen uptake curve after the NADH has been consumed (experiment 1). If NADH is omitted completely, the same result obtains, i.e. there is no effect on succinate-supported respiration over a broad range of drug concentration.

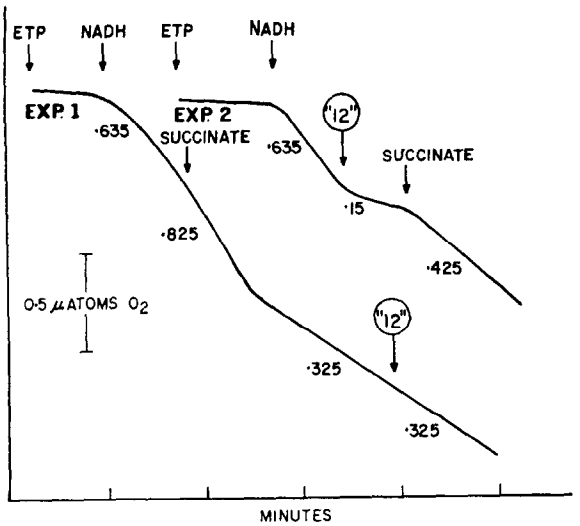


FIG. 6. The effect of *N,N'*-bis(dichloroacetyl)-1,12-dodecanediamine on oxygen uptake by ETP. Numbers adjacent to the oxygen traces refer to the specific activity of the oxygen uptake rates in μmoles/min/mg of protein. The number 12 refers to the dodecanediamide at a concentration of 5 μM. Details of the polarographic assay of NADH-oxidase are given in the text.

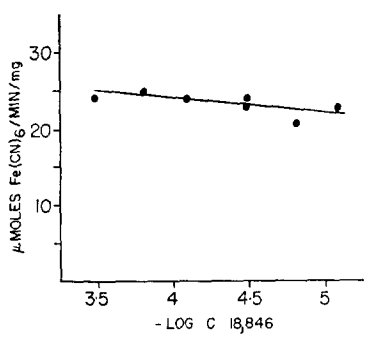


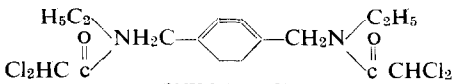
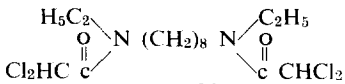
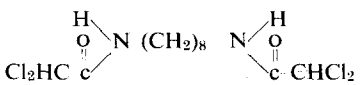
FIG. 7. Oxidation of NADH in the presence of *N,N'*-bis(dichloroacetyl)-1,9-nonanediamine (18,846) using ferricyanide as the electron acceptor. Ferricyanide reduction was determined spectrophotometrically by a modification of the method of Minakami *et al.*[12] as described in the text.

These results suggest that the dichloroacetamides block at some point in the substrate portion of the respiratory chain prior to the common pathway of electron flow, i.e. at a site similar to that blocked by amytal and rotenone.[15] The data of Fig. 7 and the results obtained in the NADH-coenzyme Q_1 assays suggest this as the site of inhibition since, like rotenone and amytal, the dichloroacetamides did not affect ferricyanide activity, but NADH-coenzyme Q_1 activity was markedly affected. In the latter assay, activities of 0·64, 0·43 and 0·13 μmole NADH oxidized per min per mg of protein were measured in the presence of none, 5 μM and 50 μM *N,N'*-bis(dichloroacetyl)-1,9-nonanediamine respectively. In the ferricyanide assays, activity tended to be increased slightly, a result one might expect if the block were beyond the site of ferricyanide reduction. In this test a concentration of 0·3 mM *N,N'*-ethyl-substituted

nonamethylene diamide, which had no inhibitory effect on NADH oxidation, the drug to protein ratio was 33 μmoles/mg of protein. Using the same drug and the same preparation of ETP, a drug to protein ratio of 0·37 μmole/mg of protein resulted in 60 per cent inhibition in a spectrophotometric assay of NADH oxidase. Parenthetically, although this inhibition of pyridine nucleotide-linked electron transport resembles that exhibited by tetraethylthiuram disulfide, the inhibitory activity of the latter compound can be prevented by addition of 2-mercaptoethanol to the mitochondrial particle suspension,[14] but this thiol has no effect on dichloroacetamide inhibition.

In Table 1 the data from two experiments which gave indirect evidence for binding

TABLE 1. INHIBITION OF NADH-OXIDASE BY BOUND BIS-DICHLOROACETAMIDES*

Exp.	Addition	Activity	% Inhibition
Exp. I	None	0·585	
Expt. I	 (WIN 13,099)	0·491	16
Expt. I		0·392	33
Expt. II	None	0·712	
	 (WIN 18,446)	0·448	37

* For each drug, 44 μmoles/mg of protein was added to rapidly stirring solutions of electron transfer particles. Evidence for binding is taken as an inhibition of NADH-oxidase activity after two washings by centrifugation in sucrose. Other details of the assay are given in the text.

are presented. Treatment with polymethylene or aromatic bis-dichloroacetamides resulted in inhibition of pyridine nucleotide oxidase activity after two washings by centrifugation in sucrose. In each case the level of drug added (44 μmoles/mg protein) was greater than any of the electron transfer components. If one assumes that the dichloroacetamides inhibit electron transport in a manner similar to that of rotenone, then this suggests either that only a small amount of the drug added is bound or that a considerable amount of nonspecific binding occurs. The latter is very likely, since the addition of untreated particles to drug-treated and washed particles results in a rate of oxygen uptake less than that which one would obtain from simple addition of rates, suggesting that nonspecific binding and re-equilibration with the fresh particles can occur. This explanation is speculative and the binding and localization of the dichloroacetamides are now under study using radioactive dichloroacetamides synthesized in our laboratory.

Effects on coupled respiration in intact mitochondria. The inhibitory effects on electron transport could also be verified in intact phosphorylating mitochondria. Table 2 shows that two of the more common and particularly potent antispermatogenic bis-dichloroacetamides, WIN 13,099 and WIN 18,446, both inhibit state 3

TABLE 2. UNCOUPLING OF RESPIRATION IN INTACT BEEF HEART MITOCHONDRIA BY WIN 13,099 AND WIN 18,446

Substrate	Addition*	Sp. act. state 3†	P/O
1. Pyruvate + malate	WIN 13,099 (5×10^{-5} M)	0·10	1·9
	WIN 18,446 (1×10^{-4} M)	0·11	1·6
	DMSO	0·18	2·5
	None	0·17	2·7
2. Succinate	None	0·28	1·8
	WIN 13,099 (5×10^{-5} M)	0·26	1·4

* WIN 13,099 is *N,N'*-1,4-xylylene-bis(*N*-ethyldichloroacetamide) and WIN 18,446 is *N,N'*-bis(dichloroacetyl)-1,8-octamethylene diamine. The effect of each dichloroacetamide was determined on intact mitochondria polarographically by the method of Chance and Williams[8] as described under Assays.

† Values are expressed as μatoms oxygen/min/mg protein.

pyridine nucleotide-linked respiration, whereas respiration supported by succinate was essentially unaffected. The efficiency of phosphorylation is also diminished to some extent in the drug-containing reaction mixtures. In extensive tests it was found that the polymethylene diamides are in general more potent uncouplers than are the phenyl-substituted diamides of roughly comparable size and lipid solubility. For this reason we investigated polymethylene diamides of longer chain length than WIN 18,446 and found these to be more active as uncouplers of oxidative phosphorylation than the shorter chain or the phenyl-substituted derivatives. This effect occurs with either pyridine nucleotide or flavin-linked substrates, but because of the inhibition of electron transport supported by pyridine nucleotide-linked substrates, the results are somewhat clearer with succinate as substrate.

It is clear from the oxygen uptake traces shown in Fig. 8 that the dodecamethylene

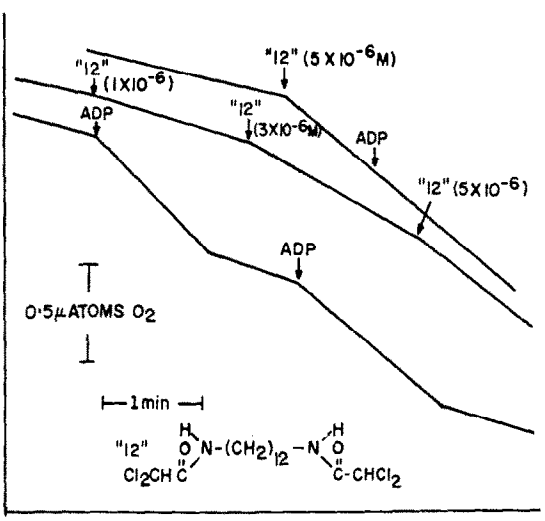


FIG. 8. The release of state 4 inhibited respiration by *N,N'*-bis(dichloroacetyl)dodecanediamine. The substrate in each case was succinate. The lower oxygen uptake trace is a control run using successive 1 μmole additions of ADP. In the middle trace, three successive additions of the dichloroacetamide were made, to give 1, 3 and finally 12 μM drug. In the upper trace, 5 μM diamide was added followed by 1 μmole ADP at the points indicated.

diamide begins to exhibit some uncoupling effects on succinate-linked oxidative phosphorylation at a concentration of about 10^{-6} M (2–4 nmoles drug/mg of protein). It is also clear that the uncoupling effect is essentially an immediate one and prevents the usual response to the addition of ADP. Much the same results are obtained with dichloroacetyloctamethylamine, indicating that, like the inhibition of pyridine nucleotide-linked electron transport, the bis configuration is not an absolute requirement for these effects on coupled systems.

A further indication of the uncoupling activity of the dichloroacetamides is the release from oligomycin inhibition (Fig. 9). It is well known that mitochondria do not

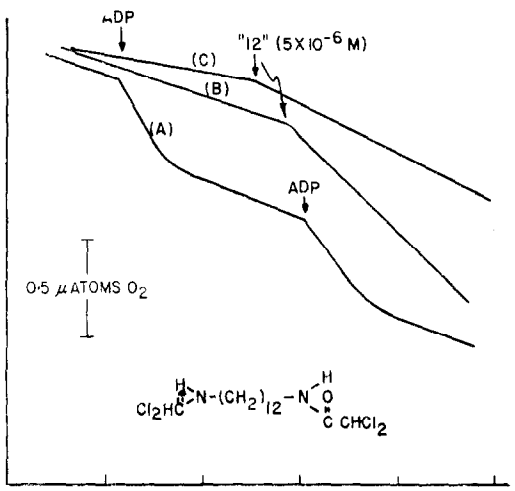


FIG. 9. Release of oligomycin-blocked respiration by *N,N'*-bis(dichloroacetyl)dodecanediamide. The lower trace is a control with pyruvate-malate as substrate. The middle and upper traces are test runs using succinate and pyruvate-malate respectively. In the test runs, sufficient oligomycin was present to prevent any response to the addition of ADP. Drug was added in dimethylsulfoxide at the places indicated.

respond to the addition of ADP by increased oxygen utilization in the presence of oligomycin. Mitochondria blocked in this manner react immediately to an addition of the dodecamethylene diamide with an increase in oxygen uptake, in a manner similar to that displayed by DNP. This response occurs with succinate or pyruvate plus malate as the substrate, but gain, due to the inhibitory effect on pyridine nucleotide-linked respiration, the effect is somewhat clearer with succinate-supported respiration.

In the experiments described by Figs. 8 and 9, the release of respiratory control with the higher level of the dodecanediamide (5 μM) appears to be essentially complete. This could be due to complete uncoupling of phosphorylation from oxidation or to a turnover of ATP sufficient to support a state 3 respiration. In order to test these possibilities, Mg^{2+} and DNP-stimulated ATPase determinations were made along with direct chemical measurements of the disappearance of phosphate coupled to oxidation, and these data appear in Figs. 10 and 11. Figure 10 shows that the same level of drug resulting in uncoupling of oxidative phosphorylation causes an immediate release of the inhibition of state 4 respiration, as one would expect of an uncoupler. Indeed the

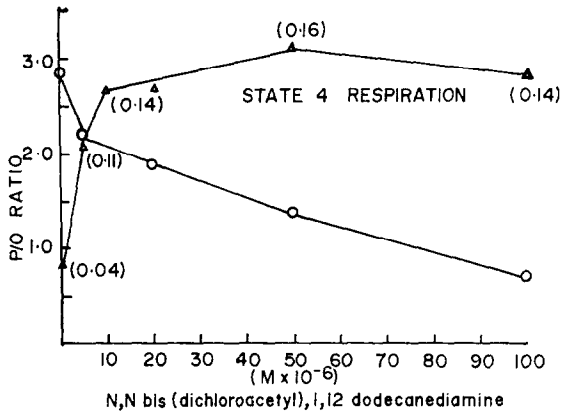


FIG. 10. Titration of *N,N'*-bis(dichloroacetyl)-1,12-dodecanediamine against oxidative phosphorylation and release of state 4 inhibited respiration. Oxidative phosphorylation was measured using a hexokinase–glucose trap as described in the text. Separate determinations were made to determine the extent of the release of state 4 respiration in a medium identical to that used for phosphorylation, except that the hexokinase–glucose trap was omitted. Numbers adjacent to the respiration traces refer to the rate of oxygen uptake in μatoms/min/mg of protein.

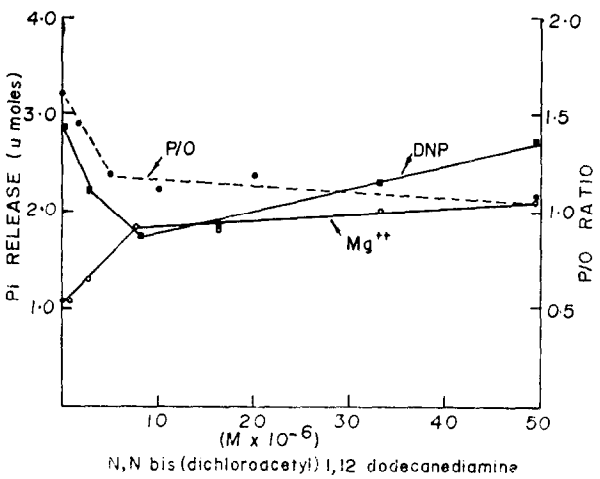


FIG. 11. The effect of *N,N'*-bis(dichloroacetyl)-1,12-dodecanediamine on Mg^{2+} (open circles) and DNP (closed squares) ATPase activities. The dotted lines represent the P/O ratio as measured by the disappearance of inorganic phosphate in a separate experiment using a hexokinase–glucose trap. For the ATPase assays, the drug was added in 10 μl DMSO and controls were run with and without dimethylsulfoxide, which had no effect on the ATPase activities at this level. ATPase activity was measured as described by Lindberg and Ernster[9] and phosphorus was determined as described by Martin and Doty.[10]

same results are obtained using dinitrophenol at nearly the exact concentrations as those used in this experiment, with the difference that dinitrophenol does not exhibit the marked curvilinear effect with respect to the phosphate esterification. Uncoupling is complete under these conditions using 35 μM DNP, whereas the decrease in phosphorylation is gradual using the dodecanediamide, and the particles are still phos-

phorylating to some extent at 100 μM while respiration is stimulated maximally at approximately 10 μM. Figure 11 shows that the most noticeable effects of the dodecanediamide on DNP- and Mg^{2+}-stimulated ATPase activity also obtain in the concentration range of 1–10 μM. In this case, the Mg^{2+} ATPase is stimulated to about double the base value without drug, while the DNP ATPase is inhibited at lower concentrations, but the activity slowly rises again at concentrations above 10 μM. Phosphorylation coupled to the oxidation of succinate is also plotted on the same graph for comparison. In each case illustrated in Fig. 11, it is clear that the concentration of drug necessary for the half maximal response is roughly the same, i.e. about 5 μM, suggesting an interrelationship between these effects.

DISCUSSION

The results presented above indicate that the antispermatogenic bis-dichloroacetamides affect both mitochondrial electron transport and the energy coupling systems associated with electron transport. The inhibition in the electron transfer system is strikingly similar to that reported for disulfiram (tetraethylthiuram disulfide) by Hassinen[14] in that oxidation of pyridine nucleotide-linked respiration is inhibited while oxidation of succinate is essentially unaffected. This parallelism is especially interesting since the dichloroacetamides also possess a disulfiram-like activity with respect to alcohol intolerance and, like disulfiram, block the oxidation *in vitro* of acetaldehyde.[3] There are, however, very clearcut differences between these agents. Disulfiram does not have marked effects on the P/O ratio and the effects of disulfiram can be prevented by 2-mercaptoethanol,[14] while the dichloroacetamides have a marked uncoupling effect on phosphorylation and the electron transport cannot be protected by 2-mercaptoethanol.

It is interesting that the mitochondrion offers a fairly good parallel to the antispermatogenic properties of the bis-dichloroacetamides. For example, the *N,N'*-ethoxyethyl derivative of the xylylene diamide is a potent amebicide, but lacks antispermatogenic activity and is without activity in the mitochondrial assays. Indeed all dichloroacetamides tested which have a bulky group substituted on the amide nitrogen are without activity, suggesting that the chemically active site involves an unhindered dichloroacetyl group. Further justification for this conclusion stems from the consideration that secondary as well as tertiary amides are active, eliminating the necessity for having an alkyl substituent on the amide nitrogen and the bridge joining the two amide portions of the compounds can be either aromatic or aliphatic. It may be recalled that the antibiotic chloramphenicol also contains the dichloroacetamide group, and it has been reported that this compound acts as an uncoupling agent in maize mitochondria[16] and can also inhibit the oxidation of NADH in mammalian mitochondria.[17] Taken together, these reports suggest that chloroamphenicol acts on mitochondria in a manner similar to the compounds reported here, although at considerably higher concentrations than the more active dichloroacetamides.

When the electron transport inhibiting activity is compared with the lipid solubility of the dichloroacetamides, a plateau is reached with some evidence for a biphasic response. These data do not appear here and will be reported separately; however, it is interesting to note that similar results are obtained in the antispermatogenic tests, but the optimal chain length is shorter, i.e. the best activity occurs with polymethylene

chain lengths of 6 through 9.[4] This may be a reflection of the basic differences in the two systems in that one is *in vivo* and the other is *in vitro*. We do know that the shorter chain derivatives are absorbed well, since these are potent drug metabolism inhibitors *in vivo*,[5] but we have no data concerning the absorption of highly lipid-soluble derivatives, although they show low or no activity in any of the tests *in vivo* reported thus far, except antiamebic activity, and presumably in this case rapid absorption is not a requirement for activity. Parenthetically, it is of interest that while we show here that activity in the electron transport system is directly proportional to chain length, this is not necessarily true when one investigates the interaction of the dichloroacetamides with microsomal systems.* The decamethylene and dodecamethylene derivatives are almost without activity in the drug metabolism system, and the short chain derivatives are the more active members, contrary to that which obtains in the mitochondrial system.

The mitochondrial effects of the dichloroacetamides, especially the long chain polymethylene derivatives, also resemble piericidin A in that both electron transport and energy conservation processes are affected. More work will have to be done to determine the extent of this resemblance. At the present time we can conclude that the dichloroacetamides possess the interesting properties of specifically inhibiting NADH-linked electron transport without affecting succinate-linked electron transport and uncoupling phosphorylation from oxidation of the succinate or pyridine nucleotide-linked substrates.

Finally, it is apparent that the list of drugs, toxins and other agents which affect mitochondrial function is very long and is still growing. It is therefore of some concern to distinguish between effects which are related to mechanism of action of these drugs and those which are nonspecific effects. We know that the concentration of drug which reaches the testes compares favorably with the concentrations which are effective *in vitro* and it has been suggested recently that *N,N'*-bis-(dichloroacetyl)-1,8-diamine may affect the energy-producing system in the testicular fluid.[18] Further work is needed before this point is established, but even if this is not the case, this group of drugs should prove to be a useful addition to the chemical tools available for the study of the energy-transducing systems and should be helpful in the clarification of the process. More work directed to these points is now in progress.

Acknowledgements—The expert technical assistance of Mrs. Marianne Jurkowitz is gratefully acknowledged.

* A. J. Merola, A. L. Beyler, G. O. Potts and J. D. Turnbull, unpublished results.

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