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STRUCTURAL REQUIREMENTS IN THE UNCOUPLING OF OXIDATIVE PHOSPHORYLATION BY *N,N'*-BIS(DICHLOROACETYL) DIAMINES*

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Abstract—The nature and extent of the disruption of mitochondrial function by bis-dichloroacetamides depends on their chemical nature and lipid solubility (partition coefficient). Secondary and tertiary amides are active in the inhibition of electron transport as are mono-, di- and trichloroacetamides. Uncoupling of oxidative phosphorylation is very much more dependent on chemical structure and it is shown that only lipid soluble secondary dichloroacetamides are active in affecting energy-linked mitochondrial function. Uncoupling phosphorylation from the oxidation of TMPD-ascorbate at the third site of phosphorylation appears to be less extensive than that supported by succinate or pyruvate plus malate. An apparant alkalization of the medium occurs when mitochondria are treated with the dichloroacetamide but not when treated with inactive acetamides. These data are discussed in light of the known respiratory effects of the dichloroacetamides on rodent testes.

CERTAIN *N,N'*-bis(dichloroacetyl) diamines inhibit spermatogenesis,[1] block alcohol metabolism,[2] and possess potent amebicidal activity.[3] We have shown that some of these compounds also inhibit drug metabolism *in vitro* and *in vivo*.[4] Recently, we reported that several members of this group of compounds are inhibitors of pyridine nucleotide-linked electron transport and also uncouple phosphorylation from oxidation in beef heart mitochondria.[5] Data obtained at that time and since suggest that these drugs act differently than the more classical uncouplers such as 2,4-dinitrophenol (DNP) and carbonyl cyanide *m*-chlorophenyl hydrazone (CCP). To some extent, the dichloroacetamides resemble tetraethylthiuram disulfide since this drug also inhibits pyridine nucleotide-linked oxidation with essentially no effect on succinoxidase activity.[6] This similarity in effects induced by the dichloroacetamides and tetraethylthiuram disulfide goes beyond the mitochondrial systems since both induce an intolerance to alcohol, presumably because of an inhibition of aldehyde dehydrogenase,[7] and recently it has been reported that the disulfide also inhibits drug metabolism.[8] The chemical mechanisms of action may differ, however, in that mitochondria can be protected by the addition of mercaptoethanol prior to tetraethylthiuram disulfide treatment but none of several thiols or dithiols protect mitochondria from the effects because of the dichloroacetamides. In this communication, experiments describing the structural requirements for the uncoupling activity of the bis-dichloroacetamides reveal a remarkable specificity which is absent in the inhibition of electron transfer.[9]

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In the coupled system only lipophilic secondary dichloroacetamides are active. Tertiary amides or several acyl derivatives other than dichloroacetyl are all inactive. These data, together with those concerning changes in membrane permeability,* suggest that these antispermatogenic agents may be useful tools in the study of the mitochondrial energy conserving process and the study of membrane permeability in general.

MATERIALS AND METHODS

Many of the acetamides used in this study were kindly supplied by the Sterling–Winthrop Research Institute, Rensselaer, N.Y. The various secondary dodecanediamides were prepared in our laboratory essentially as described by Surrey[10] using the appropriate acylchloride as acetylating agent, except in the synthesis of the non-chlorinated acetyl derivative which was synthesized by reaction of the diamine with anhydrous acetic anhydride. After crystallization from methanol, each compound was examined by thin-layer chromatography, infra-red and nuclear magnetic resonance spectroscopy and submitted for elemental analysis of carbon, hydrogen, nitrogen and chlorine with the following results (theoretical content in parenthesis): -acetyl, C, 67·27 (67·56), H, 11·25 (11·34), N, 10·10 (9·85) and Cl 0·00 (0·00); -monochloro, C, 54·27 (54·38), H, 8·39 (8·36), N, 8·05 (7·93) and Cl, 20·31 (20·07); dichloro, C, 45·63 (45·51), H, 6·65 (6·68), N, 6·38 (6·63) and Cl, 33·41 (33·59); trichloro, C, 39·27 (39·12), H, 5·30 (5·33), N, 5·40 (5·70) and Cl, 42·94 (43·31); 3-chloropropionyl, C, 56·81 (56·68), H, 8·87 (8·98), N, 7·27 (7·34) and Cl, 18·01 (18·60). These data indicated that the acetyl, monochloro, dichloro, trichloro, and 3-chloropropionyl derivatives of 1, 12-dodecanediamine were obtained in essentially pure form.

Beef heart mitochondria were prepared by Nagarse treatment in the presence of EGTA[11] and all protein determinations were made by a biuret procedure.[12]

Oxygen uptake was measured polarographically, using a Yellow Springs Instrument Co. electrode fitted to a water jacketed reaction vessel kept at 25°. Estimations of oxidative phosphorylation were made either by the method of Chance and Williams[13] using 1 μmole of ADP or by direct measurement of inorganic phosphate remaining after reaction, by the method of Martin and Doty,[14] using a hexokinase-glucose trapping system as described previously with succinate as substrate.[5] When pyruvate plus malate were used as substrates, they were each at a final concentration of 2 mM. Oxidation and phosphorylation in the cytochrome oxidase segment of the electron transfer chain was supported by tetramethyl-*p*-phenylenediamine (TMPD) and sodium ascorbate at 100 μM and 5 mM respectively. In all cases except those employing pyruvate plus malate as substrate, mitochondria were inhibited with sufficient rotenone to completely block pyridine nucleotide-linked endogenous respiration. Respiration by electron transfer particles using NADH as substrate was measured as described previously[5] or by using alcohol dehydrogenase and alcohol as an NADH generating system.

The changes in pH induced by dichloroacetamides shortly after the depletion of oxygen in solution were monitored with a conventional pH electrode in a lightly buffered solution containing 150 mM KCl, 2 mM tris chloride and 2 mM tris succinate at pH 7·0. The medium was flushed with nitrogen and kept under nitrogen to remove

* A. J. Merola and G. P. Brierley, unpublished results.

most of the dissolved oxygen prior to use in the reaction vessel. Drug additions were made in alcohol solution and were not gassed with nitrogen; therefore, a slight addition artifact is sometimes seen in the pH trace which coincides with the addition of a small amount of oxygen as evidenced by the polarographic recording of oxygen content.

RESULTS

Secondary versus tertiary amides. Previously,[5] we presented data which showed that the polymethylene bis-dichloroacetamides inhibited pyridine nucleotide-linked electron transport and that there was little difference in the activity of secondary and ethyl substituted tertiary amides. Subsequent analyses by the methods described by Hansch[15] tend to verify this conclusion and further suggest that differences which do appear in this assay system can best be explained by differences in the lipid solubility of the various compounds.[9] Although there is some covariance between R_m and both steric and inductive effects, the significance of the parabolic relationships obtained when these parameters are excluded supports the major role of lipid solubility in determining activity.[9] Similar results have been obtained in an analysis of the activity of aromatic bis-dichloroacetamides but these compounds are not included in this study for the sake of brevity. It should be emphasized that steric effects are undoubtedly operative in this system since, as we reported previously,[5] the addition of bulky groups such as ethoxyethyl and iso-propyl as the tertiary amide substituent on dichloroacetamides results in a complete loss of activity in the inhibition of electron transport. These data were obtained using aromatic dichloroacetamides and were not included in the analyses of the homologous series in our study. Furthermore, as our major interest was in active compounds and also mainly in the coupled system, which displays a greater degree of chemical specificity, we did not pursue this problem further.

In intact coupled mitochondria there is a striking difference in response to the secondary versus the tertiary amides, a difference which cannot be attributed to lipophilic activity. Figure 1 illustrates the requirement for an unsubstituted amide for potent uncoupling activity in intact mitochondria respiring with succinate as substrate. In this figure the increase in respiratory rate over the control (State 4) rate was determined by each homologue from diaminopropane to diaminododecane at 0·1 mM, a concentration in large excess with respect to the more potent uncouplers. The response to the addition of the amides is detectable nearly immediately and under these conditions the ADP:O ratio approaches zero with 20 μM *N,N'*-dichloroacetyl-1,-12-diaminododecane, and the respiratory rate is essentially the same as that obtained with the addition of ADP. Using the ethyl-substituted derivative of the same polymethylene chain length, there is no change in the ADP:O ratio at this concentration or at concentrations up to 100 μM. The ethyl substituted decamethylene derivative does lower the ADP:O ratio by approximately 10 per cent from a control value of 1·6 at the drug levels used in this experiment and this is reflected in the slight change in the rate of oxygen uptake shown in Fig. 1. This slight effect is not accompanied by a pH shift typical of uncouplers in general and with the secondary dichloroacetamides and is considered to be a marginal effect when compared with those induced by the secondary amides. The marked difference in the release of State 4 respiration by these compounds

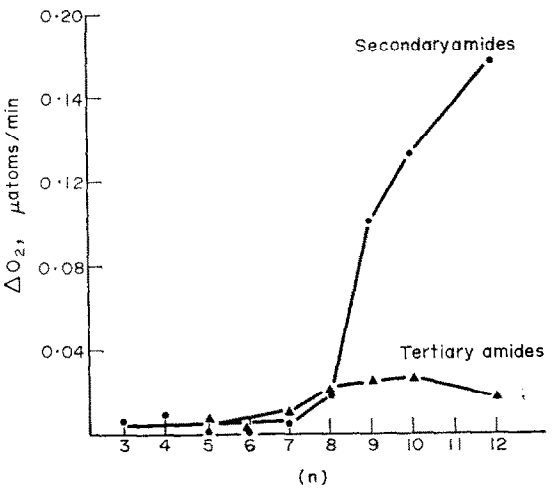

FIG. 1. Release of State 4 respiration by secondary (●——●) and ethyl substituted tertiary (▲——▲) dichloroacetamides. The medium was 0·23 M sucrose, 6 mM phosphate (K^+) pH 7·4, 6 mM $MgCl_2$ and 2 mM sodium succinate in a total volume of 5 ml. Mitochondrial protein concentration was 1 mg per ml. Addition of the various dichloroacetamides was made in 10 μl of dimethylsulfoxide to give a final concentration of 100 μM. The total increase in the rate of respiration over the rate obtained just prior to the addition of drug is plotted against the number of methylene units in the polymethylene chain.

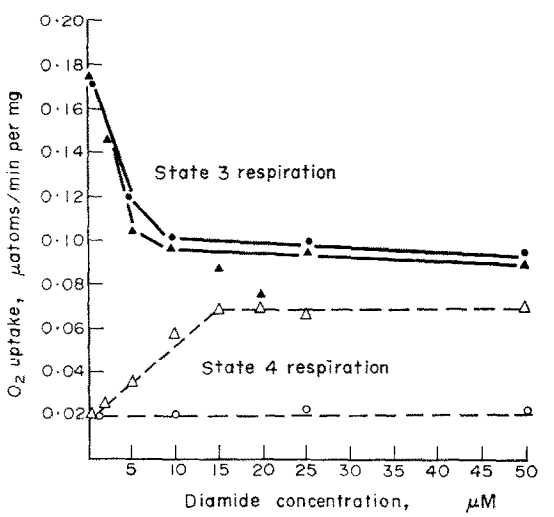

FIG. 2. Comparison of *N,N'*-bis(dichloroacetyl)-1,12-dodecanediamine and *N,N'*-bis(dichloroacetyl)-*N,N'*-diethyl-1,12-dodecanediamine in the stimulation of State 4 respiration and inhibition of State 3 respiration supported by pyruvate plus malate. The medium was the same as that of Fig. 1 except that when measuring the inhibition of State 3 respiration 1 μmole of ADP was present (closed circles and triangles). The secondary amide is plotted with circles and the ethyl substituted tertiary amide with triangles.

cannot be because of lipid solubility alone, since *N,N'*-dichloroacetyl-1,12-diaminododecane, the most potent uncoupling agent in any of these series found this far, has an R_m in 50% acetone almost identical to the *N,N'* ethyl substituted tertiary nonamethylene diamide (0·267 ± 0·0010 vs. 0·258 ± 0·033) and the latter has very little uncoupling activity.

Further evidence supporting the requirement for a secondary amide structure is presented in Figs. 2 and 3. State 3 respiration with pyruvate plus malate as substrate is inhibited nearly identically by either *N,N'* ethyl substituted or unsubstituted dodecanediamide confirming results obtained previously with submitochondrial particles.[5] In contrast, only the secondary amide releases respiration inhibited by a lack of a phosphate acceptor and in this case the half-maximal response occurs at approximately 5–6 μM, which is the same concentration necessary for half-maximal effects on ATPase activity and oxidative phosphorylation measured by uptake of inorganic phosphate.[5] Endogenous substrate-supported respiration was also markedly stimulated by secondary but not tertiary bis-dichloroacetamides (Fig. 3), verifying that the increased rate of respiration cannot be because of an increased permeability of the mitochondrial particles to substrate anions because, perhaps, of a detergent-like action of these lipophilic compounds. In this experiment, the decanediamide which was used was not as active as the dodecane derivative, but the qualitative results lead to the same

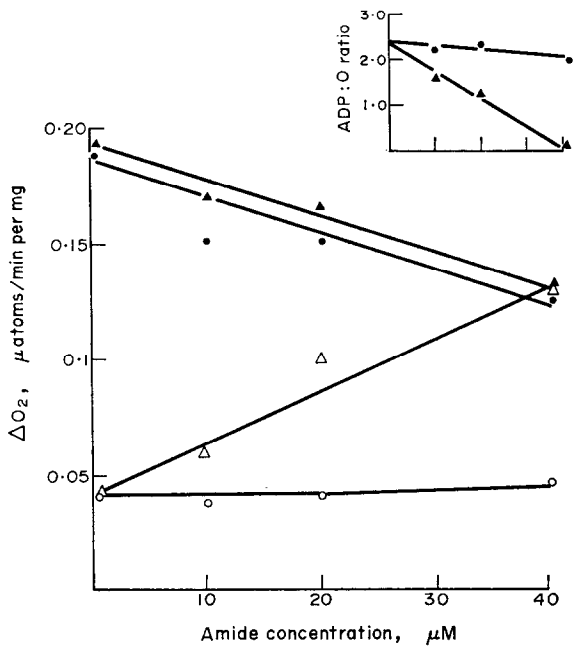

FIG. 3. Comparison of *N,N'*-bis(dichloroacetyl)-1,10-decanediamine and *N,N'*-bis(dichloroacetyl)-*N,N'*-diethyl-1,10-decanediamine in the release of State 4 respiration supported by endogenous substrate(s) and inhibition of State 3 respiration supported by pyruvate plus malate. The medium was the same as that used in Fig. 2 except that substrates were added after the drug effects on endogenous respiration were determined. The insert shows the effect of these compounds on the apparent ADP:O ratio at the same concentration. The open circles and triangles refer to the release of State 4 respiration by the tertiary and secondary amides, respectively, and the closed circles and triangles refer to the effects of these same compounds on State 3 respiration.

conclusion and at 40 μM the respiratory control was lost with the secondary amide giving an apparent ADP:O ratio approaching zero. The effect of the tertiary amide was again marginal in both the ADP:O ratio and release of State 4 respiration. Parenthetically, it should be added that none of the diamides supported respiration in the absence of exogenous substrate in rotenone-blocked mitochondria or electron transfer particles and also that the increased respiration was cyanide-sensitive in all cases.

Structural requirements in the amide group. When the inhibition of electron transport is the criterion for activity, there appears to be little specificity in the structure of the acyl group between the various chloroacetyl derivatives and the chloropropionyl derivative of diaminododecane (Fig. 4). The acetyl derivative is, however, completely inactive suggesting the necessity of having at least one chlorine on the molecule. In general these data also suggest a dependence of activity on the partition coefficient, at least at the lower drug levels, since we know that the relative lipid solubility increases as the acetyl group is substituted with chlorine. In contrast to these data, a remarkable specificity for the dichloroacetyl group is evident based on the ability of various derivatives to stimulate mitochondria respiring in State 4. These data appear in Table 1. The acetyl, monochoroacetyl, trichloroacetyl and 3-chloropropionyl derivatives are without marked activity in this system. Addition of ADP to the reaction mixture immediately after addition of the dichloroacetyl compound caused no further increase in the rate of respiration, indicating that respiration under these conditions was maximal and a polarographic P/O measurement was not possible in this system. Separate assays of oxidative phosphorylation using a hexokinase-glucose trapping system verified that only the dichloroacetyl derivative had any real effect on phosphorylation. For example, under conditions similar to those of Table 1, 10 μM

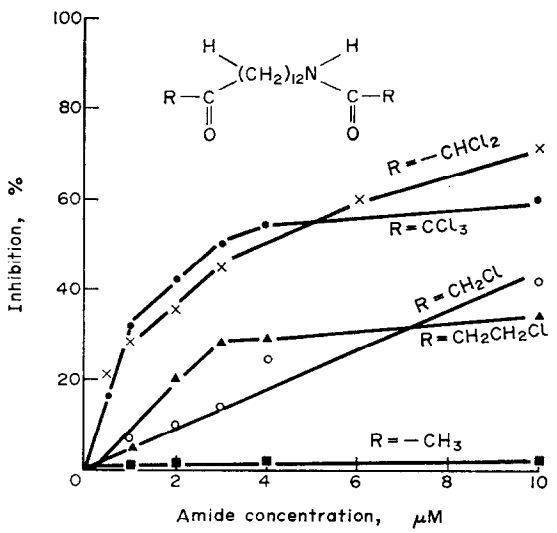

FIG. 4. Effect of changes in the acyl group on the inhibition of electron transport in submitochondrial particles. Each of the drugs was added in ethanol to the medium which contained 1·5 mg particle protein, 0·22 M sucrose, 5 mM $MgCl_2$, 4 mM phosphate (K^+) pH 7·4 and a NADH generating system consisting of alcohol dehydrogenase, alcohol and NAD sufficient to support oxygen uptake maximally.

TABLE 1. EFFECT OF THE *N,N'* AMIDE SUBSTITUENT ON STIMULATION OF STATE 4 RESPIRATION BY DODECANEDIAMIDES

Substituent	Concentration (μM)	Respiration rate*
Acetyl	0	0·050
	50	0·050
	100	0·050
Monochloro	0	0·060
	50	0·065
	100	0·068
Dichloro	0	0·045
	50	0·235
Trichloro	0	0·045
	50	0·045
	100	0·045
3-Chloropropionyl	0	0·040
	50	0·048

* Respiration rate is expressed as μatoms O_2 per minute per milligram protein. The reaction medium was the same as that of Fig. 1.

*N,N'*bis(dichloroacetyl)-1,12-diaminododecane lowered the P/O from 1·8 to 1·0 with succinate as the substrate. This is quite compatible with the data we reported previously and emphasizes the fact that the dichloroacetamides are not complete uncoupling agents. This latter point is obvious from an examination of the titration of the dichloro derivative against oxidative phosphorylation supported by pyruvate plus malate, succinate or TMPD-ascorbate seen in Fig. 5. These data should not be taken to mean that there is no effect at the third phosphorylation site but rather that phosphorylation at this site, perhaps by some peculiarity in the assay system, is least sensitive to the effects of these drugs. In line with this is the finding, not shown in the figure, that State 4 respiration coupled to the oxidation of TMPD-ascorbate is stimulated by the same concentration range of the dodecane derivative as that which stimulates respiration coupled to succinate or pyruvate plus malate oxidation. For example, about 20 μM drug nearly doubles the rate of oxygen uptake as compared to the control rate. However, oxidation of TMPD-ascorbate can be further stimulated shortly after the addition of the dodecanediamide by the addition of CCP. This is not the case when succinate is the substrate and aside from illustrating the lower sensitivity to the uncoupling activity of the bis-dichloroacetamides at site III, this also suggests that these reagents may act in a manner different from the action of CCP.

The requirement for the dichloroacetyl group can also be shown in lightly buffered media by the production of a slight but reproducible increase in pH induced by addition of drug shortly after the depletion of oxygen from the medium. As shown in Fig. 6, the dichloroacetyl derivative induces a rapid increase in pH equivalent to the disappearance of about 140 n equivalents of proton when added a few seconds after oxygen depletion. No effect is evident after the addition of the acetyl, monochloroacetyl or trichloroacetyl derivatives except for a slight inhibition of the effect because of

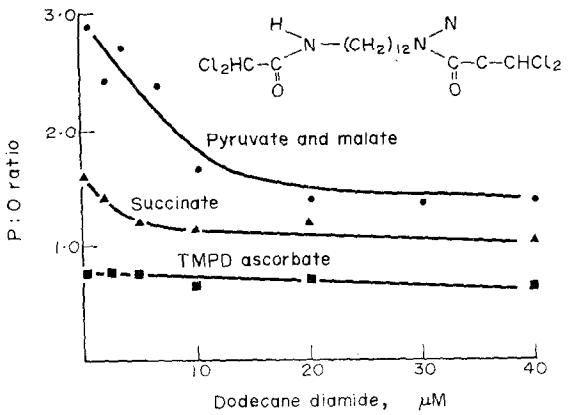

FIG. 5. Effect of *N,N'*-bis(dichloroacetyl)-1,12-dodecanediamine on oxidative phosphorylation supported by pyruvate plus malate (●——●), succinate (▲——▲) and TMPD-ascorbate (■——■). Oxygen was measured polarographically, and phosphorus esterification was determined by measuring the disappearance of inorganic phosphate according to Martin and Doty[14] in the presence of a hexokinase-glucose trapping system. Pyruvate plus malate were each 2 mM, succinate was 2 mM, TMPD was 100 μM and ascorbate was 5 mM. Mitochondrial protein was 5 mg when pyruvate plus malate and succinate were substrates and 1·25 mg when TMPD-ascorbate was the source of reducing equivalents.

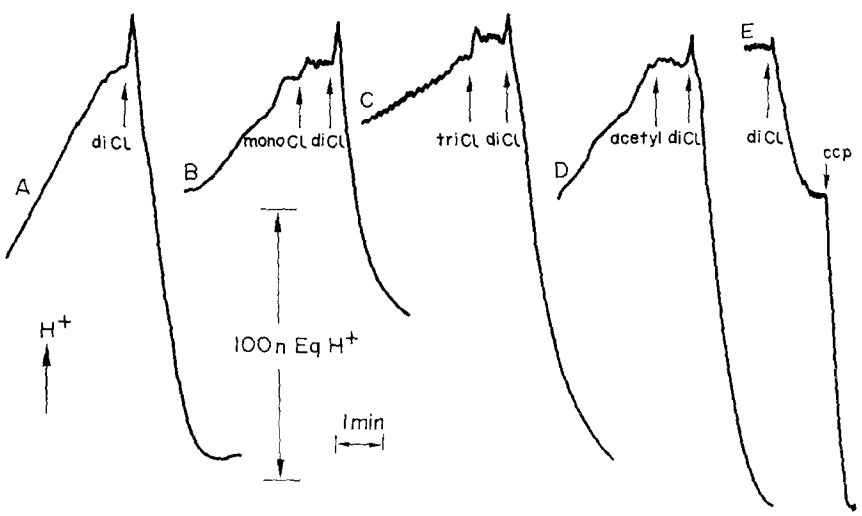

FIG. 6. Change in pH induced by *N,N'*-bis(dichloroacetyl)-1,12-dodecanediamine after depletion of oxygen (curves A–D) and under aerobic conditions (curve E). In curves A–D, 0·1 μmole of the various acetamides was added in ethanol approximately 30 sec after the depletion of oxygen. When there was no effect (monochloro, trichloro, acetyl), a second pulse containing the dichloro derivative was added. Curve E is a trace of a separate experiment under aerobic conditions. In this case 0·1 μmole of the dichloro derivative was added followed by the addition of 6 nmoles CCP after the pH trace had stabilized.

a subsequent addition of the dichloroacetyl derivative in the case of the monochloroacetamide. Results obtained with ethyl-substituted tertiary decane and dodecaneamides are not shown here, but were identical, i.e. neither induced a discernable pH shift but both inhibited to some extent the shift induced by a subsequent addition of the active dichloroacetamide. This pH effect appears to be similar both in rate and extent to those induced by CCP under the same conditions. Attention should be directed to curve E which is a trace of pH under aerobic conditions. In this case the pH change is considerably greater with CCP, again verifying the comparatively weak uncoupling effect of the dichloroacetamide under respiring conditions and perhaps, when considered with other evidence, also suggesting that a different mechanism of uncoupling action may be operative.

DISCUSSION

The mechanism of antispermatogenic action of the bis-dichloroacetamides is not known. It has been reported that they have no effect on Leydig's cells or pituitary gonadotrophic activity and in general that there is a lack of marked hormone properties.[1] In a systematic study we have accumulated a considerable amount of information on the action of these compounds in several systems where they act in either fairly low concentration *in vitro* or at dose levels quite compatible with those necessary for antispermatogenic activity. The data reported here show fairly broad structural requirements for inhibitory activity in the electron-transfer chain. Mono-, di- and trichloroacetyl derivatives, as well as the 3-chloropropionyl derivative of the dodecanediamide, all inhibit pyridine nucleotide-linked electron transport at μM concentrations. Aromatic derivatives and both tertiary and secondary amides are also active in this regard.[9] In fact, it appears that the only real requirements for activity in the inhibition of electron transport is a partition coefficient suggesting a reasonably high lipid solubility[9] and at least one chlorine substituent on the acyl group.

For uncoupling phosphorylation from oxidation there appear to be very strict requirements. Lipid solubility is, of course, important in this system also, but other parameters emerge which play a greater role in determining activity. Substitution of an alkyl group to give a tertiary amide causes a near complete loss of activity even though the compounds may possess solubilities, based on partition data, nearly identical to very active secondary amides. A very strict requirement in the nature of the acyl group also exists. Of the derivatives tested, only the dichloroacetyl compound is an active uncoupler and only this derivative stimulates Mg^{2+} ATPase[5] and induces a rapid pH change reminiscent of the change induced by DNP or CCP in weakly buffered media. Addition of chlorine to the acyl group would tend to make the carbonyl carbon even more electrophilic than it already is, and the oxygen would tend to become nucleophilic and offer a suitable site for electrophiles; for example, hydrogen ion. Proton conductance is in fact one of the proposed mechanisms for uncoupling activity. According to this reasoning, the trichloro derivative should be even more reactive with respect to the carbonyl group, but this compound is without uncoupling activity. In this case, steric effects may play a role in limiting activity. The monochloro derivative does apparently compete with the active compound, however, even though it has no uncoupling effect of its own, suggesting the competition for

some site on or in the mitochondrial coupling membrane. It follows, then, that the minimal requirements for uncoupling are shown in Fig. 7, with R apparently only lending to the molecule the proper degree of lipid solubility for interaction with the lipid rich mitochondrial particles. When this structure is compared with those known to have antispermatogenic activity, an apparent difference is evident in that the ethyl substituted amide is active in blocking spermatogenesis.[1] It follows that uncoupling or the primary chemical event leading to uncoupling can be the mechanism for antispermatogenesis only if the tertiary amides undergo dealkylation *in vivo*. While this is a fairly common route of drug metabolism, our studies thus far have identified only the dehalogenated and hydroxylated metabolite.

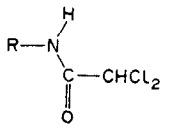

FIG. 7. Structure.

It is interesting to consider the effects on electron transport as reported previously and in this paper in the light of a report by Kar *et al.*[16] showing a marked accumulation of lactic acid in the seminiferous tubules of rats medicated with *N,N'*-bis (dichloroacetyl)-1,8-octamethylenediamine. This accumulation of lactic acid would be predicted if mitochondrial electron transport were inhibited. One might also expect a lower bicarbonate concentration if the oxidation of Krebs cycle intermediates were inhibited and a lowering of bicarbonate was also reported in the study by Kar *et al.*[16] Our data tend to support the suggestion made by these authors that lowered oxygen uptake and the resulting dependency of the tubules on anaerobic glycolysis may play a role in the arrest of spermatogenesis. It should, however, be kept in mind that we have shown these compounds to be inhibitors of microsomal oxidation at pharmacological dose levels,[4] and this activity should also be considered in any search for mechanism of action.

Finally, we would like to emphasize the possible usefulness of the dichloroacetamides as tools in the study of mitochondrial function. The compounds are fairly simple and easy to manipulate chemically. Their action on mitochondria appears to be either milder than uncouplers such as DNP or CCP or to have a different mechanism of action, so that they should be of interest from this point of view as well.

For example, under conditions described in Fig. 5, DNP or CCP would cause complete uncoupling of phosphorylation from oxidation. This is clearly not the case with the dichloroacetamides even though the pH change affected by the active members of this group of compounds is as rapid and extensive as that obtained with DNP or CCP under passive conditions and even though the stimulation of State 4 respiration is maximal. Recent studies in simple, lightly buffered salts media suggest that this incomplete uncoupling may be because of a transient response to the drugs. This transient response is also seen in the pH trace under active conditions, in the response to ADP and in the rates of respiration after addition of drug.*

* A. J. Merola and G. P. Brierley, unpublished observations.

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