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INHIBITION OF SOME MITOCHONDRIAL FUNCTIONS BY ACROLEIN AND METHYLVINYLKETONE

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Abstract—The effects of acrolein and methylvinylketone on some mitochondrial processes have been studied. The respiration with various substrates is inhibited. The sensitivity to acrolein decreases in the following order: ADP-stimulated respiration with glutamate, DNP-stimulated respiration with glutamate, ADP-stimulated respiration with succinate, DNP-stimulated respiration with succinate and respiration with NADH of aged mitochondria. It is suggested that acrolein acts on three different sites; glutamate transport, Pi transport and succinodehydrogenase. The effect on Pi transport is competitive with respect to Pi. Methylvinylketone shows the same effects as acrolein, however, it is less effective by one order of magnitude.

SPERMINE, spermidine and other polyamines are oxidized to the corresponding aldehydes by plasma amino oxidase.[1–3] These aminoaldehydes are toxic to Ehrlich ascites tumor cells,[2] bacteria,[4–7] some bacteriophages,[8–11] and viruses[12] and inhibit DNA-, RNA- and protein syntheses in *Escherichia coli*[1,6,13] and Ehrlich ascites tumor cells.[2]

The oxidized polyamines are unstable and at pH 7·0, 37° and low concentration undergo β-elimination to yield acrolein and the corresponding amine.[1,14,16] Acrolein is able to substitute for the oxidized polyamines with respect to their effects on thymidine, uracil or leucine incorporation in the corresponding macromolecules in *E. coli*.[1] From these facts it was suggested that the acrolein formed is responsible for at least a major portion of these inhibitory effects.[1]

Acrolein was also shown to affect some liver enzymes after injection or inhalation.[16,17] More recently Alarcon and Meienhofer[18] suggested, that activation of cyclophosphamide in the liver involves formation of acrolein.

The present paper deals with the action of acrolein and methylvinylketone on the respiration of isolated rat liver mitochondria with various substrates.

MATERIALS AND METHODS

Preparation of mitochondria. Rat liver mitochondria were isolated according to the method of Myers and Slater.[19]

Incubation. For oxygen uptake measurement, mitochondria were incubated at 25° in a medium of the following composition (oxygen assay medium): 6·6 mM PO_4^{3-} (KH_2PO_4/K_2HPO_4 buffer pH 7·4), 20 mM glycyl–glycine buffer pH 7·4, 1 mM

List of abbreviations: Acr. acrolein, MVK methylvinylketone, ATPase adenosinetriphosphatase, DNP 2,4-dinitrophenol, Pi inorganic phosphate, TCA trichloraceticacid.

EDTA, 80 mM KCl, 50 mM sucrose, 6·6 mM $MgCl_2$, when present 6·6 mM succinate, 6·6 mM glutamate, 3·3 mM β-hydroxybutyrate, 0·1 mM 2,4-dinitrophenol, 0·17 mM ADP, 1 μg rotenone, present only when respiration with succinate was measured; final volume 3 ml.

To study the effects of acrolein and methylvinylketone on mitochondrial respiration, mitochondria were incubated in the oxygen assay medium with the inhibitors. Three min after the addition of the inhibitor the substrate was added, followed 1 min later by ADP, followed 1 min later still by DNP.

Enzyme assay. ATPase activity was measured according to Myers and Slater in a medium containing: 4 mM ATP, 76 mM KCl, 1 mM EDTA, 108 mM sucrose, 3 mM $MgCl_2$, 20 mM Tris–HCl pH 7·4. Mitochondria were incubated in 0·1 ml ice-cold 0·25 M sucrose with or without the inhibitor for 5 min and the reaction started by the addition of the incubation mixture. The final volume was 1·5 ml. After 15 min at 25° the reaction was stopped by the addition of 0·5 ml TCA 20%.

Distribution of inorganic phosphate. To determine the Pi distribution between mitochondria and the medium after stimulation of ATPase with DNP, mitochondria were incubated with or without acrolein or methylvinylketone as described for the enzyme assay and the reaction was started by the addition of the incubation mixture to the ATPase assay medium. After 5 min at 25° the reaction was stopped by centrifugation at 8000 *g* for 3 min and the Pi content of the supernatant and the pellet was determined after deproteinization with trichloraceticacid 5%. The Pi content of the intramitochondrial space was estimated as described by Tyler.[20]

Mitochondrial swelling. Mitochondria incubated with or without the inhibitor as described above were added to a medium of the following composition: 0·1 M ammonium glutamate, 0·1 mM EGTA and 1 μg rotenone, final volume 3 ml. The rates of swelling were measured by following the decrease of optical density at 550 nm with a Zeiss spectrophotometer.

Analytical determinations. Inorganic phosphate was determined after Lindberg and Ernster.[21] The protein concentration of the mitochondrial suspension was estimated by the biuret method[22] using samples clarified with 0·2% sodium cholate as described by Tyler.[20] Oxygen uptake was measured polarographically with a Clark type electrode. Additional details are given in the Figure and Table Legends.

Chemicals. Acrolein and methylvinylketone (Schuchardt, Munich) were always freshly distilled before use. Acrolein was dissolved in water; methylvinylketone was used as an ethanolic solution. If necessary all solutions were adjusted to pH 7·4 with Tris base.

All chemical reagents were of analytical grade.

RESULTS

Effects of acrolein on mitochondrial respiration. From Table 1 it can be seen that acrolein inhibits the respiratory response to ADP + Pi with succinate or glutamate or β-hydroxybutyrate as substrates. MVK exerts the same effects.

The effect on the oxygen uptake stimulated by ADP in the presence of glutamate or β-hydroxybutyrate is cancelled by the addition of DNP only to a minor degree, whereas the inhibition of the succinate oxidation is almost abolished by the addition of DNP. Fifty per cent inhibition is brought about at 22 nmoles/mg protein for acrolein

TABLE 1. EFFECTS OF ACROLEIN ON THE RESPIRATION OF INTACT MITOCHONDRIA

Substrate	Acrolein (nmoles/mg protein)	Oxygen uptake (natoms/mg protein × min) in the presence of Pi	Pi + ADP	Pi + ADP + DNP
Succinate	0	18	76	114
	315	15	15	72
Glutamate	0	5	40	43
	60	7	8	9
β-Hydroxybutyrate	0	6	32	33
	60	8	14	20

Composition of the medium and conditions of incubation are described in materials and methods.

and 200 nmoles for MVK in the case of glutamate oxydation. β-Hydroxybutyrate oxidation seems to be a little less sensitive. ADP-stimulated respiration with succinate is significantly less sensitive. To get half maximal inhibition 75 nmoles/mg protein for acrolein and 750 nmoles/mg protein for MVK are needed.

Experiments depicted in Figs. 1–3 revealed that acrolein concentrations, causing inhibition of glutamate oxidation or succinate oxidation in the presence of ADP, have no effect on the respiration of aged mitochondria with NADH. Succinate oxidation by aged mitochondria is more sensitive than NADH oxidation (Fig. 3). Differences exist in the sensitivity to acrolein between ADP- and DNP-stimulated respiration with glutamate or succinate. In both cases the DNP-stimulated respiration is less sensitive. The DNP-stimulated respiration with glutamate, however, is more sensitive to the inhibitory effect of acrolein than the DNP-stimulated respiration with succinate (compare Figs. 1 and 2).

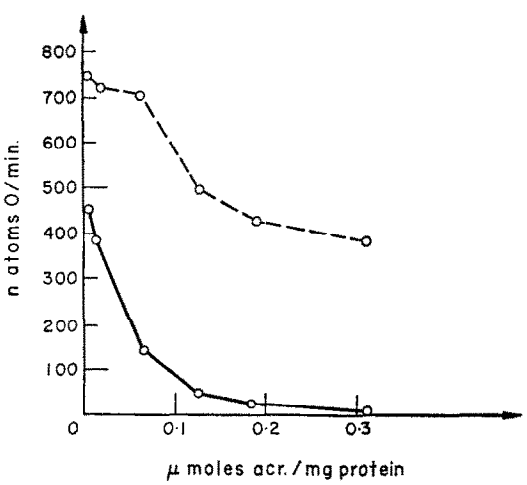

FIG. 1. ADP- and DNP-stimulated respiration with succinate in relation to acrolein concentration. ○——○ Respiration stimulated by ADP, ○- - -○ respiration stimulated by DNP. Mitochondria were suspended in the medium described in materials and methods, additional 1 μg rotenone was present. Mitochondria, 7·5 mg protein, were incubated with the inhibitor in the assay medium. Three min after the addition of acrolein 6·6 mM succinate, was added followed by mM Pi and 0·1 mM DNP at 1 min intervals.

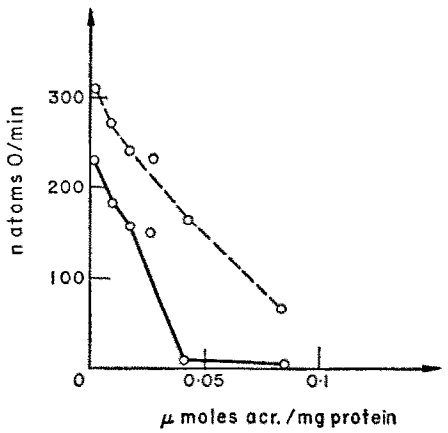

FIG. 2. ADP- and DNP-stimulated respiration with glutamate in relation to acrolein. O——O Respiration stimulated by ADP, O- - -O respiration stimulated by DNP. Experimental conditions as in Fig. 1 except that rotenone was omitted. 5·5 mg mitochondrial protein.

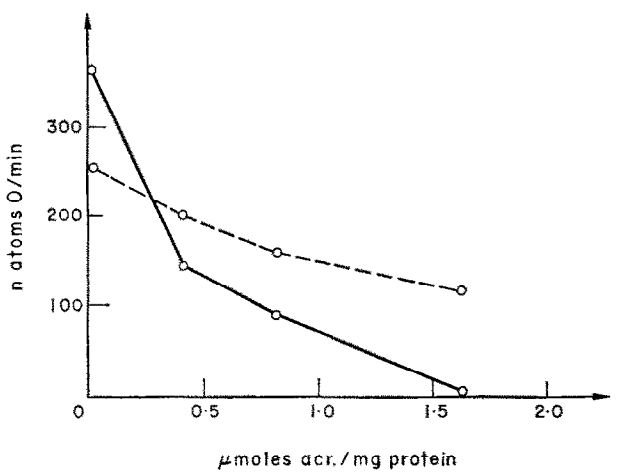

FIG. 3. Response of the respiration of aged mitochondria to acrolein. O——O Respiration with succinate, O- - -O respiration with NADH. Aged mitochondria were obtained by incubating mitochondria in water for 3 min at 37°. The assay medium was that described in materials and methods, additional 1 μg rotenone and 1 mg cytochrome *c* was present. 5·2 mg mitochondrial protein.

Effect of Pi on acrolein inhibited respiration. Figure 4 shows a Lineweaver–Burk plot of the succinate oxidation in the presence or absence of acrolein, dependent on the Pi concentration. Obviously a competition of acrolein with Pi takes place. A similar plot was obtained using MVK.

Effect of acrolein on the activity of the DNP-stimulated ATPase. Figure 5 shows the response of the DNP-stimulated ATPase-activity to increasing inhibitor concentrations. Complete inhibition could not be obtained even with high inhibitor–protein ratios.

The Pi content of inhibitor-treated mitochondria at the end of the ATPase experiment is much higher than that of untreated mitochondria (Fig. 5, trace 1).

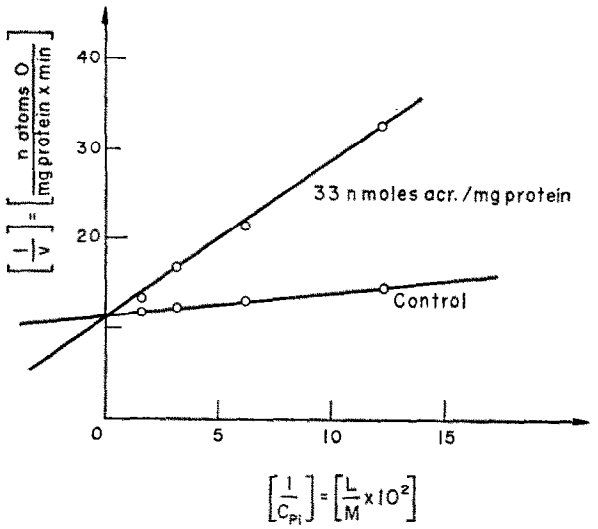

FIG. 4. Kinetics of acrolein inhibited succinate oxidation. The reaction medium had the following composition: 80 mM KCl, 50 mM sucrose, 20 mM glycyl-glycine buffer pH 7·4, 1 mM EDTA, 6·6 mM $MgCl_2$, 1 μg rotenone, 0·33 mM ADP, 3·0 mg mitochondrial protein, final volume 3·0 ml. Three min after the addition of the inhibitor 20 μM succinate was added and after another minute more Pi was added. v = Respiration rate in the presence of Pi minus respiration rate in the absence of Pi.[23]

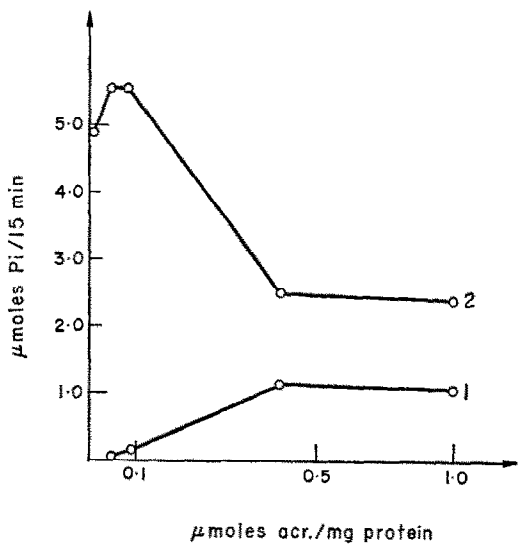

FIG. 5. The effect of acrolein on the DNP-stimulated ATPase. Trace 1, Pi content of the mitochondria, trace 2, Pi content of the supernatant. Experimental conditions are described in Methods. Mitochondrial protein 5·3 mg.

Effect of acrolein on mitochondrial swelling in ammonium–glutamate. Mitochondria swell in isotonic ammonium salt solutions if the anion is able to penetrate the mitochondrial membrane. In order to locate the point of attack of acrolein on glutamate oxidation, the effect on swelling in ammonium glutamate was studied.

TABLE 2. INHIBITION OF MITOCHONDRIAL SWELLING IN 0·1 M AMMONIUM GLUTAMATE BY ACROLEIN

Acrolein (μmoles/mg protein)	Rate of swelling (ΔE/min)*
0	0·30
0·28	0·22
0·56	0·12

* The first minute after addition of mitochondria to the assay medium was taken to calculate the rate of swelling.

Table 2 shows that acrolein inhibits mitochondrial swelling in ammonium glutamate, indicating that glutamate entry into mitochondria is affected.

DISCUSSION

The results show clearly that acrolein is a potent inhibitor of some mitochondrial processes. Of all processes investigated, the state 3 respiration with glutamate shows the greatest sensitivity to acrolein. The sensitivity decreases in the following order: ADP-stimulated respiration with glutamate, ADP-stimulated oxidation of succinate, DNP-stimulated oxidation of succinate and respiration with NADH of aged mitochondria. Though state 3 respiration with all the 3 substrates tested is inhibited, there are differences with respect to the mechanism of inhibition. DNP-stimulated respiration with glutamate as well as DNP-stimulated respiration with succinate is less sensitive to the aldehyde than the corresponding ADP-stimulated respirations. These and the accumulation of Pi within the mitochondria after ATPase has been stimulated by DNP in the presence of acrolein unequivocally indicate that the Pi transporting system is one point of attack. The same mode of action was found for some SH-reagents.[18,20,24–26] The inhibition of Pi transport is competitive with respect to Pi.

Two systems are responsible for phosphate transport in either direction across the mitochondrial membrane, the phosphate–hydroxyl exchange carrier and the dicarboxylate exchange carrier.[27] Phosphate, which is liberated by the DNP-stimulated ATPase activity, is transported from the matrix space of the mitochondria into the medium by the phosphate–hydroxyl translocator. As no dicarboxylate ions are present in the ATPase assay medium, the phosphate–dicarboxylate exchange carrier does not operate. Phosphate accumulation within MVK- or acrolein-treated mitochondria during the ATP hydrolysis indicates that the inhibitors affect the phosphate–hydroxyl exchange carrier. The present studies provide no evidence if the second phosphate translocator is affected.

Higher acrolein concentrations inhibit DNP-stimulated respiration with succinate. This effect must be due to an inhibition of the succinate dehydrogenase and not to that of the electron transport as the respiration with NADH of aged mitochondria is not affected to the same extent at this concentration.

If the inhibition of the state 3 respiration with glutamate is also due to the inhibition

of Pi transport, the acrolein concentration for half maximal inhibition must be comparable with that for the respiration with succinate. Obviously, this is not the case. Respiration with glutamate is 3–4-times more sensitive to acrolein than respiration with succinate. Therefore a further point of attack must exist. Inhibition of mitochondrial swelling in isotonic ammonium glutamate which indicates inhibition of glutamate uptake suggests that acrolein acts on glutamate oxidation at a site before the entry of NADH in the respiratory chain; presumably the glutamate carrier[28] itself is inhibited. The effect of acrolein, apparent in the inhibition of the state 3 respiration with glutamate results from the inhibition of the Pi transport plus the inhibition of the glutamate transport, and perhaps from yet unknown effects. DNP releases only that part of inhibition which is contributed by the effect on Pi transport.

The action of MVK is similar to acrolein with some quantitative differences. Substitution of the hydrogen of the aldehyde group leading to MVK alters the inhibitory effectiveness by one order of magnitude.

Many aldehydes were tested with respect to their action on mitochondrial processes but only few were effective.[28–32]

The saturated counterpart of acrolein, propionaldehyde, only inhibits respiration with pyruvate.[30] The high reactivity of acrolein is due to the activated vinyl group which has high affinity for sulfhydryl groups.[33,34]

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