Effect of Chloral Hydrate and Acetaldehyde on Mitochondrial Preparations from Sweet Potato

W. S. Pierpoint[1] and George G. Laties

Department of Botany and Plant Biochemistry, University of California, Los Angeles, California

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Summary. The inhibitory effects of chloral hydrate and acetaldehyde have been studied on oxidations performed by mitochondrial preparations of sweet potatoes (*Ipomea batatas*). With a variety of substrates, chloral acts very like amytal but only between a fifth and a tenth as effectively; it affects those reactions which would be expected to depend on the oxidation of intramitochondrial DPNH, more than the oxidation of succinate or of added DPNH. It also acts like amytal when oxygen is replaced by other electron accepting agents. It is more effective, for example, against the malate reduction of cytochrome c than against the malate reduction of 2:6-dichlorophenol-indophenol.

Inhibitions produced by acetaldehyde are more complex. Some DPN-dependent oxidations, especially those of pyruvate and α-keto-glutarate, are strongly inhibited, while that of citrate is not.

It is suggested that chloral affects the electron transport sequence of sweet potato mitochondria at a similar locus to amytal. Although the present work fails to provide unambiguous evidence that acetaldehyde acts in the same manner, experiments described in the literature have been interpreted in this way.

Introduction

It has been suggested (13) that the variety of metabolic changes that occur when thin slices of potato are incubated in air, are under the control of a volatile metabolite. The presence of the metabolite in freshly cut slices presumably prevents these developments; its loss by volatalization removes the restraint. It has also been suggested that the metabolite may be an aldehyde (13), the evidence for this being that aldehydes were the only low molecular weight carbon compounds tested that had the required properties. They reversibly inhibited the development of respiratory changes, and of auxin-induced growth (18), but they had little effect on the respiration of either fresh or aged tissue. Furthermore, acetaldehyde, which is palpably volatile, is present in potato.

Of the effective aldehydes, chloral hydrate is the most convenient to use. As part of an effort to determine its locus of action, it has been tested on various enzymic systems of the potato. The present report describes effects that chloral hydrate and acetaldehyde have on some oxidations performed by mitochondrial preparations from sweetpotato (*Ipomea batatas*) tubers. These tubers are a convenient source of phosphorylating mitochondria; discs of this tissue show a chloral-inhibited increase in respiration just as do discs from *Solanum tuberosum*.

[1] Present address: Rothamsted Experimental Station, Harpenden, Herts., Great Britain.

Materials and Methods

Mitochondrial preparations were made from sweet potatoes by the method described by Wiskich and Bonner (19). Of a variety of methods tried it was the only one which consistently produced preparations in which respiration was controlled by the availability of added ADP.

Sweet potatoes, obtained commercially, were cooled, peeled, and 150 g shredded on a plastic grater into a chilled mortar containing 300 ml of medium of composition: mannitol (0.37 M), sucrose (0.25 M), cysteine (0.4 mM) and ethylene-diamine-tetra-acetic acid (EDTA, 5 mM). The mixture was ground with acid-washed sand, during which time the initial pH of the medium (7.2–7.6) was maintained by the dropwise addition of 5.5 M KOH. The sedimentation and washing of the mitochondria were performed exactly as described by Wiskich and Bonner (19) and the final pellet suspended in 2 to 4 ml of 0.5 M mannitol.

O_2 utilization by the mitochondrial suspensions (0.1–0.2 ml) was measured polarographically with a Clark electrode in a small polyethylene chamber completely filled with 3 ml of air-saturated reaction medium of composition: mannitol (0.3 M), Tris (10 mM, pH 7.2), potassium phosphate (25 mM), magnesium chloride (8 mM), EDTA (0.2 mM) and substrate (17 mM). The concentrations of additional cofactors, when present, were: DPN, 0.17 mM; ADP, 0.85 mM; coenzyme A, 6.7 μM; thiamine diphosphate, 3.3 mM; cytochrome c 3.3 μM.

Spectrophotometric Estimations. The reduction of 2:6-dichlorophenol-indophenol was followed at a wavelength of 600 mμ in a Beckman spectrophotometer. The blank cuvette contained mannitol, Tris, potassium phosphate, DPN, ADP, and substrate, at the concentrations used in the polarographic estimations, and mitochondrial suspension usually equivalent to 0.5 g tissue. It also contained 0.33 mM KCN or, when acetaldehyde was present, 3.3 mM azide to inhibit cytochrome oxidase. The experimental cuvette contained all these reagents together with 0.04 mM 2:6-dichlorophenol-indophenol. The reaction was started by addition of substrate and followed for at least 5 minutes. During this time the decrease in OD was linear, and the rate of decrease was proportional to the amount of enzyme present. There was little or no decrease in OD in the absence of substrate.

The reduction of ferricyanide was followed at a wavelength of 420 mμ in the same manner, with 0.33 mM potassium ferricyanide replacing the 2:6-dichlorophenol-indophenol. The decrease in OD was proportional to the amount of enzyme added, and did not occur in the absence of substrate.

The reduction of cytochrome c (53 μM) was also followed in the same system by measuring the increase in absorption at 550 mμ. This increase, which was proportional to the amount of mitochondrial preparation added, occurred only slowly in the absence of substrate.

Alcohol dehydrogenase was estimated by measuring spectrophotometrically the reduction of DPN to DPNH. The reaction medium (3 ml) contained Tris (33 mM; pH 7), DPN (0.25 mM), ethanol (50 mM) and commercial yeast alcohol dehydrogenase (4.4 μg). The blank had no ethanol. The increase in OD was followed at 340 mμ for 3 to 4 minutes, and was proportional to the amount of enzyme added.

Results

Properties of the Mitochondrial Preparations. The preparations readily absorbed O_2 in the presence of malate and DPN. The rate was increased, generally 3-fold, by the addition of small amounts (0.5 μmole) of ADP. This stimulation ended in a manner suggesting that the oxidation was obligatorily coupled to phosphorylation, and that the P/O ratio was approximately 1.5. Further stimulation could be elicited by the addition of further small quantities of ADP.

Malate was oxidized at a rate of about 100 mμmoles of O_2 absorbed per minute in the presence of mitochondrial preparation corresponding to 5 g tissue. The relative rates at which a variety of other substrates were oxidized is shown in table I. The fastest was the oxidation of ascorbate in the presence of a substituted phenylene diamine, a process that probably measures cytochrome oxidase activity (19). CoA and TDP were both necessary for maximal oxidation of pyruvate and α-keto-glutarate. Cytochrome c did not increase the rate of oxidation of any of the organic acids.

Amytal, an inhibitor which mainly affects pyridine nucleotide-linked oxidations (4, 7), inhibited the oxidation of malate; a concentration of 2.5 mM halving the rate. Bonner and Wiskich (19) reported a similar sensitivity provided that amytal is added after ADP and DPN. The oxidation of DPNH (0.66 mM), and of DPN (0.17 mM) continuously reduced by an excess of ethanol and alcohol dehydrogenase, was much less sensitive to amytal. With both these systems it took about 20 mM to inhibit by 50%. This suggests that the oxidation of exogenous DPNH goes by an external pathway (14) that is less sensitive to inhibitors (6,7), although oxidation by a contaminating nonmitochondrial DPNH oxidase (10) has not been ruled out.

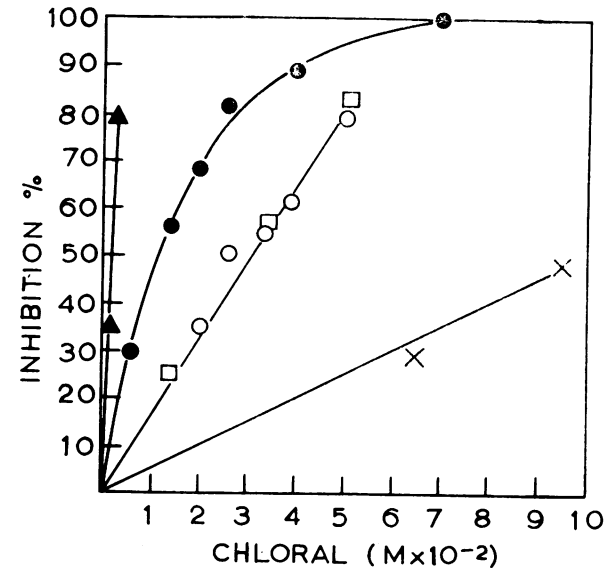
Table I. *Oxidations Catalyzed by Mitochondrial Preparations from Sweet Potato*

The substrates (17 mM unless otherwise indicated) were incubated in the standard medium together with the cofactors listed and 0.1 to 0.3 ml of mitochondrial preparation corresponding to 5 to 15 g of fresh tuber. The rates of O_2 uptake measured were the steady states observed after the addition of ADP (0.85 mM). Data are collected from a number of experiments and they are expressed relative to the uptake produced from malate by the same amount of mitochondrial preparation.

Substrate	Cofactors	Rate of O_2 uptake (malate=100)
Malate	DPN	100
Succinate	...	180
Citrate	DPN	52
Pyruvate	Malate (85 μM), DPN, CoA, TDP	45
α-keto-glutarate	DPN; CoA; TDP	170
DPNH (0.66 mM)	...	130
Ethanol (33 mM)	DPN, alcohol dehydrogenase (12 μg)	90
Ascorbate (8.3 mM)	N,N,N′-N—tetramethyl-*p*-phenylene diamine (0.83 mM)	400

Antimycin A also inhibited the oxidation of malate. The amount necessary to inhibit by 50% was about 20 μg per ml, but at this concentration the alcohol in which the inhibitor was dissolved accounted for up to half of this inhibition. These mitochondrial preparations, as were those of Wiskich and Bonner (19), are less sensitive to antimycin A than preparations from other plant material.

Effect of Chloral Hydrate on Mitochondrial Oxidations. Chloral inhibited the oxidation of malate and other substrates by mitochondrial preparations. It was between a fifth and a tenth as effective against malate oxidation as was amytal, although, like amytal, it effected DPN-dependent oxidations more than suc-



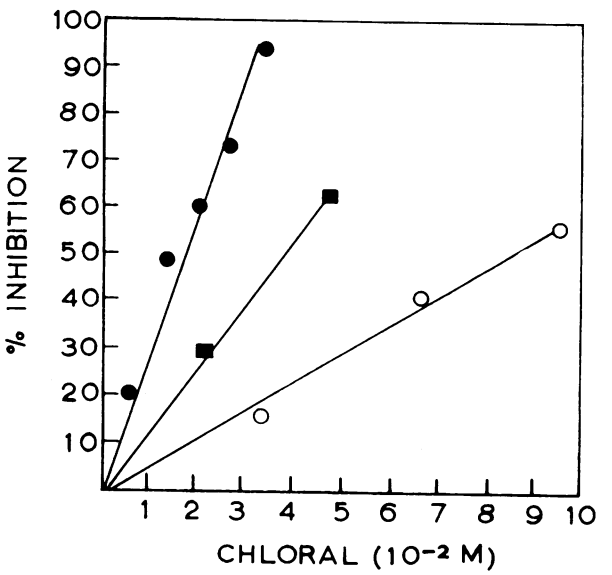


FIG. 1. (*Upper*) The effect of chloral hydrate on oxidations catalyzed by mitochondrial preparations of sweet potato. The inhibitions were produced by the addition of successive amounts of chloral to samples of a mitochondrial preparation (0.2 ml; equivalent to 10 g fresh tissue) oxidizing malate (●), pyruvate (□), citrate), (○), or succinate (×). For comparison the inhibition produced by amytal on a similar preparation oxidizing malate (▲) is plotted on the same scale.

FIG. 2. (*Lower*) Effect of chloral hydrate on the oxidation of DPNH by mitochondrial preparations from sweet potatoes, and on alcohol dehydrogenase. To samples of a mitochondrial preparation (0.1 ml; equivalent to 5 g fresh tissue) DPNH was added either in substrate amounts (○) or as a mixture of alcohol, DPN and alcohol dehydrogenase (●). The inhibition of alcohol dehydrogenase was (■) measured spectrophotometrically using a different commercial sample of alcohol dehydrogenase.

cinate oxidation (fig 1). The oxidation of α-ketoglutarate was inhibited similarly to pyruvate, although the curve was somewhat S-shaped. The oxidation of ascorbate in the presence of the substituted phenylenediamine was virtually insensitive; 0.25 M chloral only reduced it by 30 %.

Chloral inhibited the oxidation of added DPNH, although, unlike amytal, the degree of inhibition ostensibly depended on whether DPNH was presented per se, or generated. The oxidation of chemically provided DPNH was no more sensitive to chloral than succinate oxidation, whereas the oxidation of smaller amounts of DPNH, enzymically generated from DPN and alcohol, was as sensitive to chloral as was malate oxidation (compare fig 2 and fig 1). A possible cause for the anomaly was found in an inhibition of alcohol dehydrogenase by chloral (fig 2). This would reduce the rate at which DPNH was generated and hence the rate of O_2 utilization. The inhibition of alcohol dehydrogenase by chloral recorded in figure 2 is less than the inhibition of the oxidation of enzymically-generated DPNH; it was however measured in conditions somewhat different from those in the O_2 electrode and with a different commercial sample of enzyme.

The proportion of malate oxidation inhibited by a given concentration of chloral was not affected by the presence of ADP, nor of partially inhibiting amounts of amytal or antimycin A. This information is insufficient to show whether chloral acts on the same locus as amytal or, like antimycin A, on a different one. It does however illustrate a difference between chloral and amytal, as the latter has been reported not to affect malate oxidation in the absence of ADP (19).

Effect of Chloral Hydrate on Components of the Electron Transport Chain. To fix more exactly the locus of action of chloral hydrate on the oxidation of intramitochondrial DPNH, it was tested on the mitochondrial reduction of 2:6-dichlorophenol-indophenol, ferricyanide and cytochrome c respectively. These 3 pigments accept electrons from different parts of the electron transport chain of sweet potato mitochondria as indicated by effects of ADP and amytal on the reductions.

The reduction of 2:6-dichlorophenol-indophenol, with malate as reductant, was unaffected by ADP and amytal. This dye, therefore probably interacts with the electron transport chain at some point before the first phosphorylation site and the main amytal-sensitive locus (4). It is reported to interact with rat liver mitochondria in a similar manner (7). Cytochrome c reduction, on the other hand, was stimulated 3 to 4-fold by ADP and strongly inhibited by amytal (fig 3c). Exogenous cytochrome c probably accepts electrons from a level close to that of mitochondrial-bound cytochrome c.

Ferricyanide reduction is intermediate between the 2 other substances. It is stimulated 1.5 to 2-fold by added ADP, but it is only slightly affected by amytal (fig 3), suggesting that it mainly, but not

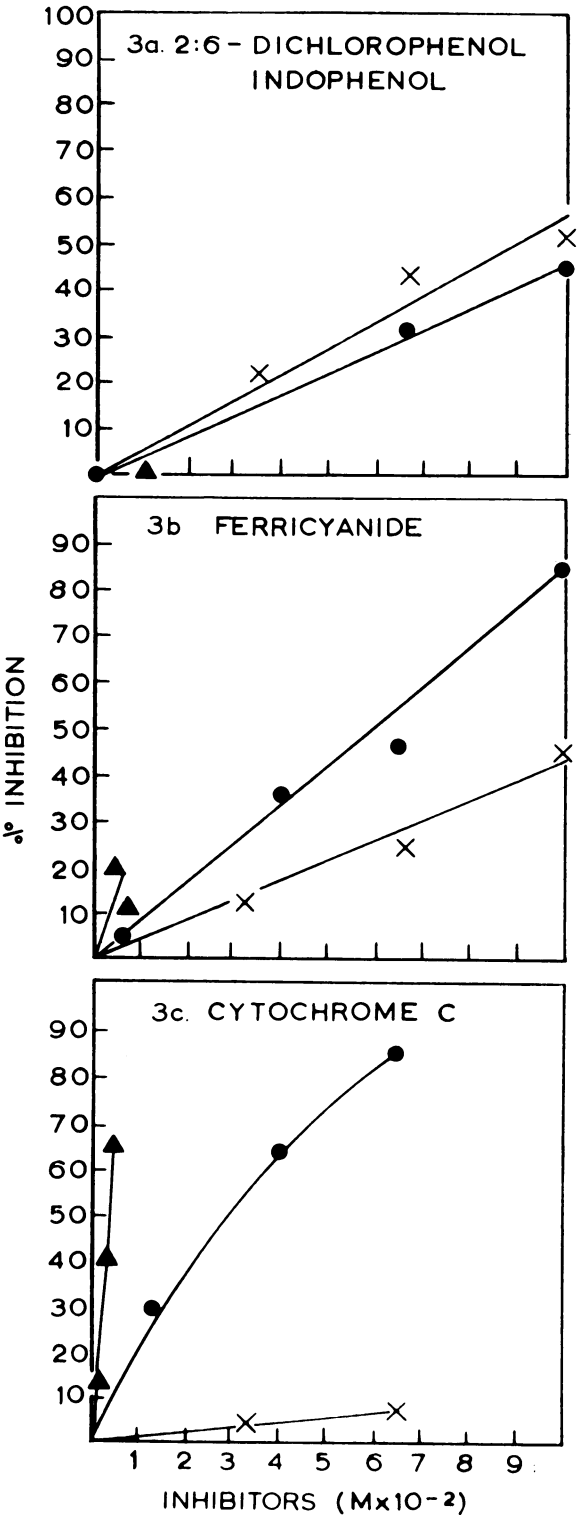


FIG. 3A. The effect of choral and amytal on the reduction of 2:6-dichlorophenol-indophenol by sweet potato mitochondria. FIG. 3B. The effect of chloral and amytal on the reduction of ferricyanide by sweet potato mitochondria. FIG. 3C. The effect of chloral and amytal on the reduction of cytochrome c by sweet potato mitochondria. In each case chloral was tested on the oxidation of malate (●) and succinate (×), and amytal was tested on the oxidation of malate (▲).

exclusively, derives electrons from a locus after the phosphorylation site which precedes the main site of amytal inhibition. Ferricyanide reduction by these preparations therefore contrasts with those systems where it received electrons from bound cytochrome c (8). It has more in common with ferricyanide reduction catalyzed by rat liver particles at high ferricyanide concentrations (16), where it is believed to interact with flavoprotein dehydrogenases. Corn root tips and mitochondrial preparations from them also appear to reduce ferricyanide at a similar level (3), although these reductions are inhibited by amytal.

The effect of chloral hydrate on the reduction of the 3 electron acceptors with both malate and succinate as substrates is shown in figure 3. The effect of amytal on malate oxidation in each system is also shown. It is clear that chloral distinguishes between malate and succinate oxidations only in those systems where amytal affects malate oxidation. Thus, in the amytal-insensitive reduction of 2:6-dichlorophenol-indophenol, chloral has virtually no effect on either succinate or malate oxidation. In the reduction of ferricyanide, malate oxidation is about twice as sensitive to chloral as is succinate oxidation. On the other hand, when cytochrome c is the electron acceptor, chloral markedly inhibits malate oxidation, in contrast to the insensitive succinate oxidation. These results suggest that chloral, like amytal, mainly affects the oxidation of intramitochondrial DPNH at a point after DPNH flavoprotein dehydrogenase (i.e. after the site of ferricyanide reduction), and before the point at which the DPNH and succinate paths converge.

Inhibitory Effects of Acetaldehyde. Acetaldehyde alone, in concentrations up to 80 mM, caused no perceptable O_2 uptake with these mitochondrial preparations. When added to mitochondrial preparations oxidizing organic acids, it caused a decrease in rate. The inhibitions were less reproduceable and more complex than those due to chloral. They varied somewhat from one preparation to another, and varied also with the amount of enzyme preparation. The oxidation of malate, α-keto-glutarate (fig 4) and pyruvate were most affected, but they were generally not completely stopped. The oxidation of citrate and succinate were less affected. An effect observed only with malate was an immediate but temporary (1 min) decrease in oxidation rate caused by small amounts of acetaldehyde. The curve shown for the inhibition of malate oxidation in figure 4 is

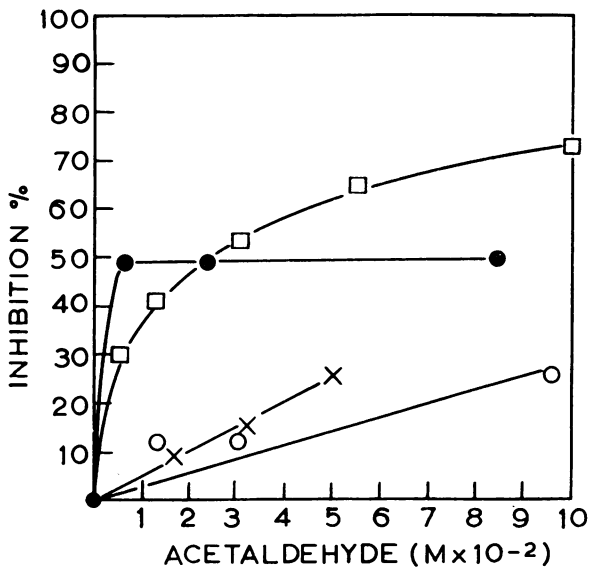


FIG. 4. Effect of acetaldehyde on the oxidation of organic acids by sweet potato mitochondria. The oxidation of malate (●) and citrate (○) were measured in the presence of 0.3 ml of a mitochondrial preparation, (equivalent to 12 g of fresh tissue) and the oxidaton of α-ketoglutarate (□) and succinate (×) in presence of 0.2 ml and 0.1 ml of different but similar preparations. Each acid was oxidized in the presence of the cofactors indicated in table I.

based on steady oxidation rates measured 1 and one-half to 2 minutes after the addition of inhibitor. Kiessling (11, 12) has described similar powerful but quickly reversed inhibitions by acetaldehyde and methyl glyoxal on pyruvate oxidation by preparations of animal mitochondria.

The effect of acetaldehyde on the reduction of 2:6-dichlorophenol-indophenol and cytochrome c was measured with azide rather than cyanide as inhibitor of cytochrome oxidase. The results are however, subject to an uncertainty introduced by the distillation of acetaldehyde from the uncovered cuvettes. It required high initial concentrations of acetaldehyde (0.06–0.1 M) to halve the rate of either reduction, although smaller concentrations had proportionally more effect. The inhibitions were similar with either succinate or malate as substrate; there was no indication that acetaldehyde resembled chloral hydrate or amytal in affecting the reduction of cytochrome c by malate more than the reduction by succinate.

Discussion

It has been appreciated since 1932 (17) that a wide range of narcotics, including chloral hydrate, affect the respiration of brain and other animal tissues, and affect DPN-dependent oxidations more than that of succinate. The probable site of action was shown for a number of these narcotics to be between flavoproteins and cytochromes (15). More recent studies on the inhibition of oxidations of mitochondrial preparations from both animal and plant tissue utilized barbiturates, especially amytal, but seldom, if ever, chloral.

Chloral is a less effective inhibitor of sweet potato mitochondria than is amytal. It was therefore tested at higher concentrations where nonspecific inhibitions may occur. Nevertheless its effects show a clear resemblance to those of amytal, suggesting that both compounds effect the electron transport chain in a similar place. The most striking difference between the 2 compounds, that chloral inhibits the oxidation of enzymically-generated DPNH more than that of added DNPH, is probably due to the inhibition of alcohol dehydrogenase. It is surprising that 2 such dissimilar compounds should have similar effects on mitochondria, and therefore of interest that formaldehyde hydrate (methylene glycol) has been shown by Chance and Hollunger (4) to inhibit the oxidation of intramitochondrial DPNH, albeit about a tenth as potently as amytal. This compound induced the same spectral changes in mitchondria as did amytal, and like amytal it also affected phosphorylation reactions, and, at higher concentrations, succinate oxidation. Chloral inhibits succinate oxidation at high concentrations (fig 1); it would be of interest to see if it mimicked the effect of the other 2 compounds on mitochondrial spectral changes and on phosphorylations.

The inhibitions produced by acetaldehyde are more complex than those produced by chloral, and more difficult to interpret. Some DPN-dependent oxidations, that of α-oxoglutarate for example, are more sensitive to small quantities of acetaldehyde than to small quantities of chloral; others, such as the oxidation of citrate, are much less so. There is, therefore, no unequivocal affirmation that acetaldehyde inhibits the oxidation of intramitochondrial DPNH. However, some effects of acetaldehyde and of other aldehydes that are recorded in the literature can be interpreted in this way.

Previous workers with animal mitochondria have stressed the inhibitory action of acetaldehyde (1,11) and methyl-glyoxal (12) on pyruvate oxidation. Kiessling (11, 12) has further argued that as neither aldehyde affects the oxidation of added DPNH, they inhibit pyruvate oxidation at a stage before the DPNH dehydrogenase. However, most of the evidence produced by these workers is consistent with aldehydes inhibiting intramitochondrial DPNH oxidation as choral and amytal do. Both the above mentioned aldehydes inhibit the DPN-dependent oxidation of glutamate but not succinate oxidation. Thus the aldehyde-resistant oxidation of added DPNH probably proceeds by an external pathway (6, 14). DPN was observed to reverse the inhibitions produced by aldehydes. Beer and Quastel (1) attributed this to the removal of acetaldehyde by oxidation, and Kiessling (12) to a replacement of DPN inactivated by aldehydes. But an alternative explanation, em-

phasized by the fact that these reversals were more marked in aged mitochondria supplemented with cytochrome c, is that DPN served to stimulate the external oxidation pathway which bypassed the amytal and aldehyde sensitive locus.

Some of the more physiological effects that have been ascribed to aldehydes, are consistent with inhibitions of the mitochondrial electron-transfer chain (2, 9). However, the effect of choral or of acetaldehyde in preventing the respiratory rise of potato discs (13) cannot easily be explained in these terms. Concentrations that repress respiratory development have little effect on O_2 uptake of either fresh or aged discs. This is unlikely to be due to a compensatory endogenous respiration, that masks a chloral inhibition; for chloral has little effect on the way discs metabolize exogenous glucose-U-C^{14}. No doubt higher concentrations of chloral will effect the respiration of aged discs, but there is clearly some system, essential to respiratory development, that is more sensitive to chloral or acetaldehyde than are the respiratory enzymes. The action of puromycin and actinomycin on respiratory development has emphasized the importance of protein synthesis in the aging of discs (5). One of us (G. G. L.) has recently obtained evidence that this may be the process which chloral represses. Chloral, in amounts sufficient to prevent the rise in respiration, inhibits the incorporation of both uracil and leucine into potato discs.

Acknowledgment

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