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Biochem. J. (1962) **82**, 164

Biochemistry of Methanol Poisoning

4. THE EFFECT OF METHANOL AND ITS METABOLITES ON RETINAL METABOLISM*

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Methanol poisoning in man, a problem of considerable toxicological interest, is characterized by an initial stage of depression of the central nervous system, which is followed by metabolic acidosis and the specific toxic effect of the oxidation product(s) of methanol on retinal cells which leads to visual degeneration. It has been claimed that swelling of the retinal ganglion cells and the rods and cones, with sparing of the optic nerve and tract, is commonly seen in human cases of methanol poisoning (Fink, 1943; Duke-Elder, 1954). However, very little information is available on the biochemical aspects of this lesion.

It is generally accepted that formaldehyde is the toxic agent in methanol poisoning. The long asymptomatic latent period of 8–36 hr. and the beneficial effects of administered ethanol, which probably acts by inhibiting the oxidation of methanol (Roe, 1946), indicate that a metabolite of methanol is probably responsible for the various manifestations of poisoning. Further support for this contention comes from the observation that formaldehyde is an extremely potent inhibitor of respiration and glycolysis in ox retina; formate exercises only weak respiratory inhibition, and methanol has no effect even at a concentration of 20M (Leaf & Zatman, 1952; Potts & Johnson, 1952). These observations on the relative magnitude of effects of methanol and its oxidation products on the metabolism of the retina *in vitro* have been corroborated by studies on the electroretinogram (Praglin, Spurney & Potts, 1955). Potts & Johnson (1952) observed that the enzymic process most susceptible to formaldehyde inhibition was anaerobic glycolysis and stated that the specific site of inhibition is the retinal hexokinase.

Cooper & Marchesi (1959) found that formaldehyde inhibited aerobic glycolysis in ox retinal homogenates with glucose as the substrate, but not

* Part 3: Kini & Cooper (1961).

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with water, was plated on tared planchets and counted in an automatic recording gas-flow counter (Nuclear–Chicago). Corrections for self-absorption were applied according to Calvin, Heidelberger, Reid, Tolbert & Yankwich (1949). [^{14}C]Lactate formed from incubation with [^{14}C]glucose was isolated by anion-exchange chromatography as follows. After incubation, 5 ml. of 95% ethanol was added to each flask to stop the reaction, and the contents of the flask were homogenized and kept in the cold for 2 hr. before centrifuging. The supernatant fluid was quantitatively transferred to a column (1·4 cm. × 12 cm.) of Dowex 1 (formate), and the [^{14}C]glucose was removed by washing the column with 50 ml. of water. The elution of the organic acids was carried out with formic acid as described by Busch, Hurlbert & Potter (1952). Fractions of 4–5 ml. were collected and evaporated to dryness *in vacuo* in a desiccator over a mixture of NaOH and $CaCl_2$ (1:2). Lactate as identified by the colorimetric method of Barker & Summerson (1941) emerged as a single sharp peak between the eleventh and fourteenth fractions. The residue in the tubes was dissolved in 2 ml. of 0·05 M-NaOH in 60% ethanol, and suitable samples were plated and counted.

$^{32}P_i$ *studies.* The ATP–$^{32}P_i$-exchange reaction was studied by the method of Nielsen & Lehninger (1955). For measurement of radioactivity, a liquid scintillation counter (Technical Measurements Corp.) was used.

All results quoted in this paper, unless otherwise stated, are representative of at least four similar experiments.

RESULTS

Effect of methanol and its metabolites on the respiration of intact ox retina in vitro. As stated in the introduction, most investigators agree that the specific toxic action on the retina in methanol poisoning is attributable to the localized effect of the oxidation products of methanol, such as formaldehyde or formate. Accordingly, the effects of methanol, formaldehyde, sodium formate and methyl formate on the respiration of ox retina were tested in the presence of [U-^{14}C]glucose (0·01 M). Methyl formate was included in these studies, since Kendal & Ramanathan (1952) found that formaldehyde could be converted into methyl formate by a hemiacetal-dehydrogenase mechanism and speculated that the preferential fat solubility of this ester might result in its being the proximal toxic agent. Results in Table 1 indicate that, among the compounds tested, formaldehyde was the most powerful inhibitor of retinal metabolism, exerting a 50% inhibition of oxygen uptake at a concentration of 3 mM, whereas 200 mM-sodium formate and 100 mM-methyl formate caused 33 and 19% inhibition of oxygen uptake respectively. However, formaldehyde at a concentration range of 1–2 mM caused a 50% inhibition of $^{14}CO_2$ formation from [^{14}C]glucose, whereas sodium formate and methyl formate produced a similar magnitude of inhibition only at concentrations of 200 and 50 mM, respectively. The relatively greater inhibition of $^{14}CO_2$ formation as compared with oxygen uptake would indicate that other substrates (endogeneous) are being preferentially oxidized. Methanol, in concentrations as high as 2 M, depressed oxygen uptake by 23% but had no effect on $^{14}CO_2$ formation from [^{14}C]glucose.

Effect of formaldehyde on the glycolysis of ox retina. The inhibitory effect exerted by formaldehyde on retinal respiration could have been

Table 1. *Effect of methanol, formaldehyde, sodium formate and methyl formate on oxygen uptake and carbon dioxide production by ox retina*

Experimental conditions were as described under Materials and Methods. Each flask contained 10 mM-[U-^{14}C]glucose (specific activity, 3333 counts/min./μmole). Final volume of incubation, 3 ml. Temperature, 37°. Time, 1 hr.

Additions	Concn. (mM)	Q_{O_2}	[U-^{14}C]Glucose converted into $^{14}CO_2$ (μm-moles/mg. of dry tissue)	Inhibition of $^{14}CO_2$ formation (%)
Nil	—	7·4	34·2	0
Methanol	1000	6·1	34·2	0
	2000	5·7	33·9	0·9
Formaldehyde	0·5	7·2	24·9	27·2
	1·0	6·7	17·7	48·2
	2·0	5·7	12·3	64·0
	3·0	3·5	8·1	76·2
	5·0	2·4	5·4	84·2
Sodium formate	10	6·5	29·4	14·0
	50	5·5	22·5	34·2
	100	4·8	17·4	49·2
	200	4·4	13·2	61·5
Methyl formate	5	7·3	28·8	15·8
	20	7·0	25·2	26·4
	50	6·6	14·1	58·8
	100	5·9	3·0	87·7

Table 4. *Effect of formaldehyde on oxidative phosphorylation in retinal mitochondria*

Experimental conditions were as described under Materials and Methods. Temperature of incubation, 25°. Time, 30 min.

Substrate	Concn. of formaldehyde (mM)	Oxygen uptake (μg.atoms)	Phosphate esterified (μmoles)	$Fe(CN)_6^{3-}$ utilized (μmoles)	P/O	P/2 $Fe(CN)_6^{3-}$
Sodium α-oxoglutarate (10 mM)	0	12·1	32·7	—	2·7	—
	0·5	9·3	25·1	—	2·7	—
	1·0	6·1	14·0	—	2·29	—
	2·0	3·4	1·2	—	0·35	—
	5·0	1·8	−0·5	—	0	—
Sodium pyruvate (10 mM) + DPN (0·67 mM) + potassium fumarate (0·67 mM)	0	8·1	15·5	—	1·95	—
	0·5	7·1	11·9	—	1·70	—
	1·0	5·1	3·9	—	0·77	—
	2·0	2·8	−0·3	—	0	—
	5·0	−2·2	−0·6	—	0	—
Potassium succinate (10 mM)	0	12·2	23·4	—	1·92	—
	0·5	10·0	9·8	—	0·98	—
	1·0	6·2	2·1	—	0·34	—
	2·0	4·0	0·2	—	0·05	—
	0	—	22·6	13·3	—	0·85
	0·5	—	8·5	8·4	—	0·50
	1·0	—	3·1	6·4	—	0·24

Table 5. *Effect of formaldehyde on oxidative phosphorylation by retinal mitochondria oxidizing cytochrome* c

Experimental conditions were as described under Materials and Methods. Each flask contained 0·5 of mitochondrial suspension (8·3 mg. of protein) in 0·25 M-sucrose. Temperature of incubation, 25°. Time, 1 hr.

Additions	Concn. (mM)	Oxygen uptake (μg.atoms)	Phosphate esterified (μmoles)	P/O	Inhibition of electron transport (%)
Nil	—	3·49	2·33	0·67	0
Formaldehyde	0·5	3·12	1·25	0·40	10·5
	1·0	2·37	0·95	0·40	32·0
	2·0	1·00	0·45	0·45	71·5

by its uncoupling action with succinate as substrate and potassium ferricyanide as electron acceptor. Potassium ferricyanide is known to accept electrons at the level of cytochrome *c* (Estabrook, 1957).

The effect of formaldehyde on phosphorylation coupled to the oxidation of ferrocytochrome *c* was then tested in the presence of ascorbate as the reducing agent. The method of Jacobs & Sanadi (1960) was used, since the procedure of Lehninger, ul Hassan & Sudduth (1954), when employed for retinal mitochondria, resulted in a total loss of the capacity for phosphorylation. Table 5 shows that when mitochondria were suspended in 0·25 M-sucrose, 40–50% inhibitions of both the oxygen uptake and phosphorylation efficiency occurred at concentrations of formaldehyde ranging from 0·5 to 2 mM. It is evident that formaldehyde inhibits both the electron transport and phosphorylation in the span of the electron-transport chain between ferrocytochrome *c* and oxygen.

As noted in Table 4, the phosphorylation associated with succinate oxidation was markedly inhibited by 0·5 mM-formaldehyde, although oxygen uptake was decreased only slightly. This relative lack of effect of formaldehyde on succinate oxidation in a phosphorylating system was further substantiated by studies on the succinoxidase of

Table 6. *Effect of formaldehyde on the mitochondrial oxidation of succinate*

The components of the medium were: KCl, 25 mM; $MgCl_2$, 10 mM; cytochrome *c*, 0·067 mM; potassium succinate, 10 mM; sodium orthophosphate buffer, pH 7·4, 20 mM; EDTA (sodium salt), 1 mM; 0·5 ml. of mitochondrial suspension (8·7 mg. of protein). Final volume, 3 ml. Temperature of incubation, 37°. Time, 40 min.

Pretreatment medium for mitochondria	Concn. of formaldehyde (mM)	Oxygen uptake (μmoles)	Inhibition (%)
Sucrose (0·25 M)	0	16·7	0
	0·5	14·9	10·8
	1·0	14·7	12·0
	2·0	11·5	31·1
Water	0	17·0	0
	0·5	15·4	9·4
	1·0	14·9	12·4
	2·0	10·7	37·1

Table 10. *Effect of formaldehyde on mitochondrial respiration in a phosphate-deficient system*

The components of the medium were: KCl, 25 mM; $MgCl_2$, 6·7 mM; ATP (sodium salt), 1·7 mM; cytochrome *c*, 0·013 mM; sodium orthophosphate buffer, pH 7·4, 1·7 mM; tris–HCl buffer, pH 7·4, 50 mM; 0·5 ml. of mitochondrial suspension. Temperature of incubation, 37°. Time, 20 min.

Additions	Oxygen uptake (μg.atoms)	Inhibition (%)
Nil	9·6	0
Formaldehyde (0·5 mM)	9·1	5
(1·0 mM)	7·8	19
(2·0 mM)	7·2	25
Dinitrophenol (0·05 mM)	11·8	0
Dinitrophenol (0·05 mM) + formaldehyde (0·5 mM)	10·2	14
Dinitrophenol (0·05 mM) + formaldehyde (1·0 mM)	9·8	17
Dinitrophenol (0·05 mM) + formaldehyde (2·0 mM)	9·0	24

phenol, which enhanced respiration in this system. These data support the premise that formaldehyde acts upon an energy-coupling process which is in functional equilibrium with the electron-transport chain, and that it acts at a point before the incorporation of P_i in this process and before the site of inhibition by dinitrophenol.

DISCUSSION

In agreement with the earlier work of Leaf & Zatman (1952) and Potts & Johnson (1952), we have observed that, among the oxidation products of methanol, formaldehyde was by far the most toxic to retinal metabolism. We also believe that formaldehyde is the proximal toxic agent in the ocular toxicity seen in methanol poisoning.

In confirmation of the observation of Potts & Johnson (1952), we found that formaldehyde is a potent inhibitor of anaerobic glycolysis in whole retinas; in addition, it also brings about a stimulation of aerobic glycolysis. In the intact retina, these strikingly different effects of formaldehyde on anaerobic and aerobic glycolysis may be a reflexion of a change in a regulatory mechanism or pacemaker in these two situations. Thus under anaerobic conditions hexokinase may be rate-limiting, and glycolysis would be subject to inhibition by formaldehyde, whereas in an aerobic medium hexokinase may not be a pacemaker. In fact, the stimulation of aerobic glycolysis by formaldehyde may be interpreted as a release of ADP by the uncoupling action of formaldehyde and a subsequent stimulation of triose phosphate dehydrogenase, the other glycolytic pacemaker (Krebs, 1956).

We could not confirm the results of Potts (1955) or of Cooper & Marchesi (1959) on the inhibition of hexokinase by formaldehyde. A possible explanation might be that hexokinase is in the particulate fraction of the retina (J. R. Cooper, unpublished observations) and the kinetics of this enzyme and, ultimately, of glycolysis, might be highly variable depending on the preparation of the homogenate.

In considering both anaerobic glycolysis in the intact retina and oxidative phosphorylation the dominant feature of the effect of formaldehyde on retinal metabolism appears to be its ability to decrease the synthesis of ATP. Although formaldehyde uncoupled the phosphorylation accompanying the oxidation by retinal mitochondria of DPN-linked intermediates in the tricarboxylic acid cycle, the phosphate esterifications coupled to the oxidation of succinate or ferrocytochrome *c* seemed to be the most sensitive. However, our results do not exclude the possibility that phosphorylation coupled to the step DPN to flavin might also be sensitive to formaldehyde. Lehninger *et al.* (1954) noted that phosphorylation coupled to the oxidation of ferrocytochrome *c* by oxygen was comparatively more sensitive to uncoupling agents than were the other sites. In our experiments, although the degree of inhibition of the phosphorylation coupled to the oxidation of succinate appeared to be greater than that of the oxidation of ferrocytochrome *c*, formaldehyde is a potent inhibitor of both the phosphorylations associated with electron transport when flavin is oxidized through ferrocytochrome *c* by oxygen.

Further effects of formaldehyde on the incorporation of P_i into ADP to form ATP during electron transport (Lehninger, 1960) may be summarized as follows: (1) with succinate as the substrate, formaldehyde lowers P/O ratios, but has little effect on oxygen uptake in either a phosphorylating or a non-phosphorylating system; (2) the ATP–$^{32}P_i$-exchange reaction is not significantly affected; (3) formaldehyde has little effect on the low adenosine triphosphatase of fresh retinal mitochondria, but inhibits the enhanced adenosine-triphosphatase activity seen upon addition of dinitrophenol; (4) in a system in which respiration is controlled by the availability of ADP, formaldehyde has little effect; however, it lowers the stimulation of respiration occurring in this system in the presence of dinitrophenol; (5) formaldehyde decreases respiration in a system deficient in P_i, the percentage of respiratory inhibition being the same even upon the addition of dinitrophenol.

Formaldehyde therefore acts primarily on some component of the intimately coupled phosphorylation mechanism and does not inhibit directly the electron-transport chain, the latter being

only a secondary effect. The work of Bernheim (1951) on the action of formaldehyde on rat-liver homogenates indicated that it affected succinoxidase, but not cytochrome oxidase; however, the concentrations of the toxic agent that he employed were considerably higher (6·2 mM) than those used in our experiments. The results of Lehninger (1960) suggest, by direct studies on ADP–ATP exchange and the ATP–$^{32}P_i$ exchange, that dinitrophenol causes a removal of the hypothetical high-energy phosphorylated intermediate. Our results suggest that formaldehyde and dinitrophenol act at different sites and indicate the presence of at least two intermediates in the reactions occurring during phosphorylation. The single site of action of formaldehyde that would account for our experimental findings is reaction (2) in the scheme of oxidative phosphorylation formulated by Slater & Hülsmann (1959):

$$A \sim I + X \rightleftharpoons X \sim I + A.$$

This equation involves the transfer of energy contained in the energy-rich compound $A \sim I$, formed during electron transfer between AH_2 and B the two adjacent members of a phosphorylative step in the respiratory chain, to a second hypothetical intermediate X, giving $X \sim I$. Respiration will be maintained only if the two hypothetical intermediates, X and I, are continuously regenerated. This is effected by P_i and ADP leading to the synthesis of ATP.

Whatever the definitive site of inhibition of formaldehyde on retinal metabolism may be, formaldehyde is a potent inhibitor of the synthesis of ATP in isolated mitochondria; as a result of what appears to be a secondary effect, the cellular respiration is depressed. In support of these conclusions, we have observed that formaldehyde at concentrations that reduced phosphorylation in mitochondria also markedly lowered the incorporation of $^{32}P_i$ into the phospholipids of the intact retina, an ATP-dependent process (Kini, King & Cooper, 1961). The relevant question is, are these results, obtained *in vitro*, consonant with the morphological organization of the retina and do they clarify the mechanism involved in the pathogenesis of methanol poisoning? Electron microscopy of rods in the retina (Sjostrand, 1953; DeRobertis, 1956) has shown a dense aggregation of slender long mitochondria, the so-called ellipsoids, in the inner rod segments. Although the role played by ATP in the transmission of the visual impulse is unknown, the close topographical arrangement between the centres concerned with visual excitation and the mitochondria in the rods and cones suggest that ATP or one of the high-energy intermediates involved in the phosphorylating mechanism may be intimately connected with the energy involved in this process. Interference by formaldehyde ultimately would cause a degeneration of the retinal cells concerned with vision and thus would lead to blindness. Hubbard & Wald (1951) showed that the formation of rhodopsin from opsin and vitamin A_1 alcohol proceeds maximally only when the system is coupled with a succinoxidase preparation from ox heart which oxidized DPNH to DPN$^+$.

Histochemical analysis of both human and rabbit retina (Kuwabara & Cogan, 1960) showed that maximal tetrazolium reduction by substrates such as pyruvate, α-oxoglutarate or succinate could be shown in the ellipsoids of rods and cones, whereas these activities were characteristically absent in the ganglion and neuronal layers of the retina, which, instead, exhibited the 'lactate–DPN' type of activity. This might be expected on the basis of the studies of Sjostrand (1953) and DeRobertis (1956) on the distribution of mitochondria in the retina. Hubbard & Wald (1951) have demonstrated that retinene reductase in the rods and cones is probably identical with liver alcohol dehydrogenase. Since alcohol dehydrogenase is the physiological mechanism catalysing the enzymic oxidation of methanol to formaldehyde (Kini & Cooper, 1961), it might be inferred that the production of the toxic agent actually takes place *in situ*. The retina also contains a specific formaldehyde dehydrogenase that catalyses the further oxidation of the toxic agent to formic acid (Kinoshita & Masurat, 1958).

The fatal dose of methanol in man is about 65 g. (Hunter & Lowry, 1956), although Duke-Elder (1954) quotes a case in which blindness occurred after the ingestion of one teaspoonful of methanol. Methanol, like ethanol, is known to become distributed uniformly in the body (Yant & Schrenk, 1937). Assuming that water represents 70 % of the body weight, ingestion of 65 g. of methanol by a 70 kg. man would result in a concentration in the body fluids of 0·042 M. Thus the amount of formaldehyde employed in our experiments appears to be pharmacologically reasonable. The action of formaldehyde in reducing oxidative phosphorylation may be just an extension of the findings of Beer & Quastel (1958) on the inhibitory effect of aliphatic aldehydes such as acetaldehyde on brain mitochondrial respiration. However, it was found that acetaldehyde (up to 5 mM) inhibited neither the respiration nor the efficiency of coupled phosphorylation in ox retinal mitochondria (M. M. Kini, unpublished observations), an observation in agreement with the work of Walkenstein & Weinhouse (1953) with liver mitochondria. Thus the toxic effect of formaldehyde on retinal metabolism seems to be a fairly specific one. If we accept the criteria laid down by Welch & Bueding

(1946), our data on the ability of formaldehyde to interfere with certain enzymic steps in the orderly flow of energy make it reasonable to assume that a similar mechanism may be operating *in vivo*. Such a biochemical mechanism is not in conflict with current information on the histochemistry and electron microscopy of the visual cell.

SUMMARY

1. Formaldehyde, as compared with sodium formate, methyl formate or methanol, was found to be a potent inhibitor, at low concentrations, of the respiration of ox retina *in vitro*. Formaldehyde depressed anaerobic glycolysis and enhanced aerobic glycolysis.

2. In an actively phosphorylating system of retinal mitochondria, the efficiency of phosphate esterification, with succinate as substrate, was more sensitive to inhibition by formaldehyde than was the oxidation of diphosphopyridine nucleotide-linked intermediates in the tricarboxylic acid cycle. Respiratory inhibition subsequently occurred on increasing the concentration of formaldehyde.

3. Analysis of the site of action of formaldehyde on the various intermediate reactions occurring during esterification of inorganic phosphate during electron transport indicated that formaldehyde had little effect on the adenosine triphosphate–[^{32}P]phosphate exchange and respiration in a system deficient in adenosine diphosphate. However, it decreased respiration in a system rate-limited by the availability of inorganic phosphate and inhibited the adenosine-triphosphatase activity induced by the addition of dinitrophenol. These results indicated that formaldehyde affected coupled phosphorylation rather than electron transport.

4. A formulation of the probable locus of action of formaldehyde on retinal metabolism *in vitro* is presented. The possibility that such a mechanism may be involved in the pathogenesis of methanol poisoning is considered in terms of the histopathology and electron microscopy of the retina.

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