mitochondria (167)(170)(171*) *ADH I think
Aerobic-anaerobic glycolysis (164) Aldehyde DH may be in retina (171*)
methyl formate fat solubility-(166)

*(166) Greater inhibition of CO_2 formation as compared to O_2 uptake

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Bat oxidation

Biochemistry of Methanol Poisoning

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

Article 11

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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, 1959). 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

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with hexose diphosphate or glucose 6-phosphate as substrate. Direct assays of retinal hexokinase revealed that formaldehyde caused a competitive inhibition of this enzyme, this inhibition being reversed by excess of glucose.

The present paper describes a detailed investigation of the effect of formaldehyde on the metabolism of the retina, to see whether this effect would afford a biochemical mechanism to explain the predisposition of the eye to injury in methanol intoxication.

MATERIALS AND METHODS

Abbreviation. P_i, inorganic phosphate.

Materials. Pyridine nucleotides, *cytochrome c* and yeast hexokinase were purchased from Sigma Chemical Co. The potassium salt of ATP was obtained from Mann Research Laboratories Inc., New York. Uniformly labelled [^{14}C]-glucose ([U-^{14}C]glucose) was obtained from New England Nuclear Corp. and [^{32}P]phosphate from the Oak Ridge National Laboratory. All other chemicals used were of reagent-grade purity.

Experimental procedures. Ox eyes were removed at the abattoir immediately after the death of the animal, placed in crushed ice and transported to the laboratory. The eyeball was opened by a circular incision along the ora serrata, the vitreous humour squeezed out and the retina gently lifted by means of a blunt forceps after its attachment to the optic disk had been severed. Approximately 300 mg. of retinal tissue was weighed in a torsion balance and suspended in incubation media contained in chilled Warburg flasks. The flasks were then attached to the manometers, transferred to a water bath at 37° and differential manometry was carried out by conventional techniques for a period of usually 1 hr. The time interval from the death of the animal to commencement of the incubation was not more than 3 hr. When [^{14}C]glucose was the substrate, the reaction was terminated by the addition of 0·2 ml. of 30% trichloroacetic acid, and shaking was continued for a period of 30 min. to ensure complete absorption of $^{14}CO_2$ by the alkali in the centre well. Dry-weight determinations indicated that 100 mg. of whole retina, wet weight, prepared as described, was equivalent to 12 mg. dry wt.

The following procedure was developed for the isolation of mitochondria from ox retina. Approximately 8–10 g. of retinal tissue was homogenized in 10 ml. of 0·25 M-sucrose in a Potter–Elvehjem homogenizer with a Teflon pestle. The homogenate was diluted to about 100 ml. with 0·25 M-sucrose and centrifuged at 1200*g* for 10 min. in a Lourdes refrigerated centrifuge. The sedimented cell debris and nuclei were discarded and the supernatant solution was centrifuged at 18 000*g* for 15 min. The sediment was washed twice by resuspension in 0·25 M-sucrose and centrifuging for further periods of 10 min. each, the 'fluffy' layer being discarded at the first washing. The resulting mitochondrial pellet was found to stain supravitally with Janus green B. The mitochondria were then suspended in 0·25 M-sucrose so that 1 ml. of the suspension contained mitochondria from approximately 1 g. of retina. To each Warburg flask was added 0·5 ml. of this suspension.

Media. In respiratory studies with intact retina, Krebs–Ringer phosphate medium of the following composition was used, the gas phase being O_2: NaCl, 128 mM; KCl, 5 mM; $CaCl_2$, 3 mM; $MgSO_4$, 1·3 mM; sodium orthophosphate–HCl buffer (pH 7·4), 10 mM (with respect to phosphate). In anaerobic experiments the same medium was used with substitution of $NaHCO_3$, 24·5 mM, for sodium phosphate buffer; the gas phase was $N_2 + CO_2$ (95:5, v/v). All concentrations are expressed as those which obtained in the final mixture. The reaction volume was 3 ml.

For assays of retinal hexokinase activity each test tube contained the following components in a final volume of 1 ml.: D-glucose, 5 mM; $MgCl_2$, 10 mM; ATP, 5 mM; tris–HCl buffer (pH 7·45), 50 mM; 0·4 ml. of an ox-retinal homogenate prepared according to Hoare & Kerly (1954). Incubations were carried out in air at 30° and terminated by the addition of 0·5 ml. of 5% $ZnSO_4$ and 0·5 ml. of 0·3% NaOH.

In experiments on oxidative phosphorylation with mitochondrial preparations, each Warburg flask contained the following basic medium in a final volume of 3 ml.: sodium orthophosphate buffer (pH 7·4), 20 mM; KF, 10 mM; ATP (potassium salt), 1 mM; $MgCl_2$, 6·7 mM; KCl, 33 mM; cytochrome *c*, 13 μM; D-glucose, 20 mM; yeast hexokinase 7·1 units, assayed by the procedure of Crane & Sols (1955); 0·5 ml. of a suspension of retinal mitochondria in 0·25 M-sucrose. Incubation times and temperatures are given in the legends of the Tables. To study the phosphorylation due to oxidation of cytochrome *c* the medium composition, in a final volume of 3 ml., was as follows: KH_2PO_4–K_2HPO_4 buffer (pH 7·4), 7·5 mM; $MgCl_2$, 7·5 mM; cytochrome *c*, 25 μM; sucrose, 0·25 M; EDTA (sodium salt), 3·3 mM; ATP (potassium salt), 1·7 mM; L-ascorbic acid (potassium salt prepared freshly before use), 10 mM; D-glucose, 20 mM; hexokinase, 7·1 units; 0·5 ml. of mitochondrial suspension in 0·25 M-sucrose.

Analytical determinations

Unless specified otherwise, all colorimetric measurements were made with a Beckman model DU spectrophotometer.

Glucose. Glucose was estimated in supernatant fluids obtained after centrifuging by the Nelson (1944) modification of the Somogyi method.

Protein. Protein was determined by the method of Lowry, Rosebrough, Farr & Randall (1951).

Oxidative phosphorylation. After thermal equilibration, 1 ml. of 10% trichloroacetic acid or 0·2 ml. of 0·3 M-glucose containing 7·1 units of hexokinase were tipped into replicates of the various Warburg flasks. Uptakes of O_2 were recorded for the periods indicated in the Tables, the vessels quickly removed and placed in crushed ice, and 1 ml. of 10% trichloroacetic acid was added. After centrifuging, phosphate determinations were made by the method of Fiske & Subbarow (1929) on suitable samples of the supernatant fluid. The difference between the initial and final concentrations of P is a measure of the P_i esterified. With ferricyanide as the electron acceptor, the phosphate determinations were carried out according to Copenhaver & Lardy (1952), ferricyanide being estimated by direct colorimetry at 420 mμ.

^{14}C studies. In order to estimate the metabolic CO_2, the contents of the centre well of the Warburg flask were quantitatively added to 0·13 m-mole of Na_2CO_3 and precipitated with excess of $BaCl_2$. The $BaCO_3$, after washing

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 2. *Effect of formaldehyde on glycolysis in ox retina*

Experimental conditions were as described under Materials and Methods. Temperature of incubation, 37°. Time, 1 hr. LA, Lactic acid.

Additions	Concn. (mM)	Anaerobic $Q^{N_2}_{LA}$	Anaerobic Inhibition (%)	Aerobic Lactic acid formed (μmole/mg. of dry tissue)	Aerobic $Q^{O_2}_{LA}$	Aerobic Stimulation (%)
Nil	—	23·9	0	0·211	4·73	0
Formaldehyde	0·5	11·5	52	—	—	—
	1·0	3·6	85	0·246	5·51	16·3
	2·0	2·7	89	0·259	5·80	22·7
	5·0	1·7	93	0·313	7·02	50·0

mediated either through a direct inhibition of one or more of the enzymes of the glycolytic pathway or possibly through a subsequent inhibition of the complex of enzymes in the tricarboxylic acid cycle. The response of anaerobic and aerobic glycolysis of ox retina to various concentrations of formaldehyde was tested in order to evaluate the former hypothesis. Lactic acid production during aerobic glycolysis could not be measured by the colorimetric method of Barker & Summerson (1941), since large and variable amounts of lactic acid accumulated during the period between the slaughter of the animal and the transport of the eyes to the laboratory, and this acid could not be removed effectively. In addition, formaldehyde, in the concentrations employed, interfered in the Barker–Summerson procedure. Hence [^{14}C]lactic acid, formed from [U-^{14}C]glucose during aerobic glycolysis, was isolated by column chromatography on Dowex 1 (formate), and the radioactivity determined. Anaerobic glycolysis was measured manometrically. The data shown in Table 2 reveal that 0·5 mM-formaldehyde depressed anaerobic glycolysis by over 50%, whereas at a concentration of 1 mM the inhibition was 85%. Under aerobic conditions, however, formaldehyde stimulated glycolysis.

Effect of formaldehyde on retinal hexokinase. The studies of Cooper & Marchesi (1959) indicated that formaldehyde inhibited aerobic glycolysis in homogenates of ox retina and that this inhibition was not observed when glucose 6-phosphate or hexose diphosphate was substituted for glucose, observations which implied that formaldehyde inhibited hexokinase. The activity of hexokinase in retinal homogenates in the presence of formaldehyde was studied by estimating the uptake of glucose under the conditions described by Cooper & Marchesi (1959). The results, illustrated in Table 3, indicate that formaldehyde exercised a slight stimulation of retinal hexokinase. These conflicting results are discussed below.

Effect of formaldehyde on respiration and phosphorylation in retinal mitochondria. The actions of

Table 3. *Effect of formaldehyde on retinal hexokinase*

Experimental conditions were as described under Materials and Methods. Temperature of incubation, 30°. Time, 15 min.

Additions	Concn. (mM)	Glucose utilized (μmoles)
Nil	—	3·15
Formaldehyde	1	3·48
	2	3·54
	5	3·62

formaldehyde in inhibiting anaerobic glycolysis and stimulating aerobic glycolysis in the intact retina are reminiscent of the effect of 2,4-dinitrophenol and the barbiturates. The effect of formaldehyde was tested in a system consisting of ox retinal mitochondria respiring with α-oxoglutarate, pyruvate or succinate as substrate (Table 4). In such a system there was invariably a decrease in the efficiency of coupled phosphorylation. With α-oxoglutarate as substrate, 1 mM-formaldehyde produced little inhibition in the P/O ratio, and oxygen uptake was reduced by 50%. However, with succinate as substrate, a similar degree of inhibition of oxygen uptake was demonstrable only at 2 mM-succinate, but phosphorylation was totally abolished by 1 mM-formaldehyde. Similar results were obtained during the complete oxidation of pyruvate effected by the presence of a 'sparker' such as fumarate. As observed with brain mitochondria (Weinhouse, 1955), retinal mitochondria exhibited a requirement for DPN for the efficient oxidation of pyruvate. With 1 mM-formaldehyde, the inhibition of the P/O ratio with pyruvate was 60%. This disproportionately greater inhibition by formaldehyde on the P/O values obtained with pyruvate as compared with α-oxoglutarate could be attributed to the fact that formaldehyde, like dinitrophenol, does not affect substrate-level phosphorylation (Hunter, 1951). That formaldehyde affects the electron-transport chain between flavin and cytochrome *c* is indicated

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

retinal mitochondria, under conditions of maximal rates of succinate oxidation, as described by Dawkins, Judah & Rees (1959). Table 6 shows that formaldehyde, at a concentration that totally abolished phosphorylation (1 mM), had little effect on the succinoxidase of both intact mitochondria and of mitochondria treated with water at 0° for 30 min. Thus it is clear that formaldehyde, while inhibiting both sites of phosphorylation involved in the oxidation of succinate, exerts little effect on the electron transport.

Action of formaldehyde on the adenosine triphosphate-[^{32}P]phosphate-exchange reaction and the dinitrophenol-stimulated adenosine triphosphatase. After it had been established that formaldehyde had little effect on the oxidation of succinate in retinal mitochondria, the effect of formaldehyde on the ATP-$^{32}P_i$-exchange reaction and on the dinitrophenol-stimulated adenosine triphosphatase was examined. These reactions were found by Cooper & Lehninger (1957) to be functionally related to coupled phosphorylation and both reactions presumably represent a reversal of the coupled phosphorylation reaction. Table 7 shows that formaldehyde, at 1 mM, exerted only a 7% inhibition of the ATP-$^{32}P_i$ exchange, whereas at 2 mM the inhibition was 26%.

Table 8 demonstrates the effects of formaldehyde, at concentrations ranging from 0·5 to 2 mM, on the dinitrophenol-stimulated adenosine triphosphatase. Formaldehyde, in increasing concentrations, caused an inhibition of the adenosine triphosphatase induced by dinitrophenol; in the absence of dinitrophenol, the adenosine-triphosphatase activity was stimulated by formaldehyde.

The relative lack of effect of formaldehyde on the ATP-$^{32}P_i$-exchange reaction and the dinitrophenol-induced adenosine triphosphatase suggested that formaldehyde interfered with a factor closely related to the step that couples oxidation with phosphorylation, inhibition of which would lead to a lowering of both respiration and phosphorylation in a phosphorylating system, but not in a non-phosphorylating one.

Effect of formaldehyde on respiration of retinal mitochondria controlled by adenosine diphosphate or inorganic phosphate. The concentrations of both P_i and ADP are important regulatory factors in controlling the rate of oxidative metabolism of tissues (Chance & Williams, 1956). The results in Table 9 demonstrate that, whereas formaldehyde had little effect on the oxygen uptake of retinal mitochondria respiring in a medium deficient in ADP, it greatly reduced the stimulation of respiration brought about on addition of dinitrophenol. Lardy & Wellman (1952) have shown that this increased respiration is due to the fact that dinitrophenol makes the system independent of ADP. Additional experiments (Table 10) carried out in a system lacking in P_i showed that formaldehyde lowered the respiration, the degree of inhibition's not being altered even after addition of dinitro-

Table 7. *Effect of formaldehyde on the adenosine triphosphate-[^{32}P]phosphate exchange in retinal mitochondria*

The components of the medium were: KCl, 25 mM; $MgCl_2$, 5 mM; sodium orthophosphate buffer, pH 7·4, 5 mM; ATP (sodium salt), 2·5 mM; $^{32}P_i$ equivalent to 247 000 counts/min.; 0·5 ml. of mitochondrial suspension (5·8 mg. of protein) in 0·25M-sucrose. Final volume, 2 ml. Temperature of incubation, 19°. Time, 20 min.

Additions	Concn. (mM)	Incorporation of $^{32}P_i$ into ATP (counts/min.)	Inhibition (%)
Nil	—	6300	0
Formaldehyde	0·5	6180	2
	1·0	5865	7
	2·0	4640	26

Table 8. *Effect of formaldehyde and dinitrophenol on the adenosine triphosphatase of mitochondria*

The components of the medium were: KCl, 27·5 mM; $MgCl_2$, 10 mM; ATP (sodium salt), 5 mM; tris-HCl buffer, pH 7·4, 50 mM; 0·5 ml. of mitochondrial suspension (2·8 mg. of protein) in 0·25M-sucrose. Final volume, 2 ml. Temperature of incubation, 25°. Time, 20 min.

Additions	Concn. of formaldehyde (mM)	P_i liberated (μmoles)
Nil	0	1·66
	0·5	1·82
	1·0	1·92
	2·0	2·20
Dinitrophenol (0·05 mM)	0	2·87
	0·5	2·32
	1·0	2·28
	2·0	2·30

Table 9. *Effect of formaldehyde on mitochondrial respiration in an adenosine diphosphate-deficient system*

The components of the medium were the same as described for oxidative phosphorylation under Materials and Methods with the following exceptions: glucose and hexokinase were omitted; potassium succinate, 10 mM; 0·5 ml. of mitochondrial suspension (3·5 mg. of protein) in 0·25M-sucrose. Final volume, 3 ml. Temperature of incubation, 37°. Time, 30 min.

Additions	Concn. of formaldehyde (mM)	Oxygen uptake (μg.atoms)	Inhibition (%)
Nil	0	6·7	0
	0·5	6·6	1·5
	1·0	5·4	20
Dinitrophenol (0·05 mM)	0	13·4	0
	0·5	9·2	31
	1·0	6·9	49

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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