REMARKS

The spectrum shows, in addition to the visually recorded α -, β - and γ -bands, a distinct δ -band, which takes the shape of a shoulder in the oxidized cytochrome *c*, and has a sharp maximum in the reduced form. This band has an isosbestic point for the ferri- and ferro-forms of cytochrome *c* near to 340 $m\mu$, which may be useful in the spectrophotometric study of oxidation and reduction of coenzyme I in systems also containing cytochrome *c*.

There is a distinct difference in the so-called 'protein' band between the reduced and oxidized

forms of cytochrome *c* around 280 $m\mu$. Since the spectra of the amino acids that absorb in this region (tyrosine, tryptophan and phenylalanine) were probably not affected by the state of oxidation or reduction of the haem, it must be assumed that the difference between the two spectra was due to a relatively large absorption of the haem in this region, which differed in the ferri- and ferro-states.

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Effect of Fluoropyruvate on the Swelling, Phosphorylative Activity and Respiration of Guinea-Pig Liver Mitochondria

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The great difference in activity between fluoropyruvate and fluoroacetate both *in vivo* (Mager & Blank, 1954) and *in vitro* (Gal, Peters & Wakelin, 1956; Avi-Dor & Mager, 1956*a*) indicates that no fluoroacetyl fragment is formed from fluoropyruvate. The finding that fluoropyruvate alkylates thiol compounds (Avi-Dor & Mager, 1956*b*; Peters & Hall, 1957) explains adequately the inhibition, by this compound, of the reactions (utilizing coenzyme A, 6:8-thioctic acid) which control the entry of pyruvate and therefore possibly of fluoropyruvate into the citric acid cycle.

The effect of fluoropyruvate on the respiration of mitochondrial preparations, on the other hand, seems to be connected more directly with a primary damage to the mitochondrial structure than with an inhibition of a particular enzyme of the respiratory chain (Avi-Dor & Mager, 1956*a*). Whether such a pattern of inhibition is characteristic of fluoropyruvate only, or is also encountered with other thiol-alkylating agents, such as iodoacetate, was therefore investigated.

A comparison has been made of the effects of fluoropyruvate and iodoacetate on the swelling, phosphorylative activity and respiration of liver mitochondria.

EXPERIMENTAL

Materials. Fluoropyruvic acid was synthesized as described by Blank, Mager & Bergmann (1955). Adenosine triphosphate (ATP) was a product of Pabst Laboratories

(Milwaukee, Wis., U.S.A.); diphosphopyridine nucleotide (DPN) (95% purity) was a product of Sigma Chemical Co. (St Louis, Mo., U.S.A.). All other chemicals were of A.R. grade.

Preparation of mitochondria. Guinea-pig liver mitochondria were prepared according to the method of Schneider (1948), as modified by Swanson (1956). Mitochondria were suspended in 0.25 M-sucrose. The final suspension (4 mg. of N/ml.) had an extinction of 0.25, when it was diluted 40 times with 0.25 M-sucrose, in a Coleman spectrophotometer at 500 $m\mu$. Unless otherwise stated in the legends, 0.5 ml. of the final suspension was used for each of the reported experiments, except for the spectrophotometric measurements, where 0.04 ml. was employed.

Methods

The extinction of the mitochondrial suspension was determined at 600 $m\mu$ in the Beckman spectrophotometer, model DU, with Corex cells of 1 cm. light path. Manometric experiments were carried out by the conventional Warburg technique. Adenosine triphosphatase (ATPase) activity was measured according to the method of Lardy & Wellman (1953). Oxidative phosphorylation was determined by the procedure described by Hunter (1955). The ATP- ^{32} P exchange reaction was estimated as described by Swanson (1956). The counts were made in a thin mica-window Geiger counter tube. Corrections were applied for self-absorption of the charcoal layer. The average specific activity of ^{32} P during the experiment was used to calculate the exchange rates, since the initial specific activity changes owing to ATPase activity. For quantitative estimation of phosphorus the method of Fiske & Subbarow (1925) was adopted. The determination of fluoropyruvate was based on the procedure described by Avi-Dor & Mager (1956*b*): the sample was incubated at 58° for 15 min. with a large excess of cysteine. The light-absorption was measured at

300 μ m in silica cells of 1 cm. light path, with the blank containing all components except fluoropyruvate. Before each experiment a calibration curve was prepared with known amounts of fluoropyruvate. The correlation between light-absorption at 300 μ m and the concentration of fluoropyruvate was linear within the range 30–120 μ m-fluoropyruvate. The millimolar extinction coefficient at 300 μ m (ϵ mm) was 5.2. In order to detect whether, during the incubation of fluoropyruvate with the mitochondria, any change had occurred in the concentration of the fluoro compound or whether any substances had been released that might affect the inhibitory action of fluoropyruvate, the following procedure was adopted: the inhibitory effect of fluoropyruvate at various time intervals was determined in the same medium as described in Table 4 with glutamate as the substrate. At the end of the incubation period the contents of the Warburg flasks were chilled to 0° and centrifuged in the cold. The concentration of fluoropyruvate in this supernatant was determined spectrophotometrically. To one sample no additional amount of fluoropyruvate was added. In another sample the concentration of fluoropyruvate was readjusted to the initial level. A fresh suspension of liver mitochondria was added to each sample and the incubation in the Warburg apparatus was resumed.

RESULTS

Effect of fluoropyruvate and iodoacetate on the translucency of mitochondrial suspensions. Isolated liver mitochondria, when suspended in isotonic sucrose solution, are known to swell at a slow rate. This swelling is indicated by a gradual decrease in light-absorption. Certain metal ions, chelating agents and anaerobic conditions retard the decrease in extinction, and agents exerting a deleterious effect on the structural integrity of the mitochondria accelerate it (Tapley, 1956; Hunter, Davis & Carlat, 1956).

In Table 1 are compared the effects of fluoropyruvate and iodoacetate on the rate of swelling of liver mitochondria under a variety of experimental conditions. The addition of these compounds to the mitochondrial suspension was associated with an increase in the rate of swelling by a factor of two

to three. On the other hand, when any of Mn^{2+} , Mg^{2+} , Zn^{2+} , ATP, ethylenediaminetetra-acetic acid (EDTA) or citrate was added (Table 1), or when the dissolved O_2 was replaced by N_2 (anaerobiosis), the swelling of the mitochondria was suppressed or completely abolished. Among the substrates of the tricarboxylic acid cycle, only citrate and isocitrate (10 mM) suppressed the swelling; all others increased the rate of swelling by 30–50 % compared with the control. In the systems thus stabilized, the effect of both fluoropyruvate and iodoacetate was markedly diminished. Furthermore, the increases in the rate of swelling, caused by the addition of Ca^{2+} ions on the one hand and by that of fluoropyruvate or iodoacetate on the other, were additive.

When both ATP and Mg^{2+} ions were added simultaneously, their combined effect depended on the ratio of their concentrations. Minimum stabilization was obtained when the concentration of Mg^{2+} ions was half of that of ATP (Fig. 1). Since ATP is known to form chelates with bivalent metals (Martel & Schwarzenbach, 1956), this seems to indicate that only free Mg^{2+} ions or ATP are effective as protecting agents, the $Mg^{2+}(ATP)_2$ complex, which according to Ernster & Löw (1955) plays an important role in the control of the physiological state of the mitochondria, being inactive.

The protection exerted by cysteine (Table 1) is probably due to its interaction with the two halogen compounds.

Effect of fluoropyruvate and iodoacetate on adenosine triphosphatase activity. An accelerating effect of fluoropyruvate and iodoacetate on the ATPase activity of rat-liver mitochondria has been reported by Fairhurst, Smith & Gal (1956). According to these authors, fluoropyruvate is more effective than iodoacetate at equimolar concentrations. In contrast with this observation, both halogen compounds showed a comparable effect in

Table 1. *Changes in the translucency of mitochondrial suspensions caused by fluoropyruvate and iodoacetate*

Each cell contained 20 mM-2-amino-2-hydroxymethylpropane-1:3-diol chloride buffer (pH 7.4), 0.25M-sucrose, 13 mM-KCl and 0.04 ml. of liver mitochondria suspension, which is added as the last component. Total vol., 3 ml. The experiment was carried out at room temperature (about 20°). Spectrophotometric readings were taken at 30 sec. intervals for a period of 10 min.

Additions or conditions	Concn. (mM)	- $\Delta E/10$ min.		
		Control	0.3 mM-Fluoropyruvate	0.3 mM-Iodoacetate
No addition	—	0.055	0.104	0.148
$MnSO_4$	3.3	0	0.016	0.010
$MgSO_4$	3.3	0.007	0.042	0.036
$ZnSO_4$	3.3	0.030	0.035	0.030
ATP	0.26	0.043	0.061	0.051
EDTA	6.6	0.032	0.062	0.070
Cysteine	3.3	0.040	0.035	0.038
Anaerobiosis	—	0.038	0.022	0.032
$CaCl_2$	6.6	0.113	0.180	0.260

guinea-pig liver mitochondria (Table 2). As can be seen from Table 2 and by comparison of Figs. 1 and 2, conditions which prevent swelling also diminish the effect of fluoropyruvate and iodoacetate on the ATPase activity. An excess of

Mg²⁺ ions in relation to ATP inhibits the ATPase activity probably not only by the suppression of swelling but also by competing with the actual substrate of the ATPase (ATP-Mg according to Kielley, 1951) for the active sites of the enzyme surface.

Effect on the exchange between ³²P and adenosine triphosphate. According to recent concepts of the

Fig. 1. Effect of Mg²⁺ ions and ATP on the swelling of mitochondria induced by fluoropyruvate or iodoacetate. The reaction mixture was the same as described in Table 1. Open symbols refer to the effect of Mg²⁺ ions without addition of ATP. Filled symbols refer to the effect of Mg²⁺ ions in the presence of 2.6 mM-ATP. ○, ●, Control; △, ▲, with addition of 0.3 mM-fluoropyruvate; □, ■, in the presence of 0.3 mM-iodoacetate. The spectrophotometric readings were taken at room temperature (approx. 20°) at 30 sec. intervals for a period of 10 min.

Fig. 2. Effect of ATP:Mg²⁺ ion ratio on the ATPase activity of mitochondria. The reaction mixture was the same as described in Table 1, but for the addition of 2.6 mM-ATP and Mg²⁺ ions as indicated in the figure. ●, Control; ▲, 0.3 mM-fluoropyruvate; ■, 0.3 mM-iodoacetate. The experiments were carried out at 28°. Duration of experiment: 15 min.

Table 2. *Effect of fluoropyruvate and iodoacetate on adenosine triphosphatase activity*

The reaction mixture was the same as described in Table 1, except for the addition of 2.6 mM-ATP and 1.3 mM-MgSO₄. Liver mitochondria suspension: 0.5 ml. Total vol., 3 ml. Temp. of incubation 28°.

Additions	Concn. (mM)	Inorganic phosphate released (μg. atoms/15 min.)		
		Control	0.3 mM- Fluoropyruvate	0.3 mM- Iodoacetate
None	—	2.2	7.0	7.5
2:4-Dinitrophenol	0.1	11.7	—	—
EDTA	3	1.8	4.6	5.0
Citrate	10	1.3	3.1	3.5
Cysteine	3	1.2	2.5	3.0
CaCl ₂	0.066	3.2	8.5	—

nature of oxidative phosphorylation, the hydrolysis of ATP by mitochondria (ATPase) may be due to a reversal and diversion of the step which leads to the synthesis of the adenine nucleotide (Chance & Williams, 1956; Hülsmann & Slater, 1957). It has been of interest therefore to test whether fluoropyruvate and iodoacetate also affect the rate of formation of ATP. With glutamate as a substrate, fluoropyruvate and iodoacetate each caused a small but significant decrease in the P:O ratio (Table 3) both with and without 10 mM-sodium fluoride. The effect of the halogen compounds on the exchange between the terminal phosphate group of ATP and [^{32} P]phosphate, a reaction closely related to oxidative phosphorylation (Boyer, Luchsinger & Falcone, 1956), has also been assayed in the absence of substrate. At a concentration of 0.6 mM, the rate of exchange during the first 4 min. of the experiment was little affected by fluoropyruvate or iodoacetate. After prolonged incubation (more than 8 min.) some inhibition of the exchange became apparent, particularly with iodoacetate. This may have been due to a lowering of the concentration of ATP.

Effect of respiration. The pattern observed in the inhibition of the oxidation of citric acid-cycle intermediates in liver mitochondria (Table 4) was similar to that described for kidney preparations (Avi-Dor & Mager, 1956a). The oxidations of citrate, isocitrate and succinate are inhibited by the two halogen compounds to a much lower

degree than those of the other intermediaries of the cycle. Mg^{2+} ions very markedly antagonize the respiratory block induced by the two halogen compounds (Fig. 3).

Addition of 0.5 mM-EDTA during the homogenization of the tissue decreased the respiratory inhibition by 20 %. However, no complete reversal was obtained as with Mg^{2+} ions.

Reversibility of the inhibitory effect of fluoropyruvate on respiration. The inhibitory effect of fluoropyruvate on respiration is not reversed by washing the mitochondria with isotonic sucrose. Addition of glutathione (GSH) concurrently with the inhibitor abolished the inhibition. When glutathione was added after the mitochondria had been incubated with fluoropyruvate, the ability of GSH to restore the respiration was reduced. The reversal of the inhibition was more complete if DPN was added together with, or up to 10 min. after, the addition of GSH (Table 5).

The concentration of fluoropyruvate decreased slowly during the time of incubation. The rate of disappearance was not affected by the concentration of Mg^{2+} ions (Fig. 4).

In some experiments the incubation of the mitochondria with fluoropyruvate was stopped after 30 or 50 min. The contents of the Warburg flasks were centrifuged and fresh mitochondria were added to the supernatant. When the experiment was now resumed, the inhibition observed was proportional to the residual concentration of

Table 3. *Effect of fluoropyruvate and iodoacetate on oxidative phosphorylation*

Each flask contained: 6 mM-2-amino-2-hydroxymethylpropane-1:3-diol chloride buffer (pH 7.4), 10 mM-phosphate buffer (pH 7.4), 0.25 M-sucrose, 10 mM-KCl, 1.3 mM-ATP, 1.3 mM- $MgSO_4$, 33 mM-glucose, hexokinase 1 mg. [approx. 50 hexokinase units (Kunitz & McDonald, 1946)], 10 mM-NaF, 10 mM-glutamate. Mitochondrial suspension: 0.5 ml. Total vol., 3 ml. Incubation time: 30°. Equilibration time: 5 min. Time of incubation: 20 min. For details of the experiment see under Experimental.

	Control	0.3 mM- Fluoropyruvate	0.6 mM- Fluoropyruvate	0.3 mM- Iodoacetate
O (μ g. atoms)	11.0	9.0	4.8	9.0
P (μ g. atoms)	29.0	19.8	9.0	18.8
P:O	2.6	2.2	1.9	2.1

Table 4. *Effect of fluoropyruvate and iodoacetate on oxidation of different substrates*

Each flask contained the same reaction mixture as described in Table 1, except for the addition of 2.6 mM-ATP, 1.3 mM- $MgSO_4$, and a substrate at a concentration of mM. Liver mitochondria suspension (0.5 ml.) is added. Total vol., 3 ml. Gas phase: air. Incubation time: 10 min. Readings have been taken at 10 min. intervals for a period of 60 min.

Substrate	O ₂ uptake (μ moles/hr.)		
	Control	0.3 mM- Fluoropyruvate	0.3 mM- Iodoacetate
Pyruvate	12.6	3.1	4.0
α -Oxoglutarate	20.6	7.4	8.4
Malate	14.4	5.6	6.5
Glutamate	20.4	12.4	14.9
isoCitrate	22.2	18.7	15.4
Citrate	22.0	18.9	16.4
Succinate	20.8	18.3	19.0

Fig. 3. Effect of ATP: Mg ratio on respiration. Contents of Warburg flask: 17 mM-2-amino-2-hydroxymethylpropane-1:3-diol (tris) chloride buffer (pH 7.4); 0.25 M-sucrose; 13 mM-KCl; 2.6 mM-ATP; 10 mM tris glutamate. Total vol., 3 ml. Contents of centre well 0.2 ml. of 20% KOH soln. Incubation at 37°. Gas phase: air. Equilibration time: 10 min. Readings were taken at 10 min. intervals for a period of 60 min. ●, Control; ▲, 0.3 mM-fluoropyruvate; ■, 0.3 mM-iodoacetate.

Fig. 4. Effect of incubation with mitochondria on the concentration and inhibitory properties of fluoropyruvate. The reaction mixture was the same as described in Table 4, with glutamate as the substrate. For details of the experimental procedure see under Experimental. ●, Concentration of fluoropyruvate in the supernatant after various times of incubation with mitochondria; △, ▲, inhibition caused by the supernatant, separated after varying times of incubation, on the oxidation by fresh mitochondrial suspensions. In the experiments designated with a filled symbol the concentration of fluoropyruvate was readjusted to its original level (0.6 mM).

Table 5. *Reversibility of the inhibitory action of fluoropyruvate on respiration by glutathione and diphosphopyridine nucleotide*

The flasks contained the same reaction mixture as described in Table 4. Fluoropyruvate (0.3 mM) was added at zero time, and 3 mM-GSH was added at the times shown in the table; 10 min. after the addition of GSH, DPN (0.1 mM) was added. The flasks, which had been at room temp. (about 20°) up to that time, were transferred into the Warburg apparatus, at 37°. Controls, without fluoropyruvate, were run under the same conditions.

Addition	Time of addition (min.)	O ₂ uptake (μmoles/hr.)			
		Control		Fluoropyruvate	
		No DPN	DPN	No DPN	DPN
None	—	19.0	19.5	1.8	6.8
GSH	0	19.3	20.0	19.0	19.5
GSH	10	18.5	18.0	10.3	14.8
GSH	20	18.7	17.6	7.5	11.6

fluoropyruvate at 30 or at 50 min. By readjusting the concentration of fluoropyruvate to its original level its inhibitory action was restored to the initial value (Fig. 4).

It follows that neither the products which result from the decomposition of fluoropyruvate nor the substances released from the mitochondria antagonize the respiratory inhibition caused by fluoropyruvate.

DISCUSSION

The observed effects of fluoropyruvate and iodoacetate on mitochondrial systems are compatible with the assumption that these two compounds affect the structural integrity of liver mitochondria. It is recalled that activation of the latent ATPase (Kielley & Kielley, 1951) and inhibition of the oxidation of substrates linked to DPN as coenzyme (Ernster & Löw, 1955; Mager & Avi-Dor, 1956) are typical manifestations of damage to the mitochondrial structure. The hypothesis of structural damage is further supported by the finding that conditions which suppress the swelling of the mitochondria that are caused by addition of the two halogen compounds also diminish or abolish their effect on ATPase activity and on respiration.

No correlation can be found between the protective effect of the metal ions (Mg^{2+} and Ca^{2+}), and the stability constants of their complexes with thiol compounds (Gurd & Wilcox, 1956). Likewise, anaerobiosis cannot have a direct influence on the reaction between thiol groups and alkylating agents. The protecting effect of agents of widely different nature (metal ions, chelating agents) cannot be explained by the assumption that the reactivity of the available SH groups towards the halogen compounds is altered. It is conceivable, however, that the protecting agents change the number of SH groups open to attack by influencing the state of folding of the proteins, which are the bearers of the sensitive SH groups.

The difference in the extent of inhibition of the oxidation of different citric acid-cycle intermediates is much more marked in liver mitochondria than in kidney preparations. Oxidation of succinate is not inhibited, in accordance with the fact that this is not dependent on DPN. Among the DPN-linked substrates, α -oxo acids and malate are more sensitive than glutamate to the inhibitory action of both fluoropyruvate and iodoacetate. In this connexion it is relevant to recall that two thiol compounds, namely coenzyme A and 6:8-thioctic acid, are involved in the oxidation of α -oxoglutarate, pyruvate and malate (by removal of the inhibitory oxaloacetate in the last-named), whereas the oxidation of glutamate depends only on DPN. Citrate, which has a special place among the metabolites of the Krebs cycle since it is oxidized both

by di- and tri-phosphopyridine nucleotide-linked dehydrogenases, and also because of its strong protective effect on the mitochondrial structure, is nearly as resistant to inhibition as succinate.

The results of this investigation do not indicate a direct effect of thiol-alkylating reagent on oxidative phosphorylation. The small decrease observed in the P:O ratio or in the rate of incorporation of ^{32}P into ATP may well reflect the influence of secondary factors, such as the inhibiting action of adenosine diphosphate, which is released from ATP on prolonged incubation.

SUMMARY

1. The effect of fluoropyruvate and iodoacetate on swelling, phosphorylating activity and respiration of guinea-pig liver mitochondria has been compared.

2. Both compounds accelerate the rate of swelling, activate the latent adenosine triphosphatase, and inhibit the oxidation of diphosphopyridine nucleotide-linked substrates of the citric acid cycle.

3. Certain metal ions (Mg^{2+} and Mn^{2+}) and certain chelating agents (citrate and ethylenediaminetetra-acetate) protect the mitochondria from swelling. They also reverse the effect of fluoropyruvate and iodoacetate on swelling, on adenosine triphosphatase activity and on respiration.

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Studies of Invertebrate Collagen Preparations

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The collagens may be defined as a group of fibrous proteins which give a characteristic wide-angle X-ray-diffraction pattern (Astbury, 1938; Bear, 1952). Although the collagens are widely distributed throughout the animal kingdom (Bear, 1952; Rudall, 1955), physical and chemical studies have been largely confined to those of vertebrates and particularly of mammals, because of their industrial and clinical importance. The vertebrate collagens form a compact group in their amino acid composition (Eastoe & Leach, 1958), and it is of interest to extend these studies to collagens from a wider range of animals, since one might expect that the greater the range of collagens for which data are available the greater may be the chance of detecting correlations between the chemical and physical properties of these proteins. It was with this aim in view that the cuticle of the earthworm was studied by Watson (1958*a*). The present publication is concerned with studies on two other collagens, those from the cuticle of *Ascaris lumbricoides* and from the Cuvierian tubules of *Holothuria forskali*.

Ascaris cuticles were first shown by Fauré-Fremiet & Garrault (1944) to give the characteristic wide-angle X-ray-diffraction pattern of collagen. For many years previously, however, the cuticle had been studied by chemical and histological methods. It is a very complex structure, and Chitwood & Chitwood (1937) have distinguished nine morphologically distinct layers. Chitwood (1936) also describes the methods which may be used to separate the various substances in the cuticle and discusses their morphological location. Bird (1956, 1957) has examined the amino acid composition of the whole cuticle by

paper chromatography, and also of extracts prepared from it by methods based on those of Chitwood (1936), and has reported a quantitative amino acid analysis in which values are given for most of the amino acids present.

The ejected filaments of the sea cucumbers were shown to contain collagen by Astbury (1939) from the X-ray-diffraction evidence. These filaments arise by extension from the Cuvierian tubules of the animals, of which the present example is *Holothuria forskali*.

An analytical investigation of the whole tissues preceded attempts to isolate and analyse the collagen proteins, for two reasons. First, such a control is useful in deciding whether the composition of the purified collagen is representative of the bulk of the collagen present, and in estimating the yield of collagen in the purification process. Secondly, it helps to determine which amino acid values in the final analysis are apt to be greatly influenced by the presence of residual impurities. Some of the preliminary results with the filaments of *Holothuria forskali* have been reported (Watson, 1958*b*).

EXPERIMENTAL

Preparation of materials for analysis

Cuvierian tubules of Holothuria forskali. Tubules were collected from the living animals by lifting them out of the sea water in which they had been transported to the laboratory. In a few moments the tubules were extruded through the cloaca as long, intensely sticky filaments. These were washed in several changes of water, two changes of ethanol and two changes of ether, and allowed to dry in air. About 2 g. of air-dried filaments was obtained from three animals. Two such preparations were made.