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Effects of Fluorocompounds on Mechanisms Related to Mitochondrial Phosphate Transfer

While studying oxidative phosphorylation in rat liver mitochondria (*1*), we have found that the P/O ratio of such preparations is markedly lowered on addition of fluoroacetate to the system (Table 1) (*2*). The effect of this compound on the adenosine triphosphate (ATP) that is formed can be seen before any appreciable inhibition of oxygen consumption is observed, supporting the belief that processes regulating phosphate transfer have a higher sensitivity to the fluorocompound than do the respiratory steps associated with the phosphorylation. In view of these findings, the conclusion (*3*) that fluoroacetate has no effect on oxidative phosphorylation cannot be supported.

Since other experiments showed that the hexokinase used to "trap" the ATP was not affected by fluoroacetate, fluorocitrate, or fluoropyruvate, the lowered P/O ratio could be due to the effect of the fluorocompound on the synthesis of ATP or to an accelerated dephosphorylation of the ATP after it was formed. Attempts to show an effect on the synthesis of ATP by using the mitochondrial extract described by Lehninger (*4*) have been complicated by the attendant ATPase activity of the preparations, although it was shown that fluoroacetate decreases the net incorporation of P^{32} into ATP in the reaction

$$ADP + P^{32} \rightarrow ATP^{32}$$

In vivo experiments (*5*) have shown a small but significant decrease in labile nucleotide phosphorus in the brain following injection of fluoroacetate, and on comparing the effects of fluoroacetate, fluorocitrate, fluoropyruvate, and 2–4 dinitrophenol (DNP) on the release of phosphate from adenosine monophosphate (AMP), adenosine diphosphate (ADP), and ATP by liver mitochondria, we observed (Table 2) that the dephosphorylation of all three nucleotides was stimulated. Fluorocitrate was by far the most active of the fluorocompounds, showing stimulation even at $10^{-6}M$, while fluoroacetate was only slightly active. Interestingly, DNP and the fluorocompounds at very low concentrations produced consistently lower hydrolysis of the nucleotides than did the controls. It is of significance that, whereas the greatest stimulation of phosphate release by the fluorocompounds is seen when AMP is the substrate, the stimulation of dephosphorylation produced by DNP is greatest when ATP is used. Since the mitochondria are capable of rapid interconversion of these three nucleotides, as demonstrated chromatographically, the reactions undergone by a given nucleotide are numerous, and studies on the time-courses of these interconversions in the presence and absence of the fluorocompounds are currently under investigation in an attempt to identify the site(s) of action of these compounds.

That a certain degree of structural integrity of the mitochondria is necessary for these effects is demonstrated by experiments in which the mitochondria have been "pre-aged" by the method of Lardy (*6*): under these conditions, the ability of the fluorocompounds and of DNP to stimulate the dephosphorylation of the nucleotides is greatly diminished. Hunter (*7*) has shown that DNP has no action on the partially purified ATPase of potato, and we have now demonstrated that the soluble 5′-nucleotidase of rattlesnake venom (*8*) is affected by neither DNP nor fluoroacetate nor fluorocitrate.

It does not appear that the fluorocompounds exert their effects by combining with some metal within the mitochondria, for the addition of equivalent concentrations of Versene has no effect on the dephosphorylating activity of the mitochondria. Furthermore, it was found that oxalic acid ($3.7 \times 10^{-5}M$) suppresses mitochondrial ATPase.

We have found that *p*-chloromercuribenzoate (PCMB) and iodoacetate stimulate dephosphorylation of these nucleotides, iodoacetate being much less active than fluoropyruvate. This finding is of particular interest now, in view of the recently demonstrated interaction between fluoropyruvate and sulfhydryl compounds in a model system (*9*).

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Table 1. Effect of fluoroacetate on oxidative phosphorylation. The flasks contained the following: $0.02M$ tris buffer at *p*H 7.28; 6 μmole of $MgCl_2$; 120 μmole of KCl; 20 μmole of potassium fumarate; 25 μmole of KF; 0.5 μmole of ATP; 40 μmole of potassium phosphate; 1 ml of rat liver mitochondria in $0.25M$ sucrose; 744 μmole of sucrose; 30 μmole of glucose; and 0.5 mg of hexokinase added from a side arm, with a final volume of 3 ml. The contents were incubated for 15 minutes at 37°C in oxygen. The reaction was stopped by 0.5 ml of 20-percent trichloroacetic acid.

Item	Oxygen (μatoms)	Phosphate (μmole)	P/O
Control	2.58	6.39	2.48
Fluoroacetate ($3.3 \times 10^{-3}M$)	2.55	3.81	1.49

Table 2. Comparative effects on nucleotide hydrolysis. The flasks contained the following: 10 μmole of nucleotide freshly neutralized to *p*H 7.28; 744 μmole of sucrose; 6 μmole of $MgCl_2$; 120 μmole of KCl; $0.02M$ tris buffer at *p*H 7.28; and mitochondria (from approximately 300 mg of rat liver) in $0.25M$ sucrose. The final volume was 3 ml. The contents were incubated for 20 minutes at 30°C in a Dubnoff shaking incubator. Phosphate was determined by the method of Gomori (*10*). The values given have been corrected for endogenous phosphate. The figures in parentheses indicate percentage stimulation above the respective controls.

System	Phosphate (μmole) released from		
	AMP	ADP	ATP
Control	1.59	3.31	5.85
DNP ($10^{-5}M$)	2.37 (48%)	7.91 (138%)	14.16 (142%)
Control	1.19	3.76	5.99
Fluoroacetate ($10^{-3}M$)	0.97 (−19%)	4.34 (15%)	6.05 (0)
Control	0.88	2.22	4.31
Fluorocitrate ($1.3 \times 10^{-5}M$)	1.77 (100%)	3.80 (70%)	6.34 (47%)
Control	0.71	1.65	4.40
Fluoropyruvate ($10^{-4}M$)	1.31 (84%)	2.94 (73%)	5.60 (27%)
Control	1.48	3.28	5.94
Iodoacetate ($10^{-4}M$)	2.00 (35%)	4.14 (26%)	6.56 (5%)
PCMB ($10^{-4}M$)	2.57 (73%)	5.71 (74%)	9.28 (56%)

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Removal of Phosphorus from Hydrogen Peroxide by Kaolinite

Hydrogen peroxide has been used as an oxidizing agent in the determination of organic phosphorus in soils and also in the determination of phosphorus in a solution that contains interfering organic matter, such as the dithionite-citrate extraction of iron phosphate by Chang and Jackson (*1*). Hydrogen peroxide is often highly contaminated with phosphorus, for phosphoric acid is usually added as stabilizing agent. Merck chemically pure 30-percent Superoxol has a maximum PO_4 content of 0.005 percent, which is equivalent to about 16.3 ppm of phosphorus. Therefore it is necessary to remove the phosphorus from the hydrogen peroxide before it is used for the aforementioned determinations. It has been found that the phosphorus could be brought to a satisfactorily low concentration by adsorption on kaolinite (Tables 1, 2, and 3).

Dickman and DeTurk (*2*) used distillation to free hydrogen peroxide from phosphorus. This method requires a comparatively complicated set-up, constant care, and a longer period of time than the new method. Furthermore, the product obtained is only half of the original amount, and the concentration of the hydrogen peroxide is also considerably decreased.

Dickman and Bray (*3*) recommended treatment with $FeCl_3$ and $CaCO_3$ for removal of phosphorus from hydrogen peroxide. They claimed that colorimetric analysis of 5 ml of hydrogen peroxide showed 0.2 ppm of phosphorus. Since $FeCl_3$ causes vigorous decomposition of the peroxide, this procedure is not easily carried out successfully.

In the proposed procedure, 10 g of Merck's "colloid kaolin" (mainly kaolinite) is shaken by hand in 100 ml of hydrogen peroxide for 5 minutes. The suspension is allowed to stand for 1 minute and then is decanted through a Büchner funnel under gentle suction. The filtrate is again treated with kaolinite as before. The number of treatments depends on the original phosphorus content of the peroxide. Three or four (rarely five) treatments are sufficient to reduce the phorphorus content to about 0.1 to 0.2 ppm. It is not necessary to make the first and second filtrates clear. The final filtrate is made clear by centrifugation. Then 0.5 ml of concentrated HCl is added to every 100 ml of the clear hydrogen peroxide for stabilization.

The proposed procedure has the advantages of (i) being simple, (ii) being time saving, (iii) being able to reduce phosphorus to about 0.1 to 0.2 ppm, (iv) giving a high percentage of volume recovery, and (v) giving little loss of H_2O_2 concentration.

Table 1. Removal of phosphorus from hydrogen peroxide by different amounts of kaolinite.

Kaolin (g/10 ml of H_2O_2)	Resulting concn. of P (ppm)	
	Replication 1	Replication 2
0	6.5	6.5
0.5	3.35	3.4
1.0	1.9	1.9
2.0	1.65	

Table 2. Removal of phosphorus from hydrogen peroxide by successive treatments with kaolinite.

Treatment No.	Resulting concn. of P (ppm)			
	1 g kaolinite per 10 ml H_2O_2		0.5 g kaolinite per 10 ml H_2O_2	
	Replication 1	Replication 2	Replication 1	Replication 2
0	6.5	6.5	6.5	6.5
1	1.9	1.9	3.35	3.4
2	0.2	0.2	1.58	
3	0.12	0.12	0.50	0.5
4			0.12	0.12

Table 3. Volume recovery of hydrogen peroxide, phosphorus concentration, and hydrogen peroxide concentration after three treatments with kaolinite at a rate of 10 g of kaolinite per 100 ml of hydrogen peroxide. The percentage of hydrogen peroxide was determined by titration with standard 0.1*N* $KMnO_4$ according to Treadwell and Hall (*4*).

Time	Total volume (ml)	P content (ppm)	H_2O_2 (%)
Before treatment	500	6.5	28.5
After treatment	430	0.11	26.1

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Relationship between Membrane Potentials and Repolarization in the Rat Atrium

The variation in membrane potentials observed between cell penetrations in a single rat atrium might be interpreted as arising experimentally; on the other hand, such variation may indicate true differences between atrial cells. An inverse relationship between the magnitude and duration of the action potential in the normal atrium was observed.

This correlation was studied quantitatively in 25 atria, from each of which 70 to 80 readings were obtained, by tabulating durations and overshoots of the action potential for 5-mv ranges in the action-potential magnitude (*1*). Membrane potentials were determined with microelectrodes on atria that were stimulated electrically at a rate of 200 per minute as previously described (*2*). The results for 1866 atrial cell penetrations are shown in Table 1. The greater the magnitude of the action potential, the shorter is the duration, and this inverse relationship is linear over the entire range studied. The overshoot varied relatively little and was constant above an action potential of 75 mv.

It might be expected that the lower the action potential, the shorter would be the duration, assuming a constant rate of repolarization; however, just the opposite was observed. The repolarization rate was approximately five-fold greater for the highest action potentials than for the lowest. Since the overshoot was relatively constant over the range, contrary to that of Purkinje fibers (*3*), and since the entire atrial preparation was depolarized during each impulse, it would appear that the repolarization rate is independent of the degree of change from