STUDIES ON THE NON-OXIDATIVE EXCHANGE BETWEEN INORGANIC PHOSPHATE AND ATP, AS CATALYZED BY INTACT MITOCHONDRIA*

by

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The oxidizing and phosphorylating tissue granules known as mitochondria used to be considered the main locus for tissue ATP**-splitting activity. It seemed useful as a preliminary to studies on phosphorylation to characterize as completely as possible the so-called ATPases. When KIELLEY AND KIELLEY reported[1] that very gently treated mitochondria showed no ATPase activity, we, along with many other investigators, turned attention to finding which circumstances could evoke such activity. It was soon evident that whenever phosphorylating capacity was damaged, ATP-splitting activity appeared. Many relationships between "ATPase" and phosphorylations were suggested to explain the strong coincidences. We adopted the hypothesis that the "inactive" ATPase might merely be masked by concurrent ATP synthesis, and so we introduced radioactive inorganic phosphate*** into the system to test the hypothesis. This resulted in the discovery that a very rapid exchange was going on between the inorganic and nucleotide phosphates, under conditions which presumably could not support oxidative phosphorylation.

Since any phosphate turnover reactions might conceivably be related to the phosphorylation processes, it seemed worthwhile to investigate this exchange in some detail. While these experiments were in progress, BOYER *et al.*[2] reported observing the exchange phenomenon. It is not yet clear to us what may be the relationships between ATP-splitting, exchange, and phosphorylation, but the present data indicate that these reactions are somehow closely related.

EXPERIMENTAL

The mitochondria were prepared by an adaptation of the method of SCHNEIDER[3]. The adult albino rats were killed by decapitation, the livers rapidly removed and chilled in 0.25*M* sucrose.

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** The following abbreviations will be used: AMP = adenosine-5-monophosphate; ATP = adenosine triphosphate; TCA = trichloroacetic acid; EDTA = ethylenediaminetetraacetic acid; DNP = 2,4-dinitrophenol; Tris = tris(hydroxymethyl)aminomethane.

*** The radioactive phosphate was obtained on allocation by the Atomic Energy Commission.

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After mincing with scissors, the tissue was homogenized in an all-glass Potter-Elvehjem homogenizer in sucrose solution, and strained through cheese cloth. The volume was made to ten times the liver weight, and the homogenate cleared of heavy debris by centrifuging 10 minutes at 1000 × *g*. The mitochondria were then collected after 30 minutes at 3000 × *g*, and washed twice in sucrose solution, discarding the fluffy layer each time. After the final washing, the granules were taken up in isotonic sucrose to a final volume equal to the original tissue weight. All the solutions were kept at 0° C, and the centrifugations were carried out in a cold box at 0° C. The preparations usually contained about 2 mg N per ml of suspension. In all the experiments reported, the mitochondria equivalent to 100 mg of tissue (0.1 ml suspension, about 0.2 mg N) were added to each 1 ml of incubation volume. The reactions were stopped by adding 2 ml 10% TCA. After chilling, the suspensions were diluted to 5 ml with H_2O, centrifuged and suitable aliquots of the clear supernatant solution taken for analysis. The inorganic phosphate was determined by the method of FISKE AND SUBBAROW[4]; the easily hydrolyzable phosphate after 20 minutes at 100° in 1 *N* H_2SO_4. The nitrogen in the mitochondria suspensions was determined by nesslerization after digestion with H_2SO_4 and H_2O_2.

The inorganic phosphate was separated from the nucleotide phosphate over acid-washed norit A, essentially as described by CRANE AND LIPMANN[5]. In order to remove all the counts from the charcoal in the zero time samples, it proved necessary to add 0.15 mg P as KH_2PO_4 in 3 ml 4% TCA after the first round of washings, and wash twice more with H_2O. Suitable aliquots of both fractions were taken, neutralized to phenol red, and evaporated to dryness in small aluminum pans, taking care to achieve an even distribution of deposited salts and charcoal over the surface of the pans. The counts were then made using a thin-mica window Geiger-Müller tube.

The materials used were the purest currently available. Since these investigations covered a period of several years, the ATP ranged from barium ATP about 90% pure through amorphous sodium ATP to crystalline sodium ATP. Some differences in reactions were noted as the purity of the ATP samples was improved, but no differences were noted when samples of comparable purity from different suppliers were compared. All the other chemicals used were of reagent grade. The EDTA was sodium versenate, kindly furnished by the Bersworth Chemical Company of Framingham, Mass.

RESULTS

In order to make possible comparisons between experiments using different amounts of phosphate and radioactivity, we adopted the convention of calculating the nucleotide radioactivity as the per cent progress toward the specific activity it would have at complete equilibration between the inorganic P and the two terminal P of ATP.

The actual order of magnitude of the measurements made and some typical time relationships are illustrated in Table I. There appears to be no way of predicting whether the degree of exchange will continue to increase as it sometimes does for as long as 20 minutes or whethe the curve will pass through a maximum as early as at six minutes. This makes comparisons between many factors at a given time

TABLE I

TIME COURSE OF EXCHANGE REACTION

Histidine-Tris buffer, Mg^{++} = 0.005*M*; EDTA = 0.01*M*; ATP = 0.0025*M*; Temp. = 26° C; times noted. Nitrogen = 1.89 mg/ml; 0.1 ml enzyme added to each ml final volume of incubation mixture.

Incubation time minutes	*Inorganic P*		*Nucleotide P*		*% Equil.*
	Micrograms	*cts/sec*	*Micrograms*	*cts/sec*	
2	33.3	610	138	215	32.6
4	32.5	550	142	296	43.4
6	31.6	460	145	374	55.0
10	31.6	397	138	452	68.0

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rather uncertain and erratic. Yet the continuous use of time curves makes the number of comparisons possible so small that the data become practically useless for generalizations. Frequently the decline in the radioactivity of the ATP seems related to ATP-splitting activity, so that some mention should be made of the characteristics of this activity.

The rate at which ATP-splitting activity develops is influenced by a large number of factors such as the temperature, pH, nature of the buffer, etc. The influence of pH and buffer on the activity measurable in 10 minute incubations is shown in Figs. 1 and 2. The tendency of magnesium ions to suppress ATP-splitting in histidine and Tris has also been found for the peak at higher pH in glycine, glycylglycine and

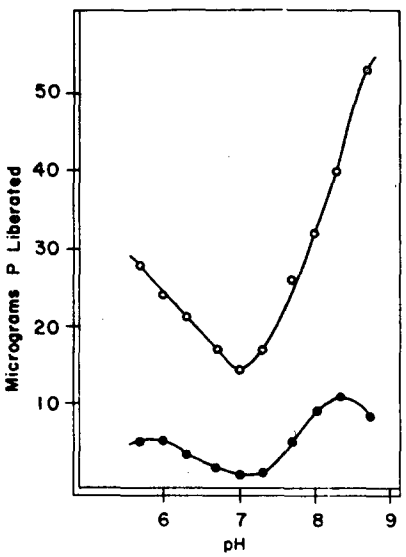

Fig. 1. Inorganic phosphate liberated from ATP in histidine buffers at 30° C, 10 minutes; ● – with Mg^{++} = 0.005*M*; ○ – No added Mg^{++}; ATP = 0.0025*M*.

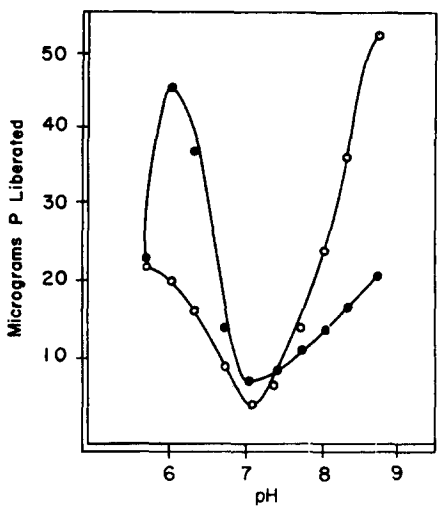

Fig. 2. Inorganic phosphate liberated from ATP in mixed succinate-Tris buffers at 30° C, 10 minutes; ● – with Mg^{++} = 0.005*M*; ○ – No added Mg^{++}; ATP = 0.0025*M*.

amino propanediol buffers. It has proven useful to adopt the appearance of magnesium stimulated ATP-splitting in histidine or Tris at pH 7.4 as a criterion for contamination of mitochondria with microsomes. The activating effect of magnesium on the peak at lower pH values in succinate can also be demonstrated in acetate, fumarate, malate and maleate buffers. Some mixtures can be made of histidine with succinate, acetate, fumarate, or malate in which magnesium has no apparent effect on ATP-splitting. Maleate, dinitrophenol, hypotonicity and aging evoke ATP-splitting which shows an optimum pH near neutrality. If the evoked activity is not too overwhelmingly great, traces of the two other peaks at pH 6 and 8 are usually visible also. The effect of calcium ion is obscured by the presence of magnesium, but when it is present alone it changes the pH-activity curves as shown in Fig. 3. The effect of Ca^{++} in Tris is similar to its effect in glycylglycine.

The effect of pH, buffer, calcium and magnesium on the exchange reaction is shown in Fig. 4, A and B. Results are here expressed as progress toward complete

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equilibration at only one time interval (10 min). Progress curves using several time intervals have given information consistent with this summary. EDTA has little

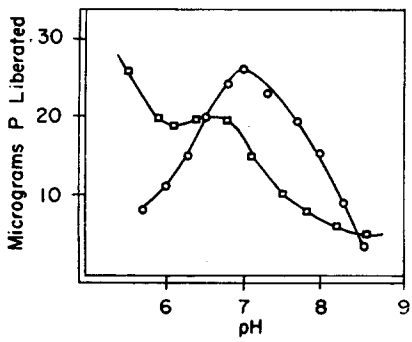

Fig. 3. Inorganic phosphate liberated from ATP in presence of calcium ions. Ca^{++} = 0.005M; ATP = 0.0025M. □ – in histidine buffers; ○ – in succinate-glycylglycine buffers. 30° C, 10 minutes.

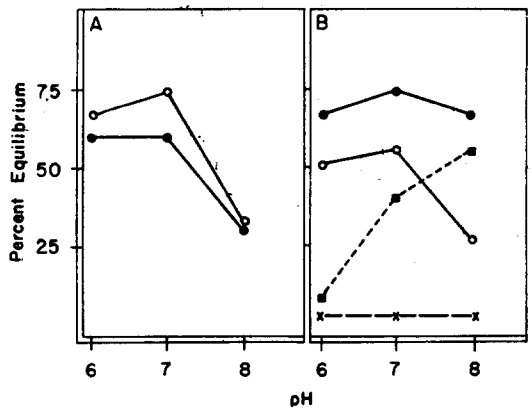

Fig. 4. Exchange between inorganic phosphate and ATP. A, in succinate-Tris buffers. B, in histidine buffers. 30°, 10 minutes. ○ – No added Mg^{++}; ● – Mg^{++} = 0.005M; × – Ca^{++} = 0.005M; ■ – Ca^{++} = 0.005M, EDTA = 0.01M; ATP = 0.0025M.

effect on the exchange reaction alone, but as shown in 4-B it relieves to some extent the inhibitory action of calcium ions. As might be expected, magnesium, calcium and hydrogen ions have effects on the exchange reaction which are the converse of their effects on ATP-splitting activity.

Several substances which have been reported to inhibit phosphorylation were tested with the exchange reaction. The effects of azide and dinitrophenol alone and in combination are shown in Fig. 5. It can be seen from this that had only the 10 minute value for azide been taken, slight inhibition would have been noted. However, for the whole time course azide at that concentration seems innocuous, or somewhat stimulatory. Even though the azide markedly inhibits the ATP-splitting usually evoked by DNP yet the ability to exchange is not restored. This suggests that the action of DNP occurs prior to the exchange, and does not consist merely of breaking down ATP after the exchange had occurred.

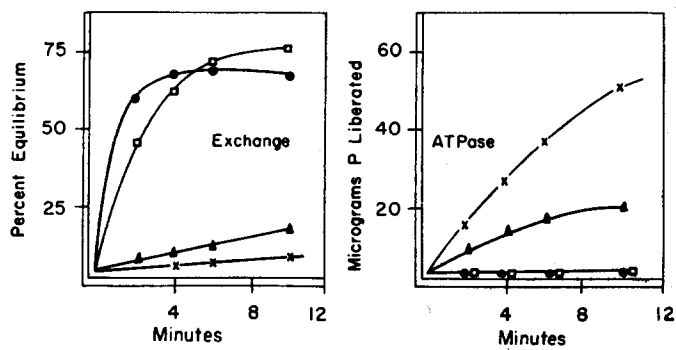

Fig. 5. Effect of dinitrophenol and azide on exchange and ATP-splitting, in Tris-histidine buffer □ – Control; ● – Azide = $2 \cdot 10^{-4}M$; × – DNP = $5 \cdot 10^{-5}M$; ▲ – Azide + DNP; ATP = 0.0025M, Mg^{++} = 0.005M; pH = 7.0; 30° C.

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The effects of arsenite and EDTA are shown in Fig. 6. This experiment represents one in which 10 minute evaluations only were made. Progress curves were run corresponding to a few of these points, and the general pattern was substantiated. When different buffer systems are used, without EDTA, arsenite has different effects, which appear to be related to the sodium and potassium concentration in the medium. We plan to discuss these effects more completely in a forthcoming publication.

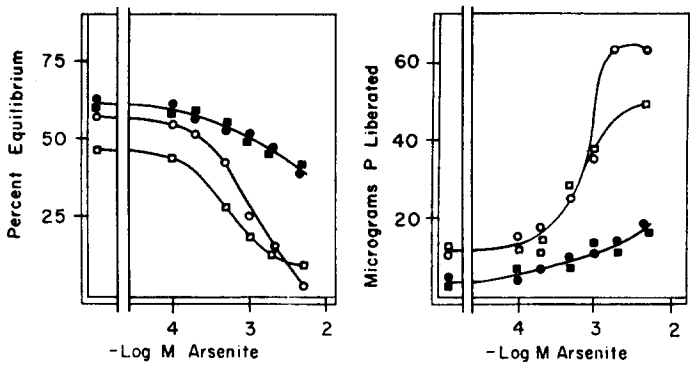

Fig. 6. Effects of arsenite and EDTA on exchange and ATP-splitting in glycylglycine buffer. □ – Control, no added Mg^{++} or EDTA; ○ – Mg^{++} = 0.005M; ■ – EDTA = 0.01M, no Mg^{++}; ● – Mg^{++} = 0.005M; EDTA = 0.01M; ATP = 0.0025M, pH = 7.0, 30° C, 10 minutes.

In order to make direct comparisons between exchange and phosphorylation it was necessary to ascertain the effects of adding oxidizable substrate. It developed that the rate of exchange is not affected by addition of substrate alone whether the mixture is incubated in an evacuated Thunberg tube, a motionless but open centrifuge tube, or shaken in a beaker in a Dubnoff incubator. Simultaneous addition of both substrate and AMP did complicate matters by allowing phosphate uptake. One striking modification was noted on the effects of arsenite in the presence of substrates as shown in Table II. The degree of protection offered by succinate seems rather impressive.

TABLE II

EFFECTS OF ARSENITE ON EXCHANGE REACTION IN DIFFERENT BUFFERS

ATP = 0.0025M; Mg^{++} = 0.005M; pH = 7.0; Temp. = 26° C; isotonic system.
Values given as % equilibration achieved in 10 minutes, taken from progress curves.

Buffer	*No Substrate*	*Citrate*	*Succinate*
Glycylglycine	58	57	56
Glycylglycine + arsenite $10^{-3}M$	41	29	56
Tris	40	46	45
Tris + arsenite $10^{-3}M$	18	17	39
Histidine	41	44	30
Histidine + arsenite $10^{-3}M$	7	8	30

When similar experiments were tried with the phosphorylating system, (that is, with AMP, substrate, and inorganic P, but no ATP) it was likewise found that the

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phosphorylation supported by succinate is far more resistant to arsenite than is that supported by other substrates as shown in Table III. Again, giving the results for a given time interval is somewhat misleading because in the presence of succinate the found degree of phosphorylation is accomplished in a much shorter time interval than is required in the presence of the other substrates.

TABLE III

EFFECT OF ARSENITE ON PHOSPHORYLATION SUPPORTED BY DIFFERENT SUBSTRATES

Phosphate uptake as micromoles per 20 minutes by 100 mg equivalent of mitochondria in Tris-histidine buffer, 30°, AMP = 0.005 M; PO_4 = 0.01 M; Mg^{++} = 0.005 M; Substrates = 0.02 M; with shaking, in air.

Conc. arsenite M	*Citrate*	*Ketoglutarate*	*Succinate*
1	4.0	8.2	9.5
10^{-5}	2.0	6.2	9.5
$5 \cdot 10^{-5}$	1.8	3.4	9.4
10^{-4}	1.6	1.7	9.1
$2 \cdot 10^{-4}$	1.2	1.7	9.0
$5 \cdot 10^{-4}$	0.6	1.3	8.9
10^{-3}	0.4	0.1	8.7
$2 \cdot 10^{-3}$	0.1	0.1	8.6
$5 \cdot 10^{-3}$	0	0	0.4

We have a variety of other comparative data on phosphate uptake which suggests that the phosphorylation supported by succinate uses mechanisms quite different from those supported by other substrates. These suggestions have not yet been well enough explored to report now but work on these points is being continued.

DISCUSSION

None of the data which we have collected, of which representative examples have been presented, is sufficient to establish the hypothesis that the exchange reaction actually plays a part in phosphorylations, but neither does any of the evidence contradict this hypothesis. In general the effects of various influences on the two kinds of reactions are so closely parallel as to suggest that if the two reactions are not part of the same sequence they are at least dependent upon the integrity of the same sensitive structure.

These studies on exchange and ATP-splitting lead to the general conclusion that a predominantly organic medium is more protective for the granules than an inorganic medium. A buffer made from Tris and histidine has a very high proportion of organic substances and appears as the most favorable medium for all the reactions we have studied. The protective action of EDTA is curious—it can hardly be completely explained by its chelating abilities. Recently EDTA, along with AMP, was reported to protect mitochondria against swelling[6], presumably thus protecting their "intactness". Histidine, Tris, AMP, and EDTA all are nitrogenous compounds, and it is tempting to think that their protective effects may be due to that type structure. It is rather interesting to note that the original observations of KIELLEY AND KIELLEY on the inactivity of mitochondrial ATPase were made in one of the very few systems

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in which complete inactivity can be observed. It seems rather strongly indicated that any biological system should be studied in several buffer solutions before any sweeping generalizations be made.

SUMMARY

Some observations are presented concerning the ability of mitochondria to exchange inorganic phosphate with adenosinetriphosphate, in the absence of oxidation. This reaction is most rapid and extensive when ATP-splitting is lowest, but if ATP-splitting activity is developed, as by dinitrophenol, the exchange reaction is not restored merely by inhibition of ATP-splitting. The exchange, ATP-splitting, and phosphorylating reactions all appear to be related to one particularly sensitive structure in the granules It is not possible to decide at this time whether the exchange reaction constitutes a part of the phosphorylating system.

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