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Studies on the Turnover Rate of ATP during Oxidative Phosphorylation *

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Investigations by Sanadi and Littlefield [1] and by Kaufman *et al.*[2,3] have demonstrated that the transfer of energy connected with the oxidation of α-ketoglutarate to succinate takes place according to the following reaction sequence:

α-ketoglutarate + DPN + CoA → succinyl ~ CoA + $DPNH_2$ + CO_2 (1)
succinyl ~ CoA + ADP + orthophosphate ⇌ succinate + CoA + ATP (2)

α-ketoglutarate + DPN + ADP + orthophosphate → succinate + $DPNH_2$ + ATP + CO_2

This reaction sequence is one of the pathways by which isolated mitochondria form ATP during respiration.

Earlier it was believed that the incorporation of orthophosphate into ATP was intrinsicly linked with oxidative energy generation and thus, that a stoichiometric relation existed between the turnover rate of ATP and the rate of oxidation. The situation becomes different, however, when, as in the case of the above mechanism, the orthophosphate enters into a preformed energy-rich bond by way of a reversible reaction.

A constant rate of oxidation and phosphorylation (by the above reaction sequence) can evidently be maintained only when ATP is removed from the system by an irreversible reaction. Under these conditions a steady state prevails; the rates of reaction (1) and of the irreversible trapping of ATP are equal. In such a system a constant level of the intermediates involved in reaction (2) is established. None of these levels could be zero, for were that the case the reaction chain would be interrupted.

Thus, in a steady state, reaction (2) must proceed in both directions. Accordingly, the rate from left to right should exceed the net rate of oxidative phosphorylation. In other words, in a steady state system the incorporation of orthophosphate into ATP must take place at a greater rate than does the net phosphate uptake.

Experiments supporting this reasoning are presented below. Some data concerning the renewal of ADP during oxidative phosphorylation are also given.

* This work has been presented at the Meeting of Swedish Biochemists, Stockholm, June 5th, 1953.

MATERIALS

Mitochondria from rat liver and rabbit heart were prepared by centrifugal fractionation of homogenates made in 0.25 *M* sucrose [4] containing 0.01 *M* versene [5]. The pH of the medium was adjusted to 7.8. The mitochondria were washed once with this solution.

Yeast hexokinase was prepared according to the method of Berger *et al.*[6], except for the cytolysis in toluene which was performed as described by Bailey and Webb [7]. The preparation obtained at step 5 was employed. This preparation contains no trace of myokinase, ATPase and phosphofructokinase, and is practically free from pyrophosphatase. It contains hexose-6-phosphate isomerase.

The reagents used were commercial products. $H_3P^{32}O_4$ was furnished by AERE Harwell, England.

EXPERIMENTAL

The mitochondria were suspended in an isotonic buffer solution (pH 7.8) containing glycyl-glycine and KCl, and brought to a final volume of 30 ml, in the case of liver, and 10 ml in the case of heart. The solution also contained α-ketoglutarate or glutamate, orthophosphate, adenylic acid, glucose, hexokinase and Mg^{++} in amounts specified under the respective experiments.

Samples of 1.5—2.0 ml of this suspension were incubated at 30° C, either in Warburg vessels or in open tubes under aeration. For chromatographic analyses, samples of 0.1 ml were removed from the open tubes at suitable intervals and fixed with 0.1 ml 10 % trichloroacetic acid containing 40 m*M* ATP.

Analytical methods

Three kinds of determinations were performed throughout the experiments: 1) oxygen consumption, 2) concentration of orthophosphate and phosphorylated intermediates, 3) turnover rate of the individual phosphate groups of ATP.

For this purpose each sample was incubated in triplicate. The first sample was incubated in a Warburg vessel where oxygen uptake was measured in the usual way. The respiration for the period of thermo-equilibration (7 minutes) was calculated by extrapolation. The second and the third samples were incubated in open vessels. Radioactive orthophosphate was added to one of these at the start. In this sample the activity contained in each intermediate formed during the experiment is a direct measure of the amount of the respective phosphate compound. In the middle of the experiment, when steady state had been established in the system, a drop of carrier-free radioactive orthophosphate was added to the second vessel, in order to compute the turnover rates of the intermediates. That identical conditions exist in the triplicates could be confirmed by determining orthopshophate colorimetrically [8] in all three samples after a given time of incubation.

The radioactivity of orthophosphate and phosphorus derivatives formed during incubation was determined after chromatographic separation of these compounds. For this purpose the samples fixed in TCA—ATP were centrifuged and the clear solutions neutralized by adding 0.2 ml of a solution containing 0.6 *M* $NaHCO_3$, 0.1 *M* glucose and 0.015 *M* $MgCl_2$. Each sample was thereafter divided into two equal aliquots. About 10 microliters of hexokinase solution was added to one aliquot. After 15 minutes at room temperature, 0.05 ml *iso*butyric acid was added to both aliquots. From each aliquot about 5 microliters was spotted onto a sheet of filterpaper. Chromatographic separation was performed with *iso*butyric acid-water-ammonia (100 : 36 : 1.36 by volume) as the solvent [9]. Blue spots, originating from the ATP added to the fixing solution, could be detected on the dried chromatograms under UV illumination. The spots from the two aliquots appeared at different heighths on the strips. The spots from aliquots containing hexokinase appeared at a level corresponding to the R_F value of ADP, whereas those from the untreated aliquots appeared at the level of ATP. This confirmed the conversion of all ATP to ADP in the aliquots containing hexokinase.

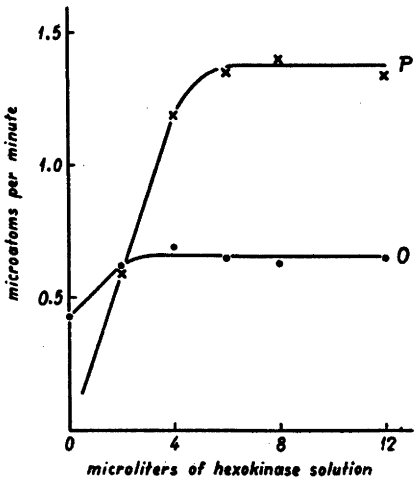

Fig. 1. Rate of oxidation and phosphorylation with varying amounts of hexokinase.

Determination of O_2 consumption: Each Warburg vessel contained: mitochondria (1/18 rat liver), glycyl-glycine (167 μmoles), KCl (130 μmoles), $MgCl_2$ (10 μmoles), K_2HPO_4 (91 μmoles), AMP (6.7 μmoles), potassium α-ketoglutarate (22.2 μmoles), glucose (185 μmoles) pH, 7.8. Hexokinase, a stock solution in 1 % glucose of about 25 ml, obtained as step 5 in a preparation starting from 11 kg yeast, added to each vessel in amounts as indicated in the figure. Final volume of each sample, 2.1 ml. Temp. 30° C. Gas phase: air.

Determination of rate of phosphorylation: Specimens of the same composition as above were incubated in open vessels from which samples were removed at different interval of time, for estimation of hexose-6-phosphate and phosphorylated intermediates.

Rates of oxidation and phosphorylation were determined by averaging the values obtained for the individual intervals from the above two series of incubation.

Also a third specimen for each hexokinase concentration was run, from which, by adding P^{32}-orthophosphate during incubation, the rate of turnover of the phosphorylated intermediates could be computed (see Fig. 2).

The strips corresponding to each aliquot were cut out and the radioactivity was measured along the entire length of the strip. This was accomplished by an automatic counter consisting of three units: a feeding device, an amplifier and a counting machine. The strips (fastened together in a long band) were wound up on the feeder-wheel and fed in half-centimeter portions per half-minute under a GM tube. The amplified impulses are registered by a decadic electron-tube system and recorded on the counting machine tape *.

Thus, for each chromatographic strip fed into the counter, a tape-record was obtained. From this record the successive peaks, corresponding to the different compounds separated by chromatography could be assessed. In this way, the activities of hexose phosphate, of orthophosphate plus ATP, and of ADP were determined. The activities of the individual labile phosphate groups of ATP were obtained from the increase in activity of hexose phosphate and ADP, respectively, in the aliquots treated with hexokinase. This method has already been described in detail [10].

* The automatic counting device was constructed and furnished by LKB-Produkter, Stockholm 12, Sweden.

Table 1. Concentration of phosphorus derivatives in three of the experiments included in Fig. 1.

	2 μl hexokinase			4 μl hexokinase			6 μl hexokinase		
Time, min.	Hexose-6-phosphate, μmoles/ml	ATP, μmoles/ml	ADP, μmoles/ml	Hexose-6-phosphate, μmoles/ml	ATP, μmoles/ml	ADP, μmoles/ml	Hexose-6-phosphate, μmoles/ml	ATP, μmoles/ml	ADP, μmoles/ml
5	1.24	1.47	0.92	1.85	1.03	1.00	1.96	0.75	0.98
10	2.32	2.57	0.38	3.66	1.63	0.92	4.49	1.04	0.98
15	3.73	2.94	0.12	5.75	2.27	0.60	7.20	1.29	1.00
20	4.98	2.93	0.13	8.22	2.56	0.35	10.16	1.44	0.93
25	6.10	2.68	0.11	10.83	2.64	0.24	12.81	1.55	0.86
30	7.40	2.95	0.13	12.79	2.80	0.17	15.40	1.66	0.77
35	8.61	2.90	0.11	15.26	2.64	0.18			
40	9.69	2.89	0.12	17.33	2.71	0.15			
45	10.71	2.84	0.11						
50	11.58	2.91	0.11						
Mean velocity of HMPformation μmoles/min	0.23			0.43			0.51		
Mean contration at steady state (N) μmoles/min		2.88	0.12		2.67	0.17		*	0.90

* The curve tends towards a level of about 1.70. In the calculation of the turnover rate for the interval 20—25 min (Fig. 2), the values between 1.44 and 1.55 were interpolated.

RESULTS

The results described here concern experiments on rat liver. Those performed on rabbit heart have given essentially the same results and will therefore only be tabulated in the next section.

When rat liver mitochondria are incubated in the presence of varying amounts of hexokinase, the rates of respiration and phosphorylation (both constant within a given experiment) vary in the way shown in Fig. 1. When no hexokinase is present all respiration is presumably dependent upon the hydrolytic breakdown of formed energy-rich phosphate, and this can be regarded as the limiting factor of oxidation. As hexokinase is introduced into the system, part of the ATP formed during respiration is converted into glucose phosphate with liberation of ADP. At hexokinase levels inducing a maximum rate of net phosphate uptake practically all the esterified phosphate is conserved as glucose phosphate, nothing being left over to be trapped by ATPase.

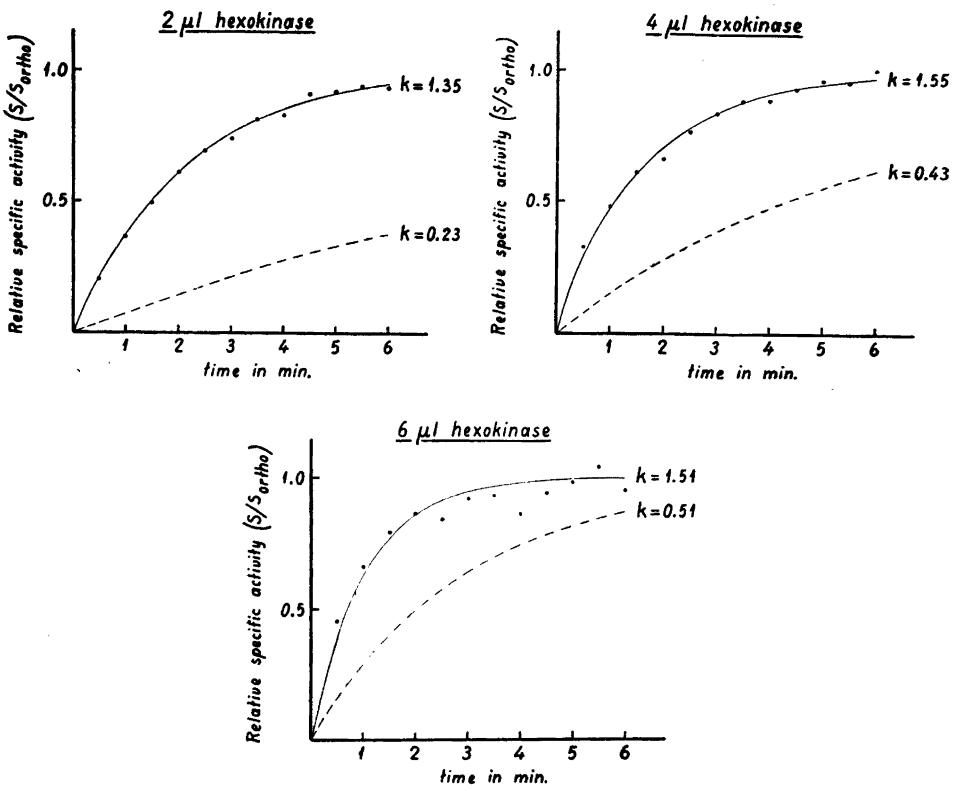

Fig. 2. Calculation of the turnover rate of the terminal phosphate group of ATP.

Points: relative spec. activity of terminal phosphate group of ATP, on the basis of values computed in Table 2.

Solid lines: theoretical curves covering the experimental points, assuming them to be fitted by equation (4) on p. 000. The values of k for these curves are the slopes of the lines obtained by plotting $-N\ln(1-S/S_{ortho})$ against t.

Dotted lines: theoretical curves corresponding to the above equation when putting k equal to the rate of hexose-6-phosphate formation in the resp. experiments (*cf.* Fig. 1).

Each point on the curve in Fig. 1 represents an individual steady-state experiment in which, as mentioned above, oxidation and phosphorylation proceed at a constant rate. In this steady state the phosphorylated intermediates, ADP and ATP, are maintained at constant concentrations, as shown in Table 1.

After these constant levels of intermediates had been attained in the three experiments shown in Table 1 (after 40, 30 and 20 minutes, respectively) radioactive orthophosphate was added to the incubation mixtures. Samples were now removed at half-minute intervals for a period of 6 minutes. In these samples the number of counts of the individual phosphate groups of ATP was determined (Table 2). The specific activity of orthophosphate during this

6-minutes period could be regarded as virtually constant. On this basis the relative specific activity of the terminal phosphate group of ATP was calculated and plotted *versus* time (Fig. 2). By taking orthophosphate as the immediate precursor of this phosphate group, the turnover rate expressed in micromoles / minute was computed from the equation:

$$N\frac{dS}{dt} = k\ (S_{ortho} - S) \tag{3}$$

which upon intergration over the period 0 to t gives,

$$S/S_{ortho} = 1 - e^{-\frac{k}{N}t} \tag{4}$$

where S designates the specific activity of the terminal phosphate group of ATP at a time t after the introduction of P^{32} into the incubation mixture (*cf.* Table 2); S_{ortho}, the specific activity of orthophosphate, taken as constant during the period concerned; N, the concentration of ATP in the steady-state system (*cf.* Table 1); and k, the turnover rate (μmoles. $ml^{-1} \cdot min^{-1}$) of the terminal phosphate group of ATP.

Table 2. Calculation of the turnover rate of the terminal phospha te group of ATP in the steady-state system.

The table refers to the same experiments as in Table 1. To each incubation, P^{32}-orthophosphate was added when steady state had well been established in the system. In the experiment with 2 μl hexokinase this was done after 40 min, with 4 μl hexokinase after 30 min, and with 6 μl after 20 min.

a. 2 μl hexokinase.

Time in seconds after addition of P^{32}	Activity (R) *Counts.* min^{-1} . 10^{-5}			Specific Activity ($S = R/N$) *Counts.* min^{-1} . $\mu M^{-1}.10^{-5}$ (N taken from Table 1)			Relative Specific Activity (S/S_{ortho}) ($S_{ortho} = 3.53$)		
	ATP terminal group	ATP middle group	ADP	ATP terminal group	ATP middle group	ADP	ATP terminal group	ATP middle group	ADP
33	2.05	0.04	0.00	0.71	0.01	...	0.20	...	...
64	3.73	0.12	0.03	1.30	0.04	...	0.37	0.01	...
94	5.00	0.16	0.03	1.74	0.06	...	0.49	0.02	...
126	6.18	0.32	0.03	2.15	0.11	...	0.61	0.03	...
154	6.99	0.40	0.05	2.43	0.14	...	0.69	0.04	...
185	7.50	0.49	0.05	2.61	0.17	...	0.74	0.05	...
215	8.20	0.61	0.07	2.86	0.21	...	0.81	0.06	...
245	8.41	0.75	0.07	2.94	0.26	...	0.83	0.07	...
280	9.19	0.85	0.08	3.20	0.30	...	0.90	0.09	...
306	9.29	0.93	0.08	3.23	0.32	...	0.91	0.09	...
334	9.51	1.06	0.08	3.31	0.37	...	0.94	0.10	...
365	9.57	1.17	0.09	3.30	0.40	...	0.93	0.11	...

b. 4 µl hexokinase.

Time in seconds after addition of P^{32}	Activity (R) *Counts. min^{-1} . 10^{-5}*			Specific Activity ($S = R/N$) *Counts. min^{-1} . µM.$^{-1}$10^{-5}* (N taken from Table 1)			Relative Specific Activity (S/S_{ortho}) ($S_{ortho} = 4.00$)		
	ATP terminal group	ATP middle group	ADP	ATP terminal group	ATP middle group	ADP	ATP terminal group	ATP middle group	ADP
36	3.46	0.23	0.06	1.30	0.09	...	0.33	0.02	...
67	5.10	0.31	0.04	1.91	0.12	...	0.48	0.03	...
97	6.47	0.44	0.05	2.43	0.17	...	0.61	0.04	...
126	7.04	0.56	0.04	2.65	0.21	...	0.66	0.05	...
156	8.13	0.85	0.10	3.05	0.32	...	0.76	0.08	...
186	8.89	1.23	0.08	3.33	0.43	...	0.83	0.11	...
216	9.38	1.26	0.11	3.51	0.48	...	0.88	0.12	...
246	9.43	1.43	0.11	3.54	0.54	...	0.88	0.14	...
277	9.88	1.72	0.08	3.70	0.65	...	0.93	0.16	...
306	10.24	1.56	0.11	3.85	0.59	...	0.96	0.15	...
336	10.19	2.27	0.14	3.82	0.86	...	0.96	0.22	...
369	10.68	2.43	0.11	4.01	0.92	...	1.00	0.23	...

c. 6 µl hexokinase.

Time in seconds after addition of P^{32}	Activity (R) *Counts. min^{-1} . 10^{-5}*			Specific Activity ($S = R/N$) *Counts. min^{-1} . µM.$^{-1}$10^{-5}* (N taken from Table 1) *			Relative Specific Activity (S/S_{ortho}) ($S_{ortho} = 3.22$)		
	ATP terminal group	ATP middle group	ADP	ATP terminal group	ATP middle group	ADP	ATP terminal group	ATP middle group	ADP
30	2.08	0.14	0.16	1.45	0.10	0.17	0.45	0.03	...
61	2.94	0.50	0.43	2.02	0.35	0.47	0.66	0.11	...
89	3.72	1.05	0.58	2.55	0.72	0.63	0.79	0.22	...
119	4.09	1.52	0.81	2.78	1.04	0.89	0.86	0.32	...
150	3.98	2.11	1.05	2.69	1.43	1.17	0.84	0.45	...
181	4.43	2.42	1.28	2.97	1.63	1.43	0.92	0.51	...
210	4.47	2.61	1.43	2.98	1.74	1.61	0.93	0.54	...
239	4.19	2.98	1.54	2.76	1.98	1.74	0.86	0.62	...
270	4.59	3.43	1.63	3.01	2.26	1.86	0.94	0.70	...
300	4.80	3.93	1.65	3.15	2.57	1.89	0.98	0.80	...
330	5.13	3.98	1.80	3.35	2.60	2.08	1.04	0.81	...
360	4.90	3.94	1.94	3.06	2.54	2.25	0.95	0.79	...

* Values of N intrapolated between 1.44 and 1.55.

The experimental points fit fairly well for $k = 1.35$, 1.55 and 1.51 μmoles $ml^{-1} \cdot min^{-1}$, respectively, in the three experiments. On the other hand, these values of k greatly exceed the actual rate at which glucose phosphate was formed in the three respective experiments. This becomes clearly evident when the experimental points are compared with the theoretical lines drawn on the basis of k-values equated to the rate of glucose phosphate formation. Thus, the incorporation of orthophosphate into the terminal group of ATP takes place at a rate considerably higher than that of the oxidative phosphate uptake. Moreover, the former rate seems to be rather independent of the latter.

Equation (3) is valid only under the condition that there is no flow of unlabeled phosphate from ADP or from the middle group of ATP to the terminal group. To take the extreme case, if there were an instantaneous equilibrium between these three phosphate groups (*i. e.*, an infinitely high myokinase activity), the value of N would be much greater than when only the terminal group is involved. The maximum error is, thus, $(N_{terminal} + N_{middle} + N_{ADP}) / N_{terminal}$, *i. e.* about 2 to 2.5 times the value obtained in the present experiments. The relative specific activities of the middle group of ATP and the labile group of ADP are, however, relatively low (Table 2). Thus, the error in the calculation of k (caused by the myokinase reaction) is insignificant.

DISCUSSION

These experimental data are in accord with the principles we have advanced in the introduction. According to these, the reversible transfer-reactions leading from the primary energy-carrier to the formation of ATP, in a respiring mitochondrial system in a steady state, proceed at a greater rate than does the oxidative formation of the primary high-energy bonds. The disparity between the velocities of the two reactions is indeed significant, as summarized in Table 3. In these systems the rate of incorporation of orthophosphate into the terminal group of ATP seems to be rather independent of the rate of oxidative phosphorylation.

Table 3. Rate of net phosphate uptake and turnover rate of the terminal group of ATP in respiring mitochondria supplemented with yeast hexokinase.

A relative amount of hexokinase = 0.5 designates the amount necessary to give half maximal net phosphate uptake.

Experimental conditions: *rat liver.* see Fig. 1; *rabbit heart,* mitochondria from one heart suspended to a final volume of 10 ml in a solution of the same composition as in the case of rat liver. As a substrate glutamate, rather than α-ketoglutarate, was used in these experiments.

RAT LIVER			RABBIT HEART		
Relative amount of hexokinase	Rate of phosphate uptake μmoles / min. per ml suspension	Rate of turnover of the terminal group of ATP μmoles/min. per ml suspension	Relative amount of hexokinase	Rate of phosphate uptake μmoles/min. per ml suspension	Rate of turnover of the terminal group of ATP μmoles/min per ml suspension
0.44	0.23	1.35	0.33	1.30	5.6
0.87	0.43	1.55	0.66	2.25	7.0
1.31	0.51	1.51			

A reasonable explanation for the fact that the turnover rate of the terminal group of ATP is higher than the rate of the oxidative phosphorylation might be that only part of the ATP is trapped by hexokinase while another part is hydrolyzed by ATPase to orthophosphate and ADP. This explanation may certainly be valid for the case when addition of more hexokinase can further increase the rate of net phosphate uptake. This situation prevailed in the first two cases recorded in Table 2. In the third case, however, hexokinase was present in an amount such that further addition of it could no longer increase the rate of net phosphate uptake. In such a system it is hardly probable that any significant leakage of ATP effected by ATPase can occur. Furthermore, in the first two cases the discrepancy between rate of phosphorylation and of ATP turnover is too great to be explained by this mechanism.

From Fig. 1 it can be seen that the maximum number of phosphate molecules esterified per atom of oxygen consumed (P/O ratio) was about 2.1 for those cases where hexokinase was present in excess. The rate of turnover of the terminal group of ATP exceeded the rate of phosphate uptake in the presence of an excess of hexokinase by a factor of about 2.5. Thus, a P/O ratio of about five would have been obtained if the ATP turnover rate had been taken as a basis for the P/O calculation. Recently Krebs *et al.*[11] used this method to determine the P/O ratio in a similar system. However, direct comparision of the present experiments with those by Krebs *et al.* is difficult, for the experimental conditions differ. The experiments by Krebs *et al.* were performed on homogenates rather than mitochondria, without the addition of hexokinase, and in the presence of about equivalent amounts of orthophosphate and adenine nucleotides. Nevertheless, these differences from the conditions of our experiments do not appear to be such as to reduce the risk of error involved in calculating the P/O ratio on the basis of ATP-turnover.

In the experiments by Krebs *et al.* no incorporation of P^{32}-orthophosphate into ATP occurred under anaerobic conditions. This circumstance is regarded by the authors as a proof that a stoichiometric relation exists between oxygen consumption and renewal of the terminal phosphate group of ATP. It is, however, questionable whether a true steady state with respect to the intermediates occurring along with the reversible energy-transferring reactions prevails in an anaerobic system. Moreover, the disparity between the rate of oxidative phosphorylation and the rate of renewal of the terminal phosphate group of ATP (as found in the present experiments) need not be restricted to the step, α-ketoglutarate → succinate; a similar disparity might also occur in phosphorylating reactions connected with the respiratory chain.

Recently it has been frequently debated whether the P/O ratio obtained for the step α-ketoglutarate → succinate is 3 or 4. Slater and Holton [12] obtained in agreement with earlier data [13–17] P/O values below or equal to 3 while Judah [18] and recently Copenhaver and Lardy [19] using the same system but apparently at a greater efficiency, obtained values indicating a P/O ratio of 4. The values calculated by Krebs *et al.*[11] would support Copenhaver and Lardy's [19] value if the turnover rate of ATP could really be regarded as an index of phosphorylation rate. Our own values might seem to support the low ratios obtained by Slater and Holton [12]. There is, however, an important aspect to be discussed in this connection.

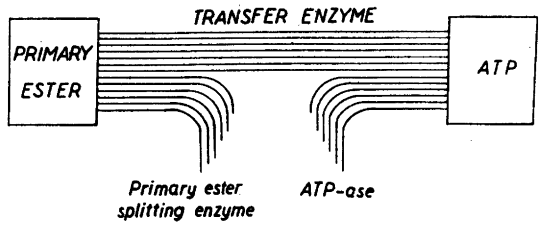

Fig. 3. Schematic illustration to a hypothesis, discussed in the text, about the way in which mitochondrial ATPase may influence P/O ratio.

In the experiment described here (see Fig. 1) we obtained a maximal P/O ratio of 2.1. This value is rather low, even when compared with those obtained by Slater and Holton [12]. In an attempt to explain this discrepancy we have carried out some preliminary experiments which we will not describe in detail here. These experiments show that the maximum P/O ratio obtainable with a sufficient excess of hexokinase is higher, the lower the capacity of the system to oxidize substrate without the addition of hexokinase. If we assume that this capacity is a direct function of the ATPase activity of the mitochondrial preparation, we can conclude that the presence of ATPase activity somehow implies a decrease of net phosphate uptake, even when a large excess of hexokinase is introduced.

It is a known fact that mitochondrial ATPase may show properties of a latent enzyme [15]. In view of this we have suggested [20] that the appearance of ATPase activity in a mitochondrial preparation during ageing or as a result of hypotonic treatment or addition of dinitrophenol, might be due to a splitting of transphosphorylation enzymes which mediate the transfer of phosphate between primary esters and ATP. It was proposed that such a splitting might result in the formation of a pair of hydrolytic enzymes, one splitting ATP and the other dephosphorylating the primary ester. If this were true, the presence of ATPase activity in a native mitochondrial preparation would imply the presence of an "equivalent" amount of primary ester-splitting enzyme. This enzyme would cause a loss of esterified phosphate, which could never be prevented by addition of hexokinase. This situation is visualized schematically in Fig. 3.

It can be readily deduced from the scheme, that in a system where the ATPase activity of the mitochondrial preparation is high, no considerable increase in the oxidation rate can be obtained by the addition of hexokinase, and the maximal P/O ratio attainable in this way is relatively low. This seems to be the case in the present experiments and perhaps also in those by Slater and Holton [12]. When the ATPase activity is low in the native preparation, a high stimulation of the oxidative rate and a high P/O ratio will be obtained by the addition of an excess of hexokinase. This situation apparently prevailed in the experiments of Copenhaver and Lardy [19]. According to this reasoning, a true value of the P/O ratio, *i. e.*, one with which leakage of phosphate does not interfere, can only be obtained in an ideal mitochondrial preparation in which ATPase activity is entirely absent.

Ever since isotopes became available for the study of metabolic processes, a great deal of work has been devoted to the estimation of the metabolic activity of different tissues by injecting P^{32}-orthophosphate into an animal and determining the renewal rate of the ATP phosphorus. This rate has been regarded as a measure of metabolic activity, *i. e.*, of energy generation in the tissue in question. From the present work it is clear that the rate of renewal of ATP in a steady-state system is largely independent of the rate of oxidation. Thus, the conclusions regarding metabolic activity which have been drawn from isotopic *in vivo* experiments of ATP labeling have to be revised. A high turnover rate of the ATP phosphorus need not indicate a corresponding high rate of oxidative energy generation in a given tissue; it merely means that the reversible reaction catalyzing the introduction of phosphate into a primary energy carrier, and its transfer to ATP is fast.

In some instances [21–24] it has been found that the renewal rate of ATP *in vivo* could be altered by the introduction of certain hormones. In connection with these experiments the question has often been raised: is it the rate of oxidation which is influenced by hormones, or is it the permeability of the cell membranes? In view of the above discussion we incline to favour the second alternative. It must be kept in mind, however, that an alteration of active permeability mechanisms implies, at least secondarily, an altered metabolic activity.

SUMMARY

Isolated mitochondria from rat liver were incubated to oxidize α-ketoglutarate in the presence of glucose and varying amounts of yeast hexokinase. In the steady state system, oxidation and phosphorylation proceed at constant rates, and constant levels of ATP and ADP become established. P^{32}-orthophosphate was added to the steady state system and the turnover rate of the terminal phosphate group of ATP determined. This was found to exceed, by a factor of about 2.5, the rate of net phosphate uptake, even when the latter was not limited by hexokinase. The middle phosphate group of ATP had a specific activity considerably lower than the terminal one throughout the experiments. Similar results have been obtained with rabbit heart mitochondria.

The disparity between the rate of net phosphate uptake and the turnover rate of the terminal ATP group is interpreted as resulting from a reversible phosphoroclastic cleavage of a primary energy carrier.

It is concluded that, in the system studied, no stoichiometric relation prevails between turnover of ATP and oxidative generation of energy. The implications of this finding as to the possibilities of estimating energy yield by determination of ATP turnover, *in vivo* or *in vitro*, are discussed.

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