Oxidative Phosphorylation Coupled with the Oxidation of α -Ketoglutarate by Heart-muscle Sarcosomes

1. KINETICS OF THE OXIDATIVE PHOSPHORYLATION REACTION AND ADENINE NUCLEOTIDE SPECIFICITY

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(Received 14 April 1953)

Ochoa (1944) showed that inorganic phosphate was esterified when α -ketoglutarate was oxidized to succinate by particulate preparations obtained from heart muscle. These particles have been identified with the sarcosomes, the granules in muscle which correspond to mitochondria in other tissues (Cleveland & Slater, 1953*a, b*; see also Harman & Feigelson, 1952).

A recent paper (Slater, 1953*b*) described a procedure for measuring the yield of oxidative phosphorylation, in which adenosine diphosphate (ADP), glucose and yeast hexokinase were added to the reaction mixture, and the hexose monophosphate (HMP) formed was determined in the deproteinized mixture by a sensitive enzymic method (Slater, 1953*a*). The reactions occurring may be written as follows, in which ATP = adenosine triphosphate:

Overall reaction

Equation (1) summarizes the reactions of oxidative phosphorylation coupled with the oxidation of α -ketoglutarate to succinate; these are brought about by the sarcosomes, while reaction (2) is catalysed by the yeast hexokinase added. In this procedure, ADP acts as a catalyst. A slow side reaction, catalysed by myokinase and hexokinase and leading to the phosphorylation of glucose by ADP (equation 4), is allowed for in a control measurement (the myokinase control) in which no oxidation occurs (AMP = adenosine monophosphate):

The method of determining HMP depends upon its enzymic conversion in the presence of excess ATP to dihydroxyacetone phosphate, which then reacts with reduced diphenylpyridine nucleotide

(reduced DPN), a reaction catalysed by glycerol phosphate dehydrogenase. The amount of reduced DPN is determined spectrophotometrically. The use of this sensitive method of measuring the HMP formed enabled the kinetics of the early stages of the overall reaction (3) to be followed. For the study of the kinetics of the oxidative phosphorylation reaction, it is necessary either that reaction (2) should be very rapid in comparison with reaction (1) or that reaction (1) should be studied separately. Ways of doing this and the results obtained are considered here.

This paper also deals with the specificity of ADP as the phosphate acceptor in reaction (1). Ochoa (1944) found that AMP as well as ADP and ATP could catalyse the uptake of inorganic phosphate while Lehninger & Smith (1949) found that AMP and ADP were equally effective. The only adenine nucleotide used in the detailed study of oxidative phosphorylation by Cross, Taggart, Covo & Green (1949) was AMP.

As already briefly mentioned in connexion with a study of the phosphorylation coupled with the reduction of cytochrome *c* by α -ketoglutarate (Slater, 1950*a*), it was found that AMP was phosphorylated by heart preparations much more slowly than ADP, showing that ADP is the preferred primary phosphate acceptor. This has been confirmed by Barkulis & Lehninger (1951) and Kielley & Kielley (1951) with liver preparations and by Lindberg & Ernster (1952) with a heart preparation.

Barkulis & Lehninger (1951) found that fluoride inhibited the phosphorylation of AMP but not of ADP, and suggested that ADP was the primary phosphate acceptor and that the phosphorylation of AMP required the participation of the fluoride-sensitive myokinase. Their experiments did not, however, exclude the possibility that AMP might still be directly phosphorylated at an appreciable rate, a reaction which could be important in initiating the uptake of inorganic phosphate. Lindberg & Ernster (1952) believe that a direct

phosphorylation of AMP does in fact occur, and that this reaction is essential for oxidative phosphorylation. The finding of Lipmann, Jones, Black & Flynn (1952) that the 'activation' of acetate by ATP involves the reversible reaction (5)

suggests the possibility of a similar reaction in the phosphorylation coupled with the oxidation of α -ketoglutarate, which also requires coenzyme A (Kaufman, 1951).

The analytical methods already mentioned (Slater, 1953*a*) permitted the direct testing of the adenine nucleotide specificity of the oxidative phosphorylation reaction. A preliminary account of this has already been published (Slater & Holton, 1953).

METHODS

Sarcosomal preparations. Two main types of preparation were used in this study.

(1) Except where stated otherwise, the preparations were made from heart immediately after killing the animal, using the isolation media and procedures described by Cleland & Slater (1953*a*). Different types of preparation within this main type are designated according to the isolation medium and the animal used.

(2) Preparations made from pig heart obtained from the slaughter house. The heart was placed in ice soon after removal from the animal and the preparation commenced about 1 hr. later.

Measurement of activity of α -ketoglutaric oxidase system and yield of oxidative phosphorylation. Except where stated otherwise, the 'isotonic reaction mixture' containing fluoride described by Slater & Cleland (1953*a*) was used. Where O_2 uptake was measured, the cold sarcosome preparation (usually 0.1–0.15 ml. in a final vol. of 1 ml.) was added at zero time to the main compartment of a 7 ml. Warburg flask which already contained all the other components of the reaction mixture at room temperature. KOH (10%, w/v) and a filter-paper roll were used to absorb CO_2 . The flask was immediately attached to the manometer and placed in a bath at 25°. Manometer readings were started 5–10 min. later and the O_2 uptake during the equilibration period determined by extrapolation. The validity of this procedure will be examined below. At the required time, the flasks were removed from the manometer and the reaction immediately stopped with trichloroacetic acid (TCA). The subsequent procedure and the methods of analysis of HMP and energy-rich phosphate ($\sim\text{P}$) have already been described (Slater, 1953*a, b*). A zero-time sample was also analysed and the values deducted from those obtained from the experimental samples to give ΔHMP and $\Delta\sim\text{P}$.

Experiments of short duration were carried out in open Erlenmeyer flasks which could be shaken in the manometric bath. By using blow-out pipettes, accurate timing of the additions of the sarcosome preparation and TCA could be made.

Activity of α -ketoglutaric-cytochrome c reductase system. The isotonic reaction mixture was used with the addition of

KCN (0.016M). The α -ketoglutarate concentration was 0.003M and the concentration of sarcosomes, 0.1 mg. protein/ml. The cyanide was added to the other components of the reaction mixture in a 1 cm. Beckman cell just before the sarcosomes which started the reaction. The total volume was 3 ml. The optical density at 550 m μ . was followed as a function of time for 3–4 min., using as reference cell a similar reaction mixture including cytochrome c and sarcosomes, but lacking cyanide and α -ketoglutarate. The sarcosomes were added to the reference cell a few minutes before the start of the reaction to allow complete oxidation of the ferrocytochrome c in the cytochrome c preparation. The temperature of the reaction mixture was determined (all were between 15° and 17°) and the measured rate corrected to 15°, assuming a temperature coefficient for the 10° range of 2. One unit of activity is defined as a rate of increase of optical density of 0.01/min., calculated from the initial rate.

Determination of α -ketoglutarate. In many of the experiments described in the present paper, the disappearance of α -ketoglutarate ($-\Delta\alpha\text{Kg}$) was also determined. An enzymic method, depending upon reaction (6),

catalysed by glutamic dehydrogenase was used (cf. Kornberg & Pricer, 1951). The amount of reduced DPN reacting with the α -ketoglutarate was estimated spectrophotometrically at 340 m μ ., using the same procedure as that described for the estimation of hexose diphosphate (HDP) (Slater, 1953*a*). The Beckman cells contained 0.05M-phosphate, pH 7.4, and 0.1M- NH_4Cl , in addition to the reduced DPN and the sample to be analysed (containing about 0.1–0.15 μmoles α -ketoglutarate). This method is very reproducible, provided care is taken with the dilution of the solution to be analysed. This is necessary, since a small error in an individual analysis of a test solution or the zero-time control causes a much larger relative error in the value of $-\Delta\alpha\text{Kg}$.

Different preparations of glutamic dehydrogenase have been used for these measurements. If the reduced DPN preparation contains ethanol (e.g. if it is prepared by reduction with ethanol and alcohol dehydrogenase), the glutamic dehydrogenase should be purified to a stage where there is little residual alcohol dehydrogenase. Otherwise, reduction of the DPN will occur after reaction (6) has gone to completion. A satisfactory preparation from ox-liver acetone powder was obtained by following the procedure of Kornberg & Pricer (1951) up to the second fractionation with $(\text{NH}_4)_2\text{SO}_4$. The precipitate obtained was dissolved in a small volume of water and stored at -15° . This preparation retained sufficient activity for several weeks. The amount of enzyme required depends upon the amount of trichloroacetate in the sample, since trichloroacetate is a strong inhibitor of reaction (6). The reaction was usually complete within 5 min. of the addition of the enzyme and the reading remained stationary for some time. No extrapolation of the type described by Slater (1953*a*) is usually necessary for this measurement. The blank obtained in the absence of α -ketoglutarate is small.

Oxaloacetate and pyruvate interfere with this measurement owing to the presence of malic and lactic dehydrogenases, respectively. However, neither of these substances is present in the solution obtained after the oxidation of α -ketoglutarate by heart-muscle sarcosomes (Slater, 1953*b*).

HDP, HMP and ~P do not interfere. The recovery of even small amounts of α -ketoglutarate in zero-time controls was excellent (Table 1).

Table 1. *Recovery of α -ketoglutarate added to reaction mixtures*

(Known amounts of α -ketoglutarate were added to the zero-time control, containing sarcosomes and the complete reaction mixture.)

α -Ketoglutarate		Recovery (%)
Added (μ moles)	Found (μ moles)	
4.90	4.80	98
7.39	7.42	100
0.89	0.84	95
0.88	0.93	105
0.93	0.93	100
1.30	1.29	99

Myokinase. This was a preparation from rabbit muscle made by the method of Colowick & Kalckar (1943). It was kindly supplied by Dr G. Cantoni.

α -Ketoglutaric acid. Since commercial samples of α -ketoglutaric acid contain up to 10% succinic acid, they were purified by dissolving in hot acetone and crystallizing by the addition of benzene, followed by solution in hot acetic acid and crystallization by the addition of benzene. These purified preparations contained about 1% succinate, as measured manometrically with the Keilin & Hartree (1947) heart-muscle preparation. In most of the experiments described in this paper, a sample of α -ketoglutaric acid containing no measurable amount of succinate was used. This sample was kindly supplied by Dr L. Levenbook.

Other materials were as described in previous papers (Slater, 1953*a*, *b*). The unit of hexokinase is that of Berger, Slein, Colowick & Cori (1946).

RESULTS

Measurement of rate of oxidation

Owing to technical difficulties, it is not possible to measure with sufficient accuracy the rate of oxygen uptake in the first few minutes of a manometric experiment. Errors can become particularly serious when the oxygen uptakes are low and carbon dioxide is produced by the reaction, as is the case during the oxidation of α -ketoglutarate to succinate (R.Q.=2). For the experiments described in this paper, it was found desirable to use differential manometers, and care was taken to provide a sufficient surface of filter paper, wet with potassium hydroxide, for the efficient absorption of carbon dioxide. Even under these circumstances, we have found that the manometric readings cannot be completely relied upon until about 7–10 min. after placing the flasks in the bath. We have, therefore, adopted the practice of extrapolating the readings to zero time in order to obtain the total oxygen uptake.

Fig. 1 describes two manometric records, curve 1 with a low concentration (0.12 mg. protein/ml.) of cat-heart sarcosomes, and curve 2 with the more usual concentration of rat-heart sarcosomes (0.60 mg. protein/ml.). The first point on each curve represents the first manometer reading. The oxygen uptake between zero time and the time of this reading was obtained by extrapolation and the value obtained added to all readings. Curve 1 illustrates the precision with which even very small oxygen uptakes can be measured with differential

Fig. 1. Course of O_2 uptake in oxidation of α -ketoglutarate by heart sarcosomes in isotonic reaction mixture. Curve 1, cat-heart sarcosomes, 0.12 mg. protein/ml. Curve 2, rat-heart sarcosomes, 0.60 mg. protein/ml. Note different scales of the ordinates. See text for description of procedure.

manometers and small flasks (constant of manometer=0.47). The cat-heart sarcosomes oxidized α -ketoglutarate at a uniform rate for at least 60 min., while the rat-heart sarcosomes showed a slight decline after about 30 min. These steady rates are obtained only if the inactivation of the α -ketoglutaric oxidase system is prevented by the addition of ethylenediaminetetraacetic acid (Versene) (Slater & Cleland, 1952).

The extrapolation procedure is valid only if the rate of oxidation during the equilibration period is the same as that measured later. In particular, it might be imagined from the frequent behaviour of the manometric readings in the first few minutes (not shown in Fig. 1) that there is an initial lag period in the oxidation. The following evidence shows that there is no lag.

(1) Replicate flasks corresponding to curve 2, Fig. 1, were removed and analysed after 15, 30 and 45 min., with the results shown in Table 2. It can be seen that the oxygen uptakes, calculated assuming

the validity of the extrapolation, agree closely with the amounts of α -ketoglutarate disappearing. Figs. 2 and 4 show the disappearance of α -ketoglutarate at a uniform rate in a short-duration experiment.

Table 2. Relationship between oxygen uptake, α -ketoglutarate disappearance and Δ HMP at different times

(Replicate flasks, identical with that whose O_2 uptake is shown in curve 2, Fig. 1, were removed at different times and treated with trichloroacetic acid.)

Time (min.)	O_2 uptake ($-\Delta O$)* (μ g. atoms)	$-\Delta\alpha$ Kg (μ moles)	Δ HMP† (μ moles)	P:O
15	1.57	1.58	4.31	2.75
30	3.13	3.19	8.46	2.71
45	4.31	4.35	11.78	2.73

* O_2 uptake in first 7 min. obtained by extrapolation.

† Corrected for myokinase control (Slater, 1953b).

(2) When cytochrome *c* was used as hydrogen acceptor instead of oxygen (in the presence of cyanide or in the absence of oxygen), there was usually no lag in the rate of reduction of cytochrome *c* (e.g. see the fig. of Slater, 1950a).

Kinetics of the oxidative phosphorylation

In earlier experiments (Slater, 1950a), it was found that phosphorylation, measured by the formation of HMP, did not begin immediately on adding the sarcosomes to the reaction mixture. An example is shown in curve 1, Fig. 2. Since there is no lag in the rate of oxidation, this lag of phosphorylation behind the hydrogen transfer must be due to the accumulation of some substance which is formed intermediate between the hydrogen-transfer reaction and the HMP. As already discussed (Slater, 1950a), this intermediate could be either one formed in the oxidative phosphorylation reaction (1) (succinyl phosphate seemed a possibility at that time) or ATP. There must, in fact, be some accumulation of ATP; its magnitude will depend upon the relative rates of reactions (1) and (2). The greater the rate of the hexokinase reaction relative to the rate of synthesis of ATP by reaction (1), the less will be the steady-state concentration of ATP.

The possibility that in addition to ATP some other intermediate product might be responsible for the lag was tested by omitting the hexokinase and measuring the rate of increase of $\sim$ P. This measures directly the rate of phosphorylation without the necessity of correcting for the myokinase activity since the myokinase reaction does not alter the total $\sim$ P content. In order to avoid the technical difficulties of carrying out experiments for very short times, a low concentration of sarcosomes

(0.12 mg. protein/ml.) was used. The results of this experiment, given in Fig. 3, curve 1, show that there is no measurable lag in $\sim$ P formation. There is, therefore, no evidence for any accumulation of an intermediate in the oxidative phosphorylation reaction itself.

Fig. 2. Kinetics of early stages of oxidative phosphorylation using small amount of hexokinase (13 units/mg. sarcosomal protein). Isotonic sucrose-ethylenediaminetetraacetate rat-heart sarcosomes, 0.72 mg. protein/ml.; initial $\sim$ P, 0.61 μ mole. Curve 1, Δ HMP; curve 2, $\Delta\sim$ P; curve 3, Δ HMP + $\Delta\sim$ P; curve 4, $-\Delta\alpha$ -ketoglutarate.

Fig. 3. Rate of phosphorylation of ADP (curve 1) and of AMP (curve 2), in the absence of hexokinase + glucose. Isotonic reaction mixture, except for omission of hexokinase and glucose and of AMP from curve 1 and ADP from curve 2. Isotonic sucrose-ethylenediaminetetraacetate cat-heart sarcosomes, 0.12 mg. protein/ml.

The P:O ratio corresponding to Fig. 3, curve 1, is 2.4, compared with 3.0 obtained in the presence of added hexokinase and glucose. The slightly lower phosphorylation yield obtained without hexokinase is certainly due to the action of ATP-ase which

would cause hydrolysis of ATP, the end product of the reaction in the absence of hexokinase. The ATP will react with glucose instead of water and accumulate as HMP when sufficient hexokinase is present.

The conclusion that the phosphorylation kinetics are perfectly linear was confirmed by measuring the course of the synthesis of HMP + $\sim P$, in the presence of hexokinase and glucose.

Fig. 2 describes an experiment in which the amount of hexokinase was purposely made low (13 units hexokinase/mg. sarcosomal protein). The separate myokinase control test (Slater, 1953*b*) was not carried out; instead, each solution was analysed for $\sim P$ as well as HMP. If no ATP accumulated, $\sim P$ would fall linearly with time as a result of the combined myokinase-hexokinase reaction (4), while HMP would increase linearly with time as a result of reactions (3) and (4). In fact, in this experiment, there was a marked lag in the HMP formation, while $\sim P$ instead of decreasing actually increased because the rate of accumulation of ATP exceeded the hexokinase reaction. The curve ($\Delta HMP + \Delta \sim P$), however, showed no lag. It is probable that the rate of phosphorylation was, in fact, somewhat greater during the first minute. This is understandable, since in the presence of only a small amount of hexokinase there will be hydrolysis of ATP by ATP-ase. While the ATP concentration is increasing, this loss of esterified phosphate will be less than when it reaches a steady state.

Fig. 4 shows an experiment with the amount of hexokinase usually employed (99 units/mg. sarcosomal protein). Although a distinct lag phase in both the ΔHMP and $\Delta \sim P$ curves is evident, the ($\Delta HMP + \Delta \sim P$) is perfectly linear for 4 min. The subsequent fall in the rate of phosphorylation in this experiment is due to exhaustion of substrate, a low concentration (0.0012*M*) of which was used (see curve 4).

By using a low concentration of sarcosomes and a high concentration of hexokinase, it is possible virtually to eliminate the initial accumulation of ATP. This is shown in the experiment described in Fig. 5, in which 510 units of hexokinase/mg. sarcosomal protein were used. Two sets of measurements were made (i) with added ADP, (ii) with added AMP and ADP. The latter is the usual procedure in the method of measuring the yield of oxidative phosphorylation previously described (Slater, 1953*b*), the AMP being added as an inhibitor of myokinase. (The addition of AMP has no effect on the P:O ratio.) With the low concentration of cat-heart sarcosomes used, there was no measurable decline of the $\sim P$ after 60 min. in the presence of AMP. In the absence of AMP, $\sim P$ declined at a slow but linear rate, due to reaction (4).

The finding that the accumulation of $\sim P$ is virtually eliminated by using a sufficient concen-

tration of hexokinase shows that the $\sim P$ compound accumulating is in fact ATP and not a compound of the type of succinyl phosphate which could conceivably be measured as $\sim P$ by the analytical method used.

Fig. 4. Kinetics of early stages of oxidative phosphorylation, using normal amount of hexokinase (99 units/mg. sarcosomal protein). Saline-ethylenediaminetetraacetate cat-heart sarcosomes, 0.64 mg. protein/ml. Curve 1, ΔHMP ; curve 2, $\Delta \sim P$; curve 3, $\Delta HMP + \Delta \sim P$; curve 4, $-\Delta \alpha$ -ketoglutarate.

Fig. 5. Kinetics of oxidative phosphorylation, using large excess of hexokinase (510 units/mg. sarcosomal protein). Curve 1, isotonic reaction mixture; curve 2, same reaction mixture, except no AMP. Same preparation and same concentration of sarcosomes as in Fig. 3.

The effect of varying the amount of hexokinase on the steady-state level of $\sim P$ was further studied in the experiment described in Fig. 6. In this experiment, the oxygen uptake substantially exceeded the α -ketoglutarate disappearance, indicating considerable oxidation past the succinate stage. Both oxygen uptake and α -ketoglutarate disappearance were inhibited by the larger amounts of hexokinase. The yield of oxidative phosphorylation has been expressed both as P:O and as P: α Kg.

The reactions taking place under the conditions of the experiment may be described by

(a)

(b)

Fig. 6. (a) Effect of amount of hexokinase on the measured yield of oxidative phosphorylation (P:O and P:αKg) and on Δ~P. Esterified P = ΔHMP + Δ~P. Saline-ethylene-diaminetetraacetate rat-heart sarcosomes, 1.45 mg. protein/ml. Initial ADP, 0.6 μmole; AMP, 0.6 μmole. Hexokinase used had specific activity of approx. 750 units/mg. protein. Horizontal arrow shows Δ~P found in a separate 'myokinase control' (see text).

(b) Same experiment, showing values obtained with 0-35 units hexokinase/mg. sarcosomal protein.

reaction of equation (8). The Δ~P found under these conditions (1.33 μmoles) is considerably greater than the ADP originally used (0.6 μmole) showing that some of the AMP (originally 0.6 μmole) has been phosphorylated via reaction (8). The total ~P found was 80% of the value which would be obtained if all the AMP and ADP had been converted to ATP.

Even small amounts of hexokinase (3.5-7 units/mg. sarcosomal protein) greatly decreased the ~P and increased the total amount of esterified P (ΔHMP + Δ~P). However, this amount of hexokinase was not sufficient to transfer all the ATP formed by reaction (1) to glucose, so that considerable amounts of ~P accumulated. Moreover, under these conditions, the total esterified P was smaller in amount than that found with larger quantities of hexokinase. This is because the ATP-ase competes with these small amounts of hexokinase and causes loss of esterified P. In this experiment, 35 units of hexokinase/mg. sarcosomal protein were sufficient to give optimum esterification of inorganic phosphate, the P:O and P:αKg remaining virtually constant when the amount of hexokinase was varied between 35 and 182 units/mg. sarcosomal protein. The Δ~P was very low under these conditions. The arrow on the lower right-hand corner of Fig. 6a shows the Δ~P found in a separate myokinase control, carried out in the presence of cyanide; this is due to the reaction shown in equation (4). The amount by which Δ~P exceeds this value (except when there was very little hexokinase) equals the amount of ATP present in the reaction mixture. With 70 or more units of hexokinase/mg. sarcosomal protein, this was only about 0.12 μmole ATP. It is noteworthy that the loss of esterified P, indicated by the point on the curves in Fig. 6 at which the P:O or P:αKg reached a maximum, ceased when the steady-state concentration of ATP had reached a very low value. This is exactly what would be expected from a consideration of equations (1), (7), (8) and (2).

The measurement of ΔHMP + Δ~P shown in Fig. 6 and in earlier experiments in this section obviates the need for the separate myokinase control, necessary in the procedure described by Slater (1953b) in which only ΔHMP is measured. It is also strictly more correct, since it allows for the ATP as well as the HMP. However, the procedure previously described is often more convenient, since it requires fewer analyses. The error is usually completely negligible, provided sufficient hexokinase is used, which is in any case essential to obtain the true value for the P:O ratio. For example, in the experiment shown in Fig. 6, with 70 units of hexokinase/mg. sarcosomal protein, the ATP concentration was only 1% of that of the HMP.

In the absence of hexokinase, the steady state concentration of ~P will be governed by the relative rates of reactions (1), (7) and (8), including the back

Adenine nucleotide specificity

Fig. 7 shows the oxygen uptake for the period 15–45 min. using a dilute sarcosomal preparation with the same concentration (about 0.0006M) of different adenine nucleotides, both in the presence

Fig. 7. Comparison of different adenine nucleotides. Isotonic sucrose-ethylenediaminetetraacetate cat-heart sarcosomes, 0.15 mg. protein/ml. Isotonic reaction mixture except for adenine nucleotide and hexokinase + glucose. Curves 1 and 5, no added nucleotide; curves 2 and 6, AMP (0.6 μ mole); curves 3 and 7, ADP (0.53 μ mole); curve 4, ATP (1.02 μ mole $\sim$ P). Without hexokinase (discontinuous lines); with 2660 units hexokinase/mg. sarcosomal protein + glucose (continuous lines). O_2 uptakes between 15 min. and 45 min. after adding sarcosomes.

and absence of hexokinase. No attempt was made to extrapolate to zero time, since in the absence of hexokinase there was a decreasing rate of oxygen uptake. It is probable that by the time the first manometric reading was taken, nearly all the ADP would be phosphorylated to ATP in the absence of hexokinase.

The first point to note is that, either in the absence of adenine nucleotide or in the presence of AMP or ADP, hexokinase stimulated the oxygen uptake, especially after 25 min. This is in agreement with several recent authors who used ATP as the adenine nucleotide (e.g. Rabinovitz, Stulberg & Boyer, 1951; Lardy & Wellman, 1952). Secondly, there was appreciable oxidation in the absence of added nucleotide, especially in the presence of hexokinase. Both AMP and ADP stimulated the oxygen uptake, but the latter was the more effective, especially in the absence of hexokinase.

The HMP and $\sim$ P contents at the end of the experiment shown in Fig. 7 are given in Table 3. The following points are worthy of comment.

(1) The ADP was completely phosphorylated. From data not shown in Table 3, it can be calculated that the ratio of $\sim$ P to total adenosine in no. 3 of Table 3 was 2.08 (cf. theoretical value for ATP, 2.00). The slight increase of $\sim$ P in no. 4 is due to the presence of some ADP in the sample of ATP used.

(2) In contrast, there was very little phosphorylation of AMP, only 0.035 μ mole $\sim$ P. Since ADP is rapidly and completely phosphorylated, it follows that in the absence of hexokinase this $\sim$ P must all be ATP; thus only 0.017 μ mole AMP was phosphorylated, which is only 0.6 % of the rate of phosphorylation of which the preparation was capable (no. 7, Table 3).

(3) The sarcosomes, even in the absence of any added adenine nucleotide, were able to synthesize appreciable amounts of HMP when hexokinase and glucose were added. Since ATP is the specific phosphate donor for hexokinase, this must be due to endogenous adenine nucleotide in the sarcosomal preparation.

(4) Added AMP was almost as effective as ADP in catalysing the oxidation and the P:O ratio was also quite high. However, only 0.01 μ mole of $\sim$ P was synthesized, i.e. the rate of phosphorylation of AMP was only 0.7 % that of the phosphorylation of hexose under these conditions. (This amount of $\sim$ P is close to the limit of sensitivity of the analytical

Table 3. *Effects of different adenine nucleotides on rate of oxidation and on phosphorylation*

(Isotonic sucrose-ethylenediaminetetraacetate cat-heart sarcosomes, 0.15 mg. protein/ml. Analyses of experiment described in Fig. 7. Reaction period, 45 min.)

No.	Nucleotide	Hexokinase + glucose	$-\Delta O$ (μ g. atoms)	Δ HMP (μ moles)	$\Delta \sim$ P (μ moles)	$\frac{(\Delta\text{HMP} + \Delta \sim \text{P})}{-\Delta O}$
1		—	0.17	.	.	.
2	AMP	—	0.35	.	0.035	.
3	ADP	—	0.55*	.	0.72	.
4	ATP	—	0.51*	.	0.07	.
5		+	0.35	0.18	.	0.50
6	AMP	+	0.72	1.51	0.01†	2.10
7	ADP	+	0.93	2.84	—0.14	2.89

* Extrapolation to zero time uncertain (see Fig. 7).

† Equivalent to a concentration of 10^{-6} M-ADP.

Table 4. *Effect of concentration of added ADP on activity of α -ketoglutaric oxidase system and on P:O ratio*
(Isotonic sucrose-ethylenediaminetetraacetate rat-heart sarcosomes, 1.20 mg. sarcosomal protein/ml.; 63 units hexokinase/mg. sarcosomal protein. AMP was added in concentration equal to that of added ADP.)

[ADP] (M $\times 10^4$)		O ₂ uptake (μ g. atoms)	Δ HMP (μ moles)	P:O
Initial	Final*			
0	0	4.37	5.40	1.24
0.98	0.28	5.75	15.6	2.72
3.0	—	5.59	15.2	2.72
6.1	4.1	5.77	15.6	2.70
12.0	—	5.09	13.9	2.74

* Determined by value of Δ HMP in myokinase control.

method and the value is therefore only approximate.) This shows unequivocally that AMP cannot be acting directly as an intermediate in the phosphorylation of glucose. For if AMP were directly phosphorylated to ATP, according to a reaction analogous to reaction (5), we should have

and there would be an increase of $\sim$ P as well as of HMP.

A possible explanation of the stimulating effect of adding the AMP is the presence of traces of $\sim$ P in the AMP solution used, which according to analysis

ADP concentration during the experiment. Both oxidation and phosphorylation were optimum when the concentration of ADP was 9.8×10^{-5} M at the beginning and 2.8×10^{-5} M at the end of the experiment. Table 3, no. 6, shows that 10^{-5} M-ADP was sufficient to give 77 % of the optimum rate of oxidation. This corresponds to a Michaelis constant of about 3×10^{-6} M-ADP (see also below).

Fig. 3 describes another experiment which directly compares the rate of phosphorylation of AMP with that of ADP, in the absence of hexokinase. The rate of increase of $\sim$ P with AMP was only about 1.5 % that with ADP, corresponding to a rate of phosphorylation of AMP of about 0.7 % that of ADP. In the presence of hexokinase (not shown in Fig. 3), no $\sim$ P could be detected (certainly less than 0.02μ mole) after 45 min., when AMP was the added nucleotide.

The kinetics of the synthesis of HMP with AMP as the only adenine nucleotide is shown in Fig. 8. Within experimental error, the rate is constant.

Fig. 9 describes a similar experiment with sarcosomes prepared from slaughter-house pig heart. These preparations differ in two important respects from the type used in the experiments described above. (1) Although they oxidize α -ketoglutarate rapidly, the P:O ratio is very low (usually less than 1), even under optimum conditions. These are the only preparations we have found with this characteristic. After a number of different types of treatment including incubation, it was the ability to oxidize α -ketoglutarate which was affected, not the P:O ratio (see Slater & Cleland, 1953*a*). (2) Pig-heart sarcosomes contain considerably more myokinase (Slater, 1953*b*).

The preparation of AMP used in this experiment (Fig. 9) contained some substance analysing as HMP. Over the first 8 min., there was actually a decline in the HMP content, due no doubt to the presence of a little phosphatase in pig-heart preparations (Slater, 1953*b*). Thereafter, the rate of phosphorylation steadily increased and was still increasing after 30 min., although the oxidation of α -ketoglutarate proceeded rapidly without any lag.

Fig. 8. Kinetics of oxidative phosphorylation with AMP as only added nucleotide. Same preparation and same conditions as in Fig. 5, except that AMP was the only added nucleotide.

of the stock solution of AMP would introduce 0.002μ mole $\sim$ P (2×10^{-6} M) into the reaction mixture. This explanation implies that a very low concentration of ADP is sufficient for optimum oxidation and phosphorylation, and has been confirmed by a different type of experiment summarized in Table 4, in which different concentrations of ADP were used. This experiment was carried out with a much higher concentration of rat-heart sarcosomes, so that the myokinase activity was appreciable, causing a considerable decline of the

The maximum P:O ratio, calculated from the tangent of the curve in Fig. 9 at 30 min. was only 0.5.

Fig. 9. Kinetics of oxidative phosphorylation, with AMP (0.001M) as the only added nucleotide and sarcosomes prepared from slaughter-house pig heart (phosphate-saline preparation). Reaction mixture did not contain malonate or fluoride. 0.58 mg. sarcosomal protein/ml.

Fig. 10 describes an experiment with a similar preparation, in which the phosphorylation of AMP is compared with that of ADP. The course of the phosphorylation with AMP (curve 1) as acceptor was the same as that in Fig. 9, but with ADP (curve 2) there was only a very slight lag, of the same type as shown in Fig. 2. The addition of myokinase did not abolish the initial lag with AMP,

but decreased the time required for phosphorylation to reach a maximum. The P:O ratio with ADP as acceptor was only 0.32 in this experiment.

Fig. 10. Comparison of AMP and ADP as primary phosphate acceptors with sarcosomes prepared from slaughter-house pig heart (phosphate-saline preparation). Reaction mixture did not contain malonate or fluoride. 0.65 mg. sarcosomal protein/ml. Curve 1, AMP (0.001M); curve 2, ADP (0.001M); curve 3, AMP + rabbit-muscle myokinase.

Effect of concentration of sarcosomes

The rate of oxidation in the absence of added nucleotide was relatively much greater in the experiment described in Table 4 (77% of the rate obtained with ADP) than in that described in Table 3 (38%). The relative P:O ratios show a similar difference. These discrepancies are due to the different concentrations of sarcosomes used, the effect of which is more fully examined in Table 5

Table 5. *Effect of concentration of sarcosomes on rate of oxidation of α -ketoglutarate and on P:O ratio, in presence and in absence of ADP*

(Isotonic sucrose-ethylenediaminetetraacetate rat-heart sarcosomes; 146 units hexokinase/mg. sarcosomal protein; ADP, where added, 6×10^{-4} M.)

	Sarcosomal protein (mg./ml.)			
	0.12	0.31	0.62	1.25
O ₂ uptake (μ g. atoms)				
Without ADP (a)	0.26	0.64	1.68	4.15
With ADP (b)	0.70*	1.76	3.56	6.26†
100 $\times$ a/b	37	36	47	63
Δ HMP (μ moles)				
Without ADP (c)	0	0.19	1.24	4.47
With ADP (d)	1.82	4.36	8.46	15.8†
100 $\times$ c/d	0	4	15	28
P:O				
Without ADP (c/a)	0	0.33	0.74	1.08
With ADP (d/b)	2.59	2.48	2.38	2.52

* O₂ uptake not accurately measured; this is interpolated value.

† This was the only concentration which showed some decline in the rate of O₂ uptake towards the end of the experiment, the final O₂ uptake being 7.5% less than that which would have been obtained had the initial rate been maintained. For comparison with other sets of values, the experimental values have been multiplied by the factor 100/92.5.

and Fig. 11. In the presence of ADP, both the oxygen uptake and the Δ HMP were approximately proportional to the concentration of sarcosomes. (The departure from linearity at the highest concentration is probably due to inhibitory substances in the hexokinase preparation, which was added in amounts proportional to the concentration of

Fig. 11. Effect of concentration of sarcosomes on rate of oxidation of α -ketoglutarate and on Δ HMP, in presence and in absence of ADP. For experimental conditions, see Table 5.

sarcosomes.) The P:O ratio was, therefore, independent of the concentration of sarcosomes. In the absence of ADP, however, the oxygen uptake and more especially the Δ HMP were relatively much smaller at low concentrations of sarcosomes and the P:O ratio increased with increasing concentration of sarcosomes. This experiment shows that the endogenous adenine nucleotide of the sarcosomes is dissociated from the sarcosomes at high dilution.

Energy-rich P content of sarcosomes

Table 6 shows the results of three analyses of perchloric acid filtrates of freshly prepared sarcosomes. Considerable amounts of $\sim$ P (mean $0.02 \mu\text{mole/mg. sarcosomal protein}$), easily measurable by the enzymic method, were detected. This value is very close to the acid-labile P content of liver mitochondria, namely $0.023 \mu\text{mole/mg. protein}$ (Kielley & Kielley, 1951). In analysis no. 3, the most accurate of the three, the ratio of $\sim$ P to total adenosine is 0.51. Since, as is discussed below, it is most likely that the $\sim$ P compound is ADP, other adenosine compounds are also present, probably AMP and possibly some coenzymes. The absorption spectrum of the neutralized perchloric acid filtrate was perfectly symmetrical around $260 \text{ m}\mu$., showing the absence of other compounds absorbing in this region.

Table 6. Analysis of sarcosomes

(1–1.3 ml. sarcosomes (containing 4.8–11.6 mg. protein) were deproteinized with perchloric acid (final concentration 7%, w/v) and centrifuged. A sample of the supernatant was neutralized with 4N-KOH and, after standing at 0° , the precipitated KClO_4 removed by filtration. Samples of the filtrate were analysed by the procedure described by Slater (1953a). Preparation 1, isotonic saline-ethylenediaminetetraacetate rat-heart sarcosomes; preparation 2, isotonic sucrose-ethylenediaminetetraacetate cat-heart sarcosomes; preparation 3, isotonic sucrose-ethylenediaminetetraacetate rat-heart sarcosomes.)

	Preparation		
	1	2	3
Analyses ($\mu\text{moles/mg. sarcosomal protein}$)			
Total adenosine	0.031	0.048	0.034
$\sim$ P	0.02	0.027	0.018
HMP	—	0.026	—
HDP	—	—	0.002

Michaelis constant of ADP

The Michaelis constant (K_m) of the endogenous ADP may be calculated from the data of Table 5 and the $\sim$ P content of the sarcosomes, by using the expression $K_m = c(V/v - 1)$ where c is the concentration of $\sim$ P (mg. sarcosomal protein $\times 0.018$), V (b or d in Table 5) is the velocity when the system is saturated with ADP and v (a or c in Table 5) is the velocity without ADP. The values obtained from these calculations are 10^{-5} M for oxidation and approx. $5 \times 10^{-5} \text{ M}$ for phosphorylation. The Michaelis constant is defined as the concentration of ADP necessary for half maximal activity; no other physical meaning is implied. In fact, these values of K_m are calculated on the basis of initial concentrations, and it would be expected that the ADP concentration would decrease, owing to the action of myokinase and hexokinase, during the course of the experiment (35 min. at 25°).

In order to measure the affinity of the system for adenine nucleotide more accurately, it was necessary to use a shorter experimental period. In the experiment summarized in Fig. 12, the oxidation of α -ketoglutarate by cytochrome c was measured spectrophotometrically, the measurements requiring only about 3 min. at 15° . Even at the very high dilution of sarcosomes used in this test, the endogenous ADP was sufficient for 76% of the maximal activity. It was possible, however, to measure the effect of very small concentrations of added ADP and, by plotting $1/\text{activity}$ against $1/[\text{ADP}]$, according to the procedure of Lineweaver & Burk (1934), to determine if there was any appreciable difference in activity of added and endogenous ADP. If the affinities of the system for endogenous and added ADP differed appreciably, a break in the curves between no added ADP and the first

addition would appear. Since no such break is apparent in Fig. 12, it is concluded that added and endogenous ADP behave similarly. The Michaelis constant calculated from Fig. 12 (b) is 7×10^{-7} M, considerably lower than that calculated from the experiment of longer duration. This may be partly due to the fact that the rate of oxidation of α -ketoglutarate by cytochrome *c* in the concentration used

Fig. 12. Michaelis constant of ADP. The activity of the α -ketoglutarate-cytochrome *c* reductase system was determined as described in Methods. Isotonic sucrose-ethylenediaminetetraacetate rat-heart sarcosomes. The activities, both with and without ADP, declined on storage at 0° of the dilute sarcosome preparation during the course of the experiment and all activities were corrected to a common basis using appropriate decay curves. (a) The concentration of ADP provided by the sarcosomes is indicated by the vertical arrow. (b) Lineweaver-Burk (1934) plot.

in the spectrophotometric test is about 25–35 % of that by oxygen at the same temperature. The main reason, however, is probably the destruction of ADP in the longer experiment.

DISCUSSION

Adenine nucleotide specificity

The above experiments show that the rate of phosphorylation of AMP is less than 1 % of that of ADP. Since such a slow rate of phosphorylation can easily be explained by a slight residual myokinase

activity, it is reasonable to conclude that all the phosphorylation steps associated with the aerobic oxidation of α -ketoglutarate to succinate by heart-muscle sarcosomes are completely specific for ADP and do not react with AMP. The appreciable rate of phosphorylation of AMP which has been found by several previous authors cannot then be due directly to the oxidative phosphorylation, but must be entirely due to the myokinase reaction. The recent studies of Barkulis & Lehninger (1951) and of Kielley & Kielley (1951) clearly showed the participation of myokinase in the phosphorylation of AMP, but did not exclude the possibility of an additional relatively rapid direct phosphorylation of AMP. We can now see that the phosphorylation of AMP requires first the formation of ATP from ADP present either in the AMP preparation or in the sarcosomes or mitochondria and secondly sufficient myokinase to promote the phosphorylation of AMP. When hexokinase is also present, there must be a considerable myokinase activity to be able to compete with the hexokinase. This is the case with liver mitochondria which are rich in myokinase. Since the endogenous ADP is insufficient for optimal phosphorylation, the ADP formed from AMP by the myokinase reaction will stimulate the phosphorylation, thereby giving the appearance that AMP has entered into the oxidative phosphorylation reaction.

Provided there is sufficient myokinase, AMP will promote the uptake of inorganic P, either in the presence or the absence of hexokinase, without any detectable time lag. This is found with freshly prepared liver mitochondria (Kielley & Kielley, 1951). Ochoa's (1944) finding that AMP was effective with heart extracts is also understandable, since he used dialysed extracts which would be rich in myokinase. Cat or rat heart-muscle sarcosomes, however, contain only about 1 % of the myokinase activity of the extract (Cleland & Slater, 1953a). A stimulatory effect of AMP was found with pig-heart sarcosomes which are considerably richer in myokinase, but this was only obtained after a time lag. This lag was unduly prolonged in the experiments described in this paper, because the initial rate of phosphorylation of endogenous ADP was very low, due to the use of pig-heart muscle which was not completely fresh. It must be emphasized that this lag is of an entirely different order of magnitude and has quite a different explanation from that previously described (Slater, 1950a). As shown in Fig. 10, it disappears when ADP is the added nucleotide. It is thus also different from yet a third type of lag, reported by Kielley & Kielley (1951), who found that, after ageing, there was a considerable delay in reaching maximum phosphorylation rate with either AMP or ADP as acceptor. The reason for this third type of lag is

not known. Since Keilley & Keilley (1951) have measured only phosphorylation, not oxygen uptake, it is not known whether it is the rate of oxidation or the P:O ratio which is affected. Kielley & Kielley's lag may be similar to a lag of oxygen uptake found when heart-muscle sarcosomes are incubated at room temperature in the presence of ADP or ATP before addition of substrate (Slater & Cleland, 1953*b*).

The conclusion, that the oxidative phosphorylation reaction is completely specific for ADP and that the phosphorylation of AMP necessarily requires the participation of myokinase, differs from that drawn by Lindberg & Ernster (1952) from a study of the distribution of ^{32}P in various phosphorylated compounds after oxidative phosphorylation by rabbit-heart sarcosomes. These authors believe that AMP can be directly phosphorylated as well as ADP, although at only 5% of the rate of the latter. This conclusion is, however, entirely dependent upon the validity of the assumption that the hexokinase was so much in excess that the ATP would react entirely with glucose and not at all with AMP. Considering the much more concentrated preparations of sarcosomes used by these authors compared with our procedure, this seems to us to be very doubtful. The amount of hexokinase employed by Lindberg & Ernster (1952) is not stated, but it can be calculated that approximately 100 mg. of their hexokinase preparation would have been required (reaction vol. 10 ml.) to give the same ratio of hexokinase to sarcosomes as used in our experiment described in Fig. 8. It can be seen in Fig. 10 that when the relative activity of myokinase to hexokinase is increased, some ADP will be formed. We do not consider, therefore, that Lindberg & Ernster's experiments provide satisfactory evidence for the direct phosphorylation of AMP.

Krebs, Johnson, Eggleston & Hems (1951) have found by tracer experiments that the dephosphorylation and resynthesis of ATP in muscle suspensions occurred mainly by scheme 1, while scheme 2 was the chief mechanism in liver suspensions.

Scheme 1

Scheme 2

These schemes are overall mechanisms and do not necessarily represent any fundamental difference between the individual reactions in the two tissues. Both are in fact consistent with ADP being the specific primary phosphate acceptor of the oxidative phosphorylation reaction. In the presence of labelled inorganic phosphate (P^*), the following

reactions are possible ($\text{ATPP} = \text{ATP}$, $\text{APP} = \text{ADP}$, $\text{AP} = \text{AMP}$):

Thus, if ATP-ase is much more active than myokinase, the ATP will be labelled mainly in the terminal phosphate group, which was what Krebs *et al.* (1951) found with muscle suspensions. If, on the other hand, the myokinase is relatively the more active, the two terminal groups of ATP would be equally labelled, as was the case with liver mitochondria in the experiments of Krebs *et al.* (1951).

The specificity for ADP of the oxidative phosphorylation reactions associated with the oxidation of α -ketoglutarate to succinate eliminates the possible participation of reaction (5). This is in agreement with Kaufman's (1952) studies of the soluble enzyme system which couples the reduction of DPN by α -ketoglutarate with phosphorylation. It is possible that coenzyme A monophosphate is an intermediate (Kaufman, 1952).

Nature of endogenous adenine nucleotide $\sim\text{P}$

The presence of adenine nucleotide $\sim\text{P}$ in the sarcosomes is clearly indicated by the formation of HMP when adenine nucleotide is omitted from the normal reaction mixture containing hexokinase and glucose. It was confirmed by direct analysis of the sarcosomes. Since there is a substantial rate of oxidation in the absence of hexokinase and glucose, ADP must be continually supplied to the system (cf. Lardy & Wellman, 1952). It follows then that ATP formed during phosphorylation in the absence of added adenine nucleotide must be broken down again to ADP, i.e. that it must be readily accessible to ATP-ase in the preparation. This rate of breakdown is, however, the rate-limiting step in the rate of oxidation, since the addition of hexokinase and glucose increases the rate of oxidation. This finding and the formation of HMP under such conditions shows too that the ATP formed is also readily accessible to added hexokinase and glucose. The explanation given above for the phosphorylation of AMP implies that the ATP formed from endogenous adenine nucleotide must also be accessible to myokinase.

Since ATP formed from endogenous adenine nucleotide is readily accessible to ATP-ase, it follows that the preparations cannot contain any appreciable amount of ATP. The endogenous energy-rich adenine nucleotide must then be ADP. The

amounts of ADP found by direct analysis are quite high, namely $0.02 \mu\text{mole/mg. sarcosomal protein}$ or 1 molecule for every molecule of protein of molecular weight 50 000. Since so many individual enzymes have been located in the mitochondria and sarcosomes, it seems unlikely that the enzymes which specifically bind and react with ADP can comprise more than a few per cent of the total protein. Only a small fraction of the endogenous ADP is likely to be firmly bound to these enzymes. The remainder may be bound non-specifically to other sites, or may actually be concentrated in the intrasarcosomal fluid.

Fig. 13. Rate of oxidation of succinate (0.032M) by different concentrations of Keilin and Hartree heart-muscle preparation. Phosphate buffer, pH 7.4, 0.07M ; ethylenediaminetetraacetate, 0.001M . Temperature, 25° .

It was of interest to determine whether the endogenous ADP was bound more firmly than added ADP and was catalytically more active, as has been found to be the case with cytochrome *c* (Slater, 1950*b*; Tsou, 1952). The experiments described above showed, however, that unlike cytochrome *c*, ADP dissociated from the enzyme at high dilution and that the catalytic activity of the added ADP was of the same order of magnitude as that of the endogenous ADP. In contrast, Fig. 13 shows that the rate of oxygen uptake with succinate as substrate was exactly proportional to the concentration of the Keilin & Hartree (1947) preparation between $0.006 \text{ mg. protein/ml.}$ and $0.05 \text{ mg. protein/ml.}$ in the absence of added cytochrome *c* (ethylenediaminetetraacetic acid was used as the activator of the succinic oxidase system; see Bonner, 1953). If it is assumed that the concentration of cytochrome *c* is of the same order of magnitude as that of the site with which it combines on the particle and that a 10% departure from linearity at the lowest concentration would have been detected, and using the concentration of cytochrome *c* in the Keilin &

Hartree preparation previously determined (Slater, 1949), it can be calculated that the K_m of bound cytochrome *c* cannot exceed $4 \times 10^{-11}\text{M}$. The K_m of added cytochrome *c* is about $1.5 \times 10^{-5}\text{M}$ (e.g. see Fig. 3 of Tsou, 1952).

The K_m is not necessarily identical with the thermodynamic dissociation constant of the enzyme-substrate or enzyme-coenzyme compound (Briggs & Haldane, 1925; see Slater & Bonner, 1952), but it sets an upper limit for the dissociation constant. Thus, cytochrome *c* is extremely firmly bound to the particles of the Keilin & Hartree heart-muscle preparation, at least in the presence of ethylenediaminetetraacetic acid. Although the affinity of ADP for sarcosomes cannot compare with that of cytochrome *c* for the Keilin & Hartree preparation, it is still very high (K_m of about $7 \times 10^{-7}\text{M}$) and much higher than would be suggested by the amounts of adenine nucleotide usually added in studies of oxidative phosphorylation. The requirement for the larger amounts is probably due to the instability of ADP in most types of enzyme preparations used in such studies.

The lower P:O ratio obtained in the absence of added ADP might indicate that, in the absence of an optimal concentration of ADP, some of the hydrogen-transferring steps involved in the α -ketoglutaric oxidase system proceed without phosphorylation.

Kinetics of oxidative phosphorylation

The experiments described on pp. 533–4 show that the oxidative phosphorylation reaction proper does not lag behind the hydrogen transfer. The lag of HMP synthesis behind hydrogen transfer, previously reported (Slater, 1950*a*) is due to the building up of a steady-state concentration of ATP. Kielley & Kielley (1951) have compared a lag of phosphorylation which they found after incubation of liver mitochondria with the one found by Slater (1950*a*). Since Kielley & Kielley (1951) followed phosphorylation by measuring disappearance of inorganic P, the accumulation of ATP would not cause any time lag. Kielley and Kielley's lag is, therefore, not the same as the one reported by Slater (1950*a*).

The perfectly linear kinetics obtained even during the first minute with a very low concentration of sarcosomes (Fig. 3) makes it unlikely that an intermediate of the type of succinyl phosphate is involved in oxidative phosphorylation. It is more probable that the concentration of any phosphorylated intermediate is of the same order of magnitude as that of the sarcosomal enzymes or coenzymes. This conclusion is in agreement with Kaufman's (1952) studies of his soluble enzyme system, which eliminate succinyl phosphate as an intermediate.

Measurement of yield of oxidative phosphorylation

The shape of the curves in Fig. 6 clearly show that, provided at least 35 units of hexokinase/mg. sarcosomal protein are used, the maximum P:O ratio is measured. The flat portion of the curve indicates that the hexokinase is reacting with all the ATP, none of which reacts with ATP-ase. As a routine, we use at least 100 units of hexokinase/mg. sarcosomal protein. Under such conditions, underestimation of the yield of oxidative phosphorylation due to the presence of ATP-ase can be neglected (cf. Slater, 1953b).

A possible objection to this conclusion is that ATP formed in the sarcosome might react more readily with ATP-ase in the sarcosome than with hexokinase in solution. We reject this interpretation for the following reasons. (1) The curves in Fig. 6 clearly reveal a competition phenomenon between ATP-ase and hexokinase. The interpretation just mentioned would not give curves of this type. (2) Reasons have already been given for believing that ATP formed even from the endogenous ADP of the sarcosomes is relatively free and capable of reacting with the soluble proteins myokinase and hexokinase as well as with the ATP-ase in the sarcosomes.

SUMMARY

1. Both oxidation and oxidative phosphorylation proceed at a uniform rate without any detectable time lag from the moment when heart-muscle sarcosomes are added to a suitable reaction mixture containing α -ketoglutarate.

2. Unless a very high concentration of hexokinase is used, there will be a slight lag in the synthesis of hexose monophosphate, due to the accumulation of adenosine triphosphate.

3. The maximum yield of oxidative phosphorylation (P:O) was obtained with rat-heart sarco-

somes isolated in saline when 35 units or more of hexokinase/mg. sarcosomal protein were used.

4. Adenosine monophosphate (AMP) was phosphorylated at less than 1% of the rate of adenosine diphosphate (ADP). This slow phosphorylation of AMP probably requires first the formation of ATP from ADP present either as traces in the AMP preparation or in the sarcosomes and secondly sufficient myokinase to promote the phosphorylation of AMP. The much greater rate of phosphorylation of AMP by liver mitochondria found by other workers is very likely due to their higher content of myokinase.

5. The α -ketoglutaric oxidase system and the accompanying phosphorylation have a high affinity for ADP (Michaelis constant about 7×10^{-7} M).

6. Freshly prepared sarcosomes contain 0.02 μ -mole energy-rich phosphate/mg. sarcosomal protein (0.04 μ -mole total adenosine). This is probably ADP which is present in amounts sufficient to give nearly optimal rates of oxidation in the absence of added adenine nucleotide when a relatively high concentration of sarcosomes (1.25 mg. sarcosomal protein/ml.) is used. Experiments at greater dilutions gave evidence of dissociation of ADP from the α -ketoglutaric oxidase system.

7. The affinity of this endogenous ADP for the α -ketoglutaric oxidase system is of the same order of magnitude as that of added ADP. This is in striking contrast to the behaviour of endogenous and added cytochrome *c* in similar systems. From the proportionality of the rate of oxidation of succinate to the concentration of heart-muscle preparation in the absence of added cytochrome *c* it was calculated that the K_m of bound cytochrome cannot exceed 4×10^{-11} M.

This work was carried out on behalf of the Agricultural Research Council. We wish to thank Prof. D. Keilin, F.R.S., for his interest and advice.

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