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

3. EXPERIMENTS WITH FERRICYTOCHROME *c* AS HYDROGEN ACCEPTOR*

By E. C. SLATER

Molteno Institute, Downing Street, University of Cambridge

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Belitzer & Tsibakowa (1939) and Ochoa (1940, 1941) found that more than one atom of phosphorus is esterified for each atom of oxygen consumed by respiring tissue preparations, and concluded that the phosphorylation could not all be associated with the initial dehydrogenation of the substrate, but must occur also at some point in the respiratory chain of catalysts over which the hydrogen atoms of the substrate (or their electrons) pass on their way to oxygen. Ochoa's (1943) attempts to demonstrate phosphorylation in the respiratory chain directly by the use of a reduced hydrogen carrier, reduced diphosphopyridine nucleotide (DPNH), as a substrate were unsuccessful. Using improved techniques, Friedkin & Lehninger (1949) were able to obtain phosphorylation coupled with the oxidation of DPNH, and in later papers by Lehninger and his co-workers (Lehninger, 1949; Lehninger & Smith, 1949; Lehninger, 1951*a*), it was shown that in the oxidation of β -hydroxybutyrate to acetoacetate all the phosphorylation occurs in the reaction between DPNH and oxygen, and none between substrate and diphosphopyridine nucleotide.

Friedkin & Lehninger (1949) and Lehninger (1949) attempted to locate the phosphorylation steps in the respiratory chain more closely by experiments in which different parts of the chain were studied in isolation. In the first set of experiments, DPNH or β -hydroxybutyrate was used as the hydrogen donor with ferricytochrome *c* as the stoichiometric hydrogen acceptor. In the second set of experiments, ferrocytochrome *c* was not used as a stoichiometric hydrogen donor, but, instead, catalytic amounts of cytochrome *c* were used in the presence of different substances which directly reduce cytochrome *c* (Keilin & Hartree, 1938). No phosphorylation was obtained in the first set of experiments while, in the second, the only phosphorylation found was a small amount with ascorbic acid as the reducing agent. Lehninger was unable to draw any conclusions from these experiments concerning the localization of the phosphorylation in the respiratory chain.

Phosphorylation was first demonstrated with ferricytochrome *c* as the hydrogen acceptor in 1950,

using α -ketoglutarate (α -oxoglutarate) as substrate, heart-muscle granules (sarcosomes, mitochondria) as the enzyme system and ferricytochrome *c*, in much smaller amounts than used by Lehninger, as hydrogen acceptor (Slater, 1950).

The P:O ratio (number of atoms of phosphorus esterified per oxygen atom equivalent of the ferricytochrome *c*) was 2.2, and there was reason to believe that this value might be underestimated. Since the P:O ratio with oxygen as hydrogen acceptor under the same conditions was 2.5, it was concluded that all the phosphorylation steps occurred between substrate and ferricytochrome *c*, implying that none occurred between ferrocytochrome *c* and oxygen.

This conclusion has been challenged on two grounds.

(1) Hunter (1951) and Krebs, Ruffo, Johnson, Eggleston & Hems (1953) have concluded, on the basis of thermodynamic calculations, that not more than three energy-rich phosphate bonds could be synthesized in the reaction between α -ketoglutarate and cytochrome *c* or more than one bond in the reaction between succinate and cytochrome *c*. Since these workers believe that the maximum P:O ratios in the aerobic oxidation of α -ketoglutarate and succinate are 4 and 2 respectively, it is argued that at least one phosphorylation must occur between cytochrome *c* and oxygen.

(2) Judah (1951) and Copenhaver & Lardy (1952) have found considerable esterification of inorganic phosphate during the oxidation of ascorbic acid by cytochrome *c* and liver mitochondria, suggesting that phosphorylation must occur in the cytochrome oxidase reaction. Nielsen & Lehninger (1954) have now reported phosphorylation with stoichiometric amounts of ferrocytochrome *c* as hydrogen donor.

Lindberg & Ernster (1954) have made more specific criticisms of the earlier experiments (Slater, 1950).

The present paper describes the earlier experiment in greater detail, as well as additional experiments carried out under more strictly controlled conditions. These additional experiments support the validity of the previous measurements in demonstrating phosphorylation with ferricyto-

* Part 2, Slater & Holton (1954).

chrome *c* as hydrogen acceptor. However, they do not confirm the conclusion that the phosphorylation ratios with added ferricytochrome *c* as hydrogen acceptor is as high as with oxygen. Experiments of this type do not give a definite answer to the question of whether all phosphorylation occurs between substrate and ferricytochrome *c*.

METHODS

Sarcosomes

The suspensions of sarcosomes were prepared from cat, pig or rat heart, using the isolation media and the procedure described by Cleland & Slater (1953). Different types of preparations are designated according to the isolation medium.

Measurement of phosphorylation ratios with oxygen as hydrogen acceptor

The procedure adopted was that described by Slater & Holton (1954). Except where stated otherwise, the composition of the reaction mixture was as specified under 'standard conditions' in Table 10 of that paper.

*Measurement of phosphorylation ratios with ferricytochrome *c* as hydrogen acceptor*

Cytochrome *c*. Several different preparations of cytochrome *c* were used in this study. Some, made by the method of Keilin & Hartree (1945), contained 0.34% iron. Others, many of which were kindly supplied by Dr E. Margoliash, were purified by the procedure of Margoliash (1954) and contained 0.45% iron. Since the cytochrome *c* in these preparations was partially reduced, the concentration of ferricytochrome *c* was determined immediately before use by the following method. The optical density at 550 m μ . of 3 ml. of a suitably diluted solution in 0.05M phosphate buffer, pH 7.3, was measured (D_1) in a 1 cm. cell against a reference cell containing 3 ml. of the phosphate buffer alone. An excess of $K_3Fe(CN)_6$ (in 0.1 ml.) was added to each cell and the optical density again measured (D_2 , after correction for the change of volume). A few crystals of dithionite (sufficient to decolorize the $K_3Fe(CN)_6$) were then added to each cell and the optical density was read a third time (D_3 , after the same correction as above). The molar concentration of ferricytochrome *c* in the diluted sample equals $3.62 \times 10^{-5} \times D_3(D_3 - D_1)/(D_2 - D_1)$.

The reduction of ferricytochrome *c* by α -ketoglutarate in the presence of the sarcosomes was followed spectrophotometrically in a Beckman or a Hilger Uvispek spectrophotometer. The concentration of cytochrome *c* was so great that the usual wavelength for studying cytochrome *c* (550 m μ ., the position of the α -band) could not be used. In some experiments, the maximum wavelength of the β -band (520 m μ .) was used. In others, the decrease of the absorption at 580 m μ . on reduction was more convenient. The reoxidation of ferrocytochrome *c* was prevented either by adding cyanide or by using completely anaerobic conditions.

Cyanide experiment. The procedure adopted in the experiment previously reported (Slater, 1950) and summarized in Table 1 and Fig. 1 will be described in detail,

although this does not include various improvements in technique which were introduced in later experiments.

Three Beckman cells (1.0 cm. light path) were filled as follows:

Beckman cell no. ...	1	2	3
Reaction mixture	+	+	+
Cytochrome <i>c</i>	+	+	+
α -Ketoglutarate	-	+	+
Final volume (ml.)	2.8	2.5	2.77

The composition of the reaction mixture is given in Table 1. Four conical flasks were filled as Beckman cell no. 3, except that in two flasks water replaced the cytochrome *c*. A few minutes before the experiment was begun, 0.2 ml. sarcosomal suspension and 0.1 ml. 0.15M- $MgCl_2$ were added to Beckman cell no. 1 and the optical density (at 520 m μ .) followed until it was constant. This cell was used as the reference for following the reduction of cytochrome *c* in cells nos. 2 and 3.

KCN (0.3 ml. of 0.1M) was added to Beckman cell no. 2, followed almost immediately, at zero time, by 0.1 ml. 0.15M- $MgCl_2$ and 0.2 ml. of the sarcosomal suspension. The optical density was followed (see curve 1, Fig. 1) until a stationary reading was obtained. Trichloroacetic acid (0.5 ml. of 40%, w/v) was added at 8.2 min.

Beckman cell no. 3 was then treated in exactly the same way, except that only 0.03 ml. 0.1M-KCN was used. Trichloroacetic acid (TCA) was added to this cell after 7.6 min.

The solutions in the conical flasks were treated in the same way, except that the optical density changes were not followed. The times of addition of TCA are shown in Table 1.

Anaerobic experiments. Three types of apparatus were used to exclude oxygen.

(i) **Manometric flask.** This was used in only one experiment, the one described in the preliminary communication (Slater, 1950; see lines 6 and 7 of the table) and in Table 4 of the present paper. This experiment was carried out with the same preparation and at the same time as the cyanide experiment described above. A Warburg manometric flask with two side bulbs and a centre well was used. The main compartment contained the reaction mixture while 0.2 ml. of the sarcosomal preparation was placed in one side-bulb and 0.5 ml. 40% TCA in the other. The air in the flask was swept out with nitrogen and a stick of yellow phosphorus placed in the centre well. The flask was rapidly attached to a manometer and thoroughly flushed through with N_2 while being gently shaken. The taps were closed and the shaking continued for another 5 min. During all this period, the side bulb containing the sarcosomal preparation was immersed in ice. The sarcosomal preparation was then mixed with the reaction mixture in the main compartment and the manometer placed in a bath at 24°. After 8.5 min., the TCA was tipped into the main compartment to terminate the reaction. A second Warburg flask with the same solutions, except for the omission of the cytochrome *c*, was treated simultaneously in the same way.

It was not possible to observe accurately the completion of the reduction of the cytochrome *c* in this experiment. Since the cytochrome *c* was completely reduced in 5.6 min. in the presence of 0.001M-KCN (see Fig. 1), the 8.5 min. used in the anaerobic experiment included some margin of safety.

(ii) Side-arm Thunberg tubes. Thunberg tubes with a side-arm hollow stopper as well as the usual top hollow stopper have been described by Keilin & Hartree (1947*b*). The reaction mixture (2.8–2.9 ml.) was placed in the main tube, the sarcosomal preparation (0.1–0.2 ml.) in the side-arm stopper which could be immersed in an ice bath, while the top stopper contained 0.5 ml. 40% TCA. The tube was attached to a pump, and while vigorously shaken, evacuated sufficiently to make its contents boil, then filled with O_2 -free nitrogen, and the whole process repeated twice. Finally, the tube was evacuated and the stopper closed. During all this treatment, the side-stopper containing the sarcosomes was immersed in ice. The reaction was commenced by mixing the contents of the side-stopper with the main tube and terminated by adding the TCA.

The completion of the reduction of ferricytochrome *c* could not be observed accurately in these tubes. The time required for complete reduction was determined in a separate experiment with the same preparation and reaction mixture, usually in the spectrophotometric Thunberg tube, and a few minutes were allowed as a margin of safety to ensure complete reduction.

(iii) Spectrophotometric Thunberg tubes. This was the most satisfactory method. The spectrophotometric Thunberg tubes (supplied by The Thermal Syndicate Ltd., 12–14 Old Pye Street, London, S.W. 1, under the name 'Beckman-Thunberg cells in vitreosil, 10 mm.') consist of a Thunberg tube sealed on to a 1 cm. silica cell, fitting into the cell-holder of the Beckman spectrophotometer. The tube projects above the top of the cell compartment and a special hood was constructed to prevent light entering the instrument. The shape of the cell makes it impossible to remove the dissolved O_2 by vigorous shaking during evacuation, and the following procedure was found necessary. The reaction mixture with all components except the hexokinase was placed in the main cell compartment of the apparatus and O_2 -free nitrogen was gently bubbled through for about 30 min. The hexokinase (not more than 0.1 ml.) was added to the main tube, and the hollow stopper containing the enzyme inserted. The tube was then evacuated and filled with O_2 -free nitrogen as described above, the evacuation and filling repeated twice and finally evacuated. The stopper containing the sarcosomes was kept in ice during all this period. At zero time, the sarcosomes were mixed with the reaction mixture in the main compartment and the tube placed in the spectrophotometer. Readings of optical density were made against a reference cell, open to the air, containing all the components of the reaction mixture, except the cytochrome *c*. The sarcosomal preparation was added to this reference cell immediately before zero time. When the optical density was steady (an example is given in Fig. 3, curve 1), the tube was quickly removed from the instrument and filled with O_2 -free nitrogen. The stopper was removed and 0.5 ml. 40% (w/v) TCA added immediately. A negligible amount of O_2 entered the solution during this operation.

The amount of ferricytochrome *c* reduced could be calculated from the difference (ΔD_{550}) between the initial optical density (obtained by extrapolation) and the final reading, using the relationship, molar concentration of ferricytochrome *c* reduced = $3.62 \times 10^{-4} \Delta D_{550}$. The value calculated in this way agreed closely with the amount of ferricytochrome *c* added, measured as described above (p. 393). The latter value was considered the more reliable, and was used in the calculation of the P:O ratio. It was assumed that all

the ferricytochrome *c* was reduced by the α -ketoglutarate in a reaction catalysed by the sarcosomes. Control experiments showed that there was no reduction of the ferricytochrome *c* by substances present in the reaction mixture, until the sarcosomes were added.

Measurement of phosphorylation

The aim of these experiments was to compare the yield of oxidative phosphorylation obtained with ferricytochrome *c* and with oxygen as hydrogen acceptors, under otherwise identical conditions. The reactions in the presence of yeast hexokinase and glucose are

where αKg = α -ketoglutarate, *A* = hydrogen acceptor (1 atom of oxygen or two molecules of ferricytochrome *c*), ADP = adenosine diphosphate, ATP = adenosine triphosphate, HMP = hexose monophosphate.

Two methods were used to determine the yields of esterified phosphate.

Method I. The amount of HMP found was corrected for the non-oxidative phosphorylation of glucose by ADP, catalysed by myokinase and hexokinase (reaction 3; AMP = adenosine monophosphate), as described by Slater (1953*b*):

Method II. The increase of $HMP + \sim P$ (energy-rich phosphate) was measured, as described by Slater & Holton (1953). No correction for reaction 3 is necessary in method II, since ($HMP + \sim P$) is not changed by this reaction.

An example of the use of method I is shown in Table 4. The 'myokinase correction' is here insignificant, amounting to only 2% of the ΔHMP . Examples of the use of method II are given in Tables 5–9.

It has been shown in an earlier paper (Slater & Holton, 1953) that method I is likely to give low values in short-time experiments, since it does not take account of the ATP which is synthesized by reaction 1, and has not had time to react according to reaction 2. In short-time experiments, this unreacted ATP is a considerable fraction of the total esterified phosphorus.

Analytical methods. Hexose monophosphate (HMP), energy-rich phosphorus ($\sim P$) and α -ketoglutarate (αKg) were measured as described previously (Slater, 1953*a*; Slater & Holton, 1953).

Hexokinase. The unit of hexokinase activity is that used by Berger, Slein, Colowick & Cori (1946).

RESULTS

Experiments using inhibitors of cytochrome oxidase

In experiments with ferricytochrome *c* as hydrogen acceptor, it is necessary to prevent the reoxidation of the ferrocytochrome *c*. One way of doing this is to inhibit the cytochrome oxidase. Cyanide is the most effective inhibitor, but in this case it suffers from the following disadvantages: (a) it inhibits phosphorylation; (b) owing to cyanohydrin formation, the concentration of free cyanide is much less than that added, so that there is an appreciable

Table 1. *Phosphorylation coupled with the reduction of ferricytochrome c by α -ketoglutarate.*
Cyanide experiment

Fluoride, 0.039M; phosphate, pH 7.3, 0.035M; glucose, 0.016M; AMP, 0.001M; ADP, 0.001M; Mg, 0.005M; α Kg, 0.0052M; hexokinase; cat-heart sarcosomes (isolated in phosphate-saline), 1.06 mg. protein/ml. See Methods for procedure and Fig. 1 for course of reduction of ferricytochrome c. Temp., between 23.2 and 24.7°. Times corrected to correspond to 24.2°, assuming that the reaction velocity doubled for a rise in temp. of 10°.

Δ HMP, corrected = Δ HMP (in presence of cytochrome c) - Δ HMP (in absence of cytochrome c).						
Time (min.)	...	...	...	...	...	...
HCN (total, M)	...	...	...	...	...	...
Ferricytochrome c (μ atoms O equiv.)	0	0.23	0.23	0	0.23	0.23
HMP (μ moles): initial	0.16	0.16	0.16	0.16	0.16	0.16
final	0.22	0.52	0.66	0.29	0.79	0.40
Δ	0.06	0.36	0.50	0.13	0.63	0.24
Δ HMP, corrected (μ moles)	—	0.30	0.40†	—	0.50	0.22‡
P:O	—	—	—	—	2.20	—

* This value is somewhat less than that given previously (Slater, 1950), which was calculated on the assumption that the sample of α -ketoglutarate was 100% pure. The new value is based on an analysis of this sample.
† Assuming that Δ HMP in absence of cytochrome c = 0.10 (interpolated value).
‡ The value subtracted here was 0.015 μ mole, found in an anaerobic experiment in the absence of cytochrome c (see Table 4).

Fig. 1. Course of reduction of cytochrome c in the experiment described in Table 1. Curve 1: α Kg, 0.01M-KCN, 23.8°. Curve 2: α Kg, 0.001M-KCN, 24.7°. Curve 3 (shown for comparison): DPNH instead of α -ketoglutarate, 0.001M-KCN, 24.2°. The actual optical density readings have been converted to correspond to a volume of 3.0 ml. The column to the right shows the optical density change corresponding to complete reduction of the cytochrome c. Individual measurements of optical density are shown for curve 2, only.

aerobic oxidation, which has to be allowed for; (c) it is not possible to estimate the disappearance of α -ketoglutarate during the reaction, since HCN in the TCA extracts causes the destruction of α -ketoglutarate; (d) HCN forms with ferricyto-

chrome c a compound which does not react with the cytochrome c-reducing enzyme systems (Potter, 1941; Horecker & Kornberg, 1946); this means that the amount of ferricytochrome c actually reduced by the α -ketoglutarate is a little less than the total ferricytochrome c.

All these difficulties eventually led to the abandonment of this method of preventing the reoxidation of the ferrocytochrome c. However, it is necessary to refer to one experiment made by this method, since it has already been published (Slater, 1950). The results of this experiment are given in Table 1. The course of the reduction of the ferricytochrome c in the presence of both 0.01 and 0.001M-HCN is shown in Fig. 1. Since method I was used to measure the phosphorylation, it is necessary to correct the Δ HMP found for the amount which would be synthesized by the combined action of myokinase and hexokinase. This was allowed for in control measurements made at the same time, in which the cytochrome c was omitted. These controls also allowed for the HMP synthesized by that fraction of the respiration which was not inhibited by the cyanide. This correction also takes account of any phosphorylation accompanying the dismutation of α -ketoglutarate (see p. 399).

It can be seen in Table 1 that the corrected values of Δ HMP continued to increase after all the ferricytochrome c was reduced (5.6 min.). This is due mostly to a lag in the synthesis of HMP from the ATP formed by the oxidative phosphorylation reaction (Slater & Holton, 1953). From this point of view, the highest value, corresponding to a P:O ratio of 2.2, is the nearest to the true value. This is practically identical with the value obtained (2.16) when the reoxidation of the

ferrocytochrome *c* was prevented by excluding oxygen (see Table 4).

However, the calculated value of 2.2 for the P:O ratio may be slightly high, since the control measurement made in the absence of cytochrome *c* is adequate only if the respiration in the presence of cyanide is unaffected by the addition of cytochrome *c*. Experiments made both with the Keilin & Hartree (1947*a*) heart-muscle preparation oxidizing succinate and with sarcosomal preparations oxidizing α -ketoglutarate showed that the respiration was slightly increased by the addition of cytochrome *c*, but the error introduced became serious only when a considerable fraction of the respiration was unaffected by the cyanide.

The differences between the initial (obtained by extrapolation) and the final optical densities in Fig. 1 show that the ferricytochrome *c* was virtually completely reduced. This shows that there must have been little respiration in this experiment. However, attempts to repeat this experiment with different types of preparations and reaction mixtures have failed, the optical density reaching a steady value (and then often declining again) when only about 70% of the cytochrome *c* was reduced. While the reason for this is not completely clear, it is probably connected with the fact that the reaction between α -ketoglutarate and cyanide is relatively slow. With high concentrations of active sarcosomal preparations and relatively low concentrations of ferricytochrome *c*, all the latter is reduced and the experiment is finished while the concentration of free cyanide is still sufficiently high to inhibit the cytochrome oxidase almost completely.

The extent of the combination between α -ketoglutarate and cyanide was determined in the following experiment, using a Keilin & Hartree heart-muscle preparation oxidizing succinate at 25°. A curve was constructed relating the rate of oxygen uptake to the concentration of cyanide. Another flask, containing 0.005*M* α -ketoglutarate and 8.75×10^{-4} *M* total cyanide, consumed oxygen at a rate corresponding to a concentration of free cyanide of 5.95×10^{-5} *M*, i.e. 92.5% of the cyanide must have been bound by the α -ketoglutarate. The uniform rate of oxygen uptake showed that the reaction between the cyanide and α -ketoglutarate had come to equilibrium by the time the manometric readings commenced, about 20 min. after mixing the reactants.

The earlier stages of the reaction between α -ketoglutarate and cyanide were studied by a different type of experiment, which is described in Fig. 2. Curve 1 shows the rapid rate of aerobic oxidation of DPNH by the Keilin & Hartree heart-muscle preparation, and curve 2 shows the strong inhibition of this oxidation by 8.75×10^{-4} *M* cyanide. The addition of a small amount of water

had no appreciable effect on this inhibition. The addition of α -ketoglutarate (curve 3), however, caused a steadily increasing release of the cyanide inhibition. Since the reaction between cyanide and cytochrome oxidase, and its reversal, is known to be a very rapid reaction, the slow release of the inhibition must be due to the slow reaction between α -ketoglutarate and cyanide. The reactivation was hardly detectable until about 3 min. after the addition of the α -ketoglutarate and did not become appreciable for several minutes later.

Inhibition of phosphorylation by cyanide. The last column of Table 1 shows that the phosphorylation is considerably less with 0.01*M*-HCN than with 0.001*M*-HCN (0.22 μ mole HMP compared with 0.40 at approximately the same time). This suggests that cyanide is an inhibitor of oxidative phosphorylation. This was shown in another way in the experiment summarized in Table 2, in which varying concentrations of α -ketoglutarate were used in the presence of 0.001*M*-HCN. Owing to cyanohydrin formation between α -ketoglutarate and cyanide, increasing the concentration of α -ketoglutarate has the effect of decreasing the concentration of cyanide, although this effect was probably lessened to some extent by the distillation of cyanide from the centre well to the main flask. Table 2 shows that with increasing α -ketoglutarate concentration, there was an increase both of the O₂ uptake and of the P:O ratio of the residual respiration. It should be noted that varying the concentration of α -keto-

Fig. 2. The reaction between α -ketoglutarate and cyanide. The curves show the oxidation of DPNH by the Keilin & Hartree heart-muscle preparation. Phosphate, pH 7.4, 0.04*M*; ethylenediaminetetraacetate, 0.001*M*; DPNH, 8.4×10^{-4} *M*; heart-muscle preparation (0.49 mg. protein) added at zero time. Curve 1, no other addition; curve 2, 8.75×10^{-4} *M*-HCN initially present, 0.16 ml. water added at arrow; curve 3, 8.75×10^{-4} *M*-HCN initially present, 0.16 ml. α -ketoglutarate (15 μ moles) added at arrow. Optical densities corrected for change of volume after additions. Temp. 24–25°.

glutarate within this range has no appreciable effect on the P:O ratio in the absence of cyanide (Slater & Holton, 1954). The P:O ratio in the absence of cyanide was a little low in this experiment, probably because a rather small concentration of hexokinase (24 units/mg. sarcosomal protein) was used in order to decrease the possibility of the dismutation reaction, discussed below.

Effect of azide on oxidative phosphorylation. In view of the difficulties with cyanide, another inhibitor of cytochrome oxidase was sought. Sulphide could not be used because it rapidly reduces ferri-cytochrome *c* in the absence of any other enzyme. As was to be expected from Loomis & Lipmann's (1949) work, azide was unsuitable because it inhibited phosphorylation. Moreover, it was relatively ineffective as an inhibitor of the respiration of the sarcosomes. Both these effects are shown in Table 3. Since previous studies of the uncoupling action of azide on oxidative phosphorylation have been made only with ferricyanide as acceptor (Loomis & Lipmann, 1949; Judah, 1951), it is of some interest to show the same effect with oxygen as hydrogen acceptor. Table 3 shows that a concentration of azide which has no effect on the respiration markedly inhibits the phosphorylation. It was found in another experiment that azide, like dinitrophenol, does not completely uncouple the phosphorylation from respiration when α -ketoglutarate is substrate, i.e. azide is primarily an inhibitor of respiratory chain phosphorylation (cf.

Judah, 1951). The P: α Kg ratio with 0.003–0.015M azide was about the same as with 1×10^{-4} – 2.8×10^{-4} M dinitrophenol (see Slater & Lewis, 1954).

It is surprising that the oxidation of α -ketoglutarate by the sarcosomes is so insensitive to azide. The oxidation of succinate is more sensitive, 85% inhibition being found with 0.03M azide compared with 66% inhibition of the oxidation of α -ketoglutarate under the same conditions (Table 3). The rate of oxidation of succinate by the Keilin & Hartree preparation was inhibited by 92% by this concentration of azide. Except for the addition of succinate and the removal of malonate and fluoride, the reaction mixture in this experiment was the same as in Table 3 even to the extent of including α -ketoglutarate, which is not oxidized by the Keilin & Hartree preparation. Cyanide was equally inhibitory to both types of preparation oxidizing succinate.

Anaerobic experiments

The anaerobic experiment reported in the preliminary publication (Slater, 1950) is shown in greater detail in Table 4. There are a number of possible sources of error in experiments of this type, and these will now be considered in detail.

Residual oxygen. The most serious possible error in these experiments is the presence of residual oxygen. It is easy by repeated evacuation and refilling with oxygen-free nitrogen to remove practically all the oxygen from the gas phase, but special care is necessary to remove dissolved oxygen from the liquid. The reaction volume used (3 ml.) contains approx. 1.5 μ atoms of dissolved oxygen in equilibrium with air. To remove this oxygen from the side-arm Thunberg tubes, it was found essential to shake the solution vigorously during the evacuation and filling. By the following method it was found that less than 0.01 μ atom of oxygen then remained.

The main tube of a side-arm Thunberg tube contained a solution of ferrocytochrome *c* equivalent to 0.01 μ atom of oxygen in phosphate buffer, total vol., 3.0 ml. The side stopper contained 0.1 ml. of a concentrated Keilin & Hartree heart-muscle preparation. In order to simulate conditions of the oxidative phosphorylation experiments, 0.5 ml. 40% (w/v) TCA was placed in the top stopper. After the usual evacuation procedure, the contents of the main

Table 2. *Effect of cyanide on oxidative phosphorylation*

Rat-heart sarcosomes (isolated in 0.28M sucrose, 0.01M ethylenediaminetetraacetate, pH 7.4), 1.5 mg. protein/ml.; phosphate, pH 7.4, 0.03M; sucrose, 0.045M; malonate, 0.01M; fluoride, 0.04M; glucose, 0.03M; ethylenediaminetetraacetate, 0.0015M; AMP, 2×10^{-4} M; ADP, 2×10^{-4} M; Mg, 0.005M; α Kg, 0.01M; no added cytochrome *c*; hexokinase, 24 units/mg. sarcosomal protein. The centre well of the manometric flask contained a solution prepared by mixing 9 ml. 2M-KCN and 0.2 ml. 1N-KOH. 30 min., 25°.

α Kg (m-moles/l.)	...	1	3	10	10
HCN (m-moles/l.)	...	1	1	1	0
– Δ O (μ atoms)		0.26	0.47	0.65	5.33
Δ HMP (μ atoms)		0.22	0.60	1.35	13.0
P:O		0.84	1.28	2.08	2.43

Table 3. *Effect of azide on oxidative phosphorylation*

Rat-heart sarcosomes (isolated in 0.28M sucrose, 0.01M ethylenediaminetetraacetate, pH 7.4), 0.74 mg. protein/ml.; phosphate, pH 7.4, 0.03M; sucrose, 0.045M; malonate, 0.01M; fluoride, 0.04M; glucose, 0.03M; ethylenediaminetetraacetate, 0.0015M; ADP, 6×10^{-4} M; AMP, 6×10^{-4} M; Mg, 0.005M; α Kg, 0.01M; cytochrome *c*, 4.9×10^{-5} M; hexokinase, 292 units/mg. sarcosomal protein. 45 min., 25°.

Azide (M)	...	...	...	0	10^{-4}	5×10^{-4}	3×10^{-3}	1.5×10^{-2}	3×10^{-2}
– Δ O (μ atoms)				3.13	3.07	3.34	2.10	1.20	1.05
Δ HMP + Δ ~P (μ moles)				8.19	6.93	5.32	1.35	0.97	0.71
P:O				2.62	2.26	1.60	0.64	0.81	0.68

tube were mixed with the heart-muscle preparation and the suspension observed under a low-dispersion micro-spectroscope. The spectrum of ferrocytochrome *c* remained visible even after 24 hr. standing. When the experiment was repeated with different amounts of ferrocytochrome *c*, omitting the shaking during the evacuation (the solutions boiled vigorously), ferrocytochrome *c* equivalent to 0.10 μ atom oxygen was completely and instantaneously oxidized when the solutions were mixed.

Table 4. *Phosphorylation accompanying the reduction of ferricytochrome c by α -ketoglutarate. Preliminary anaerobic experiment*

Anaerobic expt. using manometric flask; see p. 393 for procedure. Fluoride, 0.039M; phosphate, pH 7.3, 0.035M; glucose, 0.016M; AMP, 0.001M; ADP, 0.001M; Mg, 0.005M; α Kg, 0.0052M; hexokinase; cat-heart sarcosomes (isolated in phosphate-saline), 1.06 mg. protein/ml. 8.5 min., 24°.

Ferricytochrome <i>c</i> (μ atoms O equiv.) ...	0.23	0
HMP (μ moles): initial	0.16	0.16
final	0.67	0.17
Δ	0.51	0.01
Δ HMP, corrected (μ moles)	0.50	—
P:O	2.16	—

The procedure used with the spectrophotometric Thunberg tubes (see p. 394) was also satisfactory. Residual oxygen can be detected in this case by a time lag in reaching the maximum rate of reduction of cytochrome *c*, as is clearly shown in Fig. 3. Curve 1 describes the course of the reduction of cytochrome *c* in the spectrophotometric Thunberg tube, the contents of which had been bubbled with oxygen-free nitrogen for some time before evacuation. Curve 2 describes an experiment with the same preparation, when the evacuation procedure was carried out without this preliminary bubbling treatment.

The analyses on these two tubes are given in Table 5. The excess of $-\Delta\alpha$ Kg over the oxygen equivalent of the ferricytochrome *c* ($0.901 - 0.469 = 0.432$) in tube 2 is a measure of the residual oxygen. If this residual oxygen phosphorylates with the same P:O ratio as in the aerobic experiment with the same preparation (2.8), then $2.8 \times 0.432 = 1.21 \mu$ atoms should be subtracted from the Δ HMP + $\Delta \sim P$ found in this experiment. This gives $1.97 - 1.21 = 0.76 \mu$ atom P esterified by the reduction of ferricytochrome *c*, not significantly different from the 0.78 obtained in the other tube.

Fig. 3. Reduction of ferricytochrome *c* by α -ketoglutarate under anaerobic conditions and in presence of a small amount of oxygen. Rat-heart sarcosomes (isolated in 0.28M-sucrose, 0.01M ethylenediaminetetraacetate, pH 7.4), 0.47 mg. protein/ml.; phosphate, pH 7.4, 0.03M; malonate, 0.01M; fluoride, 0.04M; AMP, 2×10^{-4} M; ADP, 2×10^{-4} M; α Kg, 4×10^{-4} M; glucose, 0.02M; ethylenediaminetetraacetate, 0.001M; magnesium, 0.005M; hexokinase, 151 units/mg. sarcosomal protein. Curve 1, tube completely evacuated; curve 2, tube contained residual oxygen. Ferricytochrome *c* equivalent to 0.47 μ atom oxygen was used. The curves show the amount of ferricytochrome *c* reduced, calculated from the change of optical density.

This shows (a) that residual oxygen does not affect the phosphorylation obtained by the reduction of cytochrome *c*; (b) that even when there are large amounts of residual oxygen, its effect on the phosphorylation obtained with ferricytochrome *c* can be accurately allowed for.

Phosphomonoesterase. The sarcosomal preparations contain only small amounts of phosphomonoesterase which, under conditions of active and continuous oxidation, hydrolyse an insignificant proportion of the HMP synthesized. However, under the conditions of the anaerobic experiments, even a slight phosphomonoesterase activity will cause appreciable loss of HMP if the deproteinizing is delayed after the reduction of the cytochrome *c* is complete. Since some delay was inevitable, it was not possible completely to eliminate this source of error. However, only pig preparations contain sufficient phosphomonoesterase to cause a significant error. In the experiment described in Table 6, the reduction of ferricytochrome *c* was complete in

about 4 min., but α -ketoglutarate continued to disappear, because of a reaction which will be considered below. Although this anaerobic reaction is accompanied by phosphorylation, so that in the absence of phosphomonoesterase the total esterified phosphorus would increase on standing, (Δ HMP + Δ ~P) actually decreased rapidly, owing to the action of phosphomonoesterase.

Dismutation of α -ketoglutarate. As already reported (Slater & Holton, 1954; Holton, 1954a), the yeast hexokinase preparations contain a substance which, when added to the sarcosomal preparations, promotes the disappearance of α -ketoglutarate in the absence of any other hydrogen acceptor. This reaction has been studied by Holton (1954b), who has found that it is accompanied by phosphorylation, with a P: α Kg ratio of 0.5.

Even hexokinase purified to the stage of step 5 of the procedure of Berger *et al.* (1946) contains sufficient of this substance to promote the disappearance of considerable amounts of α -ketoglutarate, in the absence of oxygen or other hydrogen acceptor. Fortunately, this reaction proceeds at an appreciable rate only in the absence of other hydrogen acceptors, and in the experiments with ferricytochrome *c*, it can be made inconsiderable if the reaction is stopped soon after the reduction of the ferricytochrome *c* is complete. This is illustrated by the two experiments summarized in Tables 6 and 7, the former with crude hexokinase, the latter with the partially purified enzyme (specific activity, 20% that of Berger *et al.*'s purified preparation). In both experiments, the disappearance of α -ketoglutarate was equivalent to the ferricytochrome *c* reduced, when the reaction was stopped shortly after the reduction was complete, but continued after all the ferricytochrome *c* was reduced. Tubes 6 and 7 of Table 7 show the very considerable anaerobic reaction in the absence of cytochrome *c*. The greater rate of a dismutation reaction in the absence of oxygen (or of cytochrome *c* which takes the place of

Table 5. *Effect of residual oxygen on the measurement of the phosphorylation accompanying the reduction of ferricytochrome c by α -ketoglutarate*

Anaerobic expt. using spectrophotometric Thunberg tube. See Fig. 3 for details.

Tube	...	...	...	1	2
Ferricytochrome <i>c</i> (μ atoms O equiv.)				0.469	0.469
α Kg (μ moles): initial				0.98	0.98
final				0.53	0.08
Δ				-0.45	-0.90
~P (μ moles): initial				0.59	0.59
final				0.43	0.40
Δ				-0.16	-0.19
HMP (μ moles): initial				0.02	0.02
final				0.96	2.18
Δ				0.94	2.16
Δ HMP + Δ ~P				0.78	1.97
P:O				1.67	—
P: α Kg				1.74	2.19

Table 6. *Measurement of phosphorylation coupled with reduction of ferricytochrome c by α -ketoglutarate. Effect of phosphomonoesterase in pig-heart sarcosomal preparations and of crude hexokinase*

Pig-heart sarcosomes (isolated in phosphate-saline), 1.3 mg. protein/ml.; phosphate, pH 7.3, 0.035M; malonate, 0.01M; fluoride, 0.04M; AMP, 4.8×10^{-4} M; ADP, 4.5×10^{-4} M; α Kg, 2.8×10^{-4} M; glucose, 0.017M; Mg, 0.005M; hexokinase, 127 units/mg. sarcosomal protein. Crude hexokinase. Side-arm Thunberg tubes used. Ferricytochrome *c* completely reduced in about 4 min., 22°.

Tube	...	...	...	...	1	2	3	4	5	6
Time (min.)	...	...	...	...	0	4	5	10	30	30
Ferricytochrome <i>c</i> (μ atoms O equiv.)					0.30	0.30	0.30	0.30	0.30	0
α Kg (μ moles)					0.84	0.57	0.43	0.23	0.04	0.04
- Δ α Kg (μ moles)					—	0.27	0.41	0.61	0.80	0.80
~P (μ moles)					1.36	1.06	—	0.69	0.47	0.42
- Δ ~P (μ moles)					—	0.30	0.35*	0.67	0.89	0.94
HMP (μ moles)					0.05	0.89	1.01	1.10	1.09	0.89
Δ HMP (μ moles)					—	0.84	0.96	1.05	1.04	0.84
Δ HMP + Δ ~P					—	0.54	0.61	0.38	0.15	-0.10

* Interpolated value.

Table 7. *Measurement of phosphorylation coupled with reduction of ferricytochrome c by α -ketoglutarate. Correction for dismutation*

Rat-heart sarcosomes (isolated in 0.28 M sucrose, 0.01 M ethylenediaminetetraacetate, pH 7.4), 0.84 mg. protein/ml.; phosphate, pH 7.4, 0.03 M; malonate, 0.01 M; fluoride, 0.04 M; AMP, 2×10^{-4} M; ADP, 2×10^{-4} M; α Kg, 3.2×10^{-4} M; glucose, 0.03 M; ethylenediaminetetraacetate, 0.0017 M; Mg, 0.005 M; hexokinase, 132 units/mg. sarcosomal protein. Cytochrome c completely reduced in approx. 9 min. Temp., 21°. Partially purified hexokinase used. Tube 2 was spectrophotometric Thunberg; Tubes 3 to 7 were side-arm Thunberg tubes.

Tube	...	...	...	1	2	3	4	5	6	7
Time (min.)	...	...	...	0	11	15	15	15	15	15
Ferricytochrome c (μ atoms O equiv.) (a)				—	0.50	0.50	0.50	0.50	0	0
α Kg (μ moles)				0.97	0.51	0.34	0.44	0.37	0.40	0.35
$-\Delta\alpha$ Kg (μ moles) (b)				—	0.46	0.63	0.53	0.60	0.57	0.62
$\sim$ P (μ moles)				0.59	0.58	0.57	0.58	0.59	0.61	0.59
$\Delta\sim$ P (μ moles) (c)				—	-0.01	-0.02	-0.01	0	0.02	0
HMP (μ moles)				0.03	0.90	1.01	0.94	0.96	0.41	0.40
Δ HMP (μ moles) (d)				—	0.87	0.98	0.91	0.93	0.38	0.37
Δ HMP + $\Delta\sim$ P (μ moles) (c + d)				—	0.86	0.96	0.90	0.93	0.40	0.37
(b - a)				—	—	0.13	0.03	0.10	—	—
0.5 (b - a)				—	—	0.06	0.01	0.05	—	—
Corrected esterified P (c + d) - 0.5 (b - a)				—	0.86	0.90	0.89	0.88	—	—

Table 8. *Measurement of phosphorylation coupled with reduction of ferricytochrome c by α -ketoglutarate. Comparison of vacuum with nitrogen gas phase*

Rat-heart sarcosomes (isolated in 0.28 M sucrose, 0.01 M ethylenediaminetetraacetate, pH 7.4), 0.60 mg. protein/ml.; phosphate, pH 7.4, 0.03 M; malonate, 0.01 M; fluoride, 0.04 M; AMP, 2×10^{-4} M; ADP, 2×10^{-4} M; α Kg, 4×10^{-4} M; glucose, 0.02 M; ethylenediaminetetraacetate, 0.001 M; Mg, 0.005 M; hexokinase, 116 units/mg. sarcosomal protein. Spectrophotometric Thunberg tubes. Tube 1 was finally evacuated; Tube 2 was finally filled with nitrogen.

Tube	...	...	...	...	1	2
Ferricytochrome c (μ atoms O equiv.)					0.464	0.464
α Kg (μ moles): initial					1.10	1.10
final					0.68	0.60
Δ					-0.42	-0.50
$\sim$ P (μ moles): initial					0.62	0.62
final					0.43	0.43
Δ					-0.19	-0.19
HMP (μ moles): initial					0.02	0.02
final					1.00	1.12
Δ					0.98	1.10
Δ HMP + $\Delta\sim$ P					0.79	0.91
P:O					1.70	—
P: α Kg					1.89	1.82

oxygen in this experiment) is well-known in biochemistry and can be explained by the competition between two pathways of hydrogen-transfer.

The contribution of the anaerobic reaction was allowed for by subtracting 0.5 ($-\Delta\alpha$ Kg - oxygen equiv. of ferricytochrome c) from the measured phosphorylation (see Table 7). The correction was a small one, which could be accurately assessed.

Effect of evacuation procedure on activity of sarcosomes. The sarcosomal suspension in the evacuated

spectrophotometric Thunberg tube often scatters less light than the identical suspension in an open spectrophotometric cell. Since Cleland (1952) has shown that swelling of the sarcosomes is associated with decreased light scatter, it is possible that the evacuation procedure causes the sarcosomes to swell. It was important to determine whether this swelling was sufficient to impair the phosphorylative activity of the sarcosomes. This was investigated in two experiments.

In the first, a side-arm Thunberg tube containing the usual reaction mixture without any cytochrome c was subjected to the usual evacuation procedure, except that some oxygen (0.25 μ atom) was allowed to remain. The P: α Kg ratio was 2.49, only slightly lower than the P:O ratio obtained in the usual way with this preparation (2.64). This indicates that the evacuation procedure does not appreciably affect the phosphorylative activity of the sarcosomes, when oxygen is the hydrogen acceptor.

This was confirmed in a second experiment, in which the top stopper was closed not after the fourth evacuation as usual but after a further filling with nitrogen. This experiment is summarized in Table 8, which shows that the P: α Kg ratio was no higher when the tubes were finally filled with nitrogen instead of being left evacuated. Tube 2 of Table 8 shows why evacuation is preferable; the nitrogen, even after passage through heated copper, contains a measurable amount of oxygen.

Effect of concentration of ADP. When method II (p. 394) is used to measure the phosphorylation, it is not possible to increase the concentration of ADP beyond about 2×10^{-4} M, without introducing appreciable errors in the measurement of $\Delta\sim$ P. This concentration of ADP is sufficient for optimum

aerobic phosphorylation (Slater & Holton, 1953), but it was necessary to check that this was also the case with ferricytochrome *c* as hydrogen acceptor. It was found that increasing the concentrations of ADP fivefold (using method I to measure the phosphorylation) had no effect on the phosphorylation (0.57 compared with 0.55 μ mole).

Effect of hypotonic pretreatment of the sarcosomes. Lehninger (1951*a, b*) has found that the rate of oxidation of DPNH by liver mitochondria was greatly increased by previously exposing the mitochondria to hypotonic conditions. The P:O ratio was also increased by this hypotonic pretreatment.

Table 9 shows that, although suspension of the sarcosomes in a hypotonic medium nearly trebled the rate of reduction of ferricytochrome *c* by α -ketoglutarate and slightly increased the rate of aerobic oxidation, the phosphorylation ratios were unaffected. The rate of oxidation of ascorbate, and especially the accompanying phosphorylation, were greatly increased by this treatment. The phosphorylation with ascorbate will be discussed in a separate paper.

Effect of 2:4-dinitrophenol. It is now well-established that one of the phosphorylation steps accompanying the oxidation of α -ketoglutarate to succinate is substrate-linked, i.e. it is associated with the initial dehydrogenation of the substrate, and that this substrate-linked phosphorylation is insensitive to dinitrophenol (see Slater & Lewis, 1954, for a review of the literature). It was of interest, therefore, to determine the effect of dinitrophenol on the phosphorylation coupled with the reduction of ferricytochrome *c*. An experiment showing this is summarized in Table 10.

It can be seen that dinitrophenol markedly lowers the P: α Kg ratios both with oxygen and with ferricytochrome *c* as hydrogen acceptor, and the P: α Kg ratios in the presence of dinitrophenol are practically the same with both hydrogen acceptors. This is a good indication that the P: α Kg ratio can be measured with ferricytochrome *c* as hydrogen acceptor without appreciable errors. The reason why the P: α Kg ratio in the presence of dinitrophenol is less than 1 is discussed by Slater & Lewis (1954).

Comparison of phosphorylation ratios with oxygen and ferricytochrome c as hydrogen acceptors

Table 11 summarizes all the experiments made with ferricytochrome *c* as hydrogen acceptor in the absence of oxygen, in which normal sarcosomal preparations were used and there was close agreement between the α -ketoglutarate disappearance and the ferricytochrome *c* used, showing the absence of residual oxygen and of the anaerobic dismutation of α -ketoglutarate. Two experiments were also carried out with preparations made in

Table 9. *Effect of hypotonic pretreatment on rate of oxidation of α -ketoglutarate by ferricytochrome c and by oxygen, on rate of oxidation of ascorbate by oxygen and on phosphorylation ratios*

Rat-heart sarcosomes isolated in 0.21M sucrose, 0.01M ethylenediaminetetraacetate, pH 7.4, and one-half suspended in this medium and the other half suspended in water. Allowing for contaminating supernatant in the sediment from the centrifuging, the tonicity of the medium in which the latter sarcosomes were suspended was equivalent to about 0.01M sucrose.

Anaerobic experiments. Spectrophotometric Thunberg tubes used. Phosphate, 0.03M; malonate, 0.01M; fluoride, 0.04M; ADP, 2×10^{-4} M with sarcosomes suspended in isolation medium, 10^{-3} M with sarcosomes suspended in water; AMP, same as ADP; glucose, 0.02M; ethylenediaminetetraacetate, 0.001M; Mg, 0.005M; α Kg, 4×10^{-4} M; sarcosomes, 0.35 mg. protein/ml. (suspended in medium) or 0.40 (sarcosomes suspended in water); hexokinase, 128 and 113 units/mg. sarcosomal protein respectively. Method II used to measure phosphorylation with sarcosomes suspended in isotonic medium, Method I with sarcosomes suspended in water.

Aerobic experiments. α -Ketoglutarate as substrate: same as anaerobic expt., except ADP=AMP, 2×10^{-4} M with both preparations; α Kg, 0.005M; cytochrome *c*, 3.4×10^{-5} M. 30 min., 25°. Method II. Ascorbate as substrate: phosphate, 0.03M; cytochrome *c*, 5.2×10^{-5} M; glucose, 0.02M; ethylenediaminetetraacetate, 0.001M; fluoride, 0.04M; ADP, 10^{-3} M; AMP, 10^{-3} M; Mg, 0.005M; ascorbate, 0.01M; sarcosomes, 0.175 mg. protein/ml. (suspended in medium) or 0.198 mg. protein/ml. (suspended in water); hexokinase, 256 or 226 units/mg. sarcosomal protein respectively. 30 min., 25°. Method I.

	Sarcosomes suspended in	
	Isolation medium	Water
<i>α-Ketoglutarate $\rightarrow$ ferricytochrome <i>c</i></i>		
Rate of reduction*	0.046	0.130
Ferricytochrome <i>c</i> (μ atoms O equiv.)	0.314	0.314
$-\Delta\alpha$ Kg (μ moles)	0.33	0.31
Δ esterified P (μ atoms)	0.57	0.56
P:O	1.82	1.77
P: α Kg	1.72	1.81
<i>α-Ketoglutarate $\rightarrow$ O₂</i>		
QO ₂ (μ l. O ₂ /hr./mg. sarcosomal protein)	86	97
$-\Delta$ O (μ atoms)	1.36	1.73
Δ esterified P (μ atoms)	3.81	4.85
P:O	2.80	2.80
<i>Ascorbate $\rightarrow$ O₂</i>		
Δ O† (μ atoms)	6.0	9.9
Δ esterified P (μ moles)	0.67	3.81
P:O	0.112	0.376

* Initial decrease of optical density at 580 m μ /min./mg. sarcosomal protein.

† Corrected for O₂ uptake in absence of sarcosomes.

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Table 10. *Effect of 2:4-dinitrophenol on oxidative phosphorylation with α -ketoglutarate as hydrogen donor and either ferricytochrome c or oxygen as hydrogen acceptor*

Rat-heart sarcosomes (isolated in 0.28 M sucrose, 0.01 M ethylenediaminetetraacetate, pH 7.4), 0.54 mg. protein/ml.; phosphate, pH 7.4, 0.03 M; malonate, 0.01 M; fluoride, 0.04 M; AMP, 2×10^{-4} M; ADP, 2×10^{-4} M; α Kg, 3×10^{-4} M; glucose, 0.02 M; ethylenediaminetetraacetate, 0.001 M; Mg, 0.005 M; hexokinase, 148 units/mg. sarcosomal protein. Dinitrophenol, 10^{-4} M, where indicated. Expts. with ferricytochrome c as hydrogen acceptor carried out in spectrophotometric Thunberg tubes.

	Oxygen as H-acceptor		Ferricytochrome c as H-acceptor	
	Without DNP	With DNP	Without DNP	With DNP
- Δ O	2.15	?	0.464	0.464
α Kg: initial	4.81	4.81	0.94	0.94
final	2.81	3.13	0.50	0.49
Δ	-2.00	-1.68	-0.44	-0.45
$\sim$ P: initial	0.19	0.19	0.58	0.58
final	0.20	0.15	0.44	0.43
Δ	0.01	-0.04	-0.14	-0.15
HMP: initial	0.01	0.01	0.02	0.02
final	5.84	1.13	0.92	0.45
Δ	5.83	1.12	0.90	0.43
Δ HMP + $\Delta \sim$ P	5.84	1.08	0.76	0.28
P:O	2.72	—	1.63	0.60
P: α Kg	2.92	0.64	1.72	0.62

Table 11. *Comparison of phosphorylation ratios with α -ketoglutarate as hydrogen donor and either oxygen or ferricytochrome c as hydrogen acceptor*

Phosphate, 0.03 M; malonate, 0.01 M; fluoride, 0.04 M; Mg, 0.005 M; AMP, 2×10^{-4} M (except Expt. 1, 4.8×10^{-4} M and one tube of Expt. 8, 10^{-3} M); ADP, same as AMP; α Kg, 3×10^{-4} – 10^{-3} M; glucose, 0.01–0.03 M; ethylenediaminetetraacetate, 0.001–0.0017 M (except Expt. 1, zero); sarcosomes, 0.39–0.84 mg. protein/ml. (except Expt. 1, 1.3 mg./ml.); hexokinase, 127–181 units/mg. sarcosomal protein. 20–25°. Sarcosomes isolated from rat heart in 0.21–0.25 M sucrose, 0.01 M ethylenediaminetetraacetate, pH 7.4 except in Expt. 1 when pig heart and phosphate-saline were used. Apparatus A, side-arm Thunberg; B, spectrophotometric Thunberg.

Expt.	O ₂ as H-acceptor P:O	Apparatus	Time (min.)	Ferricytochrome c as H-acceptor				
				$-\Delta$ O*	$-\Delta\alpha$ Kg (μ mole)	Δ esterified P (μ mole)	P:O	P: α Kg
1	2.33†	A	4	0.300	0.27	0.54	1.75	1.94
2‡	2.60	A	12	0.456	0.44	0.88	1.94	2.00
3	2.63	B	11	0.505	0.46	0.86	1.69	1.87
4	2.71	B	13	0.464	0.44	0.76	1.63	1.72
5	2.71	B	14	0.469	0.45	0.78	1.67	1.74
6	2.64	B	14	0.489	0.46	0.73	1.50	1.59
7	2.70	B	13	0.464	0.42	0.79	1.70	1.89
8‡	2.80	B	12–16	0.314	0.32	0.56	1.79	1.77
9	2.80	B	18	0.314	0.33	0.57	1.82	1.72
Means							1.72	1.80

* Oxygen equivalent of ferricytochrome c.

† Low value may be due to absence of ethylenediaminetetraacetate from reaction mixture.

‡ Mean values of two separate tubes.

such a way that the P:O ratios were low in the aerobic experiments, namely 1.77 and 1.50. The mean P:O ratios in the anaerobic experiments were 0.99 and 1.18 respectively.

Normal preparations, which in our hands give a phosphorylation ratio close to 3 in aerobic experiments (Slater & Holton, 1954), give a value just under 2 with ferricytochrome c as hydrogen acceptor.

DISCUSSION

It would be expected that some at least of the phosphorylation reactions in the respiratory chain would occur between substrate and ferricytochrome c. However, the only previous attempt to demonstrate this by direct experiment was unsuccessful (Friedkin & Lehninger, 1949; Lehninger, 1949). It is of some interest, therefore, that this phosphoryla-

tion has now been directly demonstrated. Moreover, the fact that the P:αKg ratio is much greater than unity and is sensitive to dinitrophenol shows that we are dealing not only with substrate-linked phosphorylation, but also with phosphorylation in the respiratory chain.

The demonstration of this phosphorylation was made possible by the development of a highly sensitive method of measuring oxidative phosphorylation (Slater, 1953*a*). This enabled the use of much smaller amounts of cytochrome *c* than Lehninger found necessary. This was important for two reasons: (i) even a single experiment with cytochrome *c* as hydrogen acceptor consumes large amounts of cytochrome *c* (0.5 μatom oxygen equivalent of cytochrome *c* represents 12.5 mg. protein, containing 0.45 % iron); (ii) the cytochrome *c* preparation might contain a small amount of an inhibitor. As already shown (Slater, 1950), a large amount of some preparations of cytochrome *c* (1.5×10^{-4} M) made by the method of Keilin & Hartree (1945) does inhibit the phosphorylation slightly. However, even larger concentrations (6×10^{-4} M) of highly purified cytochrome *c* have no effect (Slater & Cleland, 1953).

Certain criticisms of the method employed to demonstrate phosphorylation as reported in the preliminary communication (Slater, 1950), which have been made by Lehninger (1951*b*) and by Lindberg & Ernster (1954) must be discussed. Lehninger (1951*b*) comments that 'the analytical methods employed...measure very small exchanges which approach, on a molar basis, the molar quantities of some of the electron transport factors involved. It is conceivable that such a complex pattern of exchange is "masked" with this type of measurement.' It is not clear exactly what electron transport factors Lehninger has in mind. Presumably, it is not cytochrome *c*, since in these experiments ferricytochrome *c* is used stoichiometrically as one of the reactants, and acts as the ultimate acceptor of electrons, not an electron transport factor. Diphosphopyridine nucleotide (DPN), which is possibly an electron transport factor in the α-ketoglutaric oxidase system, is probably the respiratory catalyst in the highest concentration in the sarcosomes. Holton (1954*b*) has found that sarcosomes contain 0.006 μmole DPN/mg. sarcosomal protein, i.e. there was probably about 0.02 μmole DPN in the sarcosomes used in the experiment shown in Table 4. The reduction of this amount of DPN might be expected, at the most, to esterify 0.02 μatom phosphorus, which is only about 4 % of the phosphorus esterified by the reduction of the ferricytochrome *c*. In any case, it would be allowed for in the controls which included all the components of the reaction mixture, except the cytochrome *c*.

Lindberg & Ernster (1954), as well as repeating Lehninger's (1951*b*) criticism, comment:

Slater, in his studies on the oxidation of α-ketoglutarate, employed considerable quantities of cytochrome *c* as electron acceptor, inhibiting the oxidation of cytochrome with cyanide, or with absence of oxygen. In these experiments a P:O ratio of between 0.98 and 2.20 was found. In the oxidation of α-ketoglutarate with oxygen in the same system the value varied between 1.67 and 2.79. The similarity between the two groups of values can scarcely be taken as conclusive evidence that no phosphorylation takes place between cytochrome *c* and oxygen... It may be added here that the account of the experiments gives no direct evidence to exclude the possibility that a myokinase effect contributed to the formation of glucose 6-phosphate from the added ADP. Finally, it may be remarked that, as Hunter (1951) has suggested, cytochrome *c* may behave differently when it is introduced into the system and when it functions within the mitochondria.

The comparison between P:O ratios with oxygen and ferricytochrome *c* as hydrogen acceptors is useful only when the experiments are carried out under the same conditions. Reasons were given in the preliminary communication why it was considered that the valid comparison was between 2.5 with oxygen and 2.2 (which might be underestimated) with ferricytochrome *c*. The P:O ratio of 0.98 with ferricytochrome *c* was obtained in the presence of 0.01 M-HCN which inhibited the phosphorylation considerably. The comparison between values of 1.67–2.79 with oxygen and 0.98–2.20 with ferricytochrome *c* made by Lindberg & Ernster has no meaning and was not made in our previous paper.

Contrary to Lindberg & Ernster's statement, the published account of the preliminary experiments specifically states that the glucose 6-phosphate found was corrected for that attributable to the myokinase reaction. This has been previously discussed in detail (Slater, 1953*b*) and also in the present paper.

That added cytochrome *c* might behave differently from the endogenous cytochrome *c* of the particles has in fact been repeatedly stated in papers from this laboratory. Its relevance to the particular problem under investigation is difficult to test experimentally at the moment, but it would be somewhat surprising if there were more phosphorylation coupled with the reduction of added ferricytochrome *c* than of the endogenous cytochrome.

Although it has now been confirmed that some of the respiratory chain phosphorylation occurs before the reduction of ferricytochrome *c*, the conclusion that all the phosphorylation occurs in this part of the respiratory chain requires re-examination. The phosphorylation ratios found with ferricytochrome *c* as hydrogen acceptor in Table 11 are just under 2, compared with just over 2 in the previous paper.

Since the values in Table 11 were obtained under more strictly controlled conditions, they are to be preferred. The P:O ratio of 2.16 previously obtained in the anaerobic experiment may be slightly high because of residual oxygen, or because of a small analytical error. The two possible sources of error which could overestimate the yield of phosphorylation, namely residual oxygen or phosphorylation coupled with the dismutation reaction, have been eliminated in the present experiments.

Since the P:O ratio with oxygen is close to 3 and that with ferricytochrome *c* is just under 2, it is clear that the conclusion that the phosphorylation ratio with added cytochrome *c* is as high as with oxygen as hydrogen acceptor is not justified. However, the results now presented do not, of themselves, permit the conclusion that some of the phosphorylation in the respiratory chain occurs between cytochrome *c* and oxygen. Two explanations of the experiments are possible.

(1) Two steps in the respiratory chain between α -ketoglutarate and cytochrome *c* are associated with phosphorylation. One of these is presumably the substrate-linked phosphorylation, the other is some unidentified reaction, probably between DPNH and ferricytochrome *c*. (If, as many believe, the P:O ratio for the aerobic oxidation of α -ketoglutarate is 4, more than one reaction between DPNH and ferricytochrome *c* may be associated with phosphorylation.) There is an additional phosphorylation in the respiratory chain between cytochrome *c* and oxygen. This conclusion would agree with Nielsen & Lehninger's (1954) recent direct demonstration of phosphorylation with ferrocytochrome *c* as hydrogen donor.

(2) In the intact sarcosome, all the phosphorylation occurs between α -ketoglutarate and cytochrome *c* but when added cytochrome *c* is the hydrogen acceptor one of these phosphorylating steps is by-passed, thus

where the arrows show the direction of hydrogen (or electron) transfer and X is the immediate donor to the endogenous cytochrome *c*. It is possible that the reaction between X and endogenous cytochrome *c* is coupled with phosphorylation and that this reaction cannot be obtained with the added cytochrome *c*, perhaps because the cytochrome *c* becomes reduced during the course of the experiment. When oxygen is the hydrogen acceptor, the cytochrome *c* remains largely oxidized in the aerobic steady state.

While the first explanation is the easiest to reconcile with other work, the second should not be excluded, at least not until more is known about the mechanism of the phosphorylation obtained with ascorbic acid or ferrocytochrome *c*.

SUMMARY

1. Oxidative phosphorylation has been demonstrated with α -ketoglutarate as hydrogen donor and ferricytochrome *c* as acceptor. Since the ratio, phosphorus esterified:oxygen equivalent of ferricytochrome *c* reduced, was close to 2 and was sensitive to 2:4-dinitrophenol, respiratory chain phosphorylation must be involved.

2. These experiments were carried out under completely anaerobic conditions in order to prevent reoxidation of ferrocytochrome *c*. A sensitive enzymic method of detecting residual oxygen is described.

3. Respiratory inhibitors could not be used satisfactorily. Cyanide inhibits phosphorylation and combines with α -ketoglutarate; azide also inhibits phosphorylation and is a relatively ineffective respiratory inhibitor with intact sarcosomes. Sulphide rapidly reduces ferricytochrome *c*.

4. The combination between α -ketoglutarate and cyanide was shown to be a slow reaction.

5. The yield of oxidative phosphorylation with ferricytochrome *c* as acceptor was compared with that obtained with oxygen. The phosphorus:oxygen ratios with ferricytochrome *c* varied between 1.5 and 1.9 (mean 1.72) in nine experiments (phosphorus: α -ketoglutarate ratios, 1.6–2.0, mean 1.80), compared with just under three obtained with oxygen.

6. Two possible explanations of the results are suggested.

(i) Respiratory chain phosphorylation occurs between ferrocytochrome *c* and oxygen, as well as between substrate and ferricytochrome *c*.

(ii) All the phosphorylation occurs between substrate and the endogenous cytochrome *c* of the sarcosome, but the reaction with added ferricytochrome *c* by-passes a phosphorylating reaction.

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The Fractionation of Rabbit γ -Globulin by Partition Chromatography

By R. R. PORTER

National Institute for Medical Research, Mill Hill, London, N.W. 7

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Normal and immune rabbit γ -globulins have been examined by many techniques in attempts to relate the specific antigen-binding power of antibodies with some chemical or physical property, but no success has so far been achieved (see Porter, 1953). Thus, in the rabbit all antibodies are confined to the γ -globulin fraction and there is no difference in the electrophoretic behaviour of normal and immune γ -globulin (Tiselius & Kabat, 1939). In the ultracentrifuge they are indistinguishable, their molecular weight being about 170 000 (Kabat, 1939). Recent accurate amino acid analyses are now available for rabbit normal γ -globulin and antibodies to pneumococcal polysaccharide and they show no significant differences (Smith, McFadden, Stockell & Buettner-Janusch, 1955). The *N*-terminal pentapeptide sequence of rabbit antiovalbumin is the same as that of the inert γ -globulin with which it is associated (Porter, 1950), and more recently it has been found (McFadden & Smith, 1955) that antipneumococcal antibodies also have the same terminal pentapeptide sequence. It is therefore probable that, in the rabbit, the differences between antibody and inert γ -globulin, which are responsible for their specific binding power, are slight. It follows that any method not making use of the immunochemical specificity to separate anti-

body from inert γ -globulin would require a high resolving power. If successful, it might in turn give some indication as to the nature of the differences which must exist.

As the technique of protein chromatography with its different and perhaps higher resolving power became available it was of interest to see whether γ -globulin could be fractionated by this method and, if so, whether antibodies could be separated from inert protein.

In fact, using rabbit antiovalbumin, wholly inactive γ -globulin could be separated from the mixture but pure antibody (completely precipitable by antigen) could not be obtained. It was, however, found that the chromatographic pattern of the γ -globulin changed at different stages of immunization and, while one type of antibody was present in the earlier stages, this decreased and was replaced by a chromatographically distinct second type in the later stages.

EXPERIMENTAL

Materials

Celite 545 (a large-particle kieselguhr) was purchased from Johns-Manville Co. Ltd., Artillery Row, London. This was cleaned before use by stirring with 2*N*-HCl for 24 hr. It was