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A Method of Measuring the Yield of Oxidative Phosphorylation

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Oxidative phosphorylation may be defined as the phosphorylation of adenosinediphosphate (ADP) to adenosinetriphosphate (ATP) which accompanies the oxidation of a number of intermediary metabolites. It is now clear (Slater, 1950; Barkulis & Lehninger, 1951; Kielley & Kielley, 1951; Lindberg & Ernster, 1952; Slater & Holton, 1953) that ADP is phosphorylated much more rapidly than adenosinemonophosphate (AMP).

The overall oxidative phosphorylation reaction may be written

where SH_2 is the oxidizable substrate and S the product of the oxidation. The yield of oxidative phosphorylation is expressed by the value of x , the number of atoms of inorganic phosphorus esterified per atom of oxygen consumed, usually called the P:O ratio.

Because ATP is relatively unstable in the preparations used for these studies, many investigators

add hexokinase and glucose, so that the labile terminal phosphate group is transferred to glucose to form hexosemonophosphate (HMP) by reaction (2)

$$ATP + \text{glucose} \rightarrow ADP + \text{HMP}. \quad (2)$$

The overall reaction is now reaction (3), the sum of reactions (1) and (2)

This method has the further advantage that ADP acts as a catalyst and its concentration remains relatively constant during the measurement.

In all previous studies, the yield of oxidative phosphorylation has been measured by determining the disappearance of inorganic phosphate. While this method has the advantage of simplicity and is sufficiently precise and sensitive for most investigations, it is unsuitable for certain purposes. For example, the kinetics of the early stages of the reaction cannot be studied because the percentage disappearance of inorganic phosphate is too low

for accurate measurement. A further disadvantage is that the concentration of inorganic phosphate, one of the reactants, is largely determined by analytical considerations and decreases to a considerable degree during the course of the experiment. The methods described in this paper were developed in order to measure the phosphorylation when cytochrome *c* was used instead of oxygen as hydrogen acceptor (Slater, 1950). With reasonable amounts of cytochrome *c*, the maximum fall of inorganic phosphate concentration which could be expected was only about 10^{-4} M. For this to be measured with any degree of accuracy, an initial phosphate concentration of no more than 5×10^{-4} M would have to be used. However, the oxidation of α -ketoglutarate proceeds very slowly at this concentration of inorganic phosphate (Ochoa, 1944).

These difficulties disappear if the amount of phosphorylated product formed, either HMP if hexokinase and glucose are added, or ATP if they are omitted, is determined. Sensitive and precise methods for these measurements have been described in an earlier paper (Slater, 1953). These methods depend upon the enzymic conversion of HMP, in the presence of ATP or ADP, to dihydroxyacetonephosphate, which then reacts with reduced diphosphopyridine nucleotide (DPNH) in the presence of glycerolphosphate dehydrogenase. The amount of DPNH reacting is determined spectrophotometrically. The present paper will deal with the application of these methods to the problem of measuring the yield of phosphorylation, when hexokinase and glucose are used.

The measurement of the oxygen consumed by the oxidation of SH_2 is not always easy, since SH_2 or *S* may undergo further reactions, which may or may not consume oxygen and may or may not be linked with the phosphorylation of ADP. The method to be used depends upon the substrate and will not be discussed in this paper.

Abbreviations

The following abbreviations will be used: ATP, adenosinetriphosphate; ADP, adenosinediphosphate; AMP, adenosine-5'-phosphate or muscle adenylic acid; HMP, hexosemonophosphate (glucose-6-phosphate, fructose-6-phosphate, glucose-1-phosphate); HDP, hexosediphosphate (fructose-1:6-diphosphate); $\sim$ P, energy-rich reactive phosphate groups.

EXPERIMENTAL

Preparations

Phosphorylating preparation. The preparation used consisted of heart-muscle sarcosomes, i.e. the granules in heart muscle which correspond to mitochondria in liver and kidney. The method of preparation of these granules will be

described in detail in a forthcoming paper (Cleland & Slater, 1953). Briefly, it consists of grinding minced heart muscle with sand and approx. 2 vol. of isotonic saline or sucrose, followed by differential centrifugation to separate the sarcosomes from other constituents of the muscle. Versene (ethylenediaminetetraacetic acid) (0.005–0.01 M) was sometimes added to the solution used for isolating the sarcosomes (see Slater & Cleland, 1952). The sarcosome concentrations are expressed as mg. protein/ml.

Hexokinase. The yeast hexokinase preparations described in a previous paper (Slater, 1953) were used. Activities were determined by the method of Racker (1947) or of Berger, Slein, Colowick & Cori (1946) and are expressed in terms of the unit defined by Berger *et al.* (1946).

Adenylic acid. Two commercial preparations were used. One contained a small amount of a substance which reacted like HDP in the enzymic method. The other was free from this material. Concentrations of neutralized solutions were determined by measurement of the optical density at 260 $\text{m}\mu$, using Kalckar's (1947) value for the molar extinction coefficient.

Other preparations used were as described by Slater (1953).

Methods

Measurement of phosphorylation. Unless otherwise stated, the reaction mixture (final concn. in brackets) contained Sørensen phosphate buffer, pH 7.4 (0.03–0.05 M), sodium malonate (0.01 M), NaF (0.04 M), glucose (0.01–0.025 M), potassium ADP (4.4×10^{-4} – 6.7×10^{-4} M), potassium AMP (4.7×10^{-4} – 6.0×10^{-4} M), MgCl_2 (0.005 M), potassium α -ketoglutarate (0.003–0.01 M), cytochrome *c* (3.5 – 5.5×10^{-5} M) and hexokinase. Other additions are shown in the tables or figures. The medium was made approximately isotonic by the addition of KCl, and the final volume was either 3 or 1 ml. MgCl_2 was added immediately after the hexokinase and just before the addition of the heart-muscle sarcosomes (at zero time) in order to prevent precipitation of magnesium fluorophosphate (cf. Potter, 1947). The reaction was terminated by the addition of 1/6 vol. 40% (w/v) trichloroacetic acid (TCA). When 1 ml. reaction volume was used, 2 ml. 5% TCA were then added. The precipitated protein was removed by centrifugation and 2 ml. of the supernatant neutralized with *N*-KOH. A drop of CHCl_3 was added and the solution kept at 4° until analysed for phosphorylated sugars or $\sim$ P by the methods described previously (Slater, 1953). A zero-time control was obtained by adding TCA before the hexokinase, MgCl_2 and heart-muscle sarcosomes.

In the following tables and figures, Δ HMP refers to the increase of HMP over the small zero-time control (due to traces of HMP in the ADP preparation or to the 'blank' in the AMP) and $-\Delta \sim$ P refers to the decrease of $\sim$ P. The initial $\sim$ P is contributed almost entirely by the ADP (see p. 523).

Myokinase activity. This was measured with the same reaction mixture, except that 0.01 M-KCN was added and the α -ketoglutarate and cytochrome *c* omitted. After acidification with TCA, the solution was briefly aerated in order to remove HCN.

Adenosinetriphosphatase. This was measured with the following reaction medium: phosphate, pH 7.4 (0.034 M), MgCl_2 (0.005 M), ATP (0.6×10^{-3} – 1.3×10^{-3} M), KCN (0.01 M) and KCl (to make solution isotonic) (0.09 M). The reaction was started by the simultaneous addition of the

heart-muscle sarcosomes and MgCl_2 , and stopped with TCA as above. The ATP-ase activity is given by the difference between the $\sim\text{P}$ found in a zero-time control and in the test. Laboratory-prepared ATP (see Slater (1953) for analyses) was used.

Unless otherwise stated, all incubations were carried out at 25° .

EXAMINATION OF METHOD

Analytical

Glucose-6-phosphate is the primary product of the hexokinase reaction, but the presence of hexosephosphate isomerase in the hexokinase preparation means that a mixture of glucose-6-phosphate and fructose-6-phosphate is formed. The analytical method previously described (Slater, 1953) measures the sum of these two hexosemonophosphates (as well as any glucose-1-phosphate which may be present). It measures $0.05\text{ }\mu\text{mole}$ HMP with an accuracy of a few per cent and can determine as little as $0.01\text{ }\mu\text{mole}$ with reasonable accuracy. The concentration of adenosine-reactive phosphate ($\sim\text{P}$), i.e. the terminal phosphate group of ADP + the two terminal groups of any ATP present, can be estimated by a variation of the same method. If pyruvate or oxaloacetate are present in the final reaction mixture, they must be determined separately since a molecule of these compounds analyses as half a molecule of HMP or $\sim\text{P}$. Only insignificant traces of pyruvate or oxaloacetate have been found when α -ketoglutarate, succinate or citrate was used as substrate. Considerable amounts were found with L-malate as substrate. The heart-muscle sarcosomes from cat, rabbit or rat contained insignificant amounts of substances reacting as HMP (less than $0.01\text{ }\mu\text{mole/mg.}$ heart-muscle protein). Freshly prepared sarcosomes contained some $\sim\text{P}$, but this was only a small proportion of that added as ADP.

Table 1 shows that HMP and ADP added to zero-time controls were recovered quantitatively.

Table 1. *Recovery of HMP and ADP added to zero-time controls*

(No allowance was made for small amount of $\sim\text{P}$ contributed by sarcosomes.)

	HMP or $\sim\text{P}$		
	Added (μmoles)	Found (μmoles)	Recovery (%)
HMP	0.850	0.855	101
ADP, $\sim\text{P}$	1.37	1.33	97
	2.72	2.70	99
	1.36	1.42	105
	2.15	2.15	100
	0.564	0.575	102

Side reactions

The measurement of the yield of oxidative phosphorylation is complicated by a number of side reactions, catalysed by enzymes present in the heart-muscle or hexokinase preparations. Possible side reactions are

- $2\text{ ADP} \rightleftharpoons \text{ATP} + \text{AMP}$ (myokinase),
- $\text{ATP} + \text{H}_2\text{O} \rightarrow \text{ADP} + \text{H}_3\text{PO}_4$ (ATP-ase),
- hydrolysis of unknown phosphorylated intermediate compounds (e.g. acylphosphates) by phosphatases,

- glucose-6-phosphate $\rightleftharpoons$ fructose-6-phosphate (hexose-phosphate isomerase) followed by
fructose-6-phosphate + ATP $\rightarrow$ HDP + ADP (phospho-hexokinase),
- $\text{HMP} + \text{H}_2\text{O} \rightarrow \text{hexose} + \text{H}_3\text{PO}_4$ (HMP-ase), and
- glycolysis.

Each of these side reactions will now be considered.

(a) *Myokinase*. The equilibrium constant of this reaction is about 2 (Kalckar, 1943). Since the ATP formed either by myokinase or by oxidative phosphorylation will be rapidly removed by the hexokinase reaction, the myokinase reaction will proceed only from left to right under the conditions of the experiment. In the presence of hexokinase and glucose, the net reaction will be

the equilibrium constant of which is about 10^6 . The HMP found in an oxidative phosphorylation experiment must therefore be corrected for that formed by the myokinase reaction. This correction was determined by measuring the formation of HMP in a separate control experiment in which oxidizable substrate was omitted. In order to prevent oxidation of traces of substrate remaining in the heart-muscle preparation, the reaction was carried out either in the presence of KCN (0.01 M) or in the absence of air, and cytochrome *c* was omitted.

It can be seen from equation (4) that the myokinase reaction leads to the loss of ADP. It should be possible, therefore, to determine the myokinase correction directly by measuring the $\sim\text{P}$ content of the solution obtained in the oxidative phosphorylation experiment. However, it was found in the myokinase control test (e.g. Exp. 2, Table 3) that the loss of $\sim\text{P}$ often exceeded the formation of HMP. Since the addition of further amounts of hexokinase did not increase the amount of HMP found (see p. 526), this cannot be due to hydrolysis of ATP by ATP-ase, but must be caused by loss of ADP by some other reaction. The enzymes responsible for the loss of ADP, other than myokinase + ATP-ase, will be referred to loosely as 'ADP-ase'. The 'ADP-ase' is an impurity in the crude hexokinase preparations. There is no 'ADP-ase' activity when the hexokinase is omitted (see p. 525) and very little with purified hexokinase in the presence of fluoride (e.g. Exp. 1, Table 3).

It is desirable to keep the myokinase correction as low as possible, not only because it is a blank correction, but also in order to maintain the concentration of ADP, one of the components of the oxidative phosphorylation reaction. The washed heart-muscle sarcosomes used in the present study contained only 1% of the myokinase present in the muscle (Cleland & Slater, 1953). In most experiments, this residual myokinase activity was greatly reduced by adding two inhibitors, AMP and fluoride.

Colowick & Kalckar (1943) found that reaction (4) proceeded rapidly at first, but soon slowed down, due to inhibition of the enzyme by one of the products of the reaction, AMP. This finding of Colowick & Kalckar is confirmed by the experiment summarized in Fig. 1, which shows that the rate was already markedly decreased when less than 10% of the ADP had reacted. Table 2 shows that if AMP, approximately equal in concentration to that of the ADP, is added from the beginning, the myokinase activity is greatly inhibited.

AMP can be used as an inhibitor only if it is not itself phosphorylated at an appreciable rate. This has been found

to be the case with both α -ketoglutarate and succinate as the oxidizable substrate. The experimental evidence will be presented in later papers dealing with particular substrates (Slater & Holton, 1953).

Kotelnikova (1950) and Barkulis & Lehninger (1951) have recently shown that myokinase is strongly inhibited by fluoride. Barkulis & Lehninger (1951) were unable to measure the absolute amount of inhibition by fluoride, owing to interference by phosphatases, but showed that the activity in 0.03 M-fluoride was about half that in 0.01 M-fluoride. The inhibition is shown in more detail in Table 3. In Exp. 1, purified hexokinase containing little 'ADP-ase'

Fig. 1. Phosphorylation of glucose by ADP, catalysed by myokinase (contained in sarcosomes) and yeast hexokinase, showing the rapid fall of the rate of the reaction. The reaction mixture contained 3 μ moles ADP, 25 μ moles glucose, 0.04 M-NaF, 0.035 M-phosphate (pH 7.3), 0.005 M-MgCl₂, 0.65 mg. pig-heart sarcosomes/ml. and 76 units hexokinase/mg. sarcosomes. Temp. 24°.

Table 2. *Inhibition of myokinase by adenylic acid*

(Concentrations as in Fig. 1, except pig-heart sarcosomes (0.59 mg./ml.), hexokinase (50 units/mg. sarcosomes) and glucose (50 μ moles). 6 min. at 24°.)

AMP initially added (μ moles)	Δ HMP (μ moles)
0	0.52
2.7	0.10

was used; it can be seen from the $-\Delta \sim P$ values that the addition of increasing amounts of fluoride progressively inhibited the myokinase activity, the inhibition amounting to 53 % with 0.04 M-fluoride. The HMP formed agrees closely with the loss of $\sim P$, except in the absence of fluoride when Δ HMP is less than $-\Delta \sim P$ and is, in fact, less than in the presence of 0.01 M-fluoride. This is probably due to very slight HMP-ase activity in the rat-heart preparation used in this experiment. The discrepancy between the $-\Delta \sim P$ and Δ HMP in Exp. 2 with cat heart, even in the presence of 0.04 M-fluoride, is due to the much greater 'ADP-ase' activity of the hexokinase used in this experiment; cat-heart preparations contain no measurable HMP-ase activity.

The degree of inhibition of myokinase was similar to that obtained by Kotelnikova (1950), who found 43–48 % inhibition by 0.02 M-fluoride. Also, in agreement with Kotelnikova, Exp. 1 of Table 3 shows that the inhibition of myokinase by fluoride is independent of the phosphate concentration. In this respect, the inhibition of myokinase by fluoride differs from that of enolase (Warburg & Christian, 1942) and succinic dehydrogenase (Bonner, 1951).

Table 3. *Inhibition of myokinase by fluoride*

(Exp. 1. Rat-heart sarcosomes, 0.69 mg./ml.; hexokinase (specific activity 1110), 165 units/mg. sarcosomes; 0.0035 M-Versene; reaction vol., 1 ml.; 45 min. Exp. 2. Cat-heart sarcosomes, 0.42 mg./ml.; hexokinase (specific activity 66), 39 units/mg. sarcosomes; reaction vol., 3 ml.; 30 min. Exp. 3. Rat-heart sarcosomes, 0.90 mg./ml.; hexokinase (specific activity 900), 77 units/mg. sarcosomes; reaction vol., 1 ml.; 45 min.)

Exp. no.	[NaF] (M)	[Phosphate] (M)	Δ HMP (μ moles)	$-\Delta \sim P$ (μ moles)
1	0	0.015	0.23	0.51
		0.036	0.30	0.45
	0.01	0.015	0.35	0.39
		0.036	0.32	0.39
	0.02	0.015	0.31	0.34
		0.036	0.34	0.33
	0.04	0.015	0.17	0.24
		0.036	0.18	0.21
2	0	0.035	0.22	0.63
	0.04	0.035	0.05	0.18
3	0	0.030	0.40	—
	0.04	0.030	0.19	—

Table 4. *Magnitude of myokinase correction*

(The isolation media had the following compositions: phosphate-saline (0.051 M-KCl, 0.043 M-phosphate, pH 7.1–7.3); saline (0.135 M-KCl, 0.02 M-phosphate, pH 7.4); saline-Versene (0.115 M-KCl, 0.02 M-phosphate, 0.01 M-Versene, pH 7.4); isotonic sucrose-Versene (0.30 M-sucrose, 0.005 M-Versene, pH 7.4). Values after $\pm$ signs are standard errors of means.)

Animal	Isolation medium	No. of preparations	Myokinase correction (μ moles HMP/mg. sarcosomes/hr.)
Rabbit	Phosphate-saline	3	0.07
Cat	Phosphate-saline	5	0.13 $\pm$ 0.04
Rat	Saline	4	0.30 $\pm$ 0.03
	Saline-Versene	9	0.30 $\pm$ 0.02
	Isotonic sucrose-Versene	2	0.25 $\pm$ 0.04
Pig	Phosphate-saline	6	0.44 $\pm$ 0.05

Table 5. *Effect of KCN on myokinase correction*

(The specific activities of the hexokinase used in different experiments were as follows: Exp. 1, unknown; Exp. 2, 43; Exp. 3, 43; Exp. 4, 37; Exp. 5, 625.)

Exp. no.	Animal	KCN present		Anaerobic, no KCN		Aerobic, no KCN	
		Δ HMP (μ moles)	$-\Delta \sim P$ (μ moles)	Δ HMP (μ moles)	$-\Delta \sim P$ (μ moles)	Δ HMP (μ moles)	$-\Delta \sim P$ (μ moles)
1	Rabbit	0.07	0.17	—	0.19	0.22	0.18
2	Pig	0.74	0.86	0.84	0.94	—	—
3	Pig	0.25	0.42	0.30	0.55	—	—
4	Cat	0.09	0.35	0.11	—	—	—
5	Cat	0.19	0.35	—	—	0.19	0.28

When both AMP and fluoride were added, the myokinase correction was usually only a few per cent of the oxidative phosphorylation. There was, however, a marked species difference, rabbit- and cat-heart preparations having much less myokinase than those from rat or pig. The mean values of the myokinase correction for different preparations is given in Table 4. The isolation medium used for the preparation of the sarcosomes had little effect on the value. Since the myokinase activity of the washed sarcosomes is only about 1% of that of the total activity of the homogenate, the correction would be much greater if the whole homogenate were used.

The most convenient method of preventing oxidative phosphorylation is to omit substrate and add cyanide to prevent oxidation of residual substrates. This method is valid for measuring the myokinase correction only if cyanide has no effect on the myokinase activity. This has been shown to be the case in two ways (Table 5): (i) the value of the myokinase correction obtained with KCN is the same as that found when oxidation was prevented by removing air (using the modified Thunberg tube with side arm described by Keilin & Hartree, 1947); (ii) the loss of $\sim P$ in the usual test is the same as that obtained when KCN was omitted. The excess of the HMP found in the absence of cyanide compared with that in its presence (Exp. 1, Table 5) is a measure of the residual substrates in the preparation. For certain purposes, this is the control to use since it corrects for both myokinase and residual substrates.

Table 6 shows that the myokinase correction is relatively independent of the concentration of ADP over the range 0.0001–0.001M, showing that ADP has a high affinity for myokinase. The 'ADP-ase' ($-\Delta \sim P - \Delta$ HMP), however, increased with increasing concentration of ADP.

When the myokinase correction was fairly small, it was proportional to the concentration of sarcosomes (Table 7).

The myokinase activity was slightly stimulated by malonate (18% by 0.01M; 29% by 0.04M-malonate) and by Versene (25% by 0.002M) and was inhibited by pyrophos-

phate (38% by 0.01M). It was not affected by 0.47M-sucrose or by 0.25M-KCl; 0.88M-sucrose inhibited by 42%.

(b) *ATP-ase*. This enzyme will compete with the hexokinase for the ATP formed by the oxidative phosphorylation reaction, and thereby cause the yield to be underestimated. This error can be completely prevented by adding sufficient yeast hexokinase, which is a very active enzyme. The activity of ATP-ase was measured by determining the loss of $\sim P$ on the addition of purified ATP to the enzyme preparation. The activity measured will include 'ADP-ase' as well as ATP-ase, but not myokinase. However, the 'ADP-ase' activity of sarcosome preparations is insignificant. This was shown by omitting hexokinase and glucose from the myokinase control. Under these conditions myokinase and ATP-ase will cause loss of $\sim P$ according to the reactions

Since the ATP-ase activity is much greater than that of myokinase, the rate of the overall reaction, measured by disappearance of $\sim P$, is equal to the rate of the myokinase reaction. This is measured independently by reaction (4) (see p. 523). The fact that the two methods agree (Table 8) shows that there are no side reactions causing loss of $\sim P$

Table 7. *Effect of concentration of sarcosomes on myokinase correction*

Exp. no.	Sarcosomes (mg.)	Δ HMP	
		(μ moles)	(μ moles/mg. sarcosomes)
1	0.66	0.023	0.035
	3.3	0.112	0.034
2	1.26	0.052	0.041
	6.3	0.254	0.040

Table 6. *Effect of ADP concentration on myokinase correction*

(The concentration of AMP was 92% that of ADP in all cases.)

[ADP] (M)	Δ HMP (μ moles)	$-\Delta \sim P$ (μ moles)	$-\Delta \sim P - \Delta$ HMP (μ moles)
0.92×10^{-4}	0.056	0.13	0.07
4.6×10^{-4}	0.052	0.18	0.13
9.2×10^{-4}	0.077	0.28	0.20

Table 8. *Absence of 'ADP-ase' in sarcosomes*

(In A, conditions were those of measuring myokinase activity (see Methods); in B, glucose and hexokinase were omitted.)

Exp. no.	A		B
	Δ HMP (μ moles)	$-\Delta \sim P$ (μ moles)	$-\Delta \sim P$ (μ moles)
1	0.11	0.35	0.10
2	0.17	—	0.13

Table 9. *ATP-ase activity of sarcosome preparations*(See Table 4 for composition of isolation media. 10 min. reaction time. $Q_P = \mu\text{l. P/mg. protein/hr.}$)

Exp. no.	Animal	Isolation medium	Sarcosomes (mg.)	$\sim P$ (μmoles)			$\sim P/\text{mg. sarcosomes}$ (μmoles)	Q_P
				Initial	Final	$-\Delta$		
1	Cat	Phosphate-saline	1.55	3.94	2.06	1.88	1.21	162
2	Cat	Phosphate-saline	0.95	8.12	6.65	1.47	1.55	208
3	Cat	Saline	0.91	4.30	3.06	1.24	1.36	182
		Saline-Versene	1.24	4.30	3.74	0.56	0.45	60
4	Rat	Saline	0.56	4.06	2.30	1.76	3.15	423
5	Rat	Saline-Versene	0.68	4.40	3.44	0.96	1.41	190

from ADP, when hexokinase is omitted. As already mentioned, crude hexokinase preparations contain 'ADP-ase', but they are free from myokinase or ATP-ase.

The ATP-ase activities of different preparations are shown in Table 9. The three cat preparations shown in Table 9 gave similar activities, while the two rat preparations were considerably more active. The inclusion of Versene in the isolation medium considerably reduced the activity. This was not due to a direct action of Versene on the ATP-ase, since Versene (0.005M) added to the normal reaction mixture depressed the activity by only 5%. This small inhibition is somewhat surprising, since the amount of Versene added was equivalent to the amount of Mg present and, according to the data of Schwarzenbach & Ackermann (1947), nearly all the Mg would be chelated with the Versene. In another experiment 0.002M-Versene inhibited the ATP-ase by 9%.

It should be noted that no attempt was made to measure the ATP-ase activity under optimal conditions; the conditions of pH, phosphate and Mg concentration were those under which the oxidative phosphorylation experiments were carried out.

Table 10 shows that 0.01M-cyanide, added to prevent the oxidation of residual substrates, did not affect the ATP-ase and that 0.04M-fluoride inhibited by 45–51%.

Table 10. *Effect of cyanide and fluoride on ATP-ase activity of sarcosome preparations*

(10 min. reaction time. The values for $-\Delta \sim P$ in the absence of KCN have been corrected for the small amount of $\sim P$ synthesized by the oxidation of residual substrates. This was determined by the increased HMP obtained in the myokinase control when KCN was omitted—see Table 5. The correction amounted to 0.03 $\mu\text{mole.}$)

[KCN] (M)	[NaF] (M)	$-\Delta \sim P$ (μmoles)
0	0	2.00
0.01	0	1.75
0	0.04	0.97
0.01	0.04	0.96

Without more information, it is not profitable to attempt to calculate the amount of hexokinase which should be added to prevent any losses of ATP. This was approached experimentally by determining the concentration of hexokinase beyond which further addition did not increase the phosphorylation. The phosphorylation obtained with α -ketoglutarate as substrate was corrected for a side reaction stimulated by some substance present in crude hexokinase preparations (Slater & Holton, 1952). In a number of

experiments with cat and pig preparations isolated with isotonic phosphate-saline, optimal phosphorylation was obtained in both the myokinase controls and in oxidative phosphorylation with about 20–40 units of hexokinase/mg. sarcosomes. This matter will be further considered in a later paper (Slater & Holton, 1953).

(c) *Acylphosphatase*, etc. Possible loss due to hydrolysis of unknown phosphorylated intermediate compounds instead of transfer to the adenylic system remains the greatest possible source of error in the measurement of the yield of oxidative phosphorylation. It is likely that fluoride would at least partially inhibit these enzymes.

(d) *Phosphohexokinase*. If phosphohexokinase is present in appreciable amounts, HDP will be formed by reaction sequence (d) (p. 523). This is undesirable for two reasons: (i) Each molecule of HDP is measured in the analytical method as one molecule of HMP although two molecules of ATP are required for its formation; thus if there is any HDP formed the yield of phosphorylation will be underestimated; (ii) HDP is also measured as $\sim P$, so that estimations of this activity will also be in error.

The hexokinase preparations used are completely free from phosphohexokinase activity. The heart-muscle sarcosomes contain a little, but this can be made completely ineffective by adding sufficient hexokinase to compete with the phosphohexokinase for the ATP.

To test whether HDP was formed under the conditions of the oxidative phosphorylation experiments, a moderate concentration of hexokinase (47 units/mg. sarcosomes) was used and the experiment was continued until 95% of the glucose was utilized. Even under these conditions, the amount of HDP found was only 0.3% that of the HMP. It is concluded that errors due to the formation of HDP are negligible and analyses of HDP have not been carried out regularly.

(e) *Hexosemonophosphatase*. Under the conditions of the experiment, cat-heart sarcosomes had negligible HMP-ase activity even in the absence of fluoride. When 0.85 μmole HMP was allowed to react with the sarcosomes for 30 min. at 25° in the presence or absence of fluoride, recoveries of HMP varied between 93 and 107%. In these experiments, oxidation of residual substrates was prevented either by carrying out the experiments anaerobically or in the presence of 0.01M-KCN. Even crude preparations of hexokinase had very slight HMP-ase activity. Pig-heart sarcosomes had some activity and a trace was found in rat-heart sarcosomes (see p. 524).

(f) *Glycolysis*. All the possible side reactions considered above would lead to low estimates of the yield of oxidative phosphorylation. There are two other possible side reactions which, if present, would cause a high estimate, since

they would lead to the formation of HMP without any O_2 uptake. These are (i) glycolysis, and (ii) anaerobic reactions involving the substrate and leading to phosphorylation. The former will be considered in this paper; the latter will be considered in papers dealing with different substrates.

The experiments described above already make it extremely unlikely that glycolysis was occurring, since very little HDP was detected. (The analytical procedure for measuring HDP measures the two triosephosphates as well as HDP. This excludes the possibility that triosephosphate might be formed via 6-phosphogluconic acid instead of HDP.) This was supported by the fact that the P:O ratio was not affected by the addition of 0.001 M-iodoacetate, the well known inhibitor of glycolysis. The possibility of glycolysis was finally excluded by an experiment in which HDP was added to the usual reaction mixture, in the absence of substrates. If glycolysis were taking place, reaction (5) might be expected

The experiment, summarized in Table 11, showed that even in the presence of a large amount of crude hexokinase (a possible source of glycolytic enzymes), the amount of HDP showed no appreciable change and the amount of HMP formed was the same as that obtained in a control in which HDP was omitted (due to action of myokinase + oxidation of residual substrates). Further, the addition of HDP had no appreciable effect on the $\sim P$ content of the solution. The lower $\sim P$ value obtained when crude hexokinase was added is due to 'ADP-ase' in this hexokinase preparation.

Table 11. *Test for glycolysis under conditions of oxidative phosphorylation*

(1.83 mg. cat-heart sarcosomes; 30 min. reaction period. Components as in 'Measurement of phosphorylation' except no α -ketoglutarate. 'Pure' hexokinase had specific activity, 625; crude, 16.)

Hexokinase added:			
Pure (mg. protein)	0.13	0.13	0.13
Crude (mg. protein)	—	—	5.6
HDP added (μ moles)	1.96	0	1.96
HDP found (μ moles)	2.10	0.01	1.86
HMP added (μ moles)	0.08	0.08	0.08
HMP found (μ moles)	0.37	0.33	0.40
$\sim P$ added (μ moles)	2.15	2.15	2.15
$\sim P$ found (μ moles)	1.71	1.82	1.24

Alternatively, since fluoride present in the reaction mixture would inhibit any enolase present, one might expect reaction (6) to occur, if the necessary glycolytic enzymes were present (assuming that the P:O ratio for the oxidation of DPNH is 3, Lehninger & Smith, 1949).

If this reaction occurred, no disappearance of HDP would be detected, since two molecules of phosphoglyceric acid would be analysed as one molecule of HDP by the method used (Slater, 1953). However, the presence of phosphoglyceric acid would increase the $\sim P$ value (Slater, 1953) and would not interfere with the determination of HMP (which is estimated as the difference between two measurements, one giving HDP + HMP and the other HDP). It is clear from

the values of HMP and $\sim P$ in Table 11 that reaction (6) has not taken place and glycolysis can therefore be eliminated as a source of error in the estimation of the yield of oxidative phosphorylation with heart-muscle sarcosomes and yeast hexokinase, under the conditions used in this and related investigations. This conclusion is in agreement with that of Ochoa (1943) who found no glycolysis in heart extracts unless DPN was added, and of Rabinovitz & Boyer (1951) who have shown that there was no uptake of inorganic phosphate when heart preparations and hexokinase were incubated with HDP.

DISCUSSION

The advantages of measuring the yield of oxidative phosphorylation by determination of the amount of phosphorylated product formed, instead of by the disappearance of inorganic phosphate, have been mentioned in the Introduction. The phosphorylated product measured in the present procedure is HMP formed by the hexokinase reaction from ATP, which is the only compound definitely established as a product of the oxidative phosphorylation reaction. The method described is, therefore, relatively direct. It should be noted, however, that if any compound other than ATP is the product of the uptake of inorganic phosphate it will not be measured by the present procedure. If this is the case, the method described in this paper correctly measures oxidative phosphorylation, as defined in the Introduction, whereas the measurement of the uptake of inorganic phosphate will not. However, some may prefer to define oxidative phosphorylation as the uptake of inorganic phosphate, in which case the present procedure would be in error. A comparison of the two procedures is most valuable since it determines whether or not a significant fraction of the inorganic phosphate esterified does not appear as ATP. Inorganic pyrophosphate has been suggested as a product of oxidative phosphorylation (Cori & Ochoa, 1942; Cross, Taggart, Covo & Green, 1949; Cori, Ochoa, Slein & Cori, 1951). Cross *et al.* (1949) found inorganic pyrophosphate only in absence of added hexokinase and glucose, while Lehninger & Smith (1949) obtained none even in the absence of hexokinase, and there was no incorporation of labelled inorganic phosphate into added inorganic pyrophosphate under conditions in which extensive labelling in the adenine nucleotide acid-labile phosphorus occurred. Very recently, however, Lindberg & Ernster (1952) have found inorganic pyrophosphate, even in the presence of hexokinase, using rabbit-heart preparations similar to those used in the present paper. The inorganic pyrophosphate amounted to 3–10% of the HMP.

An alternative method of measuring oxidative phosphorylation is to omit the hexokinase and glucose, so that ATP is the final phosphorylated

product. This method has been used by Lehninger & Smith (1949), who measured the disappearance of inorganic phosphate. In a later paper (Slater & Holton, 1953), the adaptation of the enzymic methods to the direct measurement of the ATP formed will be described. Although this method is more direct than that employing hexokinase and eliminates the possibility of side reactions catalysed by impurities in the hexokinase, it is less suitable for kinetic studies than the procedure described in this paper since the concentration of phosphate acceptor (ADP) is decreasing throughout the experiment and soon becomes low. Rabinovitz, Stulberg & Boyer (1951) and Lardy & Wellman (1952) (see also Kielley & Kielley, 1951) have recently shown that the rate of oxidation is controlled by the concentration of phosphate acceptors. Provided the myokinase activity is low, the concentration of ADP is kept relatively constant in the procedure described in the present paper.

The accuracy of the enzymic analytical methods has been exhaustively tested in a previous paper (Slater, 1953) by comparison with values obtained for pure compounds by phosphorus determinations and spectrophotometric estimation of adenosine compounds. From these studies, it can be concluded that any systematic error cannot exceed a few per cent. The reproducibility is also excellent, and Table 1 of the present paper shows that there is no loss of HMP or $\sim P$ during the manipulations involved in oxidative phosphorylation experiments. The present paper shows also that possible side reactions cannot cause significant errors in the measurement of the yield of oxidative phosphorylation of heart-muscle sarcosomes. The only side reaction occurring is the synthesis of HMP by myokinase and hexokinase, and this can be allowed for in a control, which usually amounts to only a few per cent of the HMP synthesized in the oxidative phosphorylation experiment. It must be emphasized that only washed sarcosomes have been investigated. Such preparations do not contain any significant proportion of the sarcoplasm enzymes which might promote considerable side reactions. For example, the sarcoplasm contains about 100 times as much myokinase as the washed sarcosomes (Cleland & Slater, 1953), so that even in the presence of the strong inhibitors fluoride and adenylic acid, the myokinase control would be considerable if a whole-muscle homogenate were used. This means that the concentration of ADP would soon fall to a very low level.

In a previous paper (Slater, 1950), it was observed that in the first minute of an oxidative phosphorylation reaction carried out under conditions similar to those described here, with a low concentration of sarcosomes (0.53 mg./ml.), phosphorylation lagged behind oxidation. In com-

menting on this finding, Colowick & Kaplan (1951) have stated: 'Slater's observations might indicate that when ATP is generated during the oxidation of α -ketoglutarate, it would react via myokinase with adenylic acid, which is added as an 'inhibitor' of myokinase but in fact would compete with the hexokinase system. The resulting ADP would then react back to form ATP, which would then react with glucose and give the apparent lag in rate of phosphorylation.'

While a discussion of the nature of the observed lag will be reserved for a later paper, Colowick & Kaplan's interesting suggestion can be most conveniently considered here, since it includes statements at variance with the views expressed in the present paper. It seems probable that Colowick & Kaplan have in mind the experiments (Slater, 1950) in which it was found that formation of HMP began some time after reduction of cytochrome *c* had started and continued after all the cytochrome *c* had been reduced and hence oxidation had ceased (0.01M-cyanide being present). According to Colowick & Kaplan's explanation, myokinase successfully competed with hexokinase for ATP formed initially, so that the myokinase reaction

proceeded from right to left. Later when oxidation and hence ATP synthesis from oxidative phosphorylation ceased, ADP would 'react back to form ATP', presumably by the myokinase reaction proceeding from left to right.

A comparison of the relative rates of the myokinase reaction under the present conditions and the hexokinase reaction shows how unlikely it is that myokinase will successfully compete with hexokinase. The average cat-heart preparation transfers 0.13 μ moles phosphorus/hr./mg. sarcosomes (Table 4) by the myokinase reaction; the recommended amount of hexokinase (40 units/mg. sarcosomes—see p. 526) will transfer 75 μ moles phosphorus/hr./mg. sarcosomes. While the conditions of the two tests are not completely comparable, it is clear that hexokinase activity is overwhelmingly greater than that of the myokinase.

Kalckar (1943) found that the equilibrium constant of the myokinase reaction was approximately 2. Since the concentration of AMP equals that of ADP, this reaction would not proceed from right to left unless the concentration of ATP became equal to twice that of the ADP and AMP. This could only occur if the rate of phosphorylation greatly exceeded the rate of the hexokinase reaction, which is certainly to be avoided and experiments with different amounts of hexokinase confirm that this could not be the case in the experiment referred to. Furthermore, according to Colowick & Kaplan's explanation and the present

argument, at least 6 μ moles ATP (twice the amount of ADP and AMP used) would have to be present at some stage of the reaction, although the total hydrogen transfer was equivalent to only 0.15 μ g. atom of oxygen (half the number of μ moles of cytochrome *c* used). This would lead to a minimum value of the P:O ratio of 40, a highly unlikely value. Some idea of the amount of intermediate accumulated (ADP according to Colowick & Kaplan, ATP or some compound formed prior to ATP according to Slater, 1950) can be obtained by extrapolating the straight line portions of curves 2B and 1B of Slater (1950) to zero time. This value is slightly less than 0.1 μ g. atom phosphorus, or only 3 % of the concentration of ADP or AMP.

It is clear that, in the presence of hexokinase in the amounts normally used in oxidative phosphorylation experiments, the myokinase reaction will proceed only from left to right. Under such circumstances, AMP is not acting as substrate but only as an inhibitor, which is the reason for its use. This can be most clearly seen by the following formulation:

where k_1 , k_2 and k_3 are the rate constants of the reactions. In the steady state, the rate of formation of ATP equals the rate of its disappearance, i.e.

$$k_1 [\text{ADP}]^2 = (k_2 [\text{AMP}] + k_3 [\text{glucose}]) [\text{ATP}],$$

ignoring any inhibition of the enzymes by AMP.

Hence

$$[\text{ATP}] = k_1 [\text{ADP}]^2 / (k_2 [\text{AMP}] + k_3 [\text{glucose}])$$

and rate of reaction

$$= k_3 [\text{glucose}] [\text{ATP}]$$

$$= k_3 [\text{glucose}] k_1 [\text{ADP}]^2 / (k_2 [\text{AMP}] + k_3 [\text{glucose}]).$$

If the rate of the hexokinase reaction greatly exceeds the rate of the myokinase reaction, $k_3 [\text{glucose}]$ will be much greater than $k_2 [\text{AMP}]$, which is thereby eliminated and the rate of the reaction becomes $k_1 [\text{ADP}]^2$, which is independent of the concentration of AMP. Thus the finding in Table 2 that AMP does inhibit the formation of HMP shows that AMP is acting in a manner not described by the above equations; it must then be acting as an inhibitor. This conclusion is in complete accord with that of Colowick & Kalckar (1943), who showed, moreover, that it was the myokinase, not the hexokinase, which was inhibited by the AMP. It is not uncommon to find that the same substance can act both as substrate for and inhibitor of an enzyme.

A dialysed extract, containing both the granular components and the soluble proteins, was used in

Ochoa's early work on oxidative phosphorylation. Glucose was added and the inorganic phosphate taken up by the oxidative phosphorylation reaction appeared as HDP (Ochoa, 1943). Because the ATP-ase activity of Ochoa's preparation was high and the hexokinase activity relatively low, hydrolysis of the ATP formed by the oxidative phosphorylation reaction was considerable even in the presence of fluoride. Ochoa found, in fact, that the concentration of fluoride (0.025M) used in his experiments inhibited the ATP-ase by only 50%. In order to allow for the loss of phosphorylation caused by the ATP-ase activity, Ochoa measured the yield of ATP from the glycolytic phosphorylation reaction under comparable conditions. Since the theoretical yield, assuming no losses, of this reaction was known, the loss could be calculated and the yield found in the oxidative phosphorylation reaction corrected appropriately. Ogston & Smithies (1948) questioned the validity of this correction and suggested that the ATP-ase was, in fact, completely inhibited in Ochoa's experiments. They explained the apparently incomplete inhibition of ATP-ase found by Ochoa as due to the presence of pyrophosphate in the ATP used by him. This explanation was made very unlikely by Lehniger & Smith (1949), who found that liver ATP-ase was inhibited only partially by 0.02M-fluoride and that pyrophosphatase was somewhat more sensitive to fluoride. The present experiments, with the same tissue as used by Ochoa, under somewhat similar conditions, confirm that the inhibition of heart ATP-ase by fluoride is incomplete. It should be noted that, in the present study, there can be no question of pyrophosphatase being confused with ATP-ase, since the loss of $\sim$ P was followed, not the formation of inorganic phosphate. In any case the sample of ATP used contained very little inorganic pyrophosphate.

More recent studies of oxidative phosphorylation have used preparations of washed granules, which contain very little hexokinase. It has thus become the practice to add yeast hexokinase. If sufficient hexokinase is added, the ATP should react completely with the hexokinase and not at all with ATP-ase and it is now possible completely to eliminate this particular loss of phosphorylation (cf. Lindberg & Ernster, 1952). A number of possible losses or errors have been studied in this paper and shown to be of negligible importance. The only remaining possible loss is the hydrolysis of hypothetical energy-rich phosphate intermediate compounds, which cannot be tested until more is known of their nature. It is a common practice for workers on oxidative phosphorylation to assume that all losses have not been eliminated and to conclude that the true P:O ratio is the next integer above the experimentally observed values. While

there was considerable justification for this view in earlier work and also in those studies in which hexokinase was not added (e.g. Lehninger & Smith, 1949), it seems doubtful if it is any longer permissible when the major loss of phosphorylation (ATP-ase) has been eliminated (see also Rabinovitz *et al.* 1951). The well known instability of oxidative phosphorylation and the ease with which phosphorylation can be dissociated from respiration has been another reason leading to the emphasis of errors causing an underestimation of oxidative phosphorylation. As has been shown in another paper (Slater & Cleland, 1952), it is now possible greatly to stabilize oxidative phosphorylation in heart-muscle preparations. There is, therefore, little reason to believe that losses of oxidative phosphorylative ability occur during preparation of the sarcosomes. It has also been shown (Slater & Holton, 1952) that when α -ketoglutarate is the substrate, side reactions can actually cause errors in the opposite direction, so that the yield of oxidative phosphorylation is overestimated.

SUMMARY

1. The yield of oxidative phosphorylation may be measured by the enzymic determination of hexosemonophosphate, which is the end product of the reaction when hexokinase and glucose are added. This method has certain advantages over the more commonly employed procedure of measuring the disappearance of inorganic phosphate.

2. A number of side reactions, catalysed by

enzymes present in the heart-muscle sarcosomes or in the hexokinase preparations, have been investigated from the point of view of possible interference in the measurement of the phosphorylation.

3. The myokinase reaction which causes formation of hexosemonophosphate in the absence of oxidative phosphorylation can be made very slow by the addition of two inhibitors (fluoride and adenylic acid) and may be allowed for in a control.

4. Adenosinetriphosphatase activity of the sarcosomes is considerable, but can be eliminated as a source of error by the addition of sufficient hexokinase.

5. The very slight hexosemonophosphatase and phosphohexokinase activities cause negligible errors.

6. No glycolysis occurs under the conditions of the oxidative phosphorylation experiments.

7. It is concluded that the amount of hexosemonophosphate found is an accurate measure of the yield of oxidative phosphorylation.

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