replace phosphate both in the extraction of the mitochondria and in the reaction medium.

4. The reaction is dependent on the presence of oxygen, but this may be reduced to a tension of 0.5% without any effect on the reaction velocity. Experiments with cyanide, azide and carbon monoxide suggest that the cytochrome system is involved.

5. 2:4-Dinitrophenol in high concentrations does not, but adenosine triphosphate (1×10^{-3} M) does inhibit the reaction. There is no evidence to suggest the necessity for the participation of compounds containing high-energy phosphate bonds in the formation of ascorbic acid from galactono- γ -lactone.

6. The enzymic activity of mitochondria prepared from peas increased rapidly in the early stages of germination.

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

2. PHOSPHORUS:OXYGEN RATIO

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Ochoa (1944) was the first to show that the single step oxidation of α -ketoglutarate to succinate in dialysed heart extracts was accompanied by the esterification of inorganic phosphate. The number of atoms of phosphorus esterified per atom of oxygen consumed (the P:O ratio) in these experiments was 1.5, the same as obtained with pyruvate as substrate under otherwise identical conditions. Previously, Ochoa (1943) had shown that after correction for the heavy losses of esterified phosphorus due to the considerable adenosine triphosphatase and low hexokinase activities of his preparations, the P:O ratio with pyruvate was 3. Accordingly, Ochoa (1944) concluded that the corrected ratio with α -ketoglutarate as substrate was also 3.

In more recent work, the phosphorylation losses have been decreased by the addition of hexokinase and glucose. In this way Cross, Taggart, Covo & Green (1949) obtained a mean uncorrected value of 2.43 ± 0.42 (standard deviation) for 62 experiments with rabbit-kidney or rabbit-liver 'cyclophorase' preparations, while Hunter & Hixon (1949) obtained values, as amended by Hunter (1951), a little

below 3 with a liver preparation. These determinations, as well as that given by Slater (1950) for a single determination with cat heart (2.79), agreed with Ochoa's conclusions.

In recent years, however, a number of workers have obtained ratios above 3. These, together with the earlier values, are summarized in Table 1. Values obtained with and without malonate have been listed separately. Unless malonate is used there will be some oxidation of the succinate formed from the α -ketoglutarate, resulting in somewhat lower ratios, since the P:O ratio for the oxidation of succinate to fumarate is lower than that obtained in the oxidation of other substrates. As a result of these recent findings and also on the basis of indirect arguments which will be considered in the Discussion, it is now widely believed that the true P:O ratio for the oxidation of α -ketoglutarate to succinate is 4 (e.g. see Hunter, 1951; Ochoa & Stern, 1952; Krebs, Ruffo, Johnson, Eggleston & Hems, 1953). Ochoa's (1943) original data are probably not sufficiently precise to distinguish between a P:O ratio of 3 and 4.

Table 1. P:O ratios in oxidation of α -ketoglutarate

Reference	Source of enzyme	Preparation	P:O (mean)	
			Without malonate	With malonate
Ochoa (1944)	Cat heart	Dialysed extract	—	3.0*
Cross <i>et al.</i> (1949)	Rabbit kidney and liver	'Cyclophorase'	2.4	—
Hunter & Hixon (1949)†	Rat liver	Washed particles	† 3	—
Slater (1950)	Cat heart	Sarcosomes (mitochondria)	2.79	—
Judah (1951)	Rat or rabbit liver or kidney	Mitochondria	3.4	—
Kielley & Kielley (1951)	Mouse liver	Mitochondria	2.6	3.3
Barkulis & Lehninger (1951)	Rat liver	Mitochondria	—	2.9
Copenhaver & Lardy (1952)	Rat liver	Mitochondria	—	3.6
Feldott & Plaut (1953)	Guinea pig and rat heart	Mitochondria	—	3.4
Krebs <i>et al.</i> (1953)	Rat liver	Dispersion of whole tissue	3.1	—

* Corrected for dephosphorylation losses.

† Amended by Hunter (1951).

In earlier papers a number of factors which might affect the yield of phosphorylation associated with the oxidation of α -ketoglutarate to succinate by heart-muscle sarcosomes was studied (Slater, 1953*a, b*; Cleland & Slater, 1953; Slater & Cleland, 1953*a, b*; Slater & Holton, 1953). The following conclusions relevant to the subject of the present paper were drawn:

(1) *Measurement of phosphorylation.* Provided sufficient hexokinase is used, the amount of phosphate esterified is correctly measured.

(2) *Measurement of P:O ratio.* When ethylenediaminetetraacetic acid is included in the reaction mixture (Slater & Cleland, 1952), the P:O ratio is constant over a period of 45–60 min. at 25°. The oxidation proceeds at a uniform rate from the moment the sarcosomes are introduced into the reaction mixture (except for a slight fall towards the end of the period), so that the oxygen uptake can be accurately measured.

(3) *Isolation of sarcosomes.* The most active sarcosomes are obtained by isolation with isotonic media; saline and sucrose give preparations with the same P:O ratio.

(4) *Composition of reaction mixture.* The following variations of the reaction mixture had no appreciable effect on the P:O ratio, although the rate of oxygen uptake was often markedly affected: (i) pH between 6.2 and 7.7; (ii) tonicity between about half-isotonic and slightly hypertonic; (iii) phosphate concentration between 0.02 and 0.06 M; (iv) omission of cytochrome *c*; (v) concentration of adenosine diphosphate (ADP) between 10^{-4} M and 1.2×10^{-3} M; (vi) addition of adenosine monophosphate (AMP).

The reaction media used in investigations of oxidative phosphorylation contain a number of components, not all of which have been varied in the above-mentioned papers. This paper fills that gap and also considers other factors which could conceivably affect the P:O ratio. Having defined the conditions under which heart-muscle sarcosomes

give the highest P:O ratio, all our experiments in this and related investigations which satisfy these conditions are assembled in order to estimate the most probable value of the P:O ratio. Since the conditions for the optimum P:O ratio in our experiments are not critical, but in every case cover a wide range of concentrations or treatment, it is considered likely that the results obtained can be extrapolated to the conditions of the intact heart. Our findings disagree with the recent assessments, and support Ochoa's original conclusion that the P:O ratio is 3.

METHODS

Sarcosomal preparations. These were made according to the procedure of Cleland & Slater (1953), using isotonic suspending media. Different types of preparations are designated according to the isolation medium and the animal used.

Measurement of activity of α -ketoglutaric oxidase system and yield of oxidative phosphorylation. Except where stated otherwise, the reaction mixture was that listed under 'standard conditions' in Table 10 of this paper or departed only slightly from these standard conditions. The reaction volume was 1 ml. in manometer flasks of 5–7 ml. total volume. Except in one experiment (Fig. 3), the reaction temperature was 25°. The experimental procedure has been described by Slater & Holton (1953).

The yield of oxidative phosphorylation is expressed either as P:O (number of atoms of inorganic phosphorus esterified per atom of oxygen consumed) or as P: α -Kg (number of atoms of inorganic phosphorus esterified per molecule of α -ketoglutarate disappearing). The number of atoms of inorganic phosphorus esterified was determined either as Δ HMP (hexose monophosphate) which equals Δ HMP minus the myokinase control determined as described by Slater (1953*b*), or as Δ HMP plus $\Delta \sim P$ ($\sim P$ = energy-rich reactive phosphate groups), as described by Slater & Holton (1953). The symbol Δ is used to denote the change in the amount of the component in the system since zero time.

The activity of the α -ketoglutaric oxidase system is expressed as Q_{O_2} (μ l. O_2 /mg. protein/hr.). One unit of the α -ketoglutaric oxidase system gives an oxygen uptake of 1 μ l./hr.

RESULTS

Further investigations of method of preparation of sarcosomes

The method used for isolating the sarcosomes liberates only about 50–70 % of the total sarcosomes in the heart muscle; a considerable proportion remains trapped in the fragments of myofibrils separated from the dispersed tissue by a low-speed centrifugation. Cleland & Slater (1953) showed that these retained sarcosomes possessed a higher activity of the α -ketoglutaric oxidase system relative to that of cytochrome oxidase than the free sarcosomes. It was important, therefore, to determine whether they also gave a higher P:O ratio. This was investigated by measuring the P:O ratio of a dispersion of cat-heart muscle and of fractions isolated by differential centrifugation. Some combinations of fractions were also tried, in order to determine if the degree of separation of the fractions affected the P:O ratio. The supernatant obtained after sedimentation of the sarcosomes at 7500 g for 7.5 min. was recentrifuged at higher speed (14 300 g for 30 min.) in order to collect a fraction consisting of some sarcosomes and smaller granular material, possibly the microsomes (see Cleland & Slater, 1953). Although the P:O ratios obtained in this experiment (Table 2) are at the lower limit of the normal range of variation (see p. 35), it is clear that there is little difference between the ratios obtained with the dispersion and the three fractions. Certainly the sarcosomes retained in the residue had no greater phosphorylative activity than isolated sarcosomes (see also Table 3). Furthermore, addition of residue or of 'microsomes' to the sarcosomes

did not affect the P:O ratio. In fact, the actual oxygen uptake and phosphorylation found with sarcosomes plus 'microsomes' were very close to that expected from the sum of the values obtained with the fractions separately.

The effect of varying the method of dispersing the heart was also studied. Two rat hearts were chopped coarsely and combined. About one-quarter of the total mince was dispersed in sucrose-ethylenediaminetetraacetic acid medium by treatment in a loosely fitting Potter & Elvehjem (1936) glass homogenizer. The remainder was ground with sand and the same medium in a glass mortar in the usual way and samples taken after 2, 3.5 and 6 min. grinding. The four samples were fractionated in the centrifuge as usual. The activities of the α -ketoglutaric oxidase system and the P:O ratios of each sarcosomal fraction and each residue from the low-speed centrifugation are summarized in Table 3.

Table 2. P:O ratios of various fractions of cat heart muscle

(For details of fractionation, see Table 3 of Cleland & Slater (1953). Dispersion = mixture immediately before first centrifugation. Residue = sediment after two centrifugations at 700 g. Sarcosomes = sediment after two centrifugations at 7500 g. 'Microsomes' = sediment after centrifugation at 14 300 g.)

Fraction	Q_{O_2}	P:O	P: α -Kg
Dispersion	11.6	2.04	2.64
Residue	6.9	2.01	—
Sarcosomes	70	2.27	2.93
'Microsomes'	19	2.17	3.06
Sarcosomes + residue	—	2.25	2.74
Sarcosomes + 'microsomes'	—	2.33	2.88

Table 3. Effect of variations in the method of preparing dispersion of rat heart

(See text for description of experiment. S = sarcosomes; R = sediment after two centrifugations at 700 g; Sup. = supernatant from centrifugation at 7500 g. In order to allow for the fact that the amounts of tissue in the different samples were not identical, the total enzyme activities have been calculated on the basis of a total protein content (determined by the sum of the protein contents of residue, sarcosomes and supernatant) of 100 mg.)

		Treatment			
		Ground with sand			Potter & Elvehjem homogenizer
		2 min.	3.5 min.	6 min.	
Protein (%)	S	8.0	11.6	12.2	9.4
	R	68	61	52	54
	Sup.	24	23	36	37
Activity of α -ketoglutaric oxidase system					
Q_{O_2}	S	83	67	50	57
	R	24	19	8	8
Total (units)	S	665	841	615	535
	R	1660	1140	420	450
	S + R	2325	1981	1035	985
P:O	S	2.74	2.64	2.55	2.47
	R	2.33	2.33	1.96	1.07
P: α -Kg	S	3.31	3.56	2.99	3.20
	R	2.96	2.98	2.70	—

As the time of grinding with sand was increased, the specific activity of the α -ketoglutaric oxidase system (Q_{O_2}) in both the sarcosomes and residue declined; the total enzymic activity also declined. The Potter & Elvehjem homogenizer gave fractions with activities similar to those obtained after 6 min. grinding with sand. The distribution of protein in the three fractions obtained using the Potter & Elvehjem homogenizer was also similar to that obtained after 6 min. grinding.

Despite the marked effects on the activity of the α -ketoglutaric oxidase system, the P:O ratio was only slightly affected by the degree of grinding, although there was a tendency for lower ratios with longer grinding. In most of our experiments, the time of grinding was 2 min., and it never exceeded 3.5 min. It is apparent that this procedure is somewhat milder than the use of a Potter & Elvehjem homogenizer. It should be mentioned that the tissue was given only a brief treatment in the Potter-Elvehjem homogenizer, less than that usually given in this method.

Stoichiometry of the reaction

Relation between ΔO and $\Delta \alpha$ -Kg

Anaerobic reaction. For the accurate determination of the P:O ratio associated with the oxidation of α -ketoglutarate to succinate, it is necessary to remove side reactions as far as possible. As already briefly reported (Slater & Holton, 1952), crude preparations of yeast hexokinase catalyse an anaerobic reaction of α -ketoglutarate which is coupled with phosphorylation. The presence of this reaction is revealed when the disappearance of α -ketoglutarate (μ moles) exceeds the oxygen uptake (μ atoms). This can be eliminated by using hexokinase of sufficient purity. We have found it necessary to proceed to step 5 of the procedure of Berger, Slein, Colowick & Cori (1946) to eliminate this reaction. Since the yield of phosphorylation in this side reaction is small ($P:\alpha\text{-Kg}=0.5$), errors in the estimated P:O ratio will become serious only when a considerable proportion of the α -ketoglutarate which reacts does so by this anaerobic reaction. This can be the case when large amounts of crude hexokinase are used, particularly with sarcosomes of relatively low

oxidative activity, since the anaerobic reaction is relatively resistant to treatments which inactivate the α -ketoglutaric oxidase system.

Examples of the effect of this anaerobic reaction are given in Table 4. As will be shown presently, the mean value of the ratio of oxygen uptake (μ atoms): α -ketoglutarate disappearance (μ moles) in the absence of this side reaction is 1.13. Assuming that the P: α -Kg of the anaerobic reaction is 0.5, it is possible to correct the observed P:O, as is shown in Table 4.

Effect of malonate and fluoride. Malonate is the most suitable reagent for the inhibition of the oxidation of the succinate formed from the α -ketoglutarate. However, the reaction mixtures used in studies of oxidative phosphorylation often contain fluoride which in the presence of phosphate is also a competitive inhibitor of this enzyme (Slater & Bonner, 1952). This is why malonate does not have as large an effect on the P:O ratio as might be expected (e.g. see Hunter & Hixon, 1949).

The effects of various concentrations of malonate and fluoride are shown in Tables 5 and 6. Table 5 shows that, in the absence of either of these inhibitors, 2 atoms of oxygen were consumed per molecule of α -ketoglutarate, i.e. the α -ketoglutarate was probably largely oxidized to fumarate. Either malonate (0.01M) or fluoride (0.04M) alone reduced this ratio to 1.2–1.3 and the two together brought it to near 1. Up to 0.01M malonate had no inhibitory effect on the α -ketoglutaric oxidase system, measured by the disappearance of α -ketoglutarate, but a slight inhibitory action was apparent with higher concentrations.

Fluoride inhibited the α -ketoglutaric oxidase system, but had little effect on the P:O ratio. As with succinic dehydrogenase (Bonner, 1951), the inhibition by fluoride was greater at the higher concentrations of phosphate, but as the difference was more marked with 0.02M fluoride than with 0.04M fluoride, it seems likely that the effect of phosphate concentration is more complicated than with succinic dehydrogenase (see Slater & Bonner, 1952). The inhibition of phosphorylation by 0.04M fluoride in the presence of 0.015M phosphate might be due to the removal of considerable amounts of phosphate in the form of magnesium fluorophosphate.

Table 4. *Effect of anaerobic reaction of α -ketoglutarate, catalysed by crude hexokinase preparations, on estimation of P:O ratio*

$$(\Delta' \text{HMP}_{\text{corr.}} = \Delta' \text{HMP} - 0.5 (-\Delta \alpha\text{-Kg} + \Delta O/1.13); P:O_{\text{uncorr.}} = \Delta' \text{HMP} / -\Delta O; P:O_{\text{corr.}} = \Delta' \text{HMP}_{\text{corr.}} / -\Delta O.)$$

Expt.	$-\Delta O$ (μ atoms)	$-\Delta \alpha\text{-Kg}$ (μ moles)	$\Delta' \text{HMP}$ (μ moles)	$\Delta O:\Delta \alpha\text{-Kg}$	$P:O_{\text{uncorr.}}$	$P:O_{\text{corr.}}$
1	3.37	5.74	9.53	0.59	2.83	2.42
2	3.68	5.85	8.31	0.63	2.26	1.92
3	2.59	3.93	6.60	0.66	2.55	2.29

Table 5. *Effect of malonate, fluoride and pyrophosphate on the α -ketoglutaric oxidase system and on oxidative phosphorylation*

(Saline-ethylenediaminetetraacetic acid sarcosomes from rat heart.)

[Fluoride] (M)	[Malonate] (M)	[Pyrophosphate] (M)	$-\Delta O$ (μ atoms)	$-\Delta \alpha\text{-Kg}$ (μ moles)	$\Delta O:\Delta \alpha\text{-Kg}$	P:O	P: $\alpha\text{-Kg}$
0	0	0	5.66	2.77	2.04	1.71	3.50
0	0.01	0	3.78	2.99	1.26	2.06	2.60
0.04	0	0	2.75	2.11	1.30	1.82	2.37
0.04	0.005	0	2.62	2.36	1.11	2.19	2.43
0.04	0.01	0	2.48	2.42	1.02	2.22	2.28
0.04	0.02	0	2.17	2.40	0.90	2.53	2.29
0.04	0.04	0	2.10	2.12	0.99	2.40	2.38
0	0	0.01	3.98	2.27	1.75	1.62	2.84
0.04	0	0.01	1.28	1.21	1.06	1.50	1.59

Table 6. *Effect of fluoride on the α -ketoglutaric oxidase system and on oxidative phosphorylation*

(Expts. 1 and 2, saline-ethylenediaminetetraacetic sarcosomes from rat heart; Expt. 3, isotonic-sucrose sarcosomes from rat heart.)

Expt.	[Phosphate] (M)	[Fluoride] (M)	$-\Delta O$ (μ atoms)	$-\Delta \alpha\text{-Kg}$ (μ moles)	Δ' HMP (μ moles)	P:O	P: $\alpha\text{-Kg}$
1	0.036	0	3.55	3.66	8.42	2.38	2.32
	0.036	0.005	2.77	2.47	5.79	2.09	2.34
	0.036	0.01	2.24	2.17	5.60	2.50	2.58
	0.036	0.02	2.16	2.22	5.41	2.50	2.44
	0.036	0.04	1.88	1.84	4.53	2.42	2.46
	0.015	0	2.33	2.06	5.88	2.52	2.86
	0.015	0.02	1.83	1.69	4.12	2.26	2.44
2	0.04	0	6.29	—	16.5	2.62	—
	0.04	0.04	3.73	—	11.07	2.97	—
	0.02	0	6.15	—	15.5	2.53	—
	0.02	0.04	3.50	—	9.87	2.82	—
3	0.02	0	3.57	—	9.62	2.70	—
	0.03	0.04	2.24	—	5.68	2.54	—

Table 5 also compares the effect of pyrophosphate, another competitive inhibitor of succinic dehydrogenase (Leloir & Dixon, 1937; Keilin & Hartree, 1949; Tsou, 1951). This reagent was unsuitable, since it markedly inhibited both the α -ketoglutaric oxidase system and the phosphorylation, without appreciably affecting the proportion of the oxidation past succinate.

As a result of these experiments, it was decided to use 0.01M malonate and 0.04M fluoride for the measurements of the P:O ratio. Although fluoride had little effect on the P:O ratio, it was preferable to include it in order to inhibit the adenosine triphosphatase and myokinase (Slater, 1953b). These concentrations were not sufficient completely to inhibit the oxidation of succinate formed from the α -ketoglutarate. In twenty-one experiments, the ratio $\Delta O:\Delta \alpha\text{-Kg}$ ranged from 0.95 to 1.28, with a mean of 1.13.

Fig. 1 shows the oxygen uptake with equivalent amounts of α -ketoglutarate and succinate under various conditions. In the absence of either malonate or fluoride, the oxygen uptake with α -ketoglutarate proceeded rapidly without any time

lag until 2 atoms of oxygen per mole of α -ketoglutarate were consumed. It appears that the succinate is largely oxidized as soon as it is formed, so that its steady-state concentration must be quite small, in agreement with Green's cyclophorase concept. However, malate is rather slowly oxidized by heart-muscle sarcosomes and this is reflected in the slow rate of oxygen uptake after the consumption of 2 atoms of oxygen. The decline in the rate of oxygen uptake is not due to inactivation of the enzyme preparation which is stable for long periods in the presence of the reaction mixture and ethylenediaminetetraacetic acid (Slater & Cleland, 1952). In the presence of malonate and fluoride, α -ketoglutarate was rapidly oxidized until rather more than 1 atom of oxygen was consumed, after which the oxygen uptake continued at a low but appreciable rate, equal to that given with the same concentration of succinate under these conditions. By extrapolating this uniform rate to zero time, the oxygen uptake corresponding to the oxidation of α -ketoglutarate to succinate was obtained. This agreed closely with the calculated value, showing the absence of other side reactions.

Fig. 1 shows that the rate of oxygen uptake with succinate declines even before 1 atom of oxygen is consumed; this is very likely due to progressively increasing inhibition by oxaloacetate which, being a competitive inhibitor of succinic dehydrogenase, will inhibit to an increasing degree as the succinate concentration declines, even if the oxaloacetate concentration remains constant. The steadily decreasing activity of succinic dehydrogenase is probably the reason why no sharp break occurs in the rate of oxygen uptake with succinate, when 1 atom of oxygen has been consumed.

Fig. 1. Oxidation of 6.3 μ moles α -ketoglutarate or succinate by sarcosomes (sucrose-ethylenediaminetetraacetic acid preparation from rat heart) under various conditions. The horizontal lines show the O_2 uptakes corresponding to the consumption of 1, 2 and 3 atoms of oxygen per molecule of α -ketoglutarate or succinate. The reaction mixture contained 0.01M malonate and 0.04M fluoride, where indicated.

Table 7. Comparison of uptake of inorganic phosphate with esterified phosphate

(Sucrose-ethylenediaminetetraacetic acid sarcosomes from rat heart.)

Expt.	Δ' HMP (μ moles)	$-\Delta$ Inorganic phosphate (μ atoms)
1	4.1	4.2
	6.8	7.9
	9.4	10.9
	10.0	10.5
	10.6	10.5
	13.4	13.4
2	8.3	8.5
	8.2	8.2

Relationship between inorganic phosphate uptake and esterified phosphate

The following methods of measuring the yield of oxidative phosphorylation have been used: (1) measurement of Δ' HMP, which is Δ HMP minus the HMP synthesized by the non-oxidative phosphorylation of glucose by ADP, catalysed by myokinase and hexokinase (Slater, 1953b); (2) measurement of Δ HMP plus $\Delta \sim P$ (Slater & Holton, 1953). The myokinase correction in method (1) is usually only a few per cent of Δ HMP and the two methods also agree within 1-2 %. If inorganic phosphate is converted into a product not measured as HMP or $\sim P$ (e.g. inorganic pyrophosphate), these methods will not measure all the uptake of inorganic phosphate. This possibility was tested by comparing our procedures with the more commonly employed measurement of the disappearance of inorganic phosphate. Table 7 shows that Δ' HMP agrees very closely with $-\Delta$ inorganic phosphate. Thus, under the conditions of our experiments, all the inorganic phosphate which disappears is accounted for by the esterified phosphate directly measured by the analytical procedures.

Effect of added magnesium

The effect of the concentration of added magnesium on the α -ketoglutaric oxidase system and on the P:O ratio is shown in Table 8. Since 0.002M ethylenediaminetetraacetic acid was present in all experiments, and this combines firmly with an equivalent concentration of magnesium (Schwarzenbach & Ackermann, 1947), the concentration of free magnesium must have been very small when the total magnesium did not exceed 0.002M. The concentrations of free magnesium in Table 8 have been calculated on the assumption that all the ethylenediaminetetraacetic acid is bound with magnesium. An insignificant proportion would have been combined with calcium or other metals in the sarcosomes or reagents.

It is apparent from Table 8 that the maximum rate of oxidation and of phosphorylation is obtained with very low concentrations of free magnesium and that the concentration can be varied widely on either side of the standard concentration (0.005M total, 0.003M free) without affecting the P:O ratio. Even in the presence of ethylenediaminetetraacetic acid, the rate of oxidation was considerable in the absence of added magnesium. This can be explained by Cleland's (1953) finding that sarcosomes contain large amounts of magnesium even when isolated in media containing ethylenediaminetetraacetic acid. The phosphorylation was more dependent upon the addition of magnesium, but this is undoubtedly because the added yeast hexokinase requires magnesium in solution. The apparent inhibition of

Table 8. *Effect of magnesium concentration on α -ketoglutaric oxidase system and on P:O ratio*

(The free Mg^{2+} is calculated on the assumption that all the ethylenediaminetetraacetic acid is bound with magnesium, but disregarding the possible formation of other magnesium compounds, e.g. magnesium fluorophosphate. The sarcosomes were prepared from rat heart in sucrose-ethylenediaminetetraacetic acid medium (Expt. 1), isotonic sucrose (Expt. 2) and saline-ethylenediaminetetraacetic acid (Expt. 3).)

Expt.	[Added Mg^{2+}]		$-\Delta O$ (μ atoms)	$-\Delta \alpha$ -Kg (μ moles)	Δ' HMP (μ moles)	P:O
	Total (m-moles/l.)	Free (m-moles/l.)				
1	0	0	—	—	0.3	—
	2.1	0.1	—	—	14.4	—
	5.3	3.3	5.67	—	15.3	2.70
	10.7	8.7	5.77	—	15.9	2.75
2	0	0	1.63	—	—	—
	2.1	0.1	2.08	—	—	—
	5.3	3.3	2.45	—	—	—
3	0	0	1.44	1.27	0.3	0.19
	1.0	0	2.84	2.67	4.6	1.62
	2.4	0.4	3.87	3.35	8.3	2.14
	10.7	8.7	4.09	3.71	9.7	2.38
	*21.3	19.3	7.94	3.50	9.8	1.23

* Copious ppt. observed.

Table 9. *Effect of α -ketoglutarate concentration on rate of oxygen uptake and on P:O*

(Saline-ethylenediaminetetraacetic acid sarcosomes from rat heart. The $[MgCl_2]$ used in this experiment was 2.4 m-moles/l. total, 0.4 m-moles/l. free.)

	[α -Ketoglutarate] (m-moles/l.)			
	2.35	4.69	7.0	14.0
Q_{O_2} (initial rate)	44.4	45.6	50.1	49.2
$-\Delta O$ (μ atoms)	3.03	3.75	3.87	3.90
$-\Delta \alpha$ -Kg (μ moles)	2.33	3.10	3.35	3.28
$\Delta O : \Delta \alpha$ -Kg	1.30	1.21	1.16	1.19
Δ' HMP (μ moles)	6.4	8.1	8.3	8.1
P:O	2.10	2.16	2.14	2.07
P: α -Kg	2.74	2.60	2.48	2.46

Table 10. *Optimum conditions for measuring oxidative phosphorylation*

(Concentrations are m-moles/l. unless stated otherwise.)

	Optimum range	Standard conditions
pH	6.2-7.7	7.4
[Na or K phosphate]	20-60	30
[Na ₂ or K ₂ malonate]	5-40	10
[NaF or KF]	0-40	40
[MgCl ₂]	3-10	5
[K ₂ α -ketoglutarate]	2-14	5
[Glucose]	†	30
[AMP*]	0-1.2	0.6
[ADP*]	0.1-1.2	0.6
[Cytochrome c]	0-0.6	0.04
[KNa ₂ H ethylenediaminetetraacetate]	0.1-2	2
[Osmolar] (M)	0.14-0.48	0.32
[Sarcosomes] (mg. protein/ml.)	0.12-1.25	0.8
Hexokinase (units/mg. sarcosomal protein)	>40	100

* K salts.

† Sufficient to react with esterified P.

phosphorylation by 0.021 M magnesium in Expt. 3, Table 8, is probably largely due to precipitation of malonate and fluoride by this high concentration of magnesium, since $-\Delta O$ greatly exceeded $-\Delta \alpha$ -Kg under these conditions.

The high affinity of the α -ketoglutaric oxidase system and of the phosphorylating systems in the sarcosomes for magnesium shown in Table 8 does not agree with the conclusions of Paul, Fuld & Sperling (1952) and Harman & Feigelson (1952), who comment upon the unusually high requirement for magnesium of heart-muscle preparations. However, it is clear from the data of Paul *et al.* (1952) (see their Fig. 1) that magnesium did not activate the initial rate of oxidation, but stabilized the enzyme system. Such a stabilizing effect of magnesium, in the absence of ethylenediaminetetraacetic acid, has been reported by Slater & Cleland (1952).

Effect of concentration of α -ketoglutarate

The effect of varying the concentration of α -ketoglutarate on the rate of oxygen uptake and on the P:O ratio is shown in Table 9. It is clear that the α -ketoglutaric oxidase system has a high affinity for its substrate and that the P:O ratio is independent of the concentration of α -ketoglutarate over a wide range. The P:O ratios in the experiment shown in Table 9 are somewhat low, because a low concentration of magnesium chloride was used.

Optimum conditions for measuring P:O ratio

Results reported in this and in previous papers enable us to define the limits within which the components of the reaction mixture may be varied without appreciably affecting the P:O ratio of heart-muscle sarcosomes, with α -ketoglutarate as

Table 11. *Reproducibility of measurements of P:O and P: α -Kg ratios*

(Each measurement represents a single flask, each containing the same reaction mixture, the same amount of sarcosomes (sucrose-ethylenediaminetetraacetic acid preparation from rat heart) and the same number of hexokinase units. Eight different hexokinase preparations were used, varying from specific activity 174 to 750 (as defined by Berger *et al.* 1946).)

	1	2	3	4	5	6	7	8
$-\Delta O$ (μ atoms)	3.03	3.03	3.06	3.13	3.10	3.16	3.24	3.22
$-\Delta \alpha\text{-Kg}$ (μ moles)*	2.69	3.00	2.79	2.59	2.68	2.70	2.92	2.74
$\Delta O:\Delta \alpha\text{-Kg}$	1.13	1.01	1.10	1.21	1.16	1.17	1.11	1.17
Δ' HMP (μ moles)	7.99	8.34	8.24	8.60	8.40	8.41	8.70	8.75
P:O	2.64	2.75	2.69	2.75	2.71	2.66	2.69	2.72
P: α -Kg	2.97	2.78	2.96	3.32	3.14	3.11	2.98	3.19

* 7.69 μ moles initially present.

Fig. 2. A. Frequency distribution of P:O ratios obtained with thirty-two cat- and rat-heart sarcosomal preparations. Open, rat; blocked, cat. The dotted line shows the theoretical value, assuming P:O for the oxidation of α -ketoglutarate to succinate is 3, P:O for oxidation of succinate is 1, and $\Delta O:\Delta \alpha\text{-Kg}=1.13$. B. Frequency distribution of P: α -Kg ratios obtained with twenty cat- and rat-heart sarcosomal preparations. Open, rat; blocked, cat. The dotted line shows the theoretical value, making the same assumptions as in A.

substrate. These are summarized in Table 10, which also includes the 'standard conditions' adopted in most of the experiments in this paper.

The reproducibility of a single measurement of the P:O ratio of a preparation is shown in Table 11. Eight different purified hexokinase preparations were used in this experiment which may account for the slight differences in oxygen uptake, since some hexokinase preparations contain an inhibitor of the α -ketoglutaric oxidase system. However, there is very little variation in the P:O ratio. Since the measurement of $\Delta \alpha\text{-Kg}$ depends upon the difference between two separate analyses, it is subject to greater experimental error than ΔO . Thus, the reproducibility of P: α -Kg is not quite as good as that of the P:O.

The P:O ratio for the oxidation of α -ketoglutarate to succinate

Fig. 2 describes all our measurements of P:O ratio and P: α -Kg ratio on cat and rat preparations which were carried out under the conditions defined by Table 10, using hexokinase of sufficient purity, and the procedure described by Slater & Holton (1953). No measurements have been omitted. Of the thirty-two sarcosome preparations, fourteen were made with saline medium, either with or without ethylenediaminetetraacetic acid, and eighteen were made with sucrose containing ethylenediaminetetraacetic acid. The average P:O ratio of the sucrose preparations was only slightly higher than the saline, but of the nine values below 2.6, six were for saline preparations and only three (out of eighteen) for sucrose preparations, including a value of 2.59.

Using the mean value of $\Delta O:\Delta \alpha\text{-Kg}$ of 1.13 and assuming that the P:O ratio for the oxidation of succinate under the conditions of these experiments is 1.0 (Slater & Holton, unpublished experiments), it can be calculated that if the P:O ratio in the oxidation of α -ketoglutarate to succinate is 3, the observed P:O ratio would be 2.77 and the observed P: α -Kg would be 3.13. It is clear from Fig. 2 that the great majority of the observed P:O ratios are grouped very close to the theoretical value. The scatter of the P: α -Kg values is somewhat greater, probably for analytical reasons, but most of the values are only slightly below the theoretical value. The presence of a small amount of the anaerobic reaction of α -ketoglutarate would lower the P: α -Kg value somewhat without having any appreciable effect on the P:O ratio.

It is considered that the results of Fig. 2 point very strongly to a P:O ratio of 3 in the oxidation of α -ketoglutarate to succinate.

Investigation of Copenhagen & Lardy's method of measuring oxidative phosphorylation

Since our results do not agree with those of a number of other workers, it was necessary to compare our procedure with theirs. Copenhagen & Lardy's (1952) procedure, which is similar to that

Table 12. Comparison of P:O ratios obtained with our reaction mixture and that of Copenhaver & Lardy (1952)

(Copenhaver & Lardy's reaction mixture. Phosphate, 0.0135M; malonate, 0.01M; NaF, 0.01M; ATP, 0.002M; α -Kg, 0.0067M; MgSO_4 , 0.0074M; cytochrome c, 1.2×10^{-5} M in main flask. Glucose, 0.017M; hexokinase, 70 units in side bulb. Sarcosomes (0.1 ml. of sucrose-ethylenediaminetetraacetic acid preparation from rat heart, containing 1.04 mg. protein) added to main flask at zero time, attached to manometer and placed in bath at 25°. Contents of side bulb tipped into main flask 3 min. later. Different flasks removed and contents immediately deproteinized 2 min., 17 min. and 27 min. after tipping. Δ refers to change in component from 2 min. after tipping.

Our procedure. Usual procedure with usual reaction mixture, same concentration of sarcosomes and hexokinase as above. 35 min. Results are shown for duplicate flasks.)

Time after tipping (min.)	Copenhaver & Lardy			Slater & Holton	
	2	17	27	—	—
— Δ O (μ atoms)	—	1.96	2.99	4.09	4.26
α -Kg (μ moles)	5.71	4.21	3.28	1.16	1.26
— $\Delta\alpha$ -Kg (μ moles)	—	1.50	2.43	3.70*	3.60*
$\Delta\alpha$ -Kg: Δ O	—	—	1.23	1.10	1.19
$\sim$ P (μ moles)	1.98	1.37	1.19	0.68	0.69
$\Delta\sim$ P (μ moles)	—	—0.61	—0.79	0.06*	0.07*
HMP	2.39	8.31	11.6	11.7	11.8
Δ HMP (μ moles)	—	5.92	9.2	11.7	11.8
Δ HMP + $\Delta\sim$ P	—	5.31	8.4	11.7	11.8
P:O	—	2.72	2.82	2.86	2.76
P: α -Kg	—	—	3.46	3.16	3.26

* 4.86 μ moles α -Kg, 0.62 μ moles $\sim$ P initially present.

used by a number of the authors listed in Table 1, was studied in detail.

Copenhaver & Lardy use a reaction mixture which does not differ in any important respect from ours, except for the omission of ethylenediaminetetraacetic acid, and the use of ATP as the adenine nucleotide. However, their procedure is considerably different. The reaction mixture containing all components except hexokinase and glucose is chilled to 0° in the main compartment of a Warburg flask containing two side bulbs. At zero time, the chilled mitochondrial preparation is added to the main compartment of the flask which is immediately attached to the manometer and placed in a bath at 30°. After 3 min. the hexokinase and glucose are tipped into the main flask from a side bulb; 2 min. later, that is 5 min. from adding the mitochondrial preparation, the manometer fluid is adjusted to zero, the taps closed and the oxygen uptake measured for a period of 15 min. The controls which serve as a basis for calculating the disappearance of inorganic phosphate are obtained by deproteinizing the contents of a flask 2 min. after tipping in the hexokinase and glucose.

This method was compared with our own in two types of experiments. The first was planned in order to determine if the reaction mixture and tipping procedure of Copenhaver & Lardy were themselves responsible for the higher P:O ratio. Consequently, their procedure was modified slightly in order to avoid certain technical difficulties inherent in the method which will be discussed below. The flask contents were at room temperature instead of at 0°,

the bath temperature was 25° and differential manometers were used. In addition, ethylenediaminetetraacetic acid was included in the reaction mixture. None of these changes could lower the P:O ratio. Our method of extrapolating the oxygen uptake during the equilibration period was followed. Finally, the phosphorylation was determined by measuring the increase of esterified phosphate instead of the disappearance of inorganic phosphate. It has been shown above that the two methods of measuring the phosphorylation agree. It is clear from the results in Table 12 that Copenhaver & Lardy's procedure and ours give identical results.

In the second type of experiment, Copenhaver & Lardy's method of measuring the oxygen uptake was compared with ours, using the same preparations and our standard reaction mixture. Fig. 3A, curves 1a and 2a show the course of the manometric reading of duplicate flasks when Copenhaver & Lardy's procedure was followed exactly, except that water was tipped in at 3 min. instead of hexokinase and glucose which were already in the main flasks. There is apparently a considerable lag in reaching the maximum rate of oxygen uptake, although it has been shown with the identical reaction mixture and type of preparation that oxidation and phosphorylation proceed at a steady rate without any time lag (Slater & Holton, 1953). That the apparent lag is a manometric artifact is confirmed by the manometric record obtained with a control flask containing only water in the main compartment and treated in the same way as the experimental flask. It is clear that with the

apparatus used in this experiment, the procedure of Copenhagen & Lardy is inadequate to ensure temperature equilibration. When the curves 1a and 2a are corrected for the pressure changes in the control flask, the rates of oxygen uptake are practically identical with that obtained by our usual procedure, using differential manometers (curve 4, Fig. 3B). Curve 5, Fig. 3B, which like curve 3 represents the pressure changes in a control flask containing only water in the main compartment, shows that complete temperature equilibration is obtained in 10 min. with our procedure.

The magnitude of the error in measuring the oxygen uptake by direct calculation from the manometric readings, as in Copenhagen & Lardy's procedure, can be calculated from Fig. 3. The apparent oxygen uptake in 15 min. calculated in this way was 41.4 μ l. (curve 1a) and 39.0 μ l. (curve 2a) for the two flasks. The mean, 40.2 μ l. (3.59 μ atoms) is in the range of oxygen uptakes obtained by Copenhagen & Lardy (3.2–6.8, mean 4.7 μ atoms). The actual oxygen uptake in 15 min. calculated from curve 4, allowing for the threefold difference in

amounts of enzyme preparation and in volume, is 67.8 μ l. = 6.04 μ atoms. Thus the P:O ratio of 2.71 actually obtained by our procedure in this experiment would become $2.71 \times 6.04 / 3.59 = 4.55$ by Copenhagen & Lardy's procedure.

DISCUSSION

Our conclusion that the P:O ratio for the oxidation of α -ketoglutarate to succinate is 3 is at variance with the presently accepted value of 4 (see, for example, Hunter, 1951; Ochoa & Stern, 1952; Krebs *et al.* 1953). It is, however, in agreement with Ochoa's (1944) original assessment.

The difference between our findings and those of other workers could conceivably be due to: (1) a real tissue difference between heart-muscle mitochondria (sarcosomes) and liver mitochondria, which have been used in most of the other work; (2) the method of preparing the mitochondria; (3) the method of measuring the phosphorylation; (4) the method of measuring the oxygen uptake.

(1) The possibility that the P:O ratio for heart-muscle sarcosomes and liver mitochondria are different was given serious consideration, but it now seems unlikely for two reasons: (a) Feldott & Plaut (1953), from the same Institute as Copenhagen & Lardy (1952), have obtained with heart preparations P:O ratios which are essentially the same as those found by the latter with liver mitochondria; (b) our own limited experience with liver mitochondria, prepared by Copenhagen & Lardy's method, does not suggest any difference between the two preparations (P:O with rat-liver mitochondria, 2.50).

(2) The method of preparation of the sarcosomes has been examined both in previous papers (Cleland & Slater, 1953; Slater & Cleland, 1953a, b) and in this paper (p. 30). We have varied the procedure considerably without affecting the P:O ratio. The only feature of our procedure differing from those of other workers is the inclusion in the isolation medium of 0.01 M ethylenediaminetetraacetic acid, which either has no effect on the P:O ratio or raises it slightly. Attempts to reproduce as closely as possible the preparative methods of other workers yielded P:O ratios rather lower than obtained by our method.

In our experience, the activity of the α -ketoglutaric oxidase system is a much more sensitive index of the effects of various treatments of the sarcosomes than is the P:O ratio. The activity (Q_{O_2}) of our preparations is considerably higher than those of other heart preparations (see Table 13).

(3) It is clear from the experiments described in Table 7 that our method of measuring the yield of phosphorylation gives the same results as the more commonly used method of measuring the uptake of

Fig. 3. Measurement of O_2 uptake of the same sarcosomal preparation (sucrose-ethylenediaminetetraacetic acid preparation from rat heart) in the same reaction mixture by Copenhagen & Lardy's procedure (A), and by our procedure (B). The concentrations of reactants were identical in the two cases, but the reaction volume ordinates differ by a factor of 3. Bath temperature, 30°. A. Curves 1a and 2a show the O_2 uptake calculated directly from the manometric readings of duplicate flasks. Curve 3 is calculated from the pressure changes in a flask treated in the same way but containing only water in the main flask, applying the constant of the manometer used for curve 1. Curve 1b is the algebraic sum of curves 1a and 3. Similarly, curve 2b is the algebraic sum of curve 2a and a curve corresponding to curve 3, applying the constant of the manometer used for curve 2. The dotted curve shows the course of the O_2 uptake calculated from B. B. Curve 4, O_2 uptake from 10 min. to 34 min. by usual procedure. Extrapolation to zero time gives O_2 uptake between 0 and 10 min. Curve 5 is calculated from pressure changes in flask containing only water.

Table 13. Activity of α -ketoglutaric oxidase system in heart-muscle preparations

Preparation	Animal	Temp. (°)	[Fluoride] (M)	[Malonate] (M)	Q_{O_2}	Reference
Cyclophorase	Rabbit	38	0	0	33-45	Paul <i>et al.</i> (1952)
Mitochondria	Guinea pig	30	0	0	72	Plaut & Plaut (1952)
Sarcosomes (sucrose- ethylenediaminetetra- acetic acid)	Rat	25	0	0	163*	This paper
		25	0.04	0.01	79†	
	Cat	25	0.04	0.01	76‡	
	Guinea pig	25	0.04	0.01	52§	

* Calculated from mean value in presence of fluoride and malonate, using data of Table 5.

† Mean of sixteen preparations, standard error of mean = 5.5.

‡ Mean of two preparations (74 and 78).

§ Single preparation.

inorganic phosphate. Further, Table 12 shows that our reaction mixture and that of Copenhagen & Lardy (1952) give the same P:O ratio. Thus, it appears certain that the difference in the methods of measuring the phosphorylation is not responsible for the different P:O ratios obtained by the latter and by ourselves.

(4) The most likely explanation of this difference is the method of measuring the oxygen uptake. The manometric apparatus commonly used for determining the oxygen uptake by tissue preparations was designed primarily for the measurement of rates of oxygen uptake and is not very suitable for the accurate measurement of the actual amount of oxygen consumed when the instability of the system does not permit an adequate period for temperature equilibration before beginning the reaction. This difficulty was recognized by earlier workers on oxidative phosphorylation and various devices were used to allow for the oxygen uptake during the equilibration period. Lehninger & Smith (1949) overcame the difficulty by measuring the amount of oxidized product (acetoacetate) with β -hydroxybutyrate as substrate. This method is reliable only when it is certain that the oxidized product is not further metabolized. Many workers (e.g. Lehninger, 1949; Copenhagen & Lardy, 1952) allow 5 min. equilibration period before commencing the manometric readings. This period is far less than that considered desirable in monographs on manometric techniques; for example, both Dixon (1951) and Umbreit (1949) state that 10-15 min. are necessary. It appears particularly inadequate in the case of Copenhagen & Lardy's (1952) procedure, which involves warming a Warburg flask with two side bulbs from 0 to 30° in 5 min. With apparatus of this type available to us, 5 min. was not sufficient for temperature equilibration and the oxygen uptake was grossly underestimated during the first few minutes. Since the total experimental period used by Copenhagen & Lardy was only 15 min., the error in the calculation of the overall oxygen uptake was serious.

Copenhagen & Lardy (1952) specifically state that 'control experiments demonstrate that the 5 min.

pre-incubation period was adequate to obtain thermal equilibrium'. If this is so, the rate of heat transfer in their apparatus was considerably greater than in ours. This may well be the case, and the error in Copenhagen & Lardy's procedure may not be as great as suggested by Fig. 3A; for example, the error would have been considerably less in this experiment if a 6 min. instead of a 5 min. equilibration had been given. However, the long time required for the maximum rate of oxygen uptake to be reached and for a steady reading in a control flask containing only water strongly suggests that a considerable error in measuring the oxygen uptake is inherent in Copenhagen & Lardy's procedure. This is supported by the fact that the mean $\Delta O:\Delta \alpha$ -Kg in their experiments was 0.9 with fluoride, 1.0 without fluoride compared with 1.13 obtained in our experiments with fluoride. The results shown in Fig. 1 of the present paper clearly show that there is considerable oxidation past succinate.

The strongest argument that the difference between Copenhagen & Lardy's (1952) findings and our own lies in the method of measuring the oxygen uptake is the fact that the mean P: α -Kg obtained by these workers (3.3 with fluoride, 3.2 without fluoride) is only very slightly higher than our values. The P: α -Kg is independent of the manometric measurements.

Unless ethylenediaminetetraacetic acid is used, the combination of the pressure development while the flask is coming to thermal development and the progressive inactivation of the α -ketoglutaric oxidase system could conceivably result in a uniform rate of change of the manometric readings. Thus both the failure of thermal equilibration and the instability of the enzyme system might be overlooked.

The other studies which have obtained a P:O ratio greater than 3 were not as detailed as those of Copenhagen & Lardy (1952). Judah (1951) reported only one value (3.4) in the text, without experimental details. Kielley & Kielley's (1951) method of measuring the oxygen uptake is preferable to that of Copenhagen & Lardy (1952) in that the flask contents were at room temperature before adding the cold enzyme, the reaction temperature was 28° and

the equilibration period was 8 min. The reaction time was also usually somewhat longer than Copenhagen & Lardy's (1952). However, it seems possible that the oxygen uptake is also slightly underestimated in these experiments. The P:O ratio in the presence of malonate and hexokinase was determined with only two preparations, values of 3.1 and 3.5 being obtained. Although Barkulis & Lehninger (1951) state that 'the highest P:O ratios obtained for α -ketoglutarate oxidation were somewhat over 3, confirming Hunter & Hixon', the four values given by these authors range from 2.42 to 3.25, with a mean of 2.89. These values appear to be more in line with a P:O ratio of 3.

Hunter (1951) believes that an observed P:O ratio of 3 indicates a true value of 4.0, since 100% recovery of phosphorylation is not likely even in the presence of fluoride. As already discussed (Slater, 1953*b*; Slater & Holton, 1953), we do not believe that such a conclusion is justified. Provided sufficient hexokinase is added, practically all the ATP will react with this enzyme, and an insignificant proportion with the ATP-ase.

Hunter (1951) also supports the view that the P:O ratio is 4 by his finding (Hunter & Spector, 1951) that dinitrophenol eliminates about 75% of the phosphorylation due to α -ketoglutarate oxidation, leaving 25% intact, which is consistent with the idea that there are three phosphorylations sensitive to dinitrophenol and one that is unaffected. However, this is not strong evidence, since the same result would be obtained if two phosphorylations were completely inhibited and the third inhibited by 25% by the concentration of dinitrophenol used. In fact, Judah (1951) found some inhibition by dinitrophenol of the substrate-linked phosphorylation (measured by the anaerobic dismutation of α -ketoglutarate to glutamate and succinate in the presence of ammonia).

Krebs *et al.* (1953) have also concluded that the P:O ratio in the oxidation of α -ketoglutarate to succinate by rat-liver suspensions is 4. The actual values for the ratio, obtained in the absence of malonate or fluoride, were 3.0–3.2. Since an extrapolation procedure similar to that employed in this paper was used to measure the oxygen consumed in the first few minutes of the experiment it is unlikely that the oxygen uptake was underestimated in the experiments of Krebs *et al.* (1953). The cause of the disagreement probably lies in the method of measuring the rate of esterification of inorganic phosphate. Krebs *et al.* do, in fact, use a method different from other workers in this field, namely the measurement of the rate of incorporation of ^{32}P into ATP. While this method has the advantage of completely eliminating the possibility of errors due to hydrolysis of ATP, there is a serious theoretical

objection, which could cause the rate of phosphorylation to be overestimated.

It has been shown by Kaufman (1951) that one of the phosphorylation reactions coupled with the oxidation of α -ketoglutarate to succinate is described by the following reactions (CoA = coenzyme A; DPN^+ = diphosphopyridine nucleotide; DPNH = reduced diphosphopyridine nucleotide):

The P:O ratio (one oxygen atom being equivalent to the reduction of one molecule of DPN) of this part of the α -ketoglutaric oxidase system is 1, and in the steady state each molecule of succinyl-CoA formed by reaction (1) would yield 1 molecule of ATP by reaction (2). However, if k_2 and k_3 are both large in comparison with k_1 , there would be a rapid exchange between inorganic phosphorus and ATP, at a rate unrelated to the rate of synthesis of ATP. In other words, the rate of synthesis of ATP by reactions (1) and (2) (ignoring subsequent reactions of ATP, for example hydrolysis) is given by the expression rate of synthesis of $\text{ATP} = k_2 [\text{succinyl-CoA}] [\text{ADP}] [\text{H}_3\text{PO}_4] - k_3 [\text{succinate}] [\text{CoA}] [\text{ATP}]$, while rate of incorporation of $^{32}\text{P} = k_3 [\text{succinyl-CoA}] [\text{ADP}] [\text{H}_3\text{PO}_4]$. Thus the rate of incorporation of ^{32}P may greatly exceed the rate of synthesis of ATP, especially when $[\text{ATP}]$ is high, as it is in the experiments of Krebs *et al.*

This kinetic treatment is undoubtedly oversimplified, since it is unlikely that termolecular collisions are involved. It is probable that an intermediate, for example CoA-monophosphate, is involved in reaction (2). But the same general conclusion will be arrived at, independently of the detailed mechanism of reaction (2), provided that the overall reaction (2) is reversible, as has been demonstrated by Kaufman (1951).

Reaction (2) is only a particular example of a type of reaction which may be general in oxidative phosphorylation, namely the utilization of the high energy of an intermediate compound to form ATP from ADP and inorganic phosphate. It is likely that the change of free energy in such a reaction is small, i.e. the reaction will be readily reversible. When such reversible reactions involving ATP and inorganic phosphate occur, the rate of incorporation of ^{32}P into ATP will overestimate the rate of oxidative phosphorylation.

Krebs *et al.* find only slow rates of incorporation of ^{32}P into ATP under anaerobic conditions with either α -ketoglutarate or succinate as substrate. How-

ever, experiments of this sort are not adequate controls for the detection of errors of the type just discussed, since the ATP concentration declined rapidly.

A discussion of the mechanism of oxidative phosphorylation is outside the scope of this paper, the purpose of which is to establish the stoichiometry of the reaction. Our conclusions are described by the equation

SUMMARY

1. Variations in the method of preparing heart-muscle sarcosomes had little effect on the yield of oxidative phosphorylation (P:O ratio) coupled with the oxidation of α -ketoglutarate to succinate, although the Q_{O_2} of the α -ketoglutaric oxidase system was considerably affected. The P:O ratio was also little changed if the sarcosomes were mixed with other components of the muscle.

2. For accurate measurement of the P:O ratio, it is necessary to purify the yeast hexokinase pre-

paration sufficiently so that it no longer catalyses an anaerobic reaction of α -ketoglutarate.

3. The further oxidation of succinate is not stopped completely by the addition of 0.01M malonate and 0.04M fluoride. Under these conditions, an average of 1.13 atoms of oxygen were consumed per mole of α -ketoglutarate disappearing.

4. The concentration of all components of the reaction mixture can be varied considerably without affecting the P:O ratio.

5. Measurements of the P:O ratio on thirty-two sarcosomal preparations are presented and analysed. In contrast to recent measurements in other laboratories, but in agreement with Ochoa's (1944) original assessment, it is concluded that the P:O ratio for the oxidation of α -ketoglutarate to succinate is 3.

6. It is suggested that the high values obtained by other workers is due in some cases to underestimation of the oxygen uptake, and in one case to overestimation of the rate of phosphorylation.

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