The Kinetics of the Exchange of the Phosphate Groups of Adenosine Triphosphate During Oxidative Phosphorylation

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In previous studies on oxidative phosphorylation the rate of incorporation of ^{32}P -labelled orthophosphate (^{32}P) into adenosine triphosphate (ATP) was measured under conditions where the amounts of ATP and orthophosphate (P) remained constant (Krebs, Ruffo, Johnson, Eggleston & Hems, 1953; Bartley, 1953; Lee & Eiler, 1953). In some experiments of Krebs *et al.* (1953) (Table 2; Table 3, Expts. 2 and 3; Table 5), after periods of incubation greater than about 10 min., a fall in the rate of incorporation was found which could have been due either to the inactivation of the phosphorylating systems by ageing or to complicating factors not operating in the early stages of incubation.

Experiments described in this paper show that the reduction in the rate of incorporation of ^{32}P into ATP is not a result of inactivation of oxidative phosphorylation but is due to different rates of incorporation into the β and γ phosphate groups.

EXPERIMENTAL

Tissue preparations. Rat-liver mitochondria, washed twice with 0.9% (w/v) KCl, were prepared as described by Bartley & Davies (1954), except that NaOH was not added to the homogenizing medium.

Special chemicals. ATP was prepared from rabbit muscle according to LePage (1949); paper chromatographic analysis by the method of Krebs & Hems (1953) showed that the phosphorus was distributed as follows: ATP 81.5%, adenosine diphosphate (ADP) 11.2%, orthophosphate 7.3%.

All other chemicals were of 'AnalaR' standard. Carrier-free $\text{KH}_2^{32}\text{PO}_4$ (ca. 1 mc) was obtained in aqueous solution from the Atomic Energy Research Establishment, Harwell; it was diluted with water to 10 ml.

Experimental procedures. One of the objects of the experiments was to determine whether the fall in the rate of incorporation of ^{32}P into ATP with time was the result of a deterioration of the oxidative phosphorylation system. Accordingly, two series of conical Warburg manometric vessels were set up; all vessels contained the same medium, viz. 0.5 ml. of 0.02M NaATP; 0.2 ml. of 0.1M potassium phosphate buffer, pH 7.4; 0.2 ml. of 0.02M MgCl_2 ; 0.5 ml. of 0.5M sodium succinate and 0.5 ml. of water. The side arm contained 0.1 ml. of the diluted aqueous solution of $\text{KH}_2^{32}\text{PO}_4$ (ca. $10\mu\text{C}$, equivalent to 1×10^6 counts/min. under our conditions) and the centre well contained 0.2 ml. of 2N-NaOH and filter paper. The vessels were kept in a

mixture of ice and water until the mitochondrial suspension was ready, when 2 ml. of the suspension were added to each vessel. The vessels were attached to the manometers, quickly gassed with oxygen and placed in a rack in such a way that the vessels were immersed in a trough of ice and water. When all were ready they were placed in the water bath at 20° and shaken. In one set of vessels the ^{32}P was tipped at the start of incubation at 20°, whilst in the other set, the tipping was made after 30 min. incubation. The vessels of the first series were removed from the bath after approx. 0, 2, 4, 6 and 8 min. incubation; those of the second series were removed at approx. 0, 2, 4, 6, 8, 16 and 32 min. after the addition of the ^{32}P . Enzymic activities were stopped by placing the vessels in a bath of ice and water and by the immediate addition of 0.5 ml. 30% (w/v) trichloroacetic acid (TCA). The contents of the vessels were centrifuged for 10 min. at 0° and the supernatants were stored at -14° until they were analysed. The TCA extract was used for the quantitative determination of phosphate, ATP, ADP, succinate, malate + fumarate and for the measurement of the radioactivity of the phosphate fractions. The O_2 uptake was measured in all vessels of the second series and its average value was used in the calculation of the phosphorylation quotients (equivalents of organic phosphate formed/atoms of oxygen consumed).

Analytical methods. The compounds containing phosphorus were separated by paper chromatography (Krebs & Hems, 1953) and estimated after wet ashing (Hanes & Isherwood, 1949) by the method of Berenblum & Chain (1938) as modified by Bartley (1953).

Succinate in the TCA extract was estimated directly by the use of succinic dehydrogenase (Krebs, 1937). The sum of fumarate and malate was determined by the method of Nossal (1951) after boiling the extracts for 1 hr. to remove TCA.

Measurements of radioactivity were made in an M6 liquid counter (Veall, 1948) on samples of the paper digest diluted to 10 ml., and corrections were made for isotope decay, dead time and background counts.

Enzymic separation of the P^β and P^γ of ATP. The rate of incorporation of ^{32}P into ATP could not be described by one rate constant (see Fig. 2) and it was of interest, therefore, to find the radioactivity associated with the β and γ phosphorus atoms of ATP. The selective hydrolysis of the P^γ of ATP has been described by Lee & Eiler (1951), who showed that incubation of a potato 'apyrase' preparation with ATP solution at 0° resulted in the appearance of phosphate corresponding to that expected from the quantitative hydrolysis of the P^γ . Above 7°, however, a larger amount of phosphate was liberated than expected for hydrolysis to ADP.

The potato 'apyrase' preparation was made and incubated with ATP at 0 and 40° under the conditions described by Lee & Eiler (1951). Samples of the reaction mixture were removed after various times and the P-containing compounds were separated by paper chromatography and then estimated for their phosphorus content. Storage of the enzyme preparation for 8 weeks at either 2° or -18° caused no appreciable loss of activity (Tables 1 and 2). On incuba-

tion up to 80 min. at 0° the enzyme showed ATPase but no ADPase activity, the amounts of ADP and phosphate formed being approximately the same. This is also true for an incubation period of 10 min. at 40°. On longer periods of incubation at 40°, however, adenosine monophosphate (AMP) and equivalent amounts of phosphate were formed. The change in the amount of ATP was approximately equal to the sum of the changes in the amounts of ADP and AMP.

Table 1. *Hydrolysis of ATP by potato 'apyrase' (Lee & Eiler, 1951) on incubation at 0°*

The incubation mixture with the fresh enzyme contained 0.1 ml. of M sodium succinate buffer, pH 6.5; 0.5 ml. of approx. 0.01M ATP; 0.09 ml. of 0.11M-CaCl₂ and 0.05 ml. of enzyme preparation (49000 units/ml.) and 1.15 ml. of water. In the experiments with the stored enzyme preparation the incubation mixture was the same except that 1.0 ml. of approx. 0.01M ATP and 0.75 ml. of water were used. Samples (0.2 or 0.3 ml.) were removed at various times and analysed for P-containing compounds.

Enzyme preparation	Period of incubation (min.)	Amounts of P-containing compounds* present in the incubation mixture (μmoles/ml.)		
		ATP	ADP	Orthophosphate
Fresh	0	2.00	0.65	0.80
	10	1.95	0.80	0.95
	20	1.60	1.15	1.40
	40	0.85	1.45	2.25
	80	0	2.40	3.20
Stored for 8 weeks at 2°	0	6.45	2.05	1.80
	10	6.20	2.15	2.10
	20	5.80	2.30	2.70
	40	4.95	3.25	3.70
	80	3.20	5.05	5.40
Stored for 8 weeks at -18°	0	6.45	1.90	1.80
	10	5.75	2.60	1.55
	20	5.35	2.90	3.20
	40	4.50	3.60	4.30
	80	2.10	5.95	6.80

* No AMP was detectable on the chromatograms.

Table 2. *Hydrolysis of ATP by potato 'apyrase' (Lee & Eiler, 1951) on incubation at 40°*

Experimental detail as in Table 1.

Enzyme preparation	Period of incubation (min.)	Amounts of P-containing compounds present in the incubation mixture (μmoles/ml.)			
		ATP	ADP	AMP	Orthophosphate
Fresh	0	1.95	0.40	0	1.27
	10	0	2.25	0	3.40
	20	0	2.20	0	3.80
	40	0	1.80	0.40	4.35
	80	0	1.45	0.70	4.70
Stored for 8 weeks at 2°	0	6.45	1.85	0	1.90
	10	3.65	4.55	0	5.00
	20	1.60	6.30	0.2	7.35
	40	0.35	7.05	0.45	9.25
	80	0.20	6.85	0.90	9.60
Stored for 8 weeks at -18°	0	6.50	1.75	0	1.85
	10	2.45	5.60	0	6.20
	20	0.40	7.40	0.2	8.85
	40	0.40	7.25	0.45	8.80
	80	0.25	6.45	1.10	10.20

Thus, these results confirm those of Lee & Eiler (1951) and show that at 0° the only reaction that appeared to take place was

whilst at 40° reaction (1) occurred, accompanied by the reaction,

During the preparation of this paper, Nguyen-Van Thoi, Roche & Tran-Thi, An (1954) published a study of the potato 'apyrase' enzyme in which they showed by paper chromatography that at 37° the hydrolysis of ATP proceeded in two stages, the first product being ADP and the second, AMP. The formation of AMP from ADP only occurred after the total hydrolysis of the terminal phosphate of ATP. The cheapness, ease of preparation and stability of this preparation make it a more convenient tool than myosin for the hydrolysis of the P γ of ATP.

Treatment of media. ATP was separated from the reaction media and the 'apyrase' preparation was then used for the hydrolysis of the P γ in the following way. 1 ml. of the TCA extract was chromatographed as a band according to Krebs & Hems (1953). On removal from the chromatogram trough the paper was washed with dry ether to remove the isobutyric acid of the solvent and to prevent the non-enzymic hydrolysis of the ATP. After drying at room temperature to remove the ether, the area of the paper corresponding to the ATP was indicated as a dark area on illumination of the paper with the 2537 Å Hg line. The phosphate content and radioactivity of a portion of this area were determined. A convenient portion of the remainder was placed in a 10 ml. centrifuge tube with 0.1 ml. of M sodium succinate buffer, pH 6.5; 0.1 ml. of 0.11M-CaCl₂; 1.7 ml. of water and 0.1 ml. of enzyme preparation, and kept at 0° for 80 min. The reaction was then stopped with 0.2 ml. of 30% (w/v) TCA and the mixture centrifuged. The supernatant was chromatographed to separate the phosphates and the specific activities of the P β of ADP and of the inorganic phosphate (originating from the P γ of ATP) were measured.

RESULTS

Incorporation of ³²P into ATP

Washed rat-liver mitochondria were used in an experiment in which ³²P was added either at the start of incubation or after a preliminary incubation of 30 min. Table 3 shows that the amounts of ATP in the vessels remained virtually constant throughout a total incubation period of 62 min. This has also been found with a rat-liver homogenate (Krebs *et al.* 1953).

During the 10 to 60 min. period of incubation the rate of oxygen uptake was constant (0.83 μ atom/min.) (Fig. 1). The amounts of succinate removed, and of fumarate + malate formed, were approximately equivalent, and the average ratio atoms of oxygen consumed/moles of fumarate + malate formed, was 0.93 ± 0.08 (S.D., 5 observations) (Table 4). These results show that the oxygen consumed was mainly due to the reaction,

The mean specific activity of the β and γ phosphorus atoms of ATP + the β phosphorus atom of ADP increased at about the same rate both when the radioactivity was added at the start or after 30 min. incubation (Table 3). This shows that the mechanism for the exchange of the labile P did not deteriorate during this time as is also borne out by the maintenance of the ATP.

A plot of the logarithm of the function

$1 - (\text{mean specific activity of the sum of } P^\beta + P^\gamma \text{ of ATP and } P^\beta \text{ of ADP}) / (\text{mean specific activity of the sum of } P^\beta + P^\gamma \text{ of ATP, } P^\beta \text{ of ADP and inorganic P})$ against time (Fig. 2) shows that after the first 6–8 min. incubation the rate of increase was con-

Table 3. *Incorporation into ATP of ³²P added at the start of incubation of rat-liver mitochondria or after 30 min. pre-incubation*

For details see Fig. 1.

Time when KH ₂ ³² PO ₄ added	Period of incubation (min.) (sec.)	Amounts of orthophosphate in vessel (μ moles)	Total nucleotide P in vessel (μ moles)	Mean specific activity of P β + P γ of ATP + P β of ADP (counts/min./ μ mole P)	Ratio:
					counts/min./ μ mole P β + P γ of ATP + P β of ADP total counts/min./total μ moles exchangeable P
At start of incubation	0 0	13.7	22.0	0	0.00
	2 15	—	21.3	3490	0.43
	4 15	—	21.9	4760	0.60
	6 15	—	22.8	5550	0.73
	8 15	12.4	23.7	5470	0.75
After 30 min. incubation	30 0	12.3	24.3	0	0.00
	32 15	—	23.8	3060	0.43
	34 15	—	22.2	4500	0.59
	36 15	—	21.8	5730	0.71
	38 15	—	21.6	6000	0.77
	46 15	—	22.3	6620	0.88
	62 15	12.4	21.7	7300	0.94

stant but lower than initially. When a single straight line is obtained in such a graph the exchange can be described by a single first-order rate constant. Since Fig. 2 shows clearly the presence of

at least two linear components, the incorporation of ^{32}P into ATP in this experiment cannot be described by an equation containing only one rate constant as was presumed by Krebs *et al.* (1953) for the cases analysed by Stewart (1953).
To see if the P^β and P^γ of ATP exchanged at the same rate, the specific activities of the P^β and P^γ atoms of ATP were measured separately. The results given in Table 5 show that the ratio of $\text{P}^\gamma/\text{P}^\beta$ fell from 14 at the end of 2 min. to practically unity after 32 min.; hence the rate of incorporation into the β phosphorus was lower than that into the γ phosphorus of ATP.

The calculation of the rate of phosphorylation

The following treatment is an attempt to deduce the rate of exchange of the P^γ of ATP from the total rate of incorporation of ^{32}P into ATP when the

Fig. 1. Oxygen uptake of rat-liver mitochondria. The Warburg vessels contained 0.2 ml. of 0.1M potassium phosphate buffer, pH 7.4; 0.2 ml. of 0.02M-MgCl₂; 0.5 ml. of 0.5M succinate; 0.5 ml. of approx. 0.02M ATP; 0.5 ml. of water and 2.0 ml. of rat-liver mitochondria suspension (dry wt. 45 mg.) in 0.9% (w/v) KCl. The side arms contained 0.1 ml. of KH₂³²PO₄. Gas phase O₂; temp. 20°. The points represent individual values for the number of vessels shown in parenthesis. The Q_{O₂} (μl./mg. dry wt./hr.) was approximately -12.

Fig. 2. The incorporation of ^{32}P into ATP during incubation at 20° with rat-liver mitochondria. Experimental details as in Fig. 1. ^{32}P added after 30 min. incubation. The term $\log \left[1 - \frac{\text{counts/min./}\mu\text{mole } \text{P}^\beta + \text{P}^\gamma \text{ of ATP} + \text{P}^\beta \text{ of ADP}}{\text{total counts/min./}\mu\text{moles total exchangeable P}} \right]$ plotted against time gives a single straight line only when all the labile P exchanges at the same rate.

Table 4. Changes in amounts of succinate, and fumarate + malate in media after incubation of rat-liver mitochondria

Same experiment as described in Fig. 1.

Period of incubation (min.) (sec.)	Amounts found in each Warburg vessel (μmoles)		Oxygen uptake (μatoms)	Ratio: μatoms O ₂ consumed / μmoles fumarate + malate formed
	Succinate	Fumarate + malate		
0 0	239	0	0	0
30 15	—	23.3	24.0	1.0
32 15	—	28.1	26.9	0.96
34 15	—	29.7	27.8	0.94
36 15	—	29.4	27.5	0.94
38 15	—	36.7	35.1	0.96
62 15	170	69.5	53.3	0.77

Table 5. Comparison of the specific activities of P^β and P^γ atoms of ATP after incubation with rat-liver mitochondria

Samples of ^{32}P -labelled ATP from the experiment described in Fig. 1 were hydrolysed at 0° with the potato 'apyrase' of Lee & Eiler (1951). The resultant ADP and orthophosphate were separated by paper chromatography (Krebs & Hems, 1953) and their specific activities determined.

Period of incubation after addition of radioactivity (min.) (sec.)	$\text{KH}_2^{32}\text{PO}_4$ added at start of incubation			$\text{KH}_2^{32}\text{PO}_4$ added after 30 min. incubation		
	Specific activity of ATP (counts/min./ $\mu\text{mole P}$)		Ratio: Specific activity of P^γ Specific activity of P^β	Specific activity of ATP (counts/min./ $\mu\text{mole P}$)		Ratio: Specific activity of P^γ Specific activity of P^β
	P^β	P^γ		P^β	P^γ	
2 15	1060	8780	8.3	1 070	14 900	13.9
4 15	1960	10300	5.2	1 110	10 970	9.9
6 15	2420	13100	5.4	2 670	14 500	5.4
16 15	—	—	—	10 200	25 300	2.5
32 15	—	—	—	15 600	19 300	1.2

rates of incorporation of ^{32}P into the P^β and P^γ of ATP differ.

The analysis applies to a system in which:

(a) The total amounts of ATP (AdPPP), of ADP (AdPP), of AMP (AdP) and of inorganic phosphate (P) remain constant throughout the experiment. Thus the rates of phosphorylation of AdPP and of dephosphorylation of AdPPP are the same (v_1):

(b) The exchange of the P^β is attributed to the myokinase reaction proceeding at the rate v_2 :

(c) When isotopic phosphate (^{32}P) takes part in reaction (3) only the P^γ of AdPPP becomes labelled,

(d) The AdPP^{32}P formed in (5) can take part in the three reactions (6), (7) and (8) catalysed by myokinase. This leads to a shift of ^{32}P from the γ to the β positions:

If the specific activity of phosphate is low the chance of reaction (8) taking place is small. At equilibrium

the amount of AdP^{32}P which has reacted with AdPP to form AdP^{32}PP is proportional to

$$\frac{\text{AdPP}}{\text{AdPP} + \text{AdP}^{32}\text{P}};$$

the amount which has reacted to form $\text{AdP}^{32}\text{P}^{32}\text{P}$ is proportional to $\frac{\text{AdP}^{32}\text{P}}{\text{AdPP} + \text{AdP}^{32}\text{P}}$. Therefore, the

ratio of ($\beta\gamma$)-labelled to β -labelled ATP is $\frac{\text{AdP}^{32}\text{P}}{\text{AdPP}}$ and even with a specific activity of 100 000 counts/min./ $\mu\text{mole P}$ which is double that normally used in this type of experiment the number of molecules of $\text{AdP}^{32}\text{P}^{32}\text{P}$ is only 1 per 10^7 of the number of molecules of AdP^{32}PP . Therefore the amount of radioactivity of ($\beta\gamma$)-labelled ATP may be ignored.

It must be pointed out that v_1 includes aerobic and any anaerobic phosphorylation. Results presented below show, however, that the anaerobic phosphorylation with succinate as substrate is very small (less than 3%) and hence v_1 is taken to represent the rate of oxidative phosphorylation.

Let n_o = total counts in the system, n_ϕ = counts in inorganic phosphate, n_γ = counts in P^γ of ATP, n_β = counts in P^β of ATP, n_δ = counts in P^β of ADP.

Then,

$$n_o = n_\phi + n_\gamma + n_\beta + n_\delta \quad (9)$$

The terminal phosphate of ADP equilibrates with the P^β of ATP by (6) and (7) and in the following it is assumed that the specific activities of the terminal P of ADP and the P^β of ATP are equal throughout the experiment; the error involved in this procedure is discussed later. From reactions (3)–(7) and the condition $n_\beta/\text{ATP} = n_\delta/\text{ADP}$ it follows that:

$$\left. \begin{aligned} \frac{d}{dt}(n_\beta + n_\delta) &= v_2 \left(\frac{n_\gamma}{\text{ATP}} - \frac{n_\beta + n_\delta}{\text{ATP} + \text{ADP}} \right), \\ \frac{d}{dt}n_\gamma &= v_1 \left(\frac{n_\phi}{\text{P}} - \frac{n_\gamma}{\text{ATP}} \right) - v_2 \left(\frac{n_\gamma}{\text{ATP}} - \frac{n_\beta + n_\delta}{\text{ATP} + \text{ADP}} \right). \end{aligned} \right\} \quad (10)$$

and

The solution of the system (10), n_ϕ being eliminated by eqn. 9, is

$$\left. \begin{aligned} \frac{n_\gamma}{n_o} &= c_1 \cdot e^{m_1 t} + c_2 \cdot e^{m_2 t} + \frac{\text{ATP}}{2\text{ATP} + \text{ADP} + \text{P}}, \\ \frac{n_\beta + n_\delta}{n_o} &= c_3 \cdot e^{m_1 t} + c_4 \cdot e^{m_2 t} + \frac{\text{ATP} + \text{ADP}}{2\text{ATP} + \text{ADP} + \text{P}}, \end{aligned} \right\} \quad (11)$$

where m_1 and m_2 are the roots of the quadratic equation

$$m^2 + m \left(\frac{v_1 + v_2}{\text{ATP}} + \frac{v_2}{\text{ATP} + \text{ADP}} + \frac{v_1}{\text{P}} \right) + \frac{v_1 v_2}{\text{ATP} + \text{ADP}} \left(\frac{1}{\text{ATP}} + \frac{1}{\text{P}} \right) + \frac{v_1 v_2}{\text{P} \times \text{ATP}} = 0. \quad (12)$$

Since

$$\frac{n_\gamma}{n_o} = \frac{n_\beta + n_\delta}{n_o} = 0 \text{ at } t = 0,$$

$$c_1 + c_2 = -\frac{\text{ATP}}{2\text{ATP} + \text{ADP} + \text{P}} \quad \text{and} \quad c_3 + c_4 = -\frac{\text{ATP} + \text{ADP}}{2\text{ATP} + \text{ADP} + \text{P}}. \quad (13)$$

So that (11) may be written

$$\left. \begin{aligned} \frac{n_\gamma}{n_o} &= c_1 \cdot e^{m_1 t} - \left[c_1 + \frac{\text{ATP}}{2\text{ATP} + \text{ADP} + \text{P}} \right] e^{m_2 t} + \frac{\text{ATP}}{2\text{ATP} + \text{ADP} + \text{P}}, \\ \frac{n_\beta + n_\delta}{n_o} &= c_3 \cdot e^{m_1 t} - \left[c_3 + \frac{\text{ATP} + \text{ADP}}{2\text{ATP} + \text{ADP} + \text{P}} \right] e^{m_2 t} + \frac{\text{ATP} + \text{ADP}}{2\text{ATP} + \text{ADP} + \text{P}}, \end{aligned} \right\} \quad (14)$$

and the total uptake of radioactivity by the ATP and ADP is described by the equation,

$$\frac{n_t}{n_o} = \frac{n_\gamma + n_\beta + n_\delta}{n_o} = (c_1 + c_3) e^{m_1 t} - \left[c_1 + c_3 + \frac{2\text{ATP} + \text{ADP}}{2\text{ATP} + \text{ADP} + \text{P}} \right] e^{m_2 t} + \frac{2\text{ATP} + \text{ADP}}{2\text{ATP} + \text{ADP} + \text{P}}. \quad (15)$$

From eqns. 10 and 13, it follows that

$$\left. \begin{aligned} \left[\frac{dn_\gamma}{dt} \right]_{t \rightarrow 0} &= \frac{v_1}{\text{P}} n_o = n_o \left[c_1(m_1 - m_2) - m_2 \frac{\text{ATP}}{2\text{ATP} + \text{ADP} + \text{P}} \right], \\ \left[\frac{d(n_\beta + n_\delta)}{dt} \right]_{t \rightarrow 0} &= 0 = n_o \left[c_3(m_1 - m_2) - m_2 \left(\frac{\text{ATP} + \text{ADP}}{2\text{ATP} + \text{ADP} + \text{P}} \right) \right], \end{aligned} \right\} \quad (16)$$

so that

$$\frac{v_1}{\text{P}} = (c_1 - c_3)(m_1 - m_2) + m_2 \frac{\text{ADP}}{2\text{ATP} + \text{ADP} + \text{P}}. \quad (17)$$

The phosphorylation velocity, v_1 , may thus be determined from the constants c_1 , c_3 , m_1 and m_2 , and the values of P, ADP and ATP. However, the last term in eqn. 17 is very small compared with the first term so that in practice it may be neglected, hence

$$\frac{v_1}{\text{P}} = (c_1 - c_3)(m_1 - m_2). \quad (18)$$

The constants are determined as follows:

If $\log \left[\frac{n_t}{n_o} - \frac{2\text{ATP} + \text{ADP}}{2\text{ATP} + \text{ADP} + \text{P}} \right]$ is plotted against time, the slope of the straight line, for values of t such that $e^{m_1 t} \ll e^{m_2 t}$, gives the value of m_2 . From eqn. 15, this straight line when extrapolated to zero time gives the value

$$\log I = \log \left[c_1 + c_3 + \frac{2\text{ATP} + \text{ADP}}{2\text{ATP} + \text{ADP} + \text{P}} \right]$$

(where $\log I$ is the intercept) which allows the calculation of $(c_1 + c_3)$ by difference. A plot of

$$\log \left[\frac{n_o}{n_t} - \frac{2\text{ATP} + \text{ADP}}{2\text{ATP} + \text{ADP} + \text{P}} + I e^{m_2 t} \right]$$

against time gives a straight line of slope m_1 . With the values of m_2 and m_1 known, c_3 is calculated from the second eqn. in 16 and c_1 from the values of I and c_3 . The calculation of the four relevant quantities of (18) requires the knowledge of

$$\frac{2\text{ATP} + \text{ADP}}{2\text{ATP} + \text{ADP} + \text{P}},$$

which can always be replaced by $(n_i/n_o)_{t \rightarrow \infty}$, the fraction of total counts in the adenosine polyphosphates when radioactive equilibrium is reached.

Precision and errors. A systematic error may be introduced by assuming the terminal phosphate of ADP to be in equilibrium with the P^β of ATP throughout the experiment.

If the uptake of ^{32}P into the adenosine phosphates is described with sufficient accuracy by a sum of two exponential terms, and if ADP is small compared with ATP—always the case in practice—it may be assumed for the purpose of description of the uptake, that there are two distinct fractions of ADP, one (f) in equilibrium with the P^γ of ATP throughout, the other $(1-f)$ in equilibrium with the

P^β of ATP. Proceeding as in eqns. 10 to 17 it may be shown that

$$\frac{v_1}{P} = \frac{(c_1 - c_3)(m_1 - m_2)}{1 + f \text{ADP/ATP}}. \quad (19)$$

Since ADP reaches equilibrium with the P^γ of ATP through v_2 and with the P^β of ATP through both v_1 and v_2 , f may be replaced by $v_2/(v_1 + 2v_2)$. So if $v_1 = nv_2$, the assumption $f=0$ leads to an overestimation of v_1 by the fraction $\frac{1}{n+2} \cdot \frac{\text{ADP}}{\text{ATP}}$, n being given by

$$n = - \frac{\left(\frac{P}{\text{ATP}} + \frac{P}{\text{ATP} + \text{ADP}} \right)}{\left(\frac{(m_1 + m_2)}{(m_1 - m_2)(c_1 - c_3)} - \frac{\text{ATP} + P}{\text{ATP}} \right)}$$

from eqns. 12 and 18.

Thus if $n \geq 5$ and $\text{ATP} \geq 5 \text{ ADP}$ the overestimation is smaller than 3%. This requirement is met by all the experimental phosphorylation velocities quoted here, as calculated by eqn. 18.

Random errors. It appears from eqn. 18 that the error in v_1 will be that due to the errors in the constants. In practice, if m_1 is at least a few times m_2 , and c_1 a few times c_3 the error in v_1 is given by

$$\frac{\Delta v_1}{v_1} \approx \left\{ \left(\frac{\Delta c_1}{c_1} \right)^2 + \left(\frac{\Delta m_1}{m_1} \right)^2 \right\}^{\frac{1}{2}} \\ \approx \left\{ \left(\frac{\Delta \log I}{I - \frac{2\text{ATP} + \text{ADP}}{2\text{ATP} + \text{ADP} + P}} \right)^2 + \left(\frac{\Delta m_1}{m_1} \right)^2 \right\}^{\frac{1}{2}}.$$

Thus from $\Delta \log I$ and Δm_1 , the errors in $\log I$ and m_1 estimated from the graphs, the error in v_1 and hence in the phosphorylation quotient may be computed. In the experiments considered it was about 5–10% in the different cases.

Experimental verification of the method. A test of the validity of the mathematical treatment is provided by a comparison of the amounts of radioactivity found separately in the P^β and P^γ atoms of ATP (by use of the potato apyrase) with those predicted by the mathematical treatment based on the total radioactivity of ATP.

The curves shown in Fig. 4 represent the calculated values of the ratios, n_t/n_o , n_γ/n_o and $(n_\beta + n_\delta)/n_o$ derived only from experimental values of n_t/n_o by the application of eqns. 14 and 15 (for data on constants see Fig. 3). The points represent experimental values, those for n_γ/n_o and $(n_\beta + n_\delta)/n_o$ being obtained by the enzymic hydrolysis of ATP and they are in reasonably good agreement with the calculated values shown by the curves. Thus, the assumptions made in the mathematical analysis are justified as a first approximation.

Fig. 3. Method of obtaining the rates of incorporation of ^{32}P into ATP. Values of $\log \left[\frac{n_t}{n_o} - \frac{2\text{ATP} + \text{ADP}}{2\text{ATP} + \text{ADP} + P} \right]$ are shown plotted against time. In the upper graph the linear part of the curve for times greater than 10 min. has slope m_2 and, when it is extrapolated to zero time, the intercept on the ordinate is

$$\log \left[c_1 + c_3 + \frac{2\text{ATP} + \text{ADP}}{2\text{ATP} + \text{ADP} + P} \right],$$

from which the value of $c_1 + c_3$ is obtained. In the lower graph, m_1 is the slope of the straight line obtained by plotting values of

$$\log \left[\frac{n_t}{n_o} - \frac{2\text{ATP} + \text{ADP}}{2\text{ATP} + \text{ADP} + P} + \left(c_1 + c_3 + \frac{2\text{ATP} + \text{ADP}}{2\text{ATP} + \text{ADP} + P} \right) e^{m_1 t} \right]$$

against time. For experimental details see text and Table 3.

Fig. 4. The distribution of ^{32}P in the P^β and P^γ of ATP after various periods of incubation with rat-liver mitochondria. The curves represent the following equations:

$$\frac{n_t}{n_o} = (c_1 + c_3) e^{m_1 t} - \left[c_1 + c_3 + \frac{2\text{ATP} + \text{ADP}}{2\text{ATP} + \text{ADP} + \text{P}} \right] e^{m_1 t} + \frac{2\text{ATP} + \text{ADP}}{2\text{ATP} + \text{ADP} + \text{P}},$$

$$\frac{n_\gamma}{n_o} = c_1 \cdot e^{m_1 t} - \left[c_1 + \frac{\text{ATP}}{2\text{ATP} + \text{ADP} + \text{P}} \right] e^{m_1 t} + \frac{\text{ATP}}{2\text{ATP} + \text{ADP} + \text{P}},$$

$$\frac{n_\beta + n_\delta}{n_o} = c_3 \cdot e^{m_1 t} - \left[c_3 + \frac{\text{ATP} + \text{ADP}}{2\text{ATP} + \text{ADP} + \text{P}} \right] e^{m_1 t} + \frac{\text{ATP} + \text{ADP}}{2\text{ATP} + \text{ADP} + \text{P}},$$

in which the constants (c_1 , c_3 , m_1 and m_2) were obtained from Fig. 3. The points show the amounts of ^{32}P in the exchangeable P of ATP and ADP as determined experimentally.

Application of the method to the results for succinate oxidation

The method developed in the preceding section has been applied to the data of Table 3. Fig. 3 shows the required plots. The values of the constants are as follows:

$$m_1 = -0.44 \text{ min.}^{-1}; \quad m_2 = -0.046 \text{ min.}^{-1}; \\ c_1 = -0.37; \quad c_3 = +0.034.$$

The value of the phosphorylation quotient obtained from this analysis of the results by the use of eqn. 18 was thus 2.4.

The fall in rate of incorporation of ^{32}P into ATP with time found with mitochondria (Table 3) was also found by Krebs *et al.* (1953) with succinate as substrate using rat-liver suspension but not when pigeon-breast muscle homogenate was used. In the

latter case the β and γ phosphate groups of ATP exchanged with inorganic phosphate at the same rate. Analogous falls in the rate of exchange which have been found previously with pyruvate and citrate as substrate (Bartley, 1953; Krebs *et al.* 1953) are reconsidered below.

Anaerobic phosphorylation with succinate. Whether v_1 is a measure of oxidative phosphorylation depends on the absence of anaerobic phosphorylation under the test conditions. The ATP content during prolonged anaerobic incubation with mitochondria falls because of the presence of phosphatases. The ATP breakdown was slight, however, during short periods of anaerobic incubation (1–2 min.) and hence these were used in measuring the anaerobic incorporation of ^{32}P into ATP. For example, in a typical experiment in which no breakdown of ATP was detectable, the anaerobic incorporation of ^{32}P into ATP in 2 min. of incubation, was only 3% of that incorporated aerobically (see also Lee & Eiler, 1953; Krebs *et al.* 1953). Thus, anaerobic phosphorylation can have contributed little to the incorporation of ^{32}P into ATP when succinate was the substrate.

Phosphorylation quotient for the oxidation of pyruvate. In an experiment with pyruvate as the substrate and sheep-kidney cyclophorase the ratio oxygen consumption/pyruvate disappearance was 2.5 indicating complete oxidation of the pyruvate removed. The experiment satisfied the necessary criteria for the steady state in that the amounts of ATP and orthophosphate remained constant, but application of the formula of Krebs *et al.* (1953) and Stewart (1953) gave values of v_1 which rapidly declined. Thus, the apparent phosphorylation quotient was about 2 at 2 min. and fell to about 1 after 10 min. incubation and plotting the function

$$\log \left[\frac{n_t}{n_o} - \frac{2\text{ATP} + \text{ADP}}{\text{P} + 2\text{ATP} + \text{ADP}} \right]$$

against time gave a graph with a change of slope at about 5 min. The application of eqns. 15 and 16 showed $c_1 = -0.18$, $c_3 = +0.012$, $m_1 = -0.47 \text{ min.}^{-1}$ and $m_2 = -0.034 \text{ min.}^{-1}$. The other values (in μmoles) were as follows: $2\text{ATP} = 9.56$; $\text{P} = 27.1$; $\text{ADP} = 0.94$, hence $\mu\text{moles P exchanged/min./vessel} = 2.42$. The oxygen uptake was constant at $0.91 \mu\text{atom/min.}$; hence the phosphorylation quotient = 2.7. This value is somewhat above that of 2.2 found by Bartley (1953) in similar experiments but it is still below the value of 3 which has been proposed on theoretical grounds (see Ochoa & Stern, 1952).

Application of the method to other results. The phosphorylation quotients given for the oxidation of succinate by Krebs *et al.* (1953) (e.g. Table 2; Table 3, Expts. 2 and 3) were lower for longer periods of incubation than for short ones. The

Table 6. *Phosphorylation quotients of succinate, pyruvate and citrate*

Besides the experiments described in this paper, experiments selected from the paper of Krebs *et al.* (1953) which showed an apparent fall in phosphorylation quotient for liver homogenates with time have been subjected to the mathematical analysis.

Substrate	Values of phosphorylation quotient Krebs <i>et al.</i> (1953)		m_1/m_2^*	Value of phosphorylation quotient calculated by method described	Reference
	Mean of first 3 values	Mean of last 3 values			
Succinate	—	—	9.5	2.4	This paper
Succinate	2.1	1.2	8.9	2.2	Krebs <i>et al.</i> (1953), table 2
Succinate	2.3	1.0	17	2.6	Krebs <i>et al.</i> (1953), table 3, expt. 3
Pyruvate	—	—	14	2.7	This paper
Citrate	3.6	2.4	6.0	4.2	Krebs <i>et al.</i> (1953), table 5, expt. 1
Citrate	3.3	3.0	6.1	3.4	Krebs <i>et al.</i> (1953), table 5, expt. 2
Citrate	3.1	2.2	4.7	3.5	Krebs <i>et al.</i> (1953), table 5, expt. 3

* See text.

uptake of radioactive P in the adenosine polyphosphates in those experiments may be described within 1% by a sum of two exponentials which was derived from a treatment of the data by the method described. Values of v_1 for these results for the oxidation of succinate to fumarate now yield phosphorylation quotients for the whole period of incubation which agree with those obtained in the present work and with that of 2.3 (mean of six values) given by Krebs *et al.* (1953) in Table 3, Expt. 1, where a decline in the phosphorylation quotient did not occur.

Similar treatment of the data of Krebs *et al.* (1953) (Table 5; Expts. 1–3) when citrate was the substrate gives phosphorylation quotients of 3.4–4.2 (Table 6).

DISCUSSION

The measurement and values of phosphorylation quotients

The results show that the fall in the rate of incorporation of ^{32}P into ATP with time is due to a slower exchange rate for the P^β atom than the P^γ atom. It is probable, therefore, that different rates of incorporation into the P^β and P^γ atoms of ATP occurred in some previous experiments from this laboratory and that uncoupling of the supply of respiratory energy from the synthesis of ATP was not the cause of the decline in the phosphorylation quotient observed by Bartley (1953) and by Krebs *et al.* (1953).

The re-calculated phosphorylation quotients for Table 2 and Table 3, Expt. 3 of Krebs *et al.* (1953), where the reaction apparently was almost completely succinate $\rightarrow$ fumarate, agree with the value reported here with a washed mitochondrial suspension. Since the experiments of Krebs *et al.* (1953) were with unwashed homogenates, the agreement illustrates the important point that no

phosphorylating activity was lost during the isolation of the mitochondria from the liver homogenate. A similar observation was made by Slater & Holton (1954) with muscle sarcosomes.

Lee & Eiler (1953) have also measured the steady-state incorporation of ^{32}P into ATP but concluded the phosphorylation quotient for succinate oxidation to be 1.0. Fluoride and phenobarbitol were present in their incubation medium, however, and may account for the low value. Employing a method measuring the net synthesis of ATP, Slater & Holton (1954) have stated that their unpublished results also show a phosphorylation quotient of 1.0 for the oxidation of succinate. It should be noted that in the experiments giving low values, fluoride was always present and this may have had unknown effects on the metabolism of the mitochondria.

The value of 2.4 for the phosphorylation quotient for succinate oxidation is significantly above 2.0. There is no *a priori* reason why the quotient should be a whole number and this result may mean that there is not a simple stoichiometric relationship between electron transport and the phosphorylation of ADP (see Davies & Krebs, 1952).

The rates of exchange of the P^β and P^γ of ATP. The ratio, m_1/m_2 , gives an estimate of the rate of incorporation of ^{32}P into the P^γ compared with that into the P^β of ATP, and Table 6 shows that the values with succinate as substrate may vary from 9 to 17 in conditions where the values of the phosphorylation quotients are very similar. This indicates differences in the rate, v_2 , which were probably a result of varying amounts of myokinase in the preparations used.

The radioactivity in the P^β of ATP may be due to the myokinase reaction. In such a case, ADP labelled in the terminal P would be an obligatory intermediate and at all times during the uptake of ^{32}P the specific activity of the P^β of ADP would be

between that of P^{β} and P^{γ} of ATP. However, if the myokinase reaction has an intermediate complex of the type AdPP-PPAd it is possible that P^{β} and P^{γ} of ATP exchange directly without free labelled AdPP being an intermediate according to the scheme

The importance of this reaction could be assessed by a study of the radioactive uptake into P^{β} of ATP and the terminal P of ADP from the P^{γ} of ATP using purified myokinase.

Applicability of the method to other systems. It is clear from the derivation of the equations that this treatment may also be applied to any similar three-compartment system in equilibrium, where one compound participates simultaneously at different rates in two reactions. Sheppard (1948) has made an analysis of the three-compartment system in which, to find two rates in one experiment, the use of two radioactive varieties of the exchanging material is required. A system in which reaction rates in equilibrium conditions could be investigated in this way is the equilibrium of citric, isocitric and aconitic acids in the presence of aconitase.

SUMMARY

1. The incorporation of ^{32}P -labelled orthophosphate into ATP in a steady state during the oxidation of succinate to fumarate by washed suspensions of rat liver mitochondria has been studied. The P^{γ} became radioactive faster than the P^{β} of ATP and the total uptake of ^{32}P could be described by the sum of two exponential terms.

2. A method for the calculation of the rate of the reaction $\text{ADP} + \text{P} \rightarrow \text{ATP}$ and the phosphorylation quotient based on the time course of the uptake of total radioactive P is described. The amount of ^{32}P

incorporated into the P^{β} and P^{γ} as calculated by this method agrees well with the quantities determined experimentally.

3. The method has been applied to the results of Krebs *et al.* (1953) for the oxidation of succinate and citrate and to those obtained in the present work for succinate and pyruvate oxidation. Within an estimated error of 5–10%, the phosphorylation quotient in the conversion of succinate into fumarate was 2.4; for the complete oxidation of pyruvate it was 2.7.

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The Determination of Iron in Plasma or Serum

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Iron circulates in blood plasma as a complex with β_1 -globulin (Schade & Caroline, 1946) which is neither dialysable nor ultrafiltrable (Barkan, 1927). Most methods for estimating this iron involve three stages, (i) incubation of the plasma or serum with dilute hydrochloric acid for periods of 15 min. to several hours to separate the iron from its protein,

(ii) precipitation of plasma proteins with trichloroacetic acid, (iii) formation of a coloured complex with reagents such as *o*-phenanthroline or 2:2'-dipyridyl (Hemmeler, 1951). Recently, Ramsay (1953) has described a method in which the plasma proteins are coagulated in the presence of a buffered solution containing a reducing agent and 2:2'-dipyridyl. In this way a coloured complex is formed under conditions which render reversibility of the

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