Effect of Adenosine Diphosphate on the Exchange of Oxygen between Inorganic Phosphate and Water Catalyzed by Digitonin Particles*

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ABSTRACT: The addition of ADP to a reaction medium containing oxidizable substrate, inorganic phosphate, $H_2{}^{18}O$, and digitonin particles from rat liver produces a large increase in the extent of the P_i-H_2O exchange. The magnitude of this increase is greater than can be accounted for by way of hydrolysis of the newly formed ATP and is attributed to a requirement for ADP in the P_i-H_2O exchange. A mechanism for oxidative phosphorylation is proposed that can readily explain both the observations presented in this paper and other previous observations that were not well understood. This mechanism suggests that the first high-energy form of phosphate produced during oxidative phosphorylation is ATP. An effort was made to prove this mechanism by use of particles free of adenine nucleotides.

Digitonin particles from rat liver mitochondria were found to contain measurable quantities of AMP, ADP, and ATP. Attempts to alter the levels of these nucleotides in a reproducible fashion were unsuccessful.

The process of oxidative phosphorylation is thought to occur in several discrete stages. All of the systems that are used to study this process can also carry out four exchange reactions that may reflect one or more of these stages. These are: (a) P_i-ATP, (b) ADP-ATP, (c) P_i-H_2O, and (d) ATP-H_2O. The chief reason for relating these exchange reactions to the events occurring during ATP synthesis is their comparable susceptibility to a variety of uncoupling agents. One drawback to this interpretation is that the rates of two of the exchange reactions (P_i-ATP and ADP-ATP) are much slower in digitonin submitochondrial particles than the maximum rate of ATP formation (Cooper and Lehninger, 1957a; Kulka and Cooper, 1962). In addition, Chance (1961a,b) has pointed out that the reversal of oxidative phosphorylation, as measured by the reduction of NAD and the simultaneous oxidation of cytochrome *c* in the presence of ATP, appears to be considerably faster in digitonin particles than is the P_i-ATP exchange.

There are reasons for not putting much emphasis on the relative rates of the P_i-ATP exchange and oxidative phosphorylation. Kaziro *et al.* (1962) have reported studies with a highly purified propionyl carboxylase that carries out the following reactions:

$$\text{ATP} + \text{CO}_2 + \text{enzyme} \overset{\text{Mg}^{2+}}{\rightleftharpoons} \text{ADP} + \text{P}_i + \text{enzyme} \sim \text{CO}_2 \quad (1)$$

$$\text{enzyme} \sim \text{CO}_2 + \text{propionyl CoA} \rightleftharpoons \text{enzyme} + \text{methylmalonyl CoA} \quad (2)$$

They found that the P_i-ATP exchange catalyzed by this enzyme was slower than the overall back reaction and attributed the slower exchange to a competition between ADP and ATP for a site on the enzyme. Scrutton *et al.* (1965) made a similar finding with pyruvate carboxylase that catalyzes reactions (3) and (4).

$$\text{ATP} + \text{CO}_2 + \text{enzyme} \underset{\text{Mg}^{2+}}{\overset{\text{acetyl CoA}}{\rightleftharpoons}} \text{ADP} + \text{P}_i + \text{enzyme} \sim \text{CO}_2 \quad (3)$$

$$\text{enzyme} \sim \text{CO}_2 + \text{pyruvate} \rightleftharpoons \text{enzyme} + \text{oxalacetate} \quad (4)$$

From these considerations it must be concluded that a direct relationship between the four exchange reactions mentioned previously and oxidative phosphorylation cannot be excluded on the basis of differences in rates and that any proposed mechanisms for the overall process must be able to explain the observations made on the exchange reactions.

The mechanism for oxidative phosphorylation receiving the widest consideration at present is shown below.

Mechanism I

$$\text{E} + \text{reduced carrier} \underset{\text{transfer}}{\overset{\text{electron}}{\rightleftharpoons}} \text{E}^* + \text{oxidized carrier} \quad \text{(I-1)}$$

* From the Department of Biochemistry, Western Reserve University, Cleveland, Ohio. *Received October 7, 1964.* This work was supported by research grants (GM-5302 and GM-10446) from the National Institutes of Health.

† Career Development Awardee, U.S. Public Health Service.

$$E^* + P_i \rightleftharpoons E \sim P + H_2O \quad \text{(I-2)}$$

$$E \sim P + ADP \rightleftharpoons E\text{-}ATP \quad \text{(I-3)}$$

$$E\text{-}ATP \rightleftharpoons E + ATP \quad \text{(I-4)}$$

Equation (I-1) is meant to represent an intimate relationship between electron transfer and the availability of E without specifying any details of this relationship.

Our findings with digitonin particles that the P_i-ATP and ADP-ATP exchanges behave in an identical fashion under all experimental circumstances and have the same rates, and that inorganic phosphate is required for ADP-ATP exchange led us to consider an alternative mechanism (Cooper and Kulka, 1961; Kulka and Cooper, 1962). This was further supported by an earlier finding that ADP may be required for the P_i-ATP exchange (Cooper and Lehninger, 1957b).

Mechanism II

$$E + \text{reduced carrier} \underset{\text{transfer}}{\overset{\text{electron}}{\rightleftharpoons}} E^* + \text{oxidized carrier} \quad \text{(II-1)}$$

$$E^* + P_i + ADP \rightleftharpoons E^{*}\begin{matrix}\diagup P_i \\ \diagdown ADP\end{matrix} \quad \text{(II-2)}$$

$$E^{*}\begin{matrix}\diagup P_i \\ \diagdown ADP\end{matrix} \rightleftharpoons E\text{-}ATP + H_2O \quad \text{(II-3)}$$

$$E\text{-}ATP \rightleftharpoons E + ATP \quad \text{(II-4)}$$

Both of these mechanisms can explain the observed exchange reactions and, if the assumption is made that reaction (I-4) or (II-4) is the rate-limiting step, both can explain the observation that the P_i-H_2O exchange is considerably more rapid than the P_i-ATP and ADP-ATP exchanges. Mechanism II, but not mechanism I, allows for a simple explanation of another experimental observation. Cohn and Drysdale (1955) and Chan *et al.* (1960) found with mitochondria and submitochondrial particles, respectively, that there is more oxygen exchanged between ATP and water than can be explained of the basis of the sequence $H_2O \rightarrow P_i \rightarrow ATP$. If the assumption is made that reaction (II-3) is the most rapid, these observations may be readily explained.

In an effort to distinguish between these two possibilities we turned to an examination of the effect of ADP on the P_i-H_2O exchange. If the first mechanism is correct, and if E* is generated by the oxidation of a substrate, then the P_i-H_2O exchange should occur via reactions (I-1) and (I-2) and no ADP should be necessary. The addition of ADP might be expected either to depress or perhaps not affect the extent of the reaction but not to stimulate it. In contrast, mechanism II requires the presence of ADP in order for the P_i-H_2O exchange to take place (via reactions II-1, II-2, and II-3). Under conditions in which the concentration of ADP is suboptimal the addition of ADP might be expected to increase the extent of this reaction. It will be shown later that under appropriate experimental conditions ADP will produce a marked stimulation of the P_i-H_2O, exchange, thus lending further support to mechanism II.

Experimental Procedures

Digitonin particles were prepared from rat liver mitochondria (Cooper and Kulka, 1961) and adjusted to contain 3.6 mg of particle protein per ml before use. Protein was determined by the method of Lowry *et al.* (1951), acetoacetate as described by Walker (1954), and dehydroascorbate according to Schaffert and Kingsley (1955). Phosphate uptake and P_i-ATP exchange were measured as previously described (Cooper and Lehninger, 1956a).

The reaction was carried out in 15 × 125-mm test tubes in a water bath at 22° with an oscillating platform. The reaction was terminated by the addition of 0.10 ml of 65% trichloroacetic acid, and the mixture was then centrifuged. Following removal of suitable aliquots for analytical estimations the remainder was extracted five times with 5-ml portions of ether. The extracted reaction medium was then placed on Dowex 1 (Cl^-) columns (0.5 × 8 cm); the columns were washed with 10 ml water and the inorganic phosphate was eluted with 4.0 ml 0.06 N HCl. When high levels of nucleotides were present (10^{-3} M and above) the eluted fraction was treated with charcoal to remove the AMP, and the charcoal was washed to remove the bulk of the absorbed inorganic phosphate. The inorganic phosphate fraction was concentrated to 0.3 ml on a Rotary Evapomix, the phosphate was precipitated as $MgNH_4PO_4$ and converted to H_3PO_4 using Dowex 50 (H^+), and the H_3PO_4 was isolated as KH_2PO_4 as described by Dempsey *et al.* (1963). The ^{18}O content of the KH_2PO_4 was determined by the guanidine hydrochloride method of Boyer *et al.* (1961) using a Consolidated Electrodynamics Corp. Model 21-130 mass spectrometer. The extent of the P_i-H_2O exchange was calculated by method 3 as described by Dempsey *et al.* (1963). No corrections were made for adenosine triphosphatase activity.

Bound nucleotides were assayed with an Aminco-Bowman spectrophotometer by a modification of the method of Imai *et al.* (1964) in the following manner. A suspension of digitonin particles containing 10–30 mg of protein was centrifuged 45 minutes at $10^5 \times g$. The pellet was extracted for 1 hour at 0° with 1.0 ml of 0.5 N $HClO_4$ per 20 mg of protein. The suspension was centrifuged, the supernatant was neutralized with KOH and chilled at 0° for 1 hour, and the precipitate of $KClO_4$ was removed by centrifugation. A control solution was prepared by carrying out the same procedure with water in place of enzyme. The supernatant was assayed for ATP, ADP, and AMP with a crude firefly luciferase prepared as described by McElroy

(1963) up to, but not including, the treatment with calcium phosphate gel. The stock enzyme was kept frozen in 1-ml aliquots and a freshly prepared solution containing 1 ml enzyme, 2 ml 0.1 M Tris, *p*H 7.5, 1 ml 0.1 M $MgSO_4$, 3 ml 1% bovine serum albumin, and water to a final volume of 10 ml was used for each assay. ATP was assayed by using a 0.3-ml aliquot of unknown in a total volume of 1.5 ml and adding 1 ml of enzyme rapidly with a syringe. A sample containing 0.3–0.5 mμmole of ATP was generally employed. A series of standards containing appropriate amounts of the control solution were assayed just prior to the unknown solution. The ADP was assayed by incubating an aliquot of extract with 0.002 M phosphoenolpyruvate (trisodium salt), 0.0025 M $MgSO_4$, 0.0075 M KCl, 0.005 M Tris, *p*H 7.5, and 2 enzyme units (μmoles/min) of pyruvic kinase in a final volume of 2.0 ml for 15 minutes at 22°. A 0.2-ml portion was removed and assayed for ATP as described above. A standard curve was made by using known amounts of ADP plus suitable amounts of control solution. The ADP content is the difference between the value obtained and the ATP content determined initially. AMP was measured by including 4 enzyme units of adenylate kinase in the incubation medium described for ADP. A standard curve was made by using known amounts of AMP (0.5–2.5 mμmoles) and ATP (0.5–2.5 mμmoles), and suitable amounts of control solution. The value obtained represents ATP + ADP + AMP. The accuracy of the method is estimated to be ±10%. Inhibition was produced by the $KClO_4$ present in the extracts and it was therefore necessary to add the control solution to the standards. Experiments were also carried out using trichloroacetic acid and known levels of nucleotides, and analysis was carried out following removal of the acid by ether extraction. A much greater variability was obtained in this case. The tricyclohexylamine salt of phosphoenolpyruvate was found to give low values and therefore the trisodium salt was used routinely. The activity of the diluted luciferase solution was found to decrease when kept at 0° for more than 4–5 hours and standards were determined before and after the unknown to be certain that low values were not obtained for this reason.

Nucleotides were obtained from Pabst Laboratories and the enzymes from California Foundation for Biochemical Research. $H_2{}^{18}O$ was purchased from Yeda Research and Development Co., Ltd., Rehovoth, Israel, and was distilled prior to use. The $H_3{}^{32}PO_4$ came from the Oak Ridge National Laboratories.

All of the data presented, except for Table I, represent the total change observed in the complete incubation medium. The values given for the P_i-$H_2{}^{18}O$ exchange refer to μmoles of H_2O exchanging with inorganic phosphate.

Results

Mechanism II requires the presence of ADP and ATP for the P_i-H_2O exchange to occur. Chan *et al.* (1960) reported earlier that a digitonin particle preparation

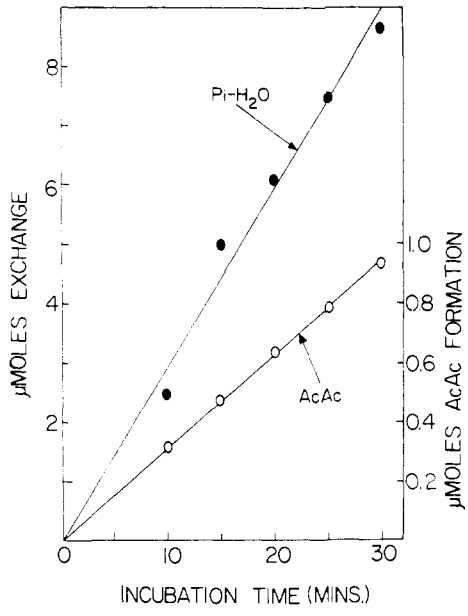

FIGURE 1: Effect of incubation time on acetoacetate formation and P_i-H_2O exchange. Medium contained 0.01 M inorganic phosphate, 0.02 M DL-β-hydroxybutyrate, and 1.80 mg particle protein in a final volume of 2.4 ml, with a final *p*H of 6.8. The water in the medium contained 1.387 atom % excess ^{18}O.

catalyzes such an exchange when supplemented with either ATP or an oxidizable substrate. In the latter case no added nucleotides were required and the authors concluded that ATP was not a requirement for the exchange. It was therefore of interest to learn (a) whether particles contain bound nucleotides, and (b) whether the exchange can occur in the complete absence of bound nucleotides.

Bound Nucleotides. Table I shows results of analyses of bound ATP, ADP, and AMP and the inability to produce significant variations in the relative amounts by some obvious treatments. The specific activity of the P_i-ATP exchange was used as an index of whether major changes were produced by the treatment employed. Attempts were also made to remove completely or alter the ratio of the bound nucleotides by methods that have been successfully employed to remove adenine nucleotides from actin (Bárány *et al.*, 1961). Treatment with charcoal and various resins produced highly variable results possibly owing to a removal of bound NAD and Mg^{2+} as well (Cooper and Lehninger, 1956b). Attempts to produce a particle devoid of bound nucleotide but still capable of oxidative phosphorylation or exchange reactions were abandoned.

Effect of Added ADP. If mechanism II is correct, and if the binding site for ADP is not saturated by bound ADP, the addition of ADP should produce an increase in P_i-H_2O exchange until saturating levels of ADP are reached. Figure 1 shows that both oxidation of β-hydroxybutyrate and P_i-H_2O exchange are linear over a 30-minute period. A 25-minute incubation period

TABLE I: Bound Adenine Nucleotide Content of Digitonin Particles. [a]

Expt	Additions	Nucleotide Content (mμmoles/mg protein) ATP	ADP	AMP	P_i-ATP Exchange (mμmoles/min per mg protein)
1	None	0.27	0.79	0.72	16.6
	0.003 M $MgSO_4$	0.29	0.77	0.65	13.3
	5×10^{-4} M dinitrophenol	0.27	0.85	0.63	14.9
2	None	0.38	0.84	1.30	20.7
	0.0025 M $MgSO_4$, 0.05 M glucose, 10 enzyme units hexokinase	0.36	0.92	1.46	13.7
3	None	0.32	0.67	1.73	22.6
	0.02 M β-hydroxybutyrate, 0.01 M inorganic phosphate	0.40	0.72	1.64	20.9

[a] Digitonin particles were incubated for 20 minutes at 22° with the additions indicated. The concentration of particles during the incubation was 2–3 mg/ml. Following the incubation the suspension was centrifuged for 30 minutes at 100,000 × g and the precipitate was assayed for nucleotide content and P_i-ATP exchange as described in the text.

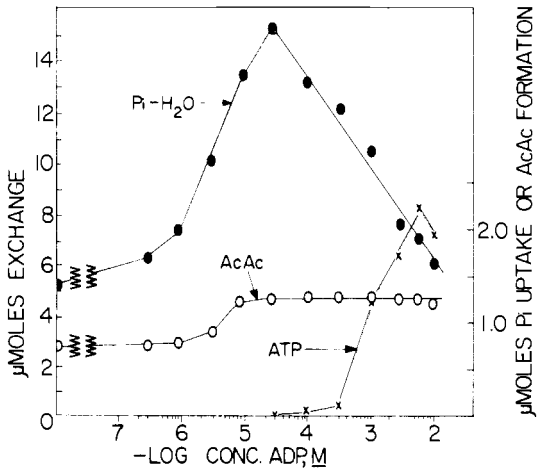

FIGURE 2: Effect of ADP concentration on P_i-H_2O exchange, acetoacetate and ATP formation for span β-hydroxybutyrate to oxygen. Medium was the same as in Figure 1 except that it also contained 7.14×10^6 cpm $^{32}P_i$.

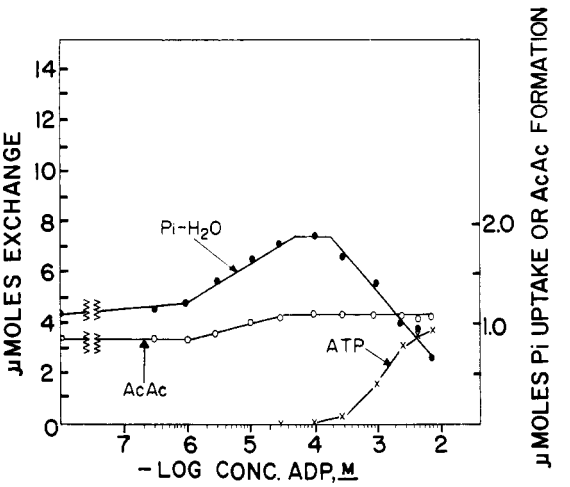

FIGURE 3: Effect of ADP concentration on P_i-H_2O exchange, acetoacetate and ATP formation for span β-hydroxybutyrate to ferricyanide. Medium was the same as in Figure 1 except that it also contained 7.33×10^6 cpm $^{32}P_i$ and 5×10^{-4} M NaCN. $K_3Fe(CN)_6$ was added to the medium in 0.5-μmole portions at 0, 5, 10, 15, and 20 minutes.

was selected for all experiments. The effect of added ADP is shown in Figure 2. The following should be noted: (1) Low levels of added ADP cause a pronounced increase in the P_i-H_2O exchange. (2) Similar amounts of ADP also produce a significant rise in acetoacetate formation thus demonstrating the existence of a degree of respiratory control in this preparation. (3) ATP does not appear until significantly higher levels of ADP are added. (4) There is a sharp decline in the P_i-H_2O exchange that coincides with the appearance of ATP.

Table II (expt 1) shows that 1.2 μg/ml of oligomycin completely abolishes the P_i-H_2O exchange and also blocks the enhancement of acetoacetate formation produced by added ADP. Experiment 2 of Table II demonstrates that ADP alone, in the absence of added substrate, produces a negligible effect on the P_i-H_2O exchange. This very slight increase is most probably attributable to ATP formation via adenylate kinase. The ATP formed in this manner may either support an exchange or undergo hydrolysis, but in either case there would be some enhancement of the ^{18}O content of the inorganic phosphate.

TABLE II: Inhibition of ADP Effect on P_i-H_2O Exchange by Oligomycin and Omission of Substrate.[a]

Expt	Additions	P_i-H_2O Exchange (μmoles)	Acetoacetate Formation (μmoles)
1	None	0.36	0.01
	0 02 M β-hydroxybutyrate	6.17	0.93
	0 02 M β-hydroxybutyrate, 3×10^{-4} M ADP	14.6	1.50
	0.02 M β-hydroxybutyrate, 3×10^{-4} M ADP, 3 μg oligomycin	0.30	1.02
2	None	0.30	0.02
	0.02 M β-hydroxybutyrate	4.83	0.74
	0 006 M ADP	0.69	0 02

[a] Medium contained 0.01 M inorganic phosphate, 1.80 mg protein, and the indicated additions in a final volume of 2.4 ml, with a final *p*H of 6.8. The water contained 1.387 atom % excess ^{18}O. Incubated 25 minutes.

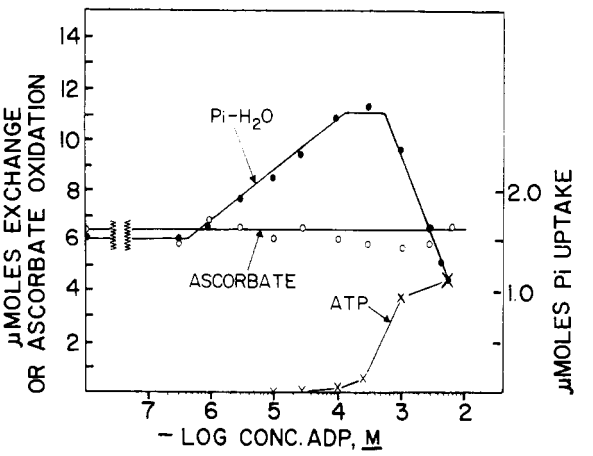

FIGURE 4: Effect of ADP concentration on P_i-H_2O exchange, ascorbate oxidation, and ATP formation for span cytochrome *c* to oxygen. Medium was the same as in Figure 1 except that 4×10^{-5} M cytochrome *c* plus 0.15 M ascorbate replaced the β-hydroxybutyrate, and 7.24×10^6 cpm $^{32}P_i$ was present.

Attempts were made to learn whether the P_i-H_2O exchange can occur when electron transport is restricted to only portions of the chain. Figure 3 shows the results obtained when β-hydroxybutyrate is oxidized in the presence of cyanide with ferricyanide serving as the electron acceptor. The same rise in P_i-H_2O exchange and acetoacetate formation is observed at low concentrations of added ADP. In addition there is the sharp decline of P_i-H_2O exchange that occurs concomitantly with the appearance of ATP. The results of an experiment in which the oxidizable substrate is ascorbate plus cytochrome *c* may be seen in Figure 4. In this case also there is a rise in P_i-H_2O exchange at low levels of added ADP and a sharp drop at the higher levels when ATP appears. In this particular experiment no stimulation of oxidation was produced by added ADP, but in other experiments, with lower initial rates of oxidation, increases varying between 40 and 100% were obtained.

It is quite clear from the results in Table III that the rise in P_i-H_2O exchange produced by the addition of low levels of ADP cannot be produced by IDP and that the addition of 3×10^{-5} M IDP along with the ADP does not produce an inhibition.

TABLE III: Inability of IDP to Produce Stimulation of P_i-H_2O Exchange.[a]

Additions	P_i-H_2O Exchange (μmoles)
None	5.9
10^{-6} M ADP	8.3
10^{-6} M IDP	6.0
10^{-6} M ADP + 3×10^{-5} M IDP	8.1
3×10^{-5} M ADP	13.3
3×10^{-5} M IDP	5.9
3×10^{-5} M ADP + 3×10^{-5} M IDP	12.9

[a] Medium contained 0.01 M inorganic phosphate, 0.02 M β-hydroxybutyrate, 1.80 mg protein, and the indicated additions in a final volume of 2.4 ml, with a final *p*H of 6.8. The water contained 1.387 atom % excess ^{18}O. Incubated 25 minutes.

Discussion

There is no question that the addition of low concentrations of ADP to the incubation medium produces a large increase in the apparent P_i-H_2O exchange. This increase begins when the concentration of added ADP is approximately equal to the concentration of bound ADP present in the reaction medium (5×10^{-7} M; see Table I). It is also clear that the occurrence of an exchange in the absence of added adenine nucleotides cannot be interpreted to mean that there is no nucleotide requirement since these preparations contain readily detectable levels of adenine nucleotides. If it were certain that the increase does reflect a requirement for ADP for the exchange it would provide evidence for mechanism II. This necessitates the consideration and elimination of other possible explanations for the increased incorporation of ^{18}O from $H_2{}^{18}O$ into the inorganic phosphate.

The most obvious alternative explanation of the ob-

served results is that the addition of ADP leads to the synthesis of ATP, and this newly formed ATP is then hydrolyzed. It has been shown that the hydrolysis of ATP by mitochondrial ATPase involves the cleavage of the terminal O-P bond so that in the presence of $H_2{}^{18}O$ the ^{18}O would be introduced into the inorganic phosphate and not the ADP (Boyer, 1958). This possibility is described in equations (5) and (6).

$$\beta\text{-hydroxybutyrate} + H_3PO_4 + 3\ ADP + {}^1/_2O_2 \xrightarrow[\text{phosphorylation}]{\text{oxidative}} \text{acetoacetate} + 3\ ATP + H_2O \quad (5)$$

$$Ad{-}O{-}P(O)(OH){-}O{-}P(O)(OH){-}O + P(O)(OH){-}OH + H_2{}^{18}O \longrightarrow$$

$$Ad{-}O{-}P(O)(OH){-}O{-}P(O)(OH){-}OH + H{}^{18}O{-}P(O)(OH){-}OH \quad (6)$$

In this situation the ^{18}O in the inorganic phosphate would be expected to rise as the enzyme(s) become saturated with ADP and ATP synthesis is increased. This would be expected to correspond with the concentrations of added ADP necessary to produce an increased substrate utilization. The drop in ^{18}O incorporation observed at higher concentrations of ADP could result from an inhibition of mitochondrial ATPase by ADP. This inhibition has been well documented (Kielley and Kielley, 1953; Cooper and Lehninger, 1957b; Cooper, 1958). The exchange observed in the absence of added ADP can also be explained on the same basis if the assumption is made that the bound ADP can act as an acceptor.

It is very difficult to rule out this possibility in a direct fashion. It is not known whether ATP formed at low ADP concentrations is available as a substrate for ATPase and, if so, whether it is completely hydrolyzed. Chan *et al.* (1960) have reported that 2×10^{-4} M ATP can support a P_i-H_2O exchange in the absence of oxidizable substrate, and we have confirmed this using 1×10^{-4} M ATP, indicating that these low levels of ATP are not hydrolyzed so rapidly that they cannot provide the necessary energy for the exchange.

These considerations therefore necessitate the use of a more indirect approach. One obvious way of trying to answer this question is to set an upper limit on the amount of ^{18}O that could be incorporated into inorganic phosphate as a result of reactions (5) and (6). This can be done by assuming a maximum P/O ratio and measuring the oxygen utilization. In Figure 2, for example, approximately 1.3 μmoles of acetoacetate is formed, and 1.3 μatoms of oxygen are utilized. If a maximum P/O ratio of 3 is assumed, then the maximum amount of ^{18}O that could be incorporated into phosphate if all of this ATP were formed and broken down would be 3×1.3 or 3.9 μmoles. In actual fact, the addition of ADP causes an increase in 9.9 μmoles (15.2–5.3), a value in considerable excess over the 3.9 μmoles that could be attributed to reactions (5) and (6). This comparison ignores the exchange obtained in the absence of added ADP and also considers the entire oxygen consumption to be involved, thus presenting the least favorable set of circumstances. There have been recent reports that the maximal P/O ratio for this span be in excess of 3 and may be as high as 6 (Boyer, 1963; Smith and Hansen, 1964). If the P/O ratio really is 6 then the maximal incorporation via reactions (5) and (6) would be 6×1.3 or 7.8 μmoles. This is still significantly less than the observed increase of 9.9 μmoles. Similar results have been obtained in three other experiments.

Estimates may be made for the span β-hydroxybutyrate to ferricyanide and cytochrome *c* to oxygen. If a maximum P/O ratio of 2 is assumed for the former then the increase produced by added ADP exceeds that which would be produced by synthesis plus hydrolysis. From Figure 3 it can be calculated that a maximum increase of 2.2 (1.1×2) would be expected whereas an increment of 3.2 is actually observed. In the experiment shown in Figure 4 this is not the case, however. Although a substantial increase in P_i-H_2O exchange is observed (5.2 μmoles), this does not exceed the calculated maximum that could be obtained by synthesis and hydrolysis if a P/O ratio of 1 is assumed (6.5 μmoles). A number of experiments have been carried out with this system and similar results have always been obtained. The failure to exceed the calculated maximum for this portion of the electron-transport chain is not interpreted to mean that there is no effect of ADP on the P_i-H_2O exchange associated with this phosphorylation site but rather that the failure is caused by technical difficulties. This system is complicated in that the P/O ratios obtained were all quite low (0.1–0.3). In order to obtain good P/O ratios special conditions are necessary (Cooper and Lehninger, 1956b). Unfortunately these conditions lead to a diminished P_i-H_2O exchange and a corresponding decrease in accuracy, and were therefore not feasible.

It will be fruitful to assess the experimental observations presented in this paper in terms of mechanism II. The following findings presented in Figure 2 require explanation: (1) a stimulation of acetoacetate formation occurring between 10^{-6} and 10^{-5} M ADP; (2) an increase in the P_i-H_2O exchange that is synchronized with the stimulation of acetoacetate formation; and (3) an inhibition of the P_i-H_2O exchange at higher levels of ADP that occurs when large amounts of ATP are being formed. As shown in reaction (II-1), respiration is limited by the availability of E. As the concentration of ADP is raised more E* is converted to E-ATP (reactions II-2 and II-3) and consequently the level of free E will also rise. This would therefore lead to an increase in oxidation especially if the K_m for E is very low. Similarly an increase in E-ATP would also lead

to an increased P_i-H_2O exchange (reaction II-3). As was mentioned in the introduction, it is most convenient to assume that the slowest step in the whole reaction sequence is the dissociation of E-ATP (reaction II-4). As the concentration of ADP increases it could compete with ATP for E, thereby depressing the reversal of reaction (II-4) (E + ATP $\rightleftarrows$ E-ATP) and effectively increasing the dissociation of E-ATP. This effect of ADP is consistent with the observations that ADP is a competitive inhibitor for ATP in the P_i-ATP and ADP-ATP exchanges (Cooper and Kulka, 1961) and mitochondrial ATPase (Kielley and Kielley, 1953; Cooper, 1958). Such a competition would lower the E-ATP level thereby leading to a diminished P_i-H_2O exchange and a stimulation of ATP formation.

The evidence presented in this paper does not unequivocally rule out a mechanism involving a covalently bound phosphate but the effects of ADP on the P_i-H_2O exchange, the requirement of inorganic phosphate for the ADP-ATP exchange, and the rapid incorporation of ^{18}O from $H_2{}^{18}O$ into ATP relative to P_i are most readily explained by a mechanism in which the first high-energy form of phosphate is ATP. This suggested lack of a covalently bound inorganic phosphate or ADP does not necessarily mean that the binding may not be tight. Bárány *et al.* (1964) have concluded that the binding of ADP to F-actin is very tight but is not covalent. Similarly it is well known that serum albumin can bind numerous anions very tightly in noncovalent linkages (Karush, 1950; Goodman, 1958; Scatchard *et al.*, 1957).

It is not difficult to formulate additional possible explanations of the effects of added ADP on the P_i-H_2O exchange that are consistent with a covalently bound phosphate intermediate. Two obvious variants are shown:

Mechanism Ia

$$E + \text{reduced carrier} \underset{\text{transfer}}{\overset{\text{electron}}{\rightleftarrows}} E^* + \text{oxidized carrier} \quad \text{(Ia-1)}$$

$$E^* + ADP \rightleftarrows E^*\text{-}ADP \quad \text{(Ia-2)}$$

$$E^*\text{-}ADP + P_i \rightleftarrows E^{*}\!\begin{smallmatrix}\diagup ADP\\ \diagdown P_i\end{smallmatrix} \quad \text{(Ia-3)}$$

$$E^{*}\!\begin{smallmatrix}\diagup ADP\\ \diagdown P_i\end{smallmatrix} \rightleftarrows E\!\begin{smallmatrix}\diagup ADP\\ \diagdown P\end{smallmatrix} + H_2O \quad \text{(Ia-4)}$$

$$E\!\begin{smallmatrix}\diagup ADP\\ \diagdown P\end{smallmatrix} \rightleftarrows E\text{-}ATP \quad \text{(Ia-5)}$$

$$E\text{-}ATP \rightleftarrows E + ATP \quad \text{(Ia-6)}$$

The feature of this mechanism is that it prescribes a required order of addition. It would be expected that inorganic phosphate would inhibit rather than stimulate the ADP-ATP exchange and it could only be consistent with the finding that ATP becomes labeled more rapidly than inorganic phosphate by $H_2{}^{18}O$ if the reversal of reaction (Ia-3) were relatively slow.

Mechanism Ib

$$E + \text{reduced carrier} \underset{\text{transfer}}{\overset{\text{electron}}{\rightleftarrows}} E^* + \text{oxidized carrier} \quad \text{(Ib-1)}$$

$$E^* + P_i \rightleftarrows E^*\text{-}P_i \quad \text{(Ib-2)}$$

$$E^*\text{-}P_i \rightleftarrows E\!\begin{smallmatrix}\diagup P\\ \diagdown H_2O\end{smallmatrix} \quad \text{(Ib-3)}$$

$$E\!\begin{smallmatrix}\diagup P\\ \diagdown H_2O\end{smallmatrix} + ADP \rightleftarrows E\!\begin{smallmatrix}\diagup P\\ \diagdown ADP\end{smallmatrix} + H_2O \quad \text{(Ib-4)}$$

$$E\!\begin{smallmatrix}\diagup P\\ \diagdown ADP\end{smallmatrix} \rightleftarrows E\text{-}ATP \quad \text{(Ib-5)}$$

$$E\text{-}ATP \rightleftarrows E + ATP \quad \text{(Ib-6)}$$

This mechanism invokes both a required order of addition and bound water that is displaced by ADP. It could explain an inorganic phosphate stimulation of the ADP-ATP exchange and the overlabeling of ATP from $H_2{}^{18}O$ provided the reversal of reaction (Ib-2) is relatively slow.

The concentration of added ADP necessary to produce an increased oxidation of β-hydroxybutyrate and a stimulation of the P_i-H_2O exchange agrees with the spectroscopic observations of Chance and Williams (1956) on the levels of ADP needed to bring about changes in the steady-state levels of components of the respiratory chain.

Acknowledgment

The author wishes to thank Miss Eileen Walters for her skillful and enthusiastic technical assistance and Dr. P. D. Boyer for making available the details of the ^{18}O analytical method prior to its publication.

References

Bárány, M., Koshland, D. E., Springham, S. S., Finkelman, F., and Therattil-Antony, T. (1964), *J. Biol. Chem. 239*, 1917.

Bárány, M., Nagy, B., Finkelman, F., and Chrambach, A. (1961), *J. Biol. Chem. 236*, 2917.

Boyer, P. D. (1958), *Symp. Enzyme Chem. (Tokyo-Kyoto) 2*, 301.

Boyer, P. D. (1963), *Science 141*, 1147.

Boyer, P. D., Graves, D. J., Suelter, C. H., and Demp-

sey, M. E. (1961), *Anal. Chem. 33*, 1906.
Chan, P. C., Lehninger, A. L., and Enns, T. J. (1960), *J. Biol. Chem. 235*, 1790.
Chance, B. (1961a), *J. Biol. Chem. 236*, 1544.
Chance, B. (1961b), *J. Biol. Chem. 236*, 1569.
Chance, B., and Williams, G. R. (1956), *J. Biol. Chem. 221*, 477.
Cohn, M., and Drysdale, G. R. (1955), *J. Biol. Chem. 216*, 831.
Cooper, C. (1958), *Biochim. Biophys. Acta 30*, 529.
Cooper, C., and Kulka, R. G. (1961), *J. Biol. Chem. 236*, 2351.
Cooper, C., and Lehninger, A. L. (1956a), *J. Biol. Chem. 219*, 489.
Cooper, C., and Lehninger, A. L. (1956b), *J. Biol. Chem. 219*, 519.
Cooper, C., and Lehninger, A. L. (1957a), *J. Biol. Chem. 224*, 547.
Cooper, C., and Lehninger, A. L. (1957b), *J. Biol. Chem. 224*, 561.
Dempsey, M. E., Boyer, P. D., and Benson, E. S. (1963), *J. Biol. Chem. 238*, 2708.
Goodman, D. S. (1958), *J. Am. Chem. Soc. 80*, 3887.
Imai, S., Riley, A. L., and Berne, R. M. (1964), *Circulation Res. 15*, 443.
Karush, F. (1950), *J. Am. Chem. Soc. 72*, 2714.
Kaziro, Y., Hass, L. F., Boyer, P. D., and Ochoa, S. (1962), *J. Biol. Chem. 237*, 1460.
Kielley, W. W., and Kielley, R. K. (1953), *J. Biol. Chem. 200*, 213.
Kulka, R. G., and Cooper, C. (1962), *J. Biol. Chem. 237*, 936.
Lowry, O. H., Rosebrough, N. F., Farr, H. L., and Randall, R. J. (1951), *J. Biol. Chem. 193*, 265.
McElroy, W. (1963), *Methods Enzymol. 6*, 445.
Scatchard, G., Coleman, J. B., and Shen, A. L. (1957), *J. Am. Chem. Soc. 79*, 17.
Schaffert, R. R., and Kingsley, G. R. (1955), *J. Biol. Chem. 212*, 59.
Scrutton, M. C., Keech, B., and Utter, M. F. (1965), *J. Biol. Chem. 240* (in press).
Smith, A. K., and Hansen, M. (1964), *Biochem. Biophys. Res. Commun. 15*, 431.
Walker, P. G. (1954), *Biochem. J. 58*, 699.

Kinetic Study of the Oxidation of Ferrohemochrome by Molecular Oxygen*

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ABSTRACT: Oxidation of dipyridineferrohemochrome by molecular oxygen was studied in both aqueous and ethanol-benzene solutions containing an excess of pyridine. In the presence of a large excess of dissolved oxygen, oxidation in all the solutions studied follows first-order kinetics. In aqueous solutions, the rate data can be interpreted by a dual path mechanism. The main path involves formation of "oxyheme" followed by its redox decomposition. In ethanol-benzene solutions the rate decreases rapidly as the volume per cent of benzene is increased. The apparent activation energies are consistent with the proposed mechanism.

The hypothesis that hemoglobin and myoglobin owe their remarkable property of reversible oxygenation mainly to the hydrophobic environment of the embedded hemes in these molecules (Wang *et al.*, 1958; Wang, 1958) has recently found support in elegant X-ray work on these proteins (Perutz *et al.*, 1960; Kendrew *et al.*, 1960, 1961). In order to examine this problem further, it would be desirable to know explicitly the effect of the hydrophobic environment on the rate of oxidation of heme derivatives by molecular oxygen without possible complication by other structural factors of the protein. With this objective, the rate of oxidation of dipyridineferrohemochrome by oxygen has been studied in the present work in both aqueous solutions and various ethanol-benzene solutions containing an excess of pyridine. A possible mechanism is proposed to account for the kinetic data thus obtained.

* From the Department of Chemistry, Yale University, New Haven, Conn. *Received November 5, 1964.* Presented at the 146th Meeting of the American Chemical Society, New York City, September 1963. This work was supported in part by a grant (GM-04483) from the U.S. Public Health Service.

† This paper is based on a dissertation submitted by O. H. W. Kao to Yale University in partial fulfillment of the requirements for the Ph.D. degree (June 1963).

Experimental

Preparation of Aqueous Solutions. A 5×10^{-4} M hemin stock solution was prepared by first dissolving