The Rapid Labeling of Adenosine Triphosphate by ^{32}P-Labeled Inorganic Phosphate and the Exchange of Phosphate Oxygens as Related to Conformational Coupling in Oxidative Phosphorylation†

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ABSTRACT: Evidence is presented that extends and amplifies the concept that in oxidative phosphorylation energy input serves to bring about release of ATP formed at a catalytic site by reversal of hydrolysis. The evidence with beef heart submitochondrial particles includes additional demonstration of uncoupler insensitive $P_i \rightleftharpoons HOH$ exchange, demonstration that this exchange is sensitive to the specific phosphorylation inhibitor, oligomycin, and demonstration that the small burst of uncoupler-insensitive ATP, rapidly labeled after addition of a trace of $^{32}P_i$, behaves in a manner consistent with its participation as a membrane-bound intermediate in the $P_i \rightleftharpoons HOH$ exchange. In addition, data are presented showing that addition of hexokinase plus glucose to submitochondrial particles in presence of ADP and P_i considerably lowers the $P_i \rightleftharpoons HOH$ exchange but that further addition of cyanide or 2,4-dinitrophenol or both has little additional effect. Such data are compatible with no energy requirement for formation of bound ATP. However, with a large excess of hexokinase, the rate of the $P_i \rightleftharpoons HOH$ exchange is further depressed. This could reflect some use of energy to promote formation of ATP at the catalytic site or to maintain the integrity of the phosphorylation system. Relationships of these findings to related information in the field are discussed.

In a recent communication, we have proposed a new concept for energy coupling in oxidative phosphorylation. According to this proposal, ATP, formed at the coupling sites, with limited or perhaps no energization from electron transport, is liberated to the medium by an energy-requiring protein conformational change (Boyer *et al.*, 1973). In a broader context, such conformational coupling is proposed for energy transductions of oxidative and photosynthetic phosphorylation, muscle contraction, and active transport (Boyer, 1974a).

An important experimental finding supporting this new concept is the retention of an appreciable $P_i \rightleftharpoons HOH$ exchange activity in presence of sufficient concentrations of uncouplers to block net ATP synthesis. Another is the demonstration that addition of a trace of $^{32}P_i$ to submitochondrial particles in presence of ADP, P_i, and uncoupler results in a a rapid[1] labeling of a small amount of ATP by a reaction sensitive to oligomycin.

Our working hypothesis suggests at least two important steps in ATP formation, as depicted by eq 1 and 2, where E

$$E + ADP + P_i \leftrightarrow E{\cdot}ADP{\cdot}P_i \leftrightarrow \underset{\text{(tight complex)}}{E'{\cdot}ATP} + HOH \quad (1)$$

$$\underset{\text{(tight complex)}}{E'{\cdot}ATP} \xleftrightarrow[\text{conformational change}]{\text{energy-linked protein}} \underset{\text{(loose complex)}}{E{\cdot}ATP} \leftrightarrow E + ATP \quad (2)$$

designates the catalytic site for ATP formation and cleavage on the mitochondrial inner membrane. Each step may, of course, be divided into additional steps. Reactions of eq 1 would account for the uncoupler-insensitive $P_i \rightleftharpoons HOH$ exchange. Reactions of eq 2 are essential for continued net ATP synthesis.

The present paper has several objectives. One is to present additional data that extend the evidence for uncoupler-insensitive $P_i \rightleftharpoons HOH$ exchange and, more important, to present results not previously published on the oligomycin sensitivity of this exchange and the relative effects of oligomycin on the $P_i \rightleftharpoons ATP$, $P_i \rightleftharpoons HOH$, and $ATP \rightleftharpoons HOH$ exchanges. Another objective is to present data on various conditions affecting the amount of uncoupler-insensitive ATP that is rapidly labeled by addition of a trace of $^{32}P_i$ together with additional evidence that a significant amount of the rapidly labeled ATP is membrane bound. Also included in the paper are results of probes on the effect of substrate, cyanide, 2,4-dinitrophenol, and hexokinase plus glucose on the $P_i \rightleftharpoons HOH$ exchange capacity. These data suggest that some energy input for ATP synthesis might occur in steps of reaction 1 as well as that required in reaction 2. A final purpose of this paper is to discuss some relationships of the current hypothesis to other related findings.

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[1] The term rapid to designate the ATP labeling in this paper is used in a comparative sense to most reports on ATP labeling by mitochondria and because our results indicate that the initial rate of ATP labeling exceeds the steady-state rate. The earliest measurements reported herein were at about 5 sec. Measurements in the millisecond range have demonstrated an initially rapid ATP labeling (Boyer, 1974b).

Experimental Procedures

Materials. Reagents used were conventional commercial sources. 2,4-Dinitrophenol was purified by recrystallization from chloroform. $^{32}P_i$ was purified as described previously (Cross and Boyer, 1973).

Rat-liver mitochondria were prepared essentially as described by Johnson and Lardy (1967). Heavy beef-heart mitochondria were prepared by a modification of the method of Smith (1967) and frozen at −20° for up to 6 months. Submitochondrial particles (ETPH-Mg^{2+}-Mn^{2+}) were prepared fresh daily by sonication of heavy beef-heart mitochondria at 0° by the method of Beyer (1967). The submitochondrial particles were stored at 0° for up to 6 hr before use. Protein concentrations were estimated by the Lowry procedure with bovine serum albumin as a standard.

Measurement of Exchange Reactions and ATPase Activity. Reactions were stopped by addition of 1 ml of 0.9 M perchloric acid to a 2.0-ml reaction volume and protein was removed by centrifugation. ATPase activity was evaluated by P_i measurements using conventional procedures. Adenine nucleotides were adsorbed on 200 mg of acid-washed charcoal and the charcoal was filtered off by suction. P_i was isolated from the filtrate and analyzed for ^{18}O, as described elsewhere (Boyer and Bryan, 1967) to estimate the $P_i \rightleftharpoons$ HOH exchange. The charcoal was washed by suction with a few small portions of distilled water using a drop of ethanol occasionally to minimize spreading of the charcoal. The charcoal was suspended in 2 ml of 1 M HCl and heated in a boiling water bath for 20 min to release β- and γ-phosphoryl groups of ADP and ATP. The filtrate was used for P_i and ^{32}P determination to estimate the $P_i \rightleftharpoons$ ATP exchange and for ^{18}O determination in the P_i to estimate the ATP $\rightleftharpoons$ HOH exchange.

Calculations of total ^{18}O and $^{32}P_i$ exchanges were based on final concentrations of reactants. Corrections were made when necessary for the approach to isotopic equilibrium (Boyer and Bryan, 1967). The procedure for ^{18}O and ^{32}P analyses in ATP included both the β- and γ-phosphoryl groups, as randomization by adenylate kinase action was expected. The $P_i \rightleftharpoons$ ATP exchange is expressed as micromoles of original P_i present in the total ATP plus ADP at the end of the incubation, and the oxygen exchanges as the microatoms of oxygen originating from HOH found in the final P_i or ATP plus ADP, with correction of the P_i value for one oxygen necessarily introduced by hydrolysis. Because of adenylate kinase activity of the particles, the total labile phosphoryl groups of ADP + ATP participate in the exchanges and are available for ATPase action.

Use of the final concentrations of reactants tends to overestimate slightly the total amount of $P_i \rightleftharpoons$ HOH exchange and underestimate the $P_i \rightleftharpoons$ ATP and ATP $\rightleftharpoons$ HOH exchanges. This is because some ^{32}P and ^{18}O originally introduced into ATP has been converted to P_i by net hydrolysis. However, corrections for such effects would cause only slight modification of the patterns observed.

Measurement of Uncoupler-Insensitive ATP Formation. Submitochondrial particles were incubated and sampled as described with the figures. Unless otherwise indicated, reaction was stopped by rapid quenching with 1 ml of cold 0.375 M $HClO_4$, 2.5 mM P_i, and 1 mM ATP. Protein was removed by centrifugation at 0° for 10 min at 19,000*g*. The pH of the supernatants was adjusted to between 6.5 and 7.5 using KOH. At this point, samples were usually stored overnight at −20°. The deproteinized, neutralized extracts were thawed and centrifuged at 0° for 2 min at 2000*g* to remove solid $KClO_4$. Aliquots (0.4 ml) of the supernatants were then applied to 0.5 × 3.0 cm columns of anion exchange resin (Bio-Rad AG-1 · 4X, 400 mesh). The samples were washed into the column with 3 ml of H_2O followed by 7 ml of 60 mM HCl to elute $^{32}P_i$, ADP, and, in some experiments, glucose 6-[^{32}P]phosphate; 3.0 ml of 1.0 M HCl was added to elute [^{32}P]ATP. The ATP fraction containing about 0.1% of the original $^{32}P_i$ was collected on ice; 0.75 ml of 60 mM ammonium molybdate and 10 μl of 0.1 M P_i were added to the ATP fraction and P_i was extracted at 0° by two washes with 4 ml of cold isobutyl alcohol–benzene (1:1, v/v). Another 10 μl of 0.1 M P_i was added followed by one or two more washes with the cold isobutyl alcohol–benzene; 1 ml of the bottom aqueous layer was counted by liquid scintillation spectrometry to determine [^{32}P]ATP.

When glucose 6-[^{32}P]phosphate was to be measured, the 60 mM HCl fraction was adjusted to 1.0 M HCl and heated at 100° for 20 min. After cooling the samples on ice, molybdate and carrier P_i were added and P_i was extracted as described above.

That the ^{32}P-labeled substance, eluted from the anion exchange column with 1 M HCl, was in fact ^{32}P-γ-labeled ATP, was demonstrated by its acid lability and susceptibility to hexokinase. Heating with 1.0 M HCl at 100° for 20 min converted more than 95% of the ^{32}P to P_i. Incubation of a neutralized fraction with hexokinase and glucose converted more than 90% of the ^{32}P to a form stable in 1 M HCl at 100° for 20 min.

Results

Uncoupler and Oligomycin Effects on Exchange Rates. The resistance of the $P_i \rightleftharpoons$ HOH exchange to 2,4-dinitrophenol and to 5-chloro-3-*tert*-butyl-2′-chloro-4′ nitrosalicylanilide (S-13) (Williamson and Metcalf, 1967) forms an important basis for suggesting energy input following ATP formation (Boyer *et al.*, 1973). Extension of such measurements to include other uncouplers and assessment of the sensitivity to oligomycin seemed of importance as additional probes of the relationship of the uncoupler-insensitive $P_i \rightleftharpoons$ HOH exchange to oxidative phosphorylation.

The effects of increasing concentrations of the potent uncoupler, *m*-chlorocarbonyl cyanide phenylhydrazone (*m*-Cl-CCP[2]), on the exchange reactions and ATPase activity catalyzed by mitochondria are shown in Figure 1. In the absence of uncoupler, the relative rates of the reactions $P_i \rightleftharpoons$ HOH, ATP $\rightleftharpoons$ HOH, and $P_i \rightleftharpoons$ ATP are about 16:4:1, respectively, for the mitochondrial preparation and conditions used. At low concentrations of uncoupler, the $P_i \rightleftharpoons$ ATP and ATP $\rightleftharpoons$ HOH exchanges are much more sensitive to the uncoupler than the $P_i \rightleftharpoons$ HOH exchange. At a concentration of *m*-Cl-CCP sufficient to inhibit the $P_i \rightleftharpoons$ ATP and ATP $\rightleftharpoons$ HOH exchanges by about 50%, the $P_i \rightleftharpoons$ HOH exchange is inhibited by only about 15%. At a concentration of *m*-Cl-CCP which gives a near zero value for the $P_i \rightleftharpoons$ ATP and ATP $\rightleftharpoons$ HOH exchanges and a maximum value for the uncoupler-stimulated ATPase activity, the $P_i \rightleftharpoons$ HOH exchange is still rapid and inhibited by only 40%.

Although it has been well recognized that oligomycin will inhibit both the $P_i \rightleftharpoons$ ATP and $P_i \rightleftharpoons$ HOH exchanges of mitochondria (Lardy *et al.*, 1964) assessments of the rela-

[2] Abbreviations used are: *m*-Cl-CCP, *m*-chlorocarbonyl cyanide phenylhydrazone; Hepes, *N*-2-hydroxyethylpiperazine-*N*-2-ethanesulfonic acid.

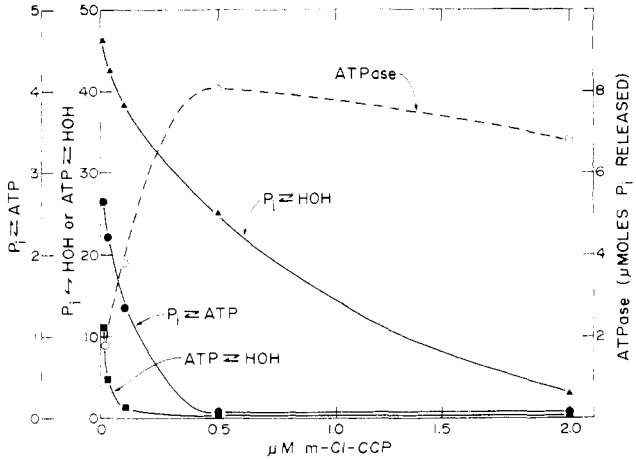


FIGURE 1: Effect of *m*-chlorocarbonyl cyanide phenylhydrazone (m-Cl-CCP) on exchange reactions and ATPase. Rat liver mitochondria (approximately 6 mg of protein) were incubated for 5 min in 2 ml total volume at pH 7.4 and 37° with 5 mM $^{32}P_i$ (4×10^6 cpm), 5 mM ATP, 5 mM Mg^{2+}, 40 mM KCl, and 40 mM Tris-Cl. The water contained 0.81 atom %excess ^{18}O. Reactions were measured and expressed as described under Experimental Procedures.

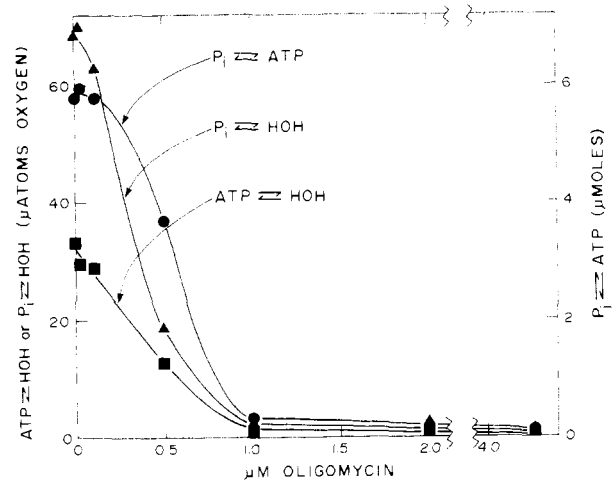


FIGURE 2: The effect of increasing concentration of oligomycin on the exchanges. Conditions and analyses were as given with Figure 1, but with increasing concentrations of oligomycin replacing m-Cl-CCP.

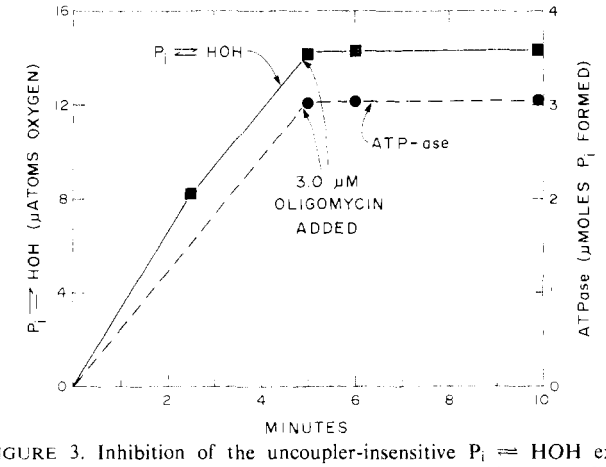


FIGURE 3. Inhibition of the uncoupler-insensitive $P_i \rightleftharpoons HOH$ exchange by oligomycin. Conditions were similar to those given with Figure 1, but with 0.1 mM 2,4-dinitrophenol present in the original incubation mixture.

tive sensitivity of these exchanges and of the $ATP \rightleftharpoons HOH$ exchange reaction to increasing concentration of oligomycin have not been reported previously.

The effects of increasing concentrations of oligomycin on the exchange reactions are shown in Figure 2. At a 0.5 μM concentration of added oligomycin, the rates of the reactions $P_i \rightleftharpoons HOH$, $ATP \rightleftharpoons HOH$, and $P_i \rightleftharpoons ATP$ are inhibited by about 70, 40, and 60%, respectively. The $P_i \rightleftharpoons HOH$ exchange thus shows the greatest relative sensitivity to oligomycin. This is in harmony with the data of Hinkle *et al.* (1967) showing that the $P_i \rightleftharpoons HOH$ exchange was more sensitive to oligomycin than the capacity for net oxidative phosphorylation. At a 1.0 μM concentration of added oligomycin, the rates of all three exchanges have values near zero. Under similar conditions 1.0 μM added oligomycin caused >95% inhibition of net oxidative phosphorylation.

These results give evidence that each of the exchange reactions measured in the absence of uncouplers under our conditions is almost totally accounted for by the partial reactions of oxidative phosphorylation. However, the possibility remains that the uncoupler-insensitive $P_i \rightleftharpoons HOH$ exchange might reflect the activity of an enzyme which is not directly related to oxidative phosphorylation but is activated in some manner by uncouplers. This possibility would appear to be ruled out by the results shown in Figure 3. In this experiment, mitochondria were incubated for 5 min in the presence of 100 μM 2,4-dinitrophenol, at which time 3.0 μM oligomycin was added. The addition of oligomycin caused the complete inhibition of the uncoupler-insensitive $P_i \rightleftharpoons HOH$ exchange as well as the uncoupler-stimulated ATPase activity. Similar results were obtained with S-13 as the uncoupler. Thus, it appears justifiable to conclude that the uncoupler-insensitive $P_i \rightleftharpoons HOH$ exchange is catalyzed by the partial reactions of oxidative phosphorylation.

2,4-Dinitrophenol Effects on Net Phosphorylation Rates and on the Rapidly Labeled ATP Fraction. When a trace of $^{32}P_i$ is added to submitochondrial particles under near steady-state conditions, in presence of substrate, O_2, ADP, P_i, and uncoupler, an initial very rapid labeling of small amounts of ATP is noted (Boyer *et al.*, 1973). The amount of this rapidly labeled fraction, estimated by extrapolation of the observed [^{32}P]ATP level to 0 time of incubation with $^{32}P_i$, is designated as $ATP_{t=0}$. As reported earlier, a significant level of $ATP_{t=0}$ is detected in the presence of sufficient 2,4-dinitrophenol to inhibit the $P_i \rightleftharpoons ATP$ exchange catalyzed by submitochondrial particles. From characteristics of its labeling and from evidence presented later in this paper, a significant amount of the $ATP_{t=0}$ appears to be bound at the catalytic site for ATP synthesis by oxidative phosphorylation.

If a major effect of an uncoupler were to block energy input for release of preformed ATP, increasing concentrations of 2,4-dinitrophenol would be expected to show a much more drastic effect on rates of net ATP synthesis than on values for $ATP_{t=0}$. Results of such an experiment are reported in Figure 4. They show that with addition of sufficient 2,4-dinitrophenol to submitochrondrial particles to decrease the rate of glucose 6-phosphate formation by over 90%, the value for $ATP_{t=0}$ is reduced by only 23%.

Effect of Addition of Hexokinase–Glucose on $ATP_{t=0}$ Values. In the course of measuring $ATP_{t=0}$ under different conditions, a marked reduction of the amount detected was noted when hexokinase plus glucose was present. Results demonstrating such an effect on the level of $ATP_{t=0}$ detected in presence of 2,4-dinitrophenol are given in Figure 5. They demonstrate a considerable decrease in the level of $ATP_{t=0}$ in presence of hexokinase plus glucose at concen-

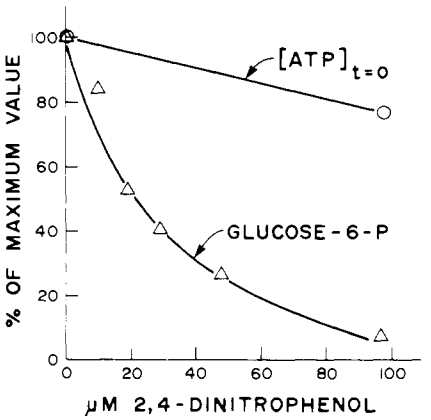


FIGURE 4: Relative effect of 2,4-dinitrophenol on glucose 6-phosphate formation and on $ATP_{t=0}$ values. Aliquots of 0.25 ml each, containing 0.25 M sucrose, 5 mM Hepes (pH 7.5), 3.5 mM $MgCl_2$, and submitochondrial particles equivalent to 3.3 mg of protein were brought to 30°. Additions were made in small volumes to give final concentrations of 11.4 mM succinate and 9.5 mM P_i at 0 sec; ±50 μg of hexokinase plus 19.3 mM glucose at 15 sec; 1.9 mM ADP plus the amount of 2,4-dinitrophenol indicated above at 30 sec; 6.4×10^9 cpm $^{32}P_i$ at 60 sec; 50 μl aliquots were withdrawn and quenched at 5-sec intervals. ATP and glucose 6-phosphate were determined as described. $ATP_{t=0}$ was measured by extrapolation to zero time of incubation with $^{32}P_i$. The blank represented 0.0035% of the $^{32}P_i$ added. The $ATP_{t=0}$ in the absence of uncoupler was measured in the presence of hexokinase plus glucose. The 100% value for $ATP_{t=0}$ was 0.53 nmol/mg of protein and the 100% value for the rate of glucose 6-phosphate formation was 133 nmol per min per mg of protein.

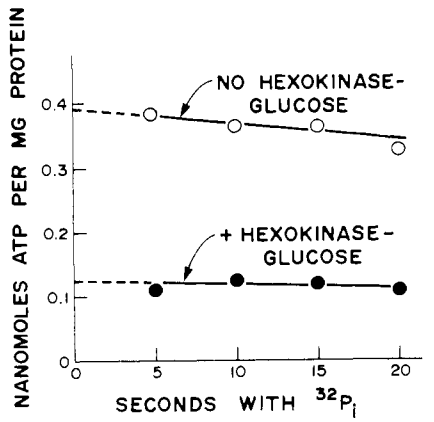


FIGURE 5: Effect of hexokinase plus glucose on $ATP_{t=0}$ values. Aliquots of 0.25 ml each, containing 0.25 M sucrose, 5 mM Hepes (pH 7.5), 3.5 mM $MgCl_2$, and submitochondrial particles equivalent to 3.3 mg of protein were brought to 30°. Additions were made in small volumes to give final concentrations of 11.4 mM succinate and 9.7 mM P_i at 0 sec; ±50 μg of hexokinase and 19.3 mM glucose at 15 sec; 1.9 mM ADP and 97 μM 2,4-dinitrophenol at 30 sec; and 6.4×10^7 cpm $^{32}P_i$ at 60 sec; 50-μl aliquots were withdrawn and quenched at the times indicated and ATP was determined by the combined column-extraction procedure. The blank represented 0.0035% of the ^{32}P added and 9% of $ATP_{t=0}$ observed in the absence of hexokinase and glucose.

trations used in net synthesis experiments. In other similar experiments, decreases of between 22 and 71% in $ATP_{t=0}$ values have been noted upon addition of hexokinase plus glucose. Additions of either hexokinase or glucose alone produced no changes, indicating that the effect of hexokinase plus glucose was caused by removal of ATP.

An important consideration is whether the levels of hexokinase used in Figure 5 and similar experiments were sufficient to effectively trap the soluble ATP formed. The ability of hexokinase to convert a small amount of medium ATP to glucose 6-phosphate was tested in separate experiments. These were run under the same conditions as the experiments reported in Figure 5 except that submitochondrial particles were omitted and [γ-^{32}P]ATP was added to a concentration equivalent to the 0.4 nmol/mg of protein observed in the upper trace of Figure 5. As anticipated, about 95% of the added [γ^{32}P]ATP was converted to glucose 6-phosphate in less than 3 sec, the shortest time period tested.

Based on kinetic characteristics of the hexokinase (Colowick, 1973) and the rate of ATP formation by the submitochondrial particles, an estimate can be made of the expected steady-state level of ATP in a given experiment. Under conditions as used in Figure 5, the expected steady state was equivalent to about 0.02 nmol/mg of protein compared to the measured $ATP_{t=0}$ value of 0.12 nmol/mg of protein. Thus, in the experiments reported in the lower trace of Figure 5, about 80% of the ATP would appear to be membrane bound.

Effect of P_i Concentration on $ATP_{t=0}$. Previous experiments have indicated that the rate of the $P_i \rightleftharpoons$ HOH exchange continues to increase with increase in P_i concentration above 5 mM (Mitchell *et al.*, 1967). If the bound component of $ATP_{t=0}$ represents ATP which participates as an intermediate in the oxygen exchange through dynamic reversal of hydrolysis, the level of $ATP_{t=0}$ should increase with increase in P_i concentration. Experiments to test this possibility showed a near linear increase in $ATP_{t=0}$ with increase in P_i concentration up to 80 mM. This indicates a low affinity of P_i for the phosphorylation site. The apparent K_m for net phosphorylation is considerably less than 80 mM, likely reflecting a rapid conversion of bound P_i to ATP. The results are consistent with but do not prove an increase in bound ATP present with increase in P_i.

Effect of Phosphorylation Modifiers on the $P_i \rightleftharpoons$ HOH Exchange. According to our present working hypothesis, the $P_i \rightleftharpoons$ HOH exchange results from dynamic reversal of the formation of bound ATP. The effect of agents that modify phosphorylation conditions on the exchange is thus of interest. Evidence that the exchange is reduced but remains rapid and prominent when electron transfer is blocked in mitochondria has been reported earlier (Boyer *et al.*, 1966). In addition, the exchange is stimulated by added ATP (Cooper, 1965, Hinkle *et al.*, 1967) and appears to have an absolute requirement for ADP (Jones and Boyer, 1969). However, it has not been ascertained whether the exchange may have a requirement for the presence of ATP. Even though additions of ADP alone suffice for detection of the exchange (Jones and Boyer, 1969) presence of ATP in ADP preparations or the action of adenylate kinase present in submitochrondrial particles could provide small amounts of ATP. To probe further at a possible ATP requirement, effects of hexokinase plus glucose to scavenge ATP was assessed.

The data of Figure 6 show the effects of addition of hexokinase plus glucose on the rate of the $P_i \rightleftharpoons$ HOH exchange catalyzed by submitochondrial particles with added ADP and P_i. The most striking effect shown in this figure is the pronounced decrease in the exchange rate as a result of addition of hexokinase plus glucose. This effect was assessed in more detail as reported below. Also of interest is the small additional effect caused by additions of cyanide or 2,4-dinitrophenol or both to the reaction mixture.

The amount of hexokinase used for the data reported in Figure 6 is larger than that commonly used for net phosphorylation assays. It seemed advisable, however, to deter-

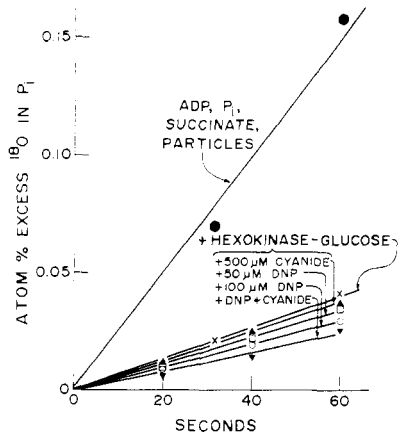


FIGURE 6: Effect of hexokinase, 2,4-dinitrophenol, and cyanide on the $P_i \rightleftharpoons HOH$ exchange. Beef heart submitochondrial particles (3.4 mg of protein) were incubated with components indicated below and in the figure in 0.8 ml total volume for 2 min at 30° and pH 7.4. Then 0.2 ml of water containing 8.3 atom % excess ^{18}O and 2,4-dinitrophenol or KCN or both as indicated in the figure to give 50 or 100 μM final 2,4-dinitrophenol, or 500 μM cyanide, or 100 μM 2,4-dinitrophenol plus 500 μM cyanide. Samples were quenched after additional incubation as indicated in the figure and ^{18}O analyses made on isolated P_i. The 0.8-ml original incubation mixture contained sufficient additions to give final concentrations, after addition of 0.2 ml of $H^{18}OH$, of 3 mM ADP, 10 mM Mg^{2+}, 40 mM Tris-Cl, 50 mM succinate, 10 mM P_i, 40 mM glucose, and 500 μg/ml of hexokinase.

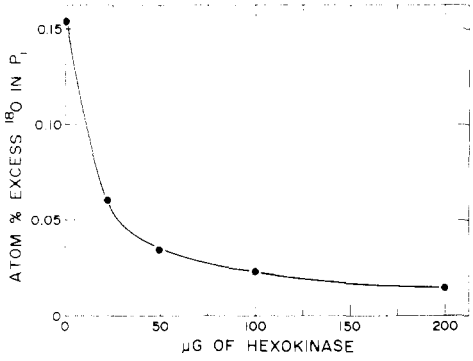


FIGURE 7: Effect of high concentrations of hexokinase on the $P_i \rightleftharpoons HOH$ exchange. Samples contained, in a 0.5-ml volume at pH 7.4 and 30°, 5 mM ADP, 10 mM $MgCl_2$, 40 mM Tris-Cl, 10 mM P_i, 40 mM glucose, beef heart submitochondrial particles (2 mg of protein), and hexokinase as indicated. After 1-min incubation, 0.5 ml of the same reaction mixture (except particles, glucose and hexokinase) in $H^{18}OH$ was added to give a final 0.75 atom % excess $H^{18}OH$. After an additional 5-min incubation, samples were quenched and analyzed in the usual manner.

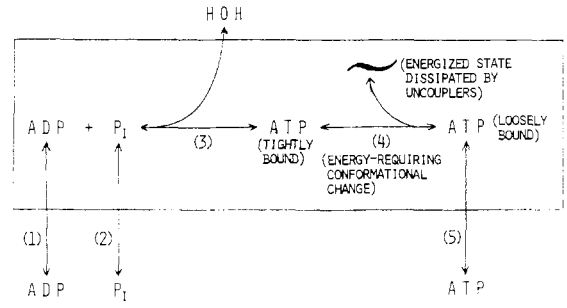
mine if increased levels of hexokinase would further inhibit the $P_i \rightleftharpoons HOH$ exchange. Results of such an experiment are reported in Figure 7. They show an increased depression of the $P_i \rightleftharpoons HOH$ exchange with increase in hexokinase concentrations to high levels. The results suggest that an even larger excess of hexokinase might inhibit the exchange completely. Caution in this interpretation is indicated, however, by the fact that about 12% of the total protein in the reaction system was hexokinase at the highest concentration used. Consideration thus needs to be given to the possibility that secondary effects might be involved.

The demonstration of the apparent absolute requirement of ADP for the $P_i \rightleftharpoons HOH$ exchange (Jones and Boyer, 1969) was made with substrate present. It was thus of interest to determine whether additions of ADP without oxidizable substrate would serve to activate the $P_i \rightleftharpoons HOH$ exchange. As shown by the results in Figure 7 and in other experiments not reported here, addition of ADP in absence of added oxidizable substrate sufficed for a rapid $P_i \rightleftharpoons HOH$ exchange.

Discussion

The results given in this paper give additional support to the hypothesis, given in the introduction, that in oxidative phosphorylation energy input causes release of ATP formed at the catalytic site by reversal of hydrolysis. For discussion of how the data fit this hypothesis, the diagram given in Scheme I is useful. Listed in Scheme I are the steps required for the exchange reactions. The dynamic reversal of step 3 that interconverts bound ATP to bound ADP and P_i is postulated as the sole means of entry of water oxygens into P_i or ATP. Steps required for the various exchanges are indicated in the scheme.

SCHEME I: A sequence for Conformation Coupling to ATP Synthesis.



STEPS REQUIRED FOR EXCHANGE REACTIONS

$P_I \rightleftharpoons HOH$	2, 3
$ATP \rightleftharpoons HOH$	3, 4, 5
$P_I \rightleftharpoons ATP$	2, 3, 4, 5

The similar sensitivity of all three exchange reactions to inhibition by oligomycin in absence of uncoupler is consistent with inhibition by oligomycin of step 3, the only step required by each of the exchanges. Such inhibition could also result from blocking of access of both P_i and ATP to the catalytic site. Data in Figure 1 show that the $P_i \rightleftharpoons HOH$ exchange is much less sensitive to uncouplers than the $P_i \rightleftharpoons ATP$ and $ATP \rightleftharpoons HOH$ exchanges. This is not consistent with energy input limited to step 3, as has been commonly assumed, because discharge of the energized state by uncouplers would allow step 3 to run only in the reverse direction causing inhibition of all exchanges. The only steps required by the two uncoupler-sensitive exchanges that are not required by the uncoupler-insensitive $P_i \rightleftharpoons HOH$ exchange are steps 4 and 5, involved in the energy-coupled release and binding of ATP.

This clearer understanding of the probable mechanism of the exchange reactions and their energy requirements has led to the present hypothesis that in oxidative phosphorylation energy input serves to drive the release of ATP that is formed at the catalytic site by reversal of hydrolysis. Further experimental findings presented in this report that support this concept include the demonstration of oligomycin sensitivity of the uncoupler-insensitive $P_i \rightleftharpoons HOH$ exchange, the rapid labeling of a small amount of ATP by addition of a trace of $^{32}P_i$ to uncoupled submitochondrial particles by an oligomycin-sensitive step, the limited effect of hexokinase plus glucose on the level of rapidly labeled ATP, and data which suggest that the rapidly labeled ATP partic-

ipates as an intermediate in the $P_i \rightleftharpoons HOH$ exchange. In future work, it will be of importance to establish whether the rate of formation and cleavage of this ATP is indeed sufficiently rapid to account for the ^{18}O exchange rate.

An important implication of the study of factors affecting the $P_i \rightleftharpoons HOH$ exchange as reported in this paper is that energy input may aid ATP formation at the catalytic site as well as promote its release. The data in Figure 6 demonstrate that additions of hexokinase plus glucose, cyanide, and 2,4-dinitrophenol together did not block the $P_i \rightleftharpoons HOH$ exchange. Such results might be taken to indicate that no energy input from oxidations or other sources was necessary for formation of bound ATP. This may indeed be the case. However, the data of Figure 7, showing that increased concentrations of hexokinase can markedly depress the $P_i \rightleftharpoons HOH$ exchange, might be explained by an increased scavenging of small remaining amounts of ATP formed by adenylate kinase. Thus, it is possible that continuation of the $P_i \rightleftharpoons HOH$ exchange depends on some energization from ATP cleavage, or that some ATP is essential to maintain the catalytic integrity of the submitochondrial particles. It is also possible that the exceptionally large amount of hexokinase added may have contained other substances capable of inhibiting the residual exchange reaction. Additional probes of the possible requirement of some medium ATP to continue the $P_i \rightleftharpoons HOH$ exchange appear desirable.

Our findings, as mentioned earlier (Boyer *et al.*, 1973), may be related to the interesting observations of Eisenhardt and Rosenthal (1964, 1968). They demonstrated that addition of ADP to mitochondria in presence of substrate, $^{32}P_i$, ATP, and O_2 gave rise to a small burst of [^{32}P]ATP formation even in presence of 2,4-dinitrophenol. This might represent increased formation of the ATP bound to the catalytic site. The Ca^{2+} sensitivity of this ATP jump could reflect (a) the displacement of Mg^{2+} from the coupling site (Le Blanc and Clauser, 1974), or (b) the need for some energy input to maintain the catalytic integrity or to favor ATP formation at the catalytic site.

Comment is also desirable on the possible relationship of the bound ATP proposed as an intermediate in the $P_i \rightleftharpoons HOH$ exchange and oxidative phosphorylation, and on the tightly bound ATP detected in perchloric acid precipitates of mitochondria by Cross and Boyer (1973). Both types of observations may reflect ATP bound to the phosphorylation complex. But in contrast to the apparent level of bound ATP reported in this paper, the level of bound ATP in the perchloric acid precipitate was lowered considerably by 2,4-dinitrophenol. At this stage, the observations appear complex and no definite relationship has been established.

That protein conformational change might serve a key role for energy transduction leading to ATP formation was first suggested by Boyer about a decade ago (Boyer, 1965). However, at that time, it was thought that protein conformational change might give rise to a high-energy precursor to ATP synthesis, such as an acyl-S linkage. Our laboratory in the interim has given only limited attention to the development of conformational coupling. In retrospect, continuing developments in the understanding of biological catalyses make the hypothesis underlying the present work quite plausible, and it appears to have warranted attention before now. In many enzyme reactions, substrate binding and release steps are rate limiting and relatively large free energy changes may be associated with the binding and release. Increased understanding of enzyme control mechanisms points to energy-requiring conformational changes accompanying ligand binding, with energy transmission between subunits. These and other considerations are developed in recent symposium papers (Boyer, 1974a,b).

Observations demonstrating conformational change in the ATPase coupling factors of oxidative and photosynthetic phosphorylation could be quite relevant to our suggested mechanism for ATP formation. In this regard, Ryrie and Jagendorf (1972) found that the chloroplast ATPase showed additional exposure of peptide hydrogens to exchange upon exposure to light, and McCarty and Fagen (1973) showed a light-dependent incorporation of *N*-ethylmaleimide by chloroplast ATPase. Chang and Penefsky (1973,1974) demonstrated conformational changes in mitochondrial ATPase as probed by fluorescence of aurovertin. Such changes appear to occur rapidly enough to be essential steps in energy coupling and could represent conformational transitions modifying ATP affinity.

Coupling factor ATPases isolated from chloroplasts (Roy and Moudrianakis, 1971) and from beef-heart mitochondria (Harris *et al.*, 1973) have bound adenine nucleotides present. Relationships of the bound nucleotides to coupled ATP synthesis is at present uncertain. Harris *et al.* (1973) suggest that tight binding of ATP may favor its formation from ADP and P_i. Valuable studies of Hilborn and Hammes (1973) on the isolated beef-heart ATPase document some nucleotide binding properties. How such binding may change during active oxidative phosphorylation merits investigation.

As noted elsewhere, the basic pattern of coupling the energy changes associated with covalent bound cleavage to protein conformational change, as suggested herein for oxidative phosphorylation, may also be operative in photophosphorylation as well as muscle contraction (Boyer, 1974a,b).

As mentioned earlier, present data are regarded by us as favoring a single point of entry of water oxygens for both the $P_i \rightleftharpoons HOH$ and $ATP \rightleftharpoons HOH$ exchanges, namely, as depicted in step 3 of Scheme I. This view is in clear contrast to that of Young *et al.* (1974). They regard their "pseudorotation mechanism" as the only mechanism proposed to date that adequately explains the oxygen exchange reactions. An important point they emphasize is earlier data of Mitchell *et al.* (1967) showing high ratios of the $P_i \rightleftharpoons HOH$ exchange to other exchanges observed under some conditions. However, such persistence of the $P_i \rightleftharpoons HOH$ exchange is readily understandable in terms of Scheme I. For example, a continued $P_i \rightleftharpoons HOH$ exchange but depressed $P_i \rightleftharpoons ATP$ and $ATP \rightleftharpoons HOH$ exchanges were observed when ADP was removed by pyruvate kinase and phosphoenolpyruvate. High ATP/ADP ratios could act as a metabolic control through decreasing the rate of release of bound ATP.

The experimental results of this paper do not shed light on how energy from oxidations may be transmitted to the catalytic machinery whose conformational change drives net ATP synthesis. One possibility is that an interlinked protein matrix couples conformational changes accompanying electron transport to ATP release through protein-protein interactions (Boyer, 1974a). There is, however, compelling evidence for the importance to energy-transducing processes of membrane potential following concepts pioneered by Peter Mitchell. Development of a potential might be a reversible alternative to ATP synthesis, might be a requisite condition for proper orientation or activation of proteins involved, or might represent a compulsory intermedi-

ate state in coupling of electron transport to ATP synthesis. Even if energy is captured as a potential gradient, conformational coupling appears to us to be a more chemically satisfying means of using a potential gradient for ATP synthesis than does addition of a proton to different ionic forms of P_i at the catalytic site for ATP synthesis as suggested by Mitchell (1974). For example, one can readily visualize that development of a negative potential could induce protein COO^- groups to be protonated and move into a membrane. The associated conformational changes might drive ATP release at a quite distal catalytic site.

The concept of energy coupling advanced in this paper obviously does not require or indicate the participation of a phosphorylated or nonphosphorylated "high-energy" intermediate. Indeed, this simple feature is an attractive part of the hypothesis and may explain why all attempts to isolate such intermediates have failed.[3] But it must be noted that the concept does not rule out the possibility of a phosphorylated intermediate. The ease of formation of an acyl phosphate accompanied by elimination of water from P_i by the transport ATPases (Dahms *et al.*, 1973) points to the possibility that similar events could accompany the formation of bound ATP found in our studies. But as in any catalytic sequence, unless there are chemically logical reasons for participation of an intermediate or intermediates, they should be considered absent until and unless proved otherwise. We thus suggest that, for the present, the very common inclusion of phosphorylated intermediates in schemes for oxidative phosphorylation be discontinued.

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[3] The laboratory of one of the authors (P.D.B.) has participated in a number of such unsuccessful attempts, the most productive of which led to the discovery of phosphohistidine as an intermediate in ATP and GTP formation by succinyl CoA synthetase (Mitchell *et al.*, 1964).