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Studies on Energy Transfer in Mitochondrial Oxidative Phosphorylation. III. On the Interaction of Adenosine Diphosphate with High-Energy Intermediates*

Rudolf H. Eisenhardt and Otto Rosenthal

ABSTRACT: The initial rapid phase of adenosine triphosphate formation (ATP jump) that is observed when adenosine diphosphate (ADP) is added to mitochondrial suspensions incubated in presence of inorganic phosphate (P_i) and substrate has been further investigated. Direct determinations of the rate and magnitude of the ATP jump are reported in the presence of β-hydroxybutyrate and of succinate, respectively. When succinate rather than β-hydroxybutrate is the substrate, the rate of the ATP jump is considerably enhanced. With either substrate, uncoupling concentrations of 2,4-dinitrophenol are without effect on the ATP jump, though the slower subsequent steady-state phosphorylation is inhibited. The results are interpreted to signify that the ATP jump is due to the rapid interaction of ADP and P_i with preformed high-energy intermediates and that it represents the actual phosphorylation reaction. Steady-state phosphorylation, which is that normally observed, is limited by the rate at which these intermediates are resynthesized. This step is considered to be rate limiting, which suggests that the intermediates are isolated from and independent of the members of the respiratory chain. It is concluded that uncouplers act on the respiratory chain side of the rate-limiting step.

The principal function of the mitochondrial electron transport system is to provide the energy required to combine ADP[1] with P_i to produce ATP. Although Boyer (1965) and Mitchell (1966) have advanced alternate hypotheses, most current theories of oxidative phosphorylation invoke the existence of some intermediary energy carriers, the so-called high-energy intermediates, to explain the transfer and subdivision of free energy from the electron transport system to the phosphorylation site.

Notwithstanding a considerable search for such intermediates, no convincing *chemical*, as contrasted to

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[1] Abbreviations used that are not listed in *Biochemistry 5*, 1445 (1966) are: dimethyl-POPOP, 1,4-bis[2-(4-methyl-5-phenyloxazolyl)]benzene; POP, 2,5-diphenyloxazole.

kinetic, evidence for their existence has been reported in mitochondria isolated from higher organisms. Most of this search has been directed at phosphorylated intermediates. However, as we have previously pointed out, evidence is against the existence of *stable* phosphorylated energy carriers on the pathway to ATP (Eisenhardt and Rosenthal, 1964). The recent isotope-exchange studies of Mitchell *et al.* (1967) support this conclusion.

This paper reports on an expansion of earlier studies of one of us (Schachinger *et al.*, 1960) on the interaction of ADP with mitochondria preincubated under state 4 conditions (Chance and Williams, 1956a). Addition of ADP to mitochondria in state 4 elicits a rapid burst of ATP formation, the so-called ATP jump, which is faster than and precedes the normally observed steady-state phosphorylation. In contrast to the earlier study the present report establishes the biphasic nature of ATP formation directly rather than by interpolation. This has enabled us to measure the rate and magnitude of the ATP jump directly. We also report on the effect of the uncoupler, DNP, on this initial part of the phosphorylation reaction.

The biphasic nature of ATP formation indicates the existence of a pool of high-energy intermediates that accumulates in mitochondria in state 4 (Eisenhardt and Rosenthal, 1962). Although kinetic evidence cannot take the place of chemical isolation and identification, any postulated high-energy intermediate must comply with these kinetic observations.

Experimental Procedure

All experiments were performed with rat liver mitochondria isolated in 0.25 M sucrose according to the method of Schneider (1948). The liver of one male Sprague–Dawley rat (200–250 g) was used per experiment. The mitochondrial pellet was washed twice with sucrose, special care being taken to exclude the fluffy layer that sediments on top of the pellet. The protein concentration was 4.9 ± 0.3 mg per ml as determined by a biuret reaction (Gornall *et al.*, 1949). Before each experiment, the mitochondria were assayed with a recording oxygen electrode under conditions closely matching those of the experiment, except for temperature, which was 25° for the assays. Only preparations with high values for respiratory control and ADP/O ratios were used in these studies. In some instances the oxygen electrode assays were supplemented by spectrophotometric ones, as described previously (Schachinger *et al.*, 1960). The actual experiments were carried out with 12–14-ml total volume of reaction medium in a water-jacketed glass chamber. The temperature in the reaction chamber was between 9 and 10° and was kept constant to within ±0.2° in each experiment. The reaction chamber, from which aliquots were obtained as single drops from an adjustable orifice, is an integral part of a rapid sampling apparatus (Eisenhardt, 1964). The rate of sampling in different experiments was in the range of one to two samples per second, though in any given experiment the rate was kept constant to ±5%. Under these conditions the drop volume was found to be uniform within 0.15%.

After a 10-min equilibration period at the temperature of the experiment, the reagents under study (such as ADP, DNP, and succinate) were added by means of remotely controlled injectors and rapidly mixed into the otherwise complete suspension. The gas phase was air and the suspension was stirred during the entire experiment, hence the oxygen tension stayed close to equilibrium with air throughout. Sampling was started a few seconds before the additions in order to establish a reference base line, the last part of which is shown in Figures 1–5 before time zero. The reaction was terminated when the samples dropped into tubes containing a large excess of rapidly stirred buffered perchloric acid (0.9 M $HClO_4$, 0.6 M Na_2SO_4, and 0.3 % Br_2). The times at which additions were made and when samples fell into the deproteinizing medium were determined to an accuracy of 0.02 sec with an Esterline-Angus ten-channel event recorder equipped with high-speed drive and a recording time signal independent of the chart paper graduations.

Radioactivity Determinations. The unreacted $^{32}P_i$ was removed as the phosphomolybdate complex by reverse-phase column chromatography on siliconized Celite (Johns-Manville). Details of this technique, a semiautomatic modification of the procedure of Hagihara and Lardy (1960), will be described elsewhere. The rate of [^{32}P]ATP formation was followed by determining the appearance of radioactivity in an aliquot of the aqueous eluate containing the nucleotide fraction. The distribution of radioactivity between the β- and γ-phosphate groups of ATP, and between ADP and ATP, respectively, was determined in selected aliquots by either paper chromatography or ion-exchange chromatography both before and after treatment with hexokinase and glucose, according to the procedures of Lowenstein (1960) and of Pressman (1960). All radioactivity determinations were carried out on a Packard Tri-Carb liquid scintillation counter Model 314 E. The scintillation medium, essentially that of Davidson (1958), contained *p*-dioxane, ethylene glycol, anisole, and 1,2-dimethoxyethane in a 6:2:1:1 volume ratio. PPO (0.25%) (w/v) and dimethyl-POPOP (0.015%) (w/v) were used as the primary and secondary scintillators, respectively. This medium (14 ml) was added to 1-ml aliquots and every sample was counted three or more times, usually to over 100,000 counts. The raw counts were corrected for self-decay, analyzed statistically, and converted into ATP concentrations by means of a digital computer. Some of the figures accompanying this report were traced originally by a plotting attachment of the computer.

Materials. $^{32}P_i$ was obtained as H_3PO_4 in HCl, initially from Oak Ridge National Laboratory, more recently from E. R. Squibb and Sons. Some of the early batches from Oak Ridge required purification, which was usually accomplished by the magnesia method (Umbreit *et al.*, 1957), occasionally by chromatography on Dowex 1 and 50 (Suelter *et al.*, 1961). ADP was obtained from the Sigma Chemical Co. as the sodium salt. The extent of AMP contamination and the actual ADP content were determined by the procedure of Adam (1963a), and the ATP content by the phosphoglycerate

kinase reaction (Adam, 1963b). Only samples containing less than 2% AMP were used. Stock solutions (0.2 M) were adjusted to pH 7.2, stored below −20°, and used only once; *i.e.*, no thawed ADP solutions were refrozen for future use. No significant deterioration occurred at either −24 or −78° within 3 months. Isoosmolar glycylglycine buffer (Nutritional Biochemicals) containing 0.012 M NaCl and 0.006 M $MgCl_2$ was adjusted to pH 7.2. All other reagents were adjusted to the same pH prior to addition, except for the radioactive, carrier-free P_i. To neutralize this material, an amount of NaOH equivalent to the HCl content was added. DNP (Eastman Organic Chemicals) was resublimed *in vacuo*, mp 114.0–114.5° uncor, and used as a 5×10^{-3} M stock solution. β-Hydroxybutyrate (Nutritional Biochemicals Corp.) and succinate (California Corp. for Biochemical Research, A grade) were used without further purification. PPO and dimethyl-POPOP were purchased from the Packard Instrument Co. *p*-Dioxane (spectral grade), anisole, and 1,2-dimethoxyethane (both reagent grade) were products of Matheson Coleman and Bell, and reagent ethylene glycol was obtained from the J. T. Baker Chemical Co.

Results

The results have been corrected for any ATP present before additions were made and only net changes are recorded. By reacting aliquots of the endogenous ATP with hexokinase and glucose and determining the radioactivity of the resultant ADP and glucose 6-phosphate, it was found that full isotopic equilibration of both β- and γ-phosphate groups with P_i had taken place during the 10-min equilibration period. Additional activity appearing after ADP addition was found to be entirely in the γ position. Endogenous ATP concentration varied from 3 to 7 μmoles per g of protein, whereas the ATP jump was generally below 1 μmole/g of protein. Since the specific radioactivity of the endogenous ATP is twice that of newly formed ATP, the ATP jump leads to an increase in radioactivity of barely 10%. It was therefore necessary to refine the analytical techniques for the determination of ATP to a considerable extent. In carefully controlled experiments it became possible to reduce the standard deviation to less than 0.3% (Eisenhardt, 1964), and 0.7% could be attained routinely.

In selected experiments, certain aliquots obtained shortly before and after ADP addition were chromatographed after removal of P_i in order to ascertain the localization of the additional radioactivity over and above the base line, since the procedure of Hagihara and Lardy (1960) does not separate ADP from ATP. No significant activity was found in the ADP fraction. The β- and γ-phosphate groups of the ATP fraction were then separated as described above. A rather accurate analysis of the additional activity was obtained by comparing the relative increase in activity of the β- and γ-phosphate groups of ATP from samples obtained before and after ADP addition. No significant change appeared in the β-phosphate group, and the total increase in radioactivity was closely paralleled by that found in the γ-phosphate group. These results rule out any role of adenylate kinase in the ATP jump. Similar results were obtained by Vignais (1963) in digitonin particles. Furthermore, no differences in the rates of ATP formation were encountered in experiments performed in the presence and absence of NaF, as inhibitor of adenylate kinase (Schachinger *et al.*, 1960). Demonstration of the persistence of the ATP jump in presence of atractylate, a specific inhibitor of the ATP–P_i-exchange reaction, by Vignais (1963), would appear to remove any remaining question about the origin of the radioactive ATP formed during the initial rapid phase.

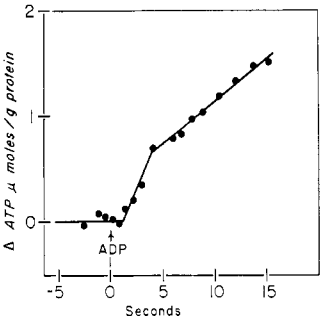

FIGURE 1: ATP jump with β-hydroxybutyrate. A suspension of rat liver mitochondria (protein concentration 4.9 mg/ml) was incubated with β-hydroxybutyrate (10 mM) and ^{32}Pi (10 mM, 0.1 mCi/ml) in glycylglycine buffer for 10 min at 10° at pH 7.0 prior to addition of ADP (0.5 mM) at time zero.

ATP Jump with β-Hydroxybutyrate. Addition of ADP to mitochondria incubated in state 4 with β-hydroxybutyrate as substrate leads to a rapid burst of ATP formation, the so-called ATP jump, followed by the normally observed steady-state phosphorylation rate. A typical experiment of this sort is shown in Figure 1. In the original study (Schachinger *et al.*, 1960), existence of the initial rapid phase could only be deduced through interpolation, even though the temperature had been reduced to 7°. The rapid automatic sampler (Eisenhardt, 1964) used in the present study permits the direct observation of the ATP jump and determination of its rate and magnitude at 10°.

Following ADP addition, delays of between 0.7 and 2.4 sec were observed in 16 out of 18 experiments. This delay probably reflects the translocation of ADP into the phosphorylating space. The rate of translocation has recently been measured under similar conditions by Heldt (1967) and compatible values have been obtained.

Effect of DNP on the β-Hydroxybutyrate-Supported ATP Jump. Figure 2 depicts a similar experiment, except that 10 μM DNP was added 1.6 sec before ADP. This concentration of DNP, the minimum concentration that uncoupled this preparation according to the oxygen electrode assay, is seen to have no effect on the ATP jump, though the subsequent steady-state phosphorylation is inhibited by about 90%. To eliminate the possibility that the persistence of the ATP jump might be the consequence of a time delay in the action of DNP, the order of addition was reversed in the experiment shown in Figure 3. The conditions are identical with those of Figure 2, except for a slightly lower protein

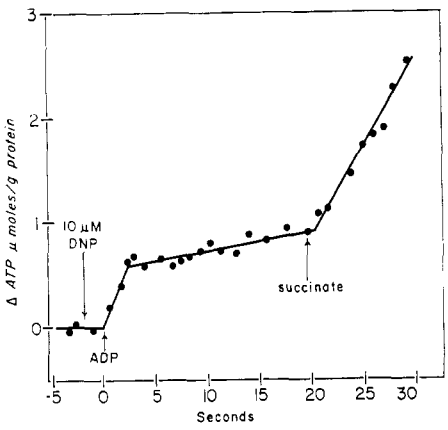

FIGURE 2: Effect of DNP on β-hydroxybutyrate-supported ATP jump (a). Conditions similar to Figure 1. Protein concentration, 5.1 mg/ml; β-hydroxybutyrate, 8 mM; ^{32}Pi, 8 mM, 0.06 mCi/ml; temperature, 9.5°; pH 7.1. DNP was added 1.6 sec *before* ADP (0.4 mM), and succinate (6.5 mM) 19.4 sec after ADP.

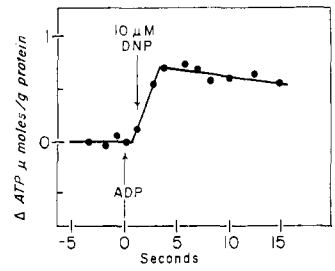

FIGURE 3: Effect of DNP on β-hydroxybutyrate-supported ATP jump (b). Conditions similar to Figure 1. Protein concentration, 4.4 mg/ml; β-hydroxybutyrate, 10 mM; ^{32}Pi, 10 mM, 0.1 mCi/ml; temperature, 10°; pH 7.0. DNP was added 1.6 sec *after* ADP (0.07 mM).

concentration, which might account for the more complete uncoupling seen in Figure 3.

ATP Jump with Succinate. Rat liver mitochondria metabolize succinate at an appreciably higher rate than β-hydroxybutyrate; steady-state phosphorylation is enhanced three- to fivefold. As shown in Figure 4, the rate of the ATP jump also increases markedly when succinate is the substrate instead of β-hydroxybutyrate. The experiment depicted in Figure 4 is the only one where an experimental point was actually obtained that unambiguously pertains to the rapid phase. In all other succinate experiments all points fell on either the preceding base line or the following steady-state line, so that the ATP jump manifested itself by means of the discontinuity between these two straight lines. The actual rate cannot be determined on the basis of such data and only a minimum rate can be deduced. The actual rate may easily be considerably higher. This is so because the beginning and end of the ATP jump as drawn in Figure 4 are taken as the last base-line point and the first steady-state line point, respectively. Clearly the base line may have extended further and the steady-state line started earlier than is shown in this figure.

While Figures 1–3 were drawn to the same scale to permit direct comparison, the ordinates of Figures 4 and 5 have been compressed by 60% because of the higher phosphorylation rate associated with succinate. The data of Figure 1 in which β-hydroxybutyrate was the substrate are replotted in Figure 4 to emphasize the large difference between the phosphorylation rates associated with these two substrates.

A more accurate determination of the succinate-supported ATP jump, as well as detailed studies of other fast substrates, awaits the availability of sampling devices with better time resolution. Such equipment is under development in this laboratory.

Effect of DNP on Succinate-Supported ATP Jump. The final portions of the experiments shown in Figures 2 and 4 show that 10 μM DNP, while effectively uncoupling β-hydroxybutyrate-supported oxidative phosphorylation, has only a small effect when succinate is the substrate. In order to investigate the effect of DNP on the succinate-supported ATP jump, higher uncoupler concentrations are required. In a series of polarographic studies (R. H. Eisenhardt, unpublished observations) it has been possible to demonstrate that for a given mitochondrial preparation there exists a direct proportionality between uncoupler concentration and oxygen consumption in the absence of phosphorylation. It is therefore probable that a similar relationship exists between uncoupler concentration and the extent to which phosphorylation becomes uncoupled. About 40 μM DNP is required to uncouple succinate-linked oxidative phosphorylation by some 90% under representative conditions. Unfortunately, concentrations much above 10 μM induce ATPase activity, which makes the observation of the ATP jump quite difficult. After a number of trials at different DNP concentrations, the conditions of the experiment shown in Figure 5 were chosen. This permitted the observation of the ATP jump in the presence of an intermediate concentration of DNP. Although addition of 17 μM DNP causes immediate ATPase activity, the subsequent addition of ADP leads to a normal ATP jump, similar in rate and magnitude to that obtained in the absence of DNP. The steady-state phosphorylation, when compared to that seen in Figure 4, appears to be uncoupled by about 50%. Again, only a lower limit can be established for the rate of the ATP jump. This minimum rate must be corrected for the ATPase activity and is several times larger than that seen with β-hydroxybutyrate.

Effect of ADP Concentration. The ADP concentration used in different experiments varied from 20 to 500 μM. There was no noticeable difference in the ATP jump within this range of concentration. At the protein concentration used in these experiments, an initial concentration of about 10 μM ADP would seem to be the usable lower limit, because at 5 mg of protein/ml, the concentration of ADP would change from 10 to 5 μM during the jump. Because the transition between the two phases of phosphorylation is not sharply defined with the analytical instrumentation presently available, it is impossible to establish the apparent K_m for ADP with any certainty. However, in as far as 20 μM appears to be as effective as higher concentrations of ADP, an

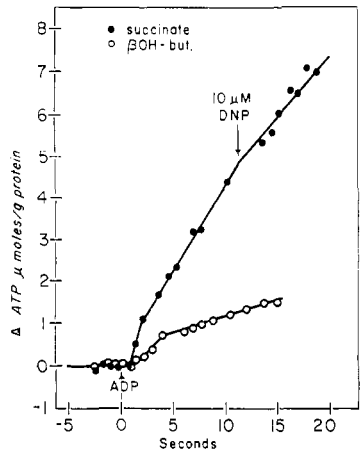

FIGURE 4: ATP jump with succinate (solid circles). Conditions similar to Figure 1 (which is repeated in this figure, open circles), except for substrate, which was succinate throughout. Protein concentration, 5.4 mg/ml; succinate, 10 mM; ^{32}Pi, 7mM, 0.05 mCi/ml; temperature, 9°; pH 7.1; ADP, 0.5 mM. DNP was added 11.3 sec after ADP.

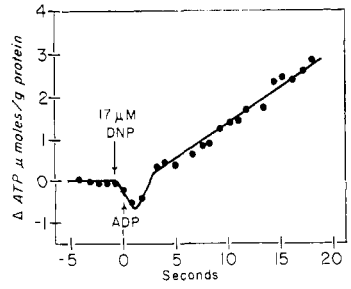

FIGURE 5: Effect of DNP on succinate-supported ATP jump. Conditions similar to Figure 1, except that succinate was the substrate throughout. Protein concentration, 5.1 mg/ml; succinate, 10 mM; ^{32}Pi, 10 mM, 0.08 mCi/ml; temperature, 10°; pH 7.1. DNP was added 0.9 sec before ADP (0.16 mM).

apparent K_m below 10^{-5} is likely. This value is lower than that obtained by Chance and Williams (1956b) which was based upon the point of half-maximal oxygen consumption.

Oscillations. Some evidence of damped oscillations immediately after the ATP jump is observable in virtually all experiments. A period of about 6 sec emerges, most clearly seen in Figure 4. The absolute magnitude is too small to permit more detailed analysis, and the observations are close to the error range.

Discussion

The occurrence of a brief period of more rapid ATP formation preceding steady-state phosphorylation is most readily interpreted by attributing this extra ATP to the interaction of ADP and P_i with a preformed energy carrier which accumulates in state 4. The concentration of this carrier decreases concomitantly with the ATP jump until it reaches a steady-state equilibrium between its formation and utilization. We have previously equated this energy carrier with the postulated high-energy intermediate X∼I (Schachinger *et al.*, 1960; Chance and Williams, 1956a). The ATP jump would thus represent the actual phosphorylation reaction, which is fast, while the subsequent slower steady-state phosphorylation is limited by the rate of resynthesis of X∼I. The synthesis of X∼I is sensitive to uncoupling agents such as DNP as was demonstrated in the preceding paper of this series (Eisenhardt and Rosenthal, 1964). These findings have now been extended. The experiments depicted in Figures 2, 3, and 5 show that the ATP jump persists in the presence of uncoupling concentrations of DNP, whereas the steady-state phase of ATP formation is inhibited, regardless of whether succinate or a pyridine nucleotide-linked substrate, such as β-hydroxybutyrate, is used.

There is no evidence that a different phosphorylation reaction may be involved in the ATP jump. Rates of phosphate incorporation during substrate-level phosphorylation under conditions directly comparable with ours were recently determined by Heldt and Schwallbach (1967). The highest rate obtained in their studies (that of incorporation of P_i into ADP) was less than 5% of that characteristic of the ATP jump. Their experiments preclude any possible contribution of succinyl-CoA to the ATP jump as well. In any case, known reactions of this type involve fairly stable phosphorylated intermediates, whereas isotope equilibration studies appear to rule out any contribution by such intermediates (Eisenhardt and Rosenthal, 1964).

Since a delay is generally observed before ADP addition elicits phosphorylation, it is necessary to show that the lack of effect of DNP on the ATP jump is not caused by a similar time lapse in DNP action. The experiment shown in Figure 3, where DNP was added during the ATP jump instead of before ADP, demonstrates that the uncoupling effect of DNP manifests itself fully within the 1.5 sec that elapse between DNP addition and the end of the ATP jump. As this time interval is shorter than the one observed between DNP addition and the onset of the ADP jump in Figures 2 and 5, it must be concluded that the insensitivity of the ATP jump to DNP is due to the lack of interaction of DNP with preformed high-energy intermediates. Our conclusions regarding the mechanism of uncoupling (Eisenhardt and Rosenthal, 1964) have recently been confirmed by Pinchot (1967) in *Alcaligenes faecalis*.

The lack of effect of DNP on the ATP jump raises questions regarding the role of the ATP jump in oxidative phosphorylation since sensitivity to DNP has been taken to be the *sine qua non* of oxidative, as contrasted to substrate-level phosphorylation. Schachinger *et al.* (1960) showed that addition of DNP 60 sec before ADP addition, as contrasted to 2 sec, as in this paper, abolished all phosphorylation, including the ATP jump. This result has been confirmed by Vignais (1963) in submitochondrial particles, where the addition of 10 μM DNP 120 sec before ADP led to over 95% inhibition of both jump and steady-state phosphorylation. Since all mitochondrial preparations have some ATPase activity (which provides ADP), the inhibition of X∼I resynthesis will eventually lead to the exhaustion of any preformed X∼I pool.

The persistence of the ATP jump in the presence of

uncoupling agents known to cause rapid shifts in the oxidation–reduction levels of the members of the electron transport system (Chance *et al.*, 1963) also suggests that, once formed, X∼I is effectively isolated from the electron transport system. Thus any association of the ATP jump with known members of the electron transport system is unlikely. It is therefore necessary to review earlier assumptions regarding stoichiometric relations between the ATP jump and the decrease in DPNH content (Schachinger *et al.*, 1960). The interpretation of this apparent stoichiometry, though founded on good experimental evidence, was always difficult. The rate of oxidation of the reduced pyridine nucleotides following ADP addition was found to be slower than the minimum rate of the ATP jump, which at the time could be determined only by interpolation. A causal relationship was therefore difficult to establish because the effect, *i.e.*, the ATP jump, preceded the probable causative action, *i.e.*, the oxidation of the reduced pyridine nucleotides. It was necessary to postulate a two-step process in which the transfer of energy from a special form of the reduced pyridine nucleotides to ATP was fast, whereas the subsequent oxidation was slow. Since the obvious energy source is the resonance energy released during the aromatization–oxidation of the pyridine ring, this postulate presented considerable conceptual difficulties. It now appears that earlier interpolated determinations of the ATP jump incorporated part of the DPNH-associated phosphorylation described by van Dam (1966).[2] This would also explain the consistent quantitative differences between the average values of the interpolated ATP jump (1.6 μmoles/g of protein) and of the directly determined values (0.8 μmoles/g of protein). Only the amount of ATP formed in excess of the steady-state phosphorylation (extrapolated to zero time) could be assigned to the interpolated ATP jump reported earlier. This does not appear to be justified for the direct determinations, as can be seen from a comparison of Figures 1–3. Not only is the rate very nearly identical in all three figures, but the magnitude of the ATP jump is remarkably uniform, *regardless of whether or not steady-state phosphorylation was inhibited.* The rate of steady-state phosphorylation is determined by the rate of synthesis of X∼I from its constituents. These will not be present at the time of ADP addition;[3] therefore, steady-state phosphorylation is initiated only during the ATP jump and does not reach its full rate until the end of the ATP jump. The contribution of steady-state phosphorylation to the rapid phase is probably of the order of 10–15%. The observation that the two phases of phosphorylation are *not* additive argues strongly against any interpretation wherein the two phases would represent independent reactions.

The stoichiometric reaction described by van Dam occurs in approximately 15 sec, the ATP jump in about 2 sec under similar conditions.

It appears that the two reactions have no close relationship and that the objections raised by Sanadi (1965) on the basis of van Dam's observations are not relevant to the ATP jump and its relationship to X∼I.

The large difference observed between the rate of the ATP jump associated with succinate and with β-hydroxybutyrate, respectively, is unexpected and not readily explained on the basis of existing theories of oxidative phosphorylation. An equally unexplainable difference between these two substrates was encountered during an entirely different type of experiment (R. H. Eisenhardt, unpublished observations). We found a consistent and reproducible difference in the ratios of relative efficiency of oxygen utilization between succinate and pyridine nucleotide-linked substrates, depending upon whether the energy was utilized in phosphorylation (when it was 2:3, in agreement with respective P/O ratios) or dissipated in uncoupling (when it was 1:2). These studies will be reported shortly. It may well be that the simplest resolution of these difficulties is to be found in a scheme that would postulate the existence of separate though interdependent components for succinate-supported and pyridine nucleotide linked oxidative phosphorylation, respectively.

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[2] The sevenfold discrepancy in the relative rates of DPNH oxidation and ATP formation given in van Dam's earlier publication (van Dam, 1964) is not present in the data presented in his dissertation (van Dam, 1966). The rates reported in the dissertation are equal; both reactions take approximately 15 sec each, as contrasted to the previously reported 5 sec for DPNH oxidation *vs.* 35 sec for the rapid phase of ATP formation.

[3] The slow turnover of X∼I in state 4 can be ignored because only net synthesis is being measured.

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A Study of the Enzymatic Reactions Involved in the Formation of 5-Hydroxy-4-ketohexanoic Acid and Its Isomer, 5-Keto-4-hydroxyhexanoic Acid*

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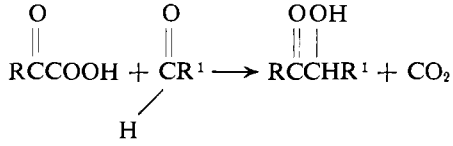
ABSTRACT: The enzymatic formation of 5-keto-4-hydroxyhexanoic acid (KHH) has been shown to occur by the condensation of succinic semialdehyde (SSA) with pyruvic acid (Pyr). This reaction was catalyzed by a beef heart particulate preparation and was markedly dependent upon the presence of thiamine pyrophosphate (TPP). Mg^{2+} was found to increase the formation of KHH by an additional 18%. In addition to kinetic data for the KHH reaction, kinetic studies of the analogous condensation of α-ketoglutaric acid (KG) with acetaldehyde (AcH) to form 5-hydroxy-4-ketohexanoic acid (HKH) are presented. The latter reaction was shown to occur also in a nonenzymatic model system with an absolute requirement for thiamine. Evidence is presented which indicates that it is likely that KHH formation is catalyzed by pyruvate decarboxylase, the same enzyme that serves as a catalyst in the formation of acetoin from Pyr and AcH. The inhibition of the KHH, HKH, and acetoin reactions by various reagents is described. The possible physiological significance of the KHH condensation as well as of KHH itself is not known at present.

The enzymatic condensation of α-keto acids with various aldehydes by the particulate fraction of mammalian tissues follows the general reaction



The reaction between pyruvate and acetaldehyde to form acetoin is well known, and the reaction between pyruvate and succinic semialdehyde to form 5-keto-4-hydroxyhexanoic acid is described in this paper. The analogous reaction between α-ketoglutarate and aldehydes was recognized recently when Bloom and Westerfeld (1966) identified 5-hydroxy-4-ketohexanoic acid as the product of the KG plus AcH[1] reaction; Koch and Stokstad (1966), Stewart and Quayle (1967),

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[1] Abbreviations used in this paper that are not defined in *Biochemistry 5*, 1445 (1966), are: HKH, 5-hydroxy-4-ketohexanoic acid; KHH, 5-keto-4-hydroxyhexanoic acid; Pyr, pyruvic acid; AcH, acetaldehyde; KG, α-ketoglutaric acid; SSA, succinic semialdehyde.