Spectrophotometric Studies of Oxidative Phosphorylation

Maynard E. Pullman and Efraim Racker

The mechanism by which mitochondria form adenosine triphosphate (ATP) (*1*) from adenosine diphosphate (ADP) and inorganic phosphate concomitant with the oxidation of carbohydrates, fatty acids, and some amino acids has been the subject of much intense investigation. The chief difficulties which have limited the experimental approach have been the lability of the phosphorylating system and the lack of a convenient, direct, and rapid method for the study of the kinetics of the phosphorylation process.

Some success in stabilization of the phosphorylating enzymes has been achieved by the use of ethylenediamine tetraacetic acid (EDTA) (*2*) and serum albumin (*3*). It has been suggested that the latter exerts its stabilizing effect by maintaining the structural integrity of the mitochondria (*4*). Until very recently, the phosphorylation processes were believed to be to a large extent dependent on the high degree of morphological organization inherent in the mitochondria, and the disruption of this organization was paralleled by an uncoupling of phosphorylation from electron-transfer processes. However, recent demonstrations of the occurrence of oxidative phosphorylation in extracts of bacteria (*5*) and of animal tissue mitochondria (*6*) have given impetus to the study of the enzyme components of the system.

A systematic study (*7*) of coupled phosphorylation has been initiated in this laboratory with the aims of (i) developing a spectrophotometric assay for the direct and continuous measurement of phosphorylation, (ii) investigating the factors responsible for the extreme lability of oxidative phosphorylation in intact mitochondria, and (iii) obtaining stable extracts of mitochondria capable of carrying out oxidative phosphorylation.

The authors are on the staff of the Division of Nutrition and Physiology of the Public Health Research Institute of the City of New York, Inc.

Spectrophotometric Assay

Mouse liver mitochondria were prepared in 0.25*M* sucrose containing 0.005*M* ethylenediamine tetraacetic acid according to a modification of the method of Schneider (*8*) similar to that used by Kielley and Kielley (*9*). Preliminary experiments demonstrated that these mitochondrial preparations were virtually unable to oxidize either reduced triphosphopyridine nucleotide (TPNH) or reduced diphosphopyridine nucleotide (DPNH) under the experimental conditions used in this study to measure oxidative phosphorylation. However, diphosphopyridine nucleotide (DPN) reduced inside the mitochondria by a diphosphopyridine nucleotide-linked substrate, such as β-hydroxybutyrate, is readily oxidized (*10*).

During this process, up to 3 moles of adenosine triphosphate are formed from adenosine diphosphate and inorganic phosphate per mole of reduced diphosphopyridine nucleotide that is oxidized (*11*). Since the adenosine triphosphate formed is available for the phosphorylation of glucose by externally added hexokinase, it was possible—by supplementing the mitochondria with adenosine diphosphate, inorganic phosphate, magnesium ion (Mg^{++}), β-hydroxybutyrate, glucose, hexokinase, glucose-6-phosphate (G-6-P) dehydrogenase, and triphosphopyridine nucleotide (TPN)—to follow the continuous formation of adenosine triphosphate in a Beckman spectrophotometer, which was equipped with a photomultiplier tube, by measuring the reduction of triphosphopyridine nucleotide at a wavelength of 340 millimicrons. The sequence of the reactions of the test system is shown at the bottom of this page.

This assay differs in principle from the ingenious methods described by Chance and Williams (*12*) in which oxidative phosphorylation is measured indirectly by rates of respiration and which, in addition, require special apparatus.

Since β-hydroxybutyrate is oxidized to acetoacetate (AcAc), which is not further metabolized in liver mitochondria, it is possible to calculate P/O ratios from acetoacetate and inorganic phosphate measurements (*13, 13a*). The physical state, and hence the light-transmitting and -scattering properties of the mitochondria, are affected by some of the constituents of the reaction mixture (14). It is therefore essential that every experimental variation of the constituents be controlled by a check cell containing all ingredients except triphosphopyridine nucleotide. Each spectrophotometric reading is taken against this control cell in check position. In the experimental values reported, a small correction has been applied for the action of mitochondrial adenylic kinase on adenosine diphosphate, which results in the formation of adenosine triphosphate and the reduction of triphosphopyridine nucleotide in the absence of inorganic phosphate or in the presence of 2,4-dinitrophenol (DNP). In order to diminish this correction factor, adenosine diphosphate was used at suboptimal concentration.

In Table 1 (column 1), it may be seen that the formation of reduced triphosphopyridine nucleotide is dependent on the presence of inorganic phosphate, adenosine diphosphate, hexokinase, and glucose-6-phosphate dehydrogenase, and partially dependent on β-hydroxybutyrate and magnesium ion. Addition of diphosphopyridine nucleotide had little or no effect. Phosphorylation is completely inhibited by $5 \times 10^{-5}M$ 2,4-dinitrophenol. The pronounced dependency on β-hydroxybutyrate that is recorded in Table 1 with aged preparations (column 2) is not usually observed with fresh mitochondria (column 1).

Restoration of Phosphorylating Activity in Aged Mitochondria

The addition of serum albumin to preparations of insect sarcosomes (*3*) or rat liver mitochondria (*15*) has been shown to maintain the phosphorylation

$$\beta\text{-hydroxybutyrate} + 3\ ADP + 3\ P_i + \tfrac{1}{2}\ O_2 \xrightarrow{\text{mitochondria}} AcAc + 3\ ATP + 4\ H_2O \quad (1)$$

$$ATP + \text{glucose} \xrightarrow{\text{hexokinase}} G\text{-}6\text{-}P + ADP + H^+ \quad (2)$$

$$G\text{-}6\text{-}P + TPN^+ \xrightarrow{\text{G-6-P dehydrogenase}} 6\text{-}PGA + TPNH + H^+ \quad (3)$$

Table 1. Spectrophotometric assay of oxidative phosphorylation in fresh and aged liver mitochondria* (*16a*).

Addition	Change in optical density in 10 min at 340 mμ × 10^3	
	Fresh preparation	Aged preparation
None	1357	87†
Bovine serum albumin (2000 μg)	1483	872
Bovine serum albumin (500 μg)	1327	814
+ Diphosphopyridine nucleotide	1405	984‡
+ 2,4-Dinitrophenol ($5 \times 10^{-5}M$)	35	0‡
+ 2,4-Dinitrophenol ($2 \times 10^{-5}M$)	381	534‡
− β-Hydroxybutyrate	602	56‡
− Orthophosphate	228	91‡
− Adenosine diphosphate	64	0‡
− Hexokinase	2	0‡
− Glucose-6-phosphate dehydrogenase	308	
− Magnesium ion (Mg^{++})	992	

* The complete system (1.0 ml) contained the following: 0.1*M* tris(hydroxymethyl)aminomethane at *p*H 7.4; 0.005*M* glucose; $1.4 \times 10^{-5}M$ adenosine diphosphate; 0.001*M* triphosphopyridine nucleotide; $3 \times 10^{-4}M$ $MgCl_2$; 0.01*M* orthophosphate at *p*H 7.4; 0.012*M* DL-β-hydroxybutyrate; 50 units of hexokinase (*17*); and 0.3 units of glucose-6-phosphate dehydrogenase (*18*). The reaction was started by the addition of mitochondria obtained from 20 mg (wet wt.) of mouse liver in a mixture of 0.25*M* sucrose and 0.005*M* ethylenediamine tetraacetic acid (480 μg of protein in column 1, 409 μg of protein in column 2) to a microcuvette with a 1-cm light path. The optical density changes at 340 mμ were recorded for 10 minutes against a control cell identical with the experimental cell except that triphosphopyridine nucleotide was omitted. Protein was determined according to Robinson and Hogden (*19*).
† The activity obtained with this preparation before aging was 1340.
‡ In these experiments, 500 μg of bovine serum albumin were present in addition to the indicated protocol.

Table 2. Correlation of spectrophotometric assay of phosphorylation with orthophosphate uptake and acetoacetate formation in an aged mitochondrial preparation.*

Additions	Glucose-6-phosphate (μmole)	Orthophosphate uptake (μmole)	Acetoacetate formed (μmole)
None	0.01	− 0.10	0
β-Hydroxybutyrate	0.07	− 0.02	0.43
Bovine serum albumin	0.10	0.17	0
β-Hydroxybutyrate + bovine serum albumin	0.56	0.62	0.36

* The complete system (3.0 ml) contained the following: 0.1*M* tris(hydroxymethyl)aminomethane at *p*H 7.4; 0.005*M* glucose; $1.4 \times 10^{-5}M$ adenosine diphosphate; 0.001*M* triphosphopyridine nucleotide; $3 \times 10^{-4}M$ $MgCl_2$; 0.001*M* orthophosphate at *p*H 7.4; 150 units of hexokinase; and 0.9 units of glucose-6-phosphate dehydrogenase. To this test system was added 0.012*M* DL-β-hydroxybutyrate and 500 μg of bovine serum albumin, as indicated. The reaction was started by the addition of aged (24 hours) mitochondria from 60 mg of mouse liver in a mixture of 0.25*M* sucrose and − 0.005*M* Versene (1.44 mg protein); readings were recorded for 20 minutes. The reaction was stopped by the addition of 0.3 ml of 50-percent perchloric acid. After centrifugation, aliquots of the supernatant were taken for orthophosphate analysis (*20*) and acetoacetate determination (*21*).

Table 3. Uncoupling of oxidative phosphorylation by mitochondrial factor and effect of bovine serum albumin.*

Additions	Glucose-6-phosphate		Orthophosphate uptake (μmole)	Acetoacetate formed (μmole)
	Direct (μmole)	Indirect (μmole)		
None	0.38	0.35	0.18	0.78
Bovine serum albumin	0.68	0.61	0.66	0.62
Inhibitor	0.09	0.06	0	0.65
Bovine serum albumin + inhibitor	0.70	0.62	0.78	0.78

* The complete system (3.0 ml) was the same as that described in Table 2, including 0.012*M* DL-β-hydroxybutyrate. To this system were added 1500 μg of bovine serum albumin and 0.3 ml of an aqueous suspension of mitochondrial factor (see text) as indicated. The reaction was started by the addition of 0.03 ml of freshly prepared mitochondrial suspension containing 1.14 mg of protein; the reaction was followed for 20 minutes. The total change in optical density for the 20-minute period is recorded in Table 3 as the direct assay of glucose-6-phosphate. The reaction was stopped by the addition of 0.3 ml of 50-percent perchloric acid to both the experimental and the check cells. After centrifugation, the supernatant solution of the check cell was neutralized with 10*N* KOH, and an aliquot was assayed spectrophotometrically for glucose-6-phosphate with triphosphopyridine nucleotide and glucose-6-phosphate dehydrogenase (*18*). These values are recorded as the indirect assay of glucose-6-phosphate in Table 3. The experimental cells in each case were used for the determination of orthophosphate and acetoacetate as described in Table 2.

system, presumably by preserving the structural integrity of the mitochondria (*4*). We have confirmed the protective action of serum albumin. Moreover, we have found that it is possible to restore phosphorylation activity, in preparations which were completely inactivated by aging at 4°C for several days, by the addition of bovine serum albumin. The degree of restoration after 24 hours has varied from 50 to 100 percent in the different mitochondrial preparations. In Table 1, column 2, it can be seen that after 24 hours of storage at 4°C, approximately 65 percent of the original activity was restored upon the addition of 500 micrograms of bovine serum albumin. After 48 hours, this particular preparation was reactivated to approximately 40 percent of its original activity by 500 micrograms of bovine serum albumin. The response of fresh preparations to serum albumin has been variable. Some preparations show no effect, while others are stimulated some twofold.

That effects of serum albumin as measured by the increases of absorption at 340 millimicrons were in fact measurements of coupled phosphorylation and not unrelated optical changes is shown in Table 2. It can be seen that in the case of active phosphorylation there is reasonably good agreement between the spectrophotometric assay and determinations of inorganic phosphate disappearance. In experiments of this type, where both spectrophotometric and chemical measurements are required, the reaction was run in 3-milliliter Beckman cells with only 10 percent of the usual concentration of phosphate (1 micromole per milliliter). The use of this smaller amount of phosphate was necessary in order to measure phosphate disappearance with some degree of accuracy, even though it diminished the rate of the reaction to about half. Since suboptimal concentrations of inorganic phosphate and adenosine diphosphate were used, it is not surprising that low P/O ratios were obtained in some experiments. As can be seen from Table 2, serum albumin activates the phosphorylation process without essentially affecting the oxidation of β-hydroxybutyrate to acetoacetate. Concerning the specificity of serum albumin in "recoupling" phosphorylation to oxidation, several other proteins were tested. Gelatin, casein, insulin, and bovine γ-globulin were inactive, while β-lactoglobulin on a molar basis has approximately 20 percent of the activity of serum albumin.

In the light of these experiments, it appeared unlikely that serum albumin acted only by preserving the structural integrity of the mitochondria; hence, a search for another mechanism was made. A possible explanation for the serum al-

bumin effect is that it combined with an uncoupler of oxidative phosphorylation which becomes manifest on aging of the mitochondria.

Release of an Uncoupler

When mitochondria prepared in the usual manner were diluted in distilled water, a substance was released that, upon addition to fresh mitochondria, produced an inhibition of aerobic phosphorylation. As in the case of aged preparations, the inhibition could be reversed by the addition of bovine serum albumin. The inhibitor was prepared as follows: freshly prepared mitochondria from 15 grams of liver were diluted with 450 milliliters of water (to give a final protein concentration of 0.1 to 0.15 percent) and kept at room temperature for 1 hour. The extract was centrifuged at 18,000*g* for 20 minutes, and the residue was discarded. The inhibitory factor was concentrated either by lyophilization or by sedimentation at 144,000*g* for 1 hour in a Spinco preparative ultracentrifuge and then suspended in water. As shown in Table 3, the factor specifically inhibits phosphorylation without affecting the oxidation of β-hydroxybutyrate and thus simulates the action of 2,4-dinitrophenol and other known uncouplers. The addition of serum albumin to this system counteracts the effect of the inhibitor.

During the course of this work, our attention was drawn to the studies of Polis and Shmukler, who reported their findings at the meetings of the American Chemical Society (*16*). These authors have isolated from liver mitochondria an electrophoretically homogeneous heme protein which inhibits aerobic phosphorylation and which is counteracted by serum albumin. They suggest that this factor participates in the process of phosphorylation as an acceptor of energy-rich phosphate.

In view of these results, it appears likely that the generally recognized lability of aerobic phosphorylation in mitochondria may be partly explained on the basis of the release of this inhibitor.

References and Notes

1. The following abbreviations are used in this article: AcAc, acetoacetate; ADP and ATP, adenosine di- and triphosphate, respectively; DPN and TPN, di- and triphosphopyridine nucleotide, respectively; DPNH and TPNH, reduced DPN and TPN, respectively; EDTA, ethylenediamine tetraacetic acid (Versene); G-6-P, glucose-6-phosphate; P_i, orthophosphate; 6-PGA, 6-phosphogluconic acid.
2. E. C. Slater and K. W. Cleland, *Biochem. J. London* 55, 566 (1953).
3. S. E. Lewis and E. C. Slater, *ibid.* 58, 207 (1954); B. Sacktor, *J. Gen. Physiol.* 37, 343 (1953–54).
4. M. I. Watanabe and C. M. Williams, *J. Gen. Physiol.* 37, 71 (1953–54).
5. A. B. Brodie and C. T. Gray, *Biochim. et Biophys. Acta* 17, 146 (1955); G. B. Pinchot, *J. Biol. Chem.* 205, 65 (1953).
6. C. Cooper, T. M. Develin, A. L. Lehninger, *Biochim. et Biophys. Acta* 18, 159 (1955); I. Raw, *J. Am. Chem. Soc.* 77, 503 (1955).
7. It is a pleasure to acknowledge the collaboration of Harvey Pinefsky in some of the work reported here, as well as the valuable technical assistance of Michael Kandrach.
8. W. C. Schneider, *J. Biol. Chem.* 176, 259 (1948).
9. W. W. Kielley and R. K. Kielley, *ibid.* 191, 485 (1951).
10. A. L. Lehninger, *Harvey Lectures Ser.* 49, 176 (1954).
11. J. H. Copenhaver and H. A. Lardy, *J. Biol. Chem.* 195, 225 (1952); A. L. Lehninger and S. W. Smith, *ibid.* 181, 415 (1949).
12. B. Chance and G. R. Williams, *ibid.* 217, 383 (1955).
13. A. L. Lehninger, *ibid.* 178, 625 (1949).

13*a*. Recently it has been possible to incorporate into this assay system the oxygen electrode described by Chance and Williams (*12*). The combined system—that is, the spectrophotometric assay of phosphorylation and the polarographic assay of oxygen concentration—makes it possible to determine P/O ratios directly.

14. R. E. Beyer *et al.*, *Exptl. Cell Research* 8, 586 (1955); F. E. Hunter and L. Ford, *J. Biol. Chem.* 216, 357 (1955).
15. H. Stern and S. Timonen, *Exptl. Cell Research* 9, 101 (1955).
16. B. D. Polis and H. W. Shmukler, *Abstr. Am. Chem. Soc. 126th Meeting,* 72c; *Abstr. Am. Chem. Soc. 128th Meeting,* 19c; H. W. Shmukler and B. D. Polis, *Abstr. Am. Chem. Soc. 124th Meeting,* 13c.

16*a*. Subsequent to the submission of this paper for publication, it was found that the addition of adenylic acid ($5 \times 10^{-4}M$), EDTA ($1 \times 10^{-2}M$) and Mg^{++} ($1 \times 10^{-2}M$) to the assay system maintained the rate of phosphorylation for longer periods and improved the reproducibility.

17. L. Berger *et al.*, *J. Gen. Physiol.* 29, 379 (1946).
18. A. Kornberg, *J. Biol. Chem.* 182, 805 (1950).
19. H. W. Robinson and C. G. Hogdin, *ibid.* 135, 727 (1940).
20. K. Lohmann and L. Jendrassik, *Biochem. Z.* 178, 419 (1926).
21. S. S. Barkulis and A. L. Lehninger, *J. Biol. Chem.* 190, 340 (1950).

Evis Water Conditioner

Graham DuShane

We feel obliged to let our readers share our interest in some of the highlights of a recent case before the Federal Trade Commission. It is in many ways parallel to that of the battery additive case [*Science* **123,** 1059 (15 June 1956) and page 1099 in this issue].

On 5 Feb. 1954, the Federal Trade Commission issued a complaint against the Evis Manufacturing Company of San Francisco, Calif. The company manufactures a product, the Evis Water Conditioner, which looks like an expanded pipe coupling with a vertical post integrally cast in the center of the internal chamber. The "conditioners" range in size from those that may be fitted into a 0.5-inch pipe to models that are intended to be fitted into large industrial or marine pipes and in price from $25 for the smallest model to $3700 for the largest bronze model. All models are made of either zinc-coated cast iron or bronze, and they are "intended to be fitted into water systems for the purpose of beneficially treating and conditioning water."

The task for the Government in pressing its charges of false advertising was made difficult by the fact that the respondents averred that treatment with the "conditioner" did not affect the chemical or physical properties of the water in any detectable way but only the *behavior of the water in use.*

Burden of Proof

The company further claimed that both castings were processed by a secret method in such a way that they differ from ordinary cast iron and bronze. Metallurgical and spectroscopic examination of the iron castings (the bronze castings were not examined) failed to show that they differed from ordinary cast iron, but the hearing examiner ruled the evidence inconclusive when a metallurgist admitted that certain minute areas in the etched surface could not be identified and the spectroscopist admitted that the presence or absence of some 26 elements