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OXIDATIVE PHOSPHORYLATION IN STABLE SONIC FRAGMENTS OF RAT-LIVER MITOCHONDRIA

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SUMMARY

The preparation of stable sonic fragments of rat-liver mitochondria capable of oxidative phosphorylation is described. These differ from sonic preparations described by others in that they are prepared under very vigorous conditions, are unexpectedly stable to freezing and thawing, and show only one phosphorylation site. They rapidly oxidize a variety of substrates to give a P/O ratio of approx. 1.0, corresponding to phosphorylation in the cytochrome *b* region of the respiratory chain. ATPase (EC 3.6.1.4), ATP–P_i exchange, and ATP–ADP exchange reactions can be demonstrated in the particles as in intact mitochondria or digitonin fragments. However, the sonic particles exhibit several unexpected properties among which are: (a) substantial Amytal-insensitive and antimycin A-insensitive respiration, (b) extremely low ATP–P_i exchange rate relative to the rate of oxidative phosphorylation, (c) inhibition of ATPase activity by Amytal or antimycin A but a large stimulation by cyanide, (d) lack of an ATP requirement for succinate oxidation after pre-treatment of the particles with uncoupling agents, and (e) failure to catalyze net reduction of NAD^+ or acetoacetate by succinate in the presence of ATP.

INTRODUCTION

A number of different submitochondrial particles have been employed in the study of oxidative phosphorylation and its partial reactions (reviewed in refs. 1–3). These preparations differ from each other in some significant respects, particularly in the number and site of the phosphorylation steps which remain functionally coupled to the respiratory chain. The digitonin particles of rat-liver mitochondria as prepared in this laboratory[4–7] are active at all three sites but are relatively deficient in phosphorylating ability in the cytochrome *b* region (Site 2). The particles prepared by KIELLEY AND BRONK[8] and by MCMURRAY, MALEY AND LARDY[9] by mild sonic disruption of rat-liver mitochondria retain much of the phosphorylating activity of Sites 1 (NAD^+–flavoprotein level) and 2 while having lost that associated with Site 3 (cytochrome oxidase (EC 1.9.3.1) level). The ETP_h particles prepared in GREEN's laboratory show similar behavior[10]. On the other hand the beef-heart particles of PULLMAN *et al.*[11] and PENEFSKY *et al.*[12] are deficient at all three sites, but the activity of Site 2 can be in part restored by a soluble ATPase (EC 3.6.1.4). Particulate preparations from

microorganisms also differ in the composition of the respiratory chain, as well as in phosphorylation sites and capacity[1-3].

In this report are described some of the properties of particles prepared by drastic sonication (up to 60 min) of rat-liver mitochondria in a hypotonic medium. These particles consistently yield a P/O ratio, with a variety of substrates, of close to 1.0, which the evidence indicates is associated with phosphorylation at Site 2. The preparation has a specific activity comparable to other submitochondrial preparations and exhibits unexpected stability. Although these particles catalyze the so-called partial reactions of oxidative phosphorylation they are strikingly different in their relative rates from those observed in digitonin particles. Presumably, these differences are reflections of the relative integrity of the different phosphorylation sites.

Two following reports deal with the control of respiration by ADP and phosphate in these particles as well as in digitonin particles[7,13]. It will be shown that there are quite extraordinary differences in the properties of these two systems which probably reflect the behavior of the different phosphorylation sites and for which existing hypotheses of the mechanism of oxidative phosphorylation do not provide satisfactory rationalization.

METHODS

Mitochondria were prepared from 20–30 g of rat liver by the method of SCHNEIDER[14], washed three times in 0.25 M sucrose and finally suspended in 25 ml 0.01 M Tris–HCl buffer (pH 7.4). Two procedures for sonic disruption of the mitochondria were used in the course of this investigation; the properties of the particles were identical by all tests applied. Initially the Raytheon 9 kc, 60 W sonic oscillator was used with four 15-min periods of sonication separated by 1- or 2-min intervals between treatments to reduce sample heating. The sample chamber was cooled with ice water at a flow rate of 500–1000 ml/min, which kept the sample temperature within the range of 0–5° throughout the procedure. The plate voltage control of the oscillator was turned to maximum and the frequency control adjusted to give a minimum on the plate voltage meter, thus yielding maximum power transfer. The oscillator was checked frequently for its ability to deliver at least 4.4 W of acoustic power under these conditions. The power output was checked as suggested by the manufacturer.

In more recent experiments we have used the MSE 60 W, 20 kc sonic oscillator and an uninterrupted treatment time of 15 min. The large probe supplied with the instrument was used. The tube containing the sample was immersed in a beaker filled with ethanol precooled to −20°. During sonication the temperature of the ethanol was kept between −5° and −10° by the occasional addition of solid carbon dioxide. The cooling bath was mounted on a magnetic stirrer and stirred vigorously during the sonic treatment. Under these conditions the sample temperature was maintained between 0° and 5°. The power output of the instrument was adjusted by varying the frequency control to give maximum plate current. The acoustic power delivered was found to raise the temperature of 25 ml of water from 20° to a final temperature of 35° in 2 min in the absence of external cooling.

The sonically treated suspension resulting from either procedure was centrifuged at 25000 × *g* for 20 min in the Spinco model L ultracentrifuge. The turbid supernatant suspension was decanted and recentrifuged at 144000 × *g* for 30 min. The

supernatant solution was discarded and the firmly packed pellet was suspended in cold water or 0.25 M sucrose by gentle homogenization.

The sonic treatment rendered more than 90% of the mitochondrial protein non-sedimentable at 25000 × *g*; the final pellet represented 15–20% of the protein of the starting material. Although the fraction which sedimented between 110000 and 144000 × *g* had a slightly higher specific activity than the heavier fractions, the specific activity and the P/O ratios obtained were essentially independent of particle size.

Oxidation of β-hydroxybutyrate was measured using the colorimetric method of WALKER[15] for determining acetoacetate accumulation or with the Clark oxygen electrode to measure oxygen uptake, essentially as described by KIELLEY AND BRONK[8]. The respiration with other substrates was measured with the Clark electrode. Phosphorylation was measured according to NIELSEN AND LEHNINGER[16], and protein was determined by the biuret method[17] with crystalline bovine serum albumin as standard. The ATP–ADP exchange reaction was assayed by measuring the [^{14}C]ATP formed after separation of ADP and ATP by high-voltage paper electrophoresis in 0.05 M citrate buffer (pH 4.5).

RESULTS AND DISCUSSION

Substrate specifictiy of sonic particles

Succinate, choline, NADH, and β-hydroxybutyrate were readily oxidized by these preparations with concomitant phosphorylation of ADP as shown in Table I. Although the oxidation rates of the substrates vary widely, the P/O ratio was remarkably constant. Added cytochrome *c* markedly stimulated the oxidation of β-hydroxybutyrate, but did not increase the rates of either succinate or choline oxidation, suggesting that the activity of the dehydrogenase was rate-limiting for oxidation of the latter substrates, rather than the respiratory chain. Added NAD^+ did not stimulate choline oxidation indicating that this oxidation does not proceed beyond betaine aldehyde[18]. These particles also oxidized proline in the presence of added NAD^+ (*cf.* ref. 9). Malate, fumarate, and citrate were not oxidized by this preparation in the presence of added NAD^+ nor were α-ketoglutarate or pyruvate in the presence of added CoA, GTP, and NAD^+. The oxidation rates with succinate, NADH, and β-hydroxybutyrate in these preparations are higher, in general, than those reported by MCMURRAY *et al.*[9], especially with NADH, which is oxidized about 5 times faster by our particles. Glutamate was also oxidized in the presence of added NAD^+ in these preparations with a P/O ratio of approx. 1.0, but the capacity to oxidize glutamate was usually low and varied greatly from preparation to preparation. The P/O ratios in Table I were obtained using rather high protein concentrations which led to a depression of the P/O ratios from their optimum values, as discussed below; normally, under optimal conditions the P/O ratio was almost exactly 1.0 in a long series of preparations, regardless of substrate.

Other experiments (Fig. 1) have shown that succinate and especially choline inhibit the oxidation of β-hydroxybutyrate, implying that these three substrates are oxidized via common or interconnected respiratory chains for which they may compete (*cf.* ref. 19).

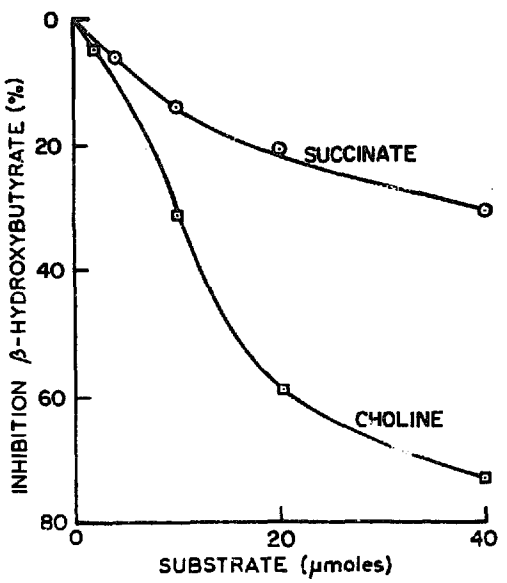

Fig. 1. Effect of succinate and choline on β-hydroxybutyrate oxidation. Conditions as in Table I except that glucose, hexokinase, and ^{32}P were omitted and each vessel contained 0.2 mg particle protein. The rate of β-hydroxybutyrate oxidation was determined by measuring acetoacetate accumulation; incubation was for 10 min at 25°.

TABLE I

OXIDATIVE PHOSPHORYLATION WITH VARIOUS SUBSTRATES

The complete system contained 10 mM substrate, 10 mM Tris–HCl buffer (pH 7.4), 0.2% bovine serum albumin, 3 mM $MgCl_2$, 3 mM glucose, 300 Kunitz–MacDonald units of hexokinase, 2.4 mM ADP, 15 mM potassium phosphate containing about 10^6 counts/min of ^{32}P, 5.2 mg particle protein and air-saturated water to a final volume of 2.0 ml. NAD^+ (0.2 mM) was added when β-hydroxybutyrate was the substrate. The vessels were incubated at 25° and respiration measured with the oxygen electrode until approx. 60% of the dissolved oxygen had been consumed, usually 1–4 min.

Substrate	*O_2 uptake (μatoms/min)*	*P/O*
β-Hydroxybutyrate	500	0.77
Succinate	250	0.67
Choline	140	0.75
NADH	710	0.77
Proline	170	—

Succinate oxidation by sonic particles

AZZONE AND ERNSTER[20] have shown that succinate oxidation in intact mitochondria is inactivated if they are preincubated with uncoupling agents and can be reactivated by the addition of ATP. We were able to duplicate the reactivation of succinate oxidation with ATP in intact mitochondria following their procedure, but the same preincubation procedure had no inhibitory effect on succinate oxidation in the sonic particles. No increase in the rate of succinate oxidation could be demonstrated on the addition of ATP either before or after preincubation with uncouplers.

The oxidation of succinate in the sonic particles was intensely inhibited by oxalacetate, as is the case for other mitochondrial systems. However, such inhibition was not relieved by addition of ATP and Mg^{2+}, which have been shown by PARDEE AND POTTER[21] and by TYLER[22] to relieve oxalacetate inhibition in tissue homogenates. The enzymic processes by which ATP can prevent inhibition of succinate dehydrogenase (EC 1.3.99.1) by oxalacetate, apparently without net destruction of the latter[22], thus do not occur in these sonic particles.

Tests of the ability of succinate to cause ATP-linked reduction of added NAD^+ in these sonic particles under anaerobic conditions showed them unable to carry out unequivocal net reduction under the conditions employed. In this respect these sonic particles of rat-liver mitochondria differ from the beef-heart preparations studied by LÖW *et al.*[23] and SANADI *et al.*[24], as well as the pigeon-breast particles of HOMMES[25] which cause net reduction of NAD^+ in the presence of succinate and ATP. The sonic particles were also unable to catalyze reduction of acetoacetate in the presence of succinate and ATP (*cf.* ref. 26), in agreement with the results on NAD^+ reduction.

Cofactors

The requirements for oxidative phosphorylation with β-hydroxybutyrate as substrate are shown in Table II. There is no oxidation or phosphorylation in the absence of added substrate or NAD^+. The requirement for Mg^{2+} for phosphorylation is usually more striking than shown in the experiment of Table II and is frequently absolute. A reconstituted system from *Micrococcus lysodeikticus* capable of oxidative phosphorylation was shown by ISHIKAWA AND LEHNINGER[27] to require Mg^{2+}, which could be replaced by spermine or spermidine. In the sonic particles, however, neither spermine nor spermidine at concentrations up to 5 mM could replace Mg^{2+}. When

TABLE II

REQUIREMENTS FOR OXIDATION AND PHOSPHORYLATION IN THE SONIC PARTICLES

Conditions were as in Fig. 1 with β-hydroxybutyrate as substrate except that each vessel contained glucose, hexokinase, and ^{32}P.

Expt.	*Component omitted*	*+Δ acetoacetate (mμmoles)*	*−ΔP_i (mμmoles)*	*P/2e*
1	None	366	339	0.93
	β-Hydroxybutyrate	0	0	—
	NAD^+	0	15	—
	Mg^{2+}	288	107	0.37
	Hexokinase, glucose	412	208	0.50
	Serum albumin	366	305	0.83
2	None	394	459	1.16
	ADP	400	0	—
	P_i	232	0	—

hexokinase (EC 2.7.1.1) is omitted from the reaction medium, the P/O ratio falls about 50 %, presumably because of the intense Mg^{2+}-stimulated ATPase (EC 3.6.1.4) activity of the particles which is discussed below. Omission of bovine serum albumin likewise reduces the P/O ratio, frequently as much as 50 %. There was no phosphorylation in the absence of added ADP and phosphate, indicating that the particles contain neither endogenous phosphate nor ADP in an amount sufficient to give measurable phosphorylation with tracer $[^{32}P]P_i$.

The specificity of the sonic particles for ADP as the phosphate acceptor in oxidative phosphorylation was also examined. IDP was about 20 % as effective as ADP (*i.e.* P/O was 0.22 for IDP and 1.09 for ADP). The other nucleoside diphosphates tested (UDP, CDP, and GDP) showed little or no activity. Addition of ADP and any one of the other nucleoside diphosphates caused a decline in the specific activity of the particles. This specificity for ADP, although striking, is somewhat less rigid than observed with the digitonin fragments[4], possibly because of the rather high level of Mg^{2+}, which has been shown to reduce the nucleotide specificity of some of the partial reactions of oxidative phosphorylations[28].

Fig. 2 shows the effect on the activity of the sonic particles of increasing the concentration of added NAD^+. The specific activity of the preparations for both oxidation and phosphorylation increased with NAD^+ concentration up to $1–2 \cdot 10^{-4}$ M. The respiration rate then continued to rise but the phosphorylation rate remained

relatively constant and then actually decreased as the concentration of NAD^+ was raised still higher. The P/O ratio thus declined with increasing concentration of NAD^+.

Particle concentration

The P/O ratio was markedly dependent on the particle concentration, the maximum ratio being obtained with low concentrations. Fig. 3 shows the rapid decline of the P/O ratio with protein concentration in 3 separate experiments. In those experiments where β-hydroxybutyrate oxidation was measured by the colorimetric method the protein concentration was 0.1 mg/ml. In experiments where the oxygen electrode was used, however, higher protein concentrations were used for experimental convenience. The P/O ratios obtained with the oxygen electrode were thus somewhat lower than obtained with the colorimetric method. Time-course studies have shown that both phosphorylation and oxidation are linear for at least 20 min at the low protein level (0.1 mg/ml).

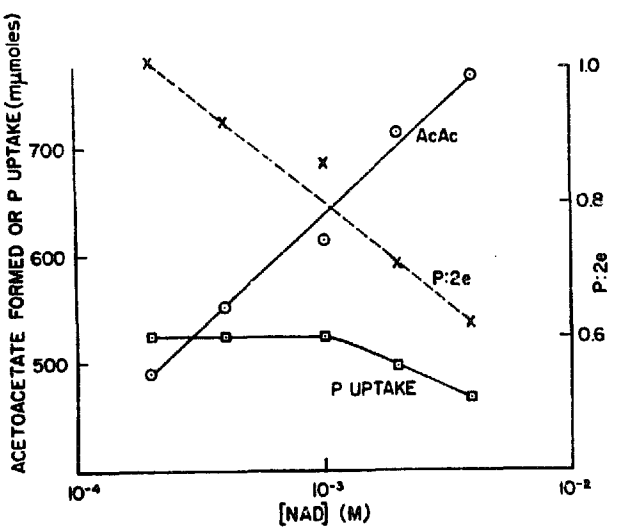

Fig. 2. The effect of added NAD^+ on the oxidation of β-hydroxybutyrate and the P/O ratio. Conditions were as in Table II. The abbreviation used is AcAc, acetoacetate.

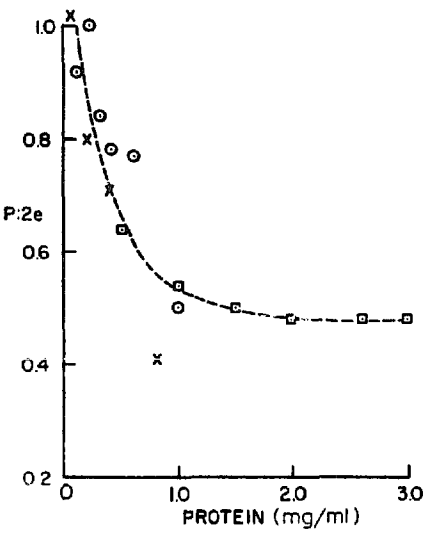

Fig. 3. The effect of protein concentration on the P/O ratio. Conditions are as in Table II. Data are presented for three experiments. Expt. 15, ⊡—⊡; Expt. 16, ⊙—⊙; Expt. 21, ×—×.

Phosphorylation sites

According to current concepts[1-3], the three sites of oxidative phosphorylation are between pyridine nucleotide and flavoprotein (Site 1), between cytochromes *b* and *c* (Site 2) and between cytochrome c and oxygen (Site 3). Pyridine nucleotide-linked substrates such as β-hydroxybutyrate or NADH itself thus give P/O ratios of nearly 3.0 in intact mitochondria while flavin-linked substrates such as succinate or choline show P/O ratios of nearly 2.0. The fact that both types of substrates give the same P/O ratio in the sonic particles indicates that the observed phosphorylation is associated with that portion of the electron transport chain common to both groups of substrates, *i.e.* the region from cytochrome *b* to oxygen and encompassing phosphorylation Sites 2 and 3. The P/O ratios of approx. 1.0 observed with either type of substrate could result either from the complete inactivation of Site 2 or Site 3 during preparation of the particles or from partial inactivation of both sites.

Site 3 can be readily isolated for study using ascorbate to reduce cytochrome *c*. Using this technique it has been shown[8,9] that the phosphorylation associated with Site 3 is completely lost in sonic particles from rat liver. This was confirmed in our preparations. Ascorbate was rapidly oxidized by our sonic particles but only barely detectable amounts of phosphorylation were obtained; the P/O ratio was usually less than 0.02.

Since only phosphorylation at Sites 2 and/or 3 could contribute to the P/O ratios observed with the various substrates, the demonstration that Site 3 was inactive in phosphorylation identified the locus of phosphorylation in the sonic particles as Site 2.

Although confirmation of this conclusion by a direct test of phosphorylation at Site 2 would be desirable, the assay of activity at the cytochrome *b*–cytochrome *c* level in submitochondrial particles, especially sonic particles, has given anomalous results[5,8,9]. In the digitonin particles[5], the reduction of cytochrome *c* with β-hydroxybutyrate as substrate gave a P/2e ratio of approx. 1.0 while a P/2e of nearly 2.0 would be expected. In the sonic particles made by the relatively gentle procedure of KIELLEY AND BRONK[8] phosphorylation could be demonstrated at Site 2. However, the anomalous observation was made that with NADH or β-hydroxybutyrate as substrates the P/2e ratio with cytochrome *c* as acceptor was 30–50 % higher than when oxygen was the electron acceptor. In particles prepared by a more vigorous sonic treatment than used by KIELLEY AND BRONK[8], MCMURRAY *et al.*[9] reported that with succinate, β-hydroxybutyrate, or NADH as substrate and either cytochrome *c* or ferricyanide as acceptor only barely significant phosphorylation was observed although the carriers were rapidly reduced. As in the particles of KIELLEY AND BRONK[8], NADH–cytochrome *c* reductase was insensitive to antimycin although the complete NADH oxidase was phosphorylating and antimycin-sensitive. The opposite is true for the digitonin particles[5].

Our observations on the direct assay of Site 2 phosphorylation parallel those of MCMURRAY *et al.*[9]. While cytochrome *c* was rapidly reduced by β-hydroxybutyrate, NADH, or succinate in these particles, there was no discernible phosphorylation associated with the reaction. It thus appears that in our sonic particles and those of MCMURRAY *et al.*[9] exogenous cytochrome *c* effectively uncouples the phosphorylation at Site 2, presumably by shunting the site of antimycin inhibition. MALEY[29] has shown that in whole mitochondria the concentrations of cytochrome *c* used here caused a drop in the P/O ratio of about 1.0 unit and a substantial reversal of the inhibition of respiration by antimycin A.

Effect of pH

Fig. 4 shows the pH-activity curve for respiration and phosphorylation with β-hydroxybutyrate as substrate. As in the sonic particles of KIELLEY AND BRONK[8] this preparation gave a pH-activity curve with a single, well-defined peak for the phosphorylation rate at pH 7.4–7.5. The respiration rate increased gradually with increasing pH throughout the range pH 6.0–8.5. In the absence of phosphate and phosphate acceptor, however, the pH-activity curve for β-hydroxybutyrate oxidation was much different than shown here (*cf.* ref. 13). The optimum pH of 7.4 for this system, which apparently shows only Site 2 phosphorylation, is thus in agreement with conclusions of HÜLSMANN AND SLATER[30] and MEYERS AND SLATER[31] on the pH-activity characteristics of the three coupling sites.

Stability

The respiratory and phosphorylative activities of the sonic particles exhibit remarkable stability toward freezing and thawing in comparison with other mitochondrial and submitochondrial preparations from rat liver[4,32,33]. The particles withstand repeated freezing and thawing up to at least 5 cycles, or frozen storage for as long as 3 weeks (Table III) under certain conditions with little loss of activity. The requirements are that the particles be frozen in rather concentrated suspension (about 40–50 mg protein/ml) in 0.25 M sucrose and that they be removed from the

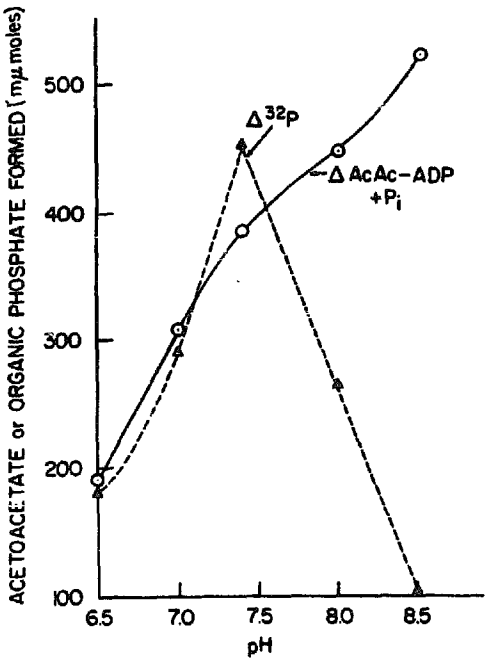

Fig. 4. The effect of pH on β-hydroxybutyrate oxidation and oxidative phosphorylation. Conditions are as in Table II.

TABLE III

STABILITY OF OXIDATION AND PHOSPHORYLATION TOWARD FREEZING AND THAWING AND FROZEN STORAGE

Conditions are as in Table II. Storage was in 0.25 M sucrose except where otherwise indicated.

Expt.	*Storage at — 20° (days)*	*Particles reisolated*	*+Δ Aceto-acetate (mμmoles)*	*—Δ P_i (mμmoles)*	*P/O*
1	Overnight in sucrose	—	179	213	1.20
	Overnight in water	—	376	125	0.33
2	0	—	276	283	1.03
	7	—	232	271	1.17
	14	—	189	180	0.95
3	0	—	276	283	1.03
	21	—	171	127	0.74
	21	+	294	250	0.85

medium in which they were stored and suspended in a fresh medium before assay. If the particles are frozen in water there is a rapid drop in activity overnight as shown in Table III. On storage at — 20° a substance is apparently released from the particles into the medium which causes a substantial reduction in the specific respiratory activity of the particle preparation and a smaller drop in the P/O ratio. When the particles were reisolated by centrifugation and suspended in fresh medium before assay, however, 85–90 % of the original activity could be recovered even after 3 weeks at — 20°. The stability of the particles is not increased by storage in a medium containing β-hydroxybutyrate, succinate, NAD^+, Tris buffer (pH 7.4), ATP, Mg^{2+}, and sucrose, a medium which stabilizes the D-β-hydroxybutyrate dehydrogenase (EC 1.1.1.30) of such particles (*cf.* ref. 34). The sonic particles thus show stability toward freezing and thawing that is of the same order as that of certain submitochondrial preparations from beef-heart mitochondria[35,36] and much greater than that of the digitonin particles from rat liver, whose activity is completely lost on freezing and thawing[4].

Inhibitors and uncoupling agents

Table IV shows the effects of inhibitors on oxidation and phosphorylation in these sonic particles. Dinitrophenol, dicumarol, and gramicidin uncouple phosphorylation completely. It is striking that significantly higher concentrations of these agents are required than are usually necessary for uncoupling in intact mitochondria or digitonin fragments[4]. Both pentachlorophenol (0.5 mM)and oligomycin (0.3 μg/ml) uncoupled completely. As in the digitonin preparations[4], Ca^{2+} does not uncouple oxidative phosphorylation in the sonic particles, when added alone or together with the supernatant fraction (*cf.* ref. 9). Antimycin A, Amytal, and sodium cyanide completely inhibited phosphorylation but inhibited the oxidation of β-hydroxybutyrate to acetoacetate only about 50 % in this experiment. More often acetoacetate formation was inhibited about 70 % by these agents.

Studies with the oxygen electrode showed good agreement between acetoacetate

TABLE IV

THE EFFECT OF INHIBITORS ON OXIDATION AND PHOSPHORYLATION

Conditions are as in Table II.

Expt.	*Additions*	*Concentration (mM)*	*+Δ Acetoacetate (mμmoles)*	*P/2e*
1	None	—	148	0.98
	Dinitrophenol	0.5	150	0.07
	Dicumarol	0.3	124	0.07
	Gramicidin D	0.001	116	0.01
	Pentachlorophenol	0.1	159	0.31
	Oligomycin	0.15 μg/ml	130	0.16
	Calcium chloride	5.0	127	0.96
	Amytal	1.0	71	0.04
	Sodium cyanide	1.0	85	0.06
	Antimycin A	0.5 μg/ml	73	0.04
2	None	—	200	0.99
	Sodium oleate	0.03	194	0.16
	Thyroxine	0.25	188	0.0

formation and oxygen uptake in the presence of Amytal and antimycin A. With cyanide, however, 90 % inhibition of oxygen uptake was obtained but only 82–84% inhibition of acetoacetate formation.

These results indicate that in the presence of Amytal or antimycin A the oxidation of β-hydroxybutyrate may continue at about 30 % of the control rate via an alternative pathway insensitive to these inhibitors. Only 10%, or less, of the normal respiration occurs by a cyanide-insensitive pathway. The "cytochrome reductase" (NADH:cytochrome *c* oxidoreductase EC 1.6.2.1) insensitive to Amytal and antimycin A may be the same pathway as that stimulated by high NAD^+ levels (Fig. 2) and which gives rise to a non-phosphorylating respiration.

It is unexpected that respiration was not completely inhibited by cyanide, even at high concentrations: this finding suggests that one of the carriers has become autoxidizable, possibly cytochrome *b* (*cf.* ref. 37). However, there was no observed increase in respiration rate on prolonged incubation with cyanide (30 min).

Sodium oleate is also a potent uncoupler of oxidative phosphorylation in the sonic particles. In agreement with the findings of BRONK[38] and of PARK *et al.*[39] on their sonic particles, it was found that thyroxine uncoupled phosphorylation in our particles. TAPLEY AND COOPER showed that thyroxine did not uncouple phosphorylation in the digitonin fragments of rat-liver mitochondria[40]. Other aspects of the uncoupling of phosphorylation in these particles are discussed in a following paper[13].

Partial reactions

ATPase activity and the ATP–P_i and ATP–ADP exchange reactions occur in the sonic particles. The relative rates of these in sonic and digitonin particles, in the presence and absence of 0.5 mM dinitrophenol are shown in Table V. In the presence of added Mg^{2+} the sonic particles show intense ATPase activity, much higher than the rate of net phosphorylation, whereas the rate of the ATP–P_i exchange reaction is only about 1 % of that of net oxidative phosphorylation. The ATPase activity in the presence of dinitrophenol, but in the absence of added Mg^{2+}, is of the same order of magnitude as the oxidative phosphorylation activity. The ATP–ADP exchange

TABLE V

COMPARISON OF PARTIAL REACTIONS

The experimental conditions are those of Table II for oxidative phosphorylation, Table VII for ATPase, Table VIII for ATP–P_i exchange, and Table IX for the ATP–ADP exchange. Mg^{2+} (1.0 mM) was present in all test systems for the sonic particles. The data for the digitonin particles were obtained in a similar system not containing added Mg^{2+}. The ATP–P_i exchange was assayed in the presence of either 0.1 mM or 10 mM inorganic phosphate. Temperature 25°. Data in μmoles per mg protein/h.

Reaction	*Sonic particles*		*Digitonin particles*	
	— 2,4-Dinitrophenol	*+ 2,4-Dinitrophenol*	*— 2,4-Dinitrophenol*	*+ 2,4-Dinitrophenol*
Oxidative phosphorylation	4.40	0.31	2.30	0.00
ATPase	15.1	15.2	2.00	6.20
ATP–P_i exchange (10 mM P_i)	0.04	0.00	2.20	0.02
ATP–P_i exchange (0.1 mM P_i)	0.00	0.00	0.52	0.01
ATP–ADP exchange	1.71	0.94	2.20	0.20

proceeds at a rate somewhat lower than the rate of net oxidative phosphorylation. In digitonin particles on the other hand the partial reactions proceed at approximately the same rates under the same test conditions (but in the absence of added Mg^{2+}). While no detailed inferences can necessarily be drawn from these rates alone with regard to the role of the partial reactions in the mechanism of oxidative phosphorylation (*cf.* ref. 3), it is striking that the one-site sonic particles differ qualitatively from the three-site digitonin particles with respect to the rate of the ATP–P_i exchange reaction, which is extremely low in the sonic particles and relatively high in the digitonin particles. The rate of this exchange is highly dependent on the concentrations of ADP and phosphate and the conditions shown are not necessarily maximal for the two systems, although they are nearly so. If the ATP–P_i exchange is measured at low phosphate concentrations (0.1 mM) then the sonic particles show no significant activity, whereas in digitonin particles it declines to about 1/4 the value shown in the Table.

As shown in Table VI the ATPase activity of the particles is 2–3-fold stimulated by dinitrophenol and about 15-fold by Mg^{2+}; it is strongly inhibited by azide. The activity in the presence of dinitrophenol and Mg^{2+} together is not significantly more than additive at the concentrations used. The ATPase activity elicited by dinitrophenol + Mg^{2+} was completely inhibited by oligomycin.

TABLE VI

STIMULATION AND INHIBITION OF ATPASE ACTIVITY

Each vessel contained 10 mM ATP, 10 mM Tris (pH 7.4), 1 mg particle protein, and water to a final volume of 2.0 ml. Incubated for 10 min at 25°.

Expt.	*Addition*	*Concentration (mM)*	*$-\Delta$ ATP (mμmoles)*
1	None	—	185
	Dinitrophenol	0.5	470
	$MgCl_2$	0.3	3000
	Sodium azide	0.1	55
2	Dinitrophenol + $MgCl_2$	0.1, 0.1	445
	+ Oligomycin	1.5 μg/ml	40

The effect of respiratory inhibitors on ATPase

The effect of the oxidation–reduction state of the respiratory carriers on the partial reactions has been the subject of a number of investigations on both digitonin particles and intact mitochondria (reviewed in refs. 2, 3). These studies have indicated that reduction of the carriers markedly inhibits the dinitrophenol-stimulated ATPase. The significance of this finding with respect to the mechanism of oxidative phosphorylation is complicated by a number of factors[41]. The partial reactions as they occur in the sonic particles are also strongly influenced by respiratory inhibitors. As shown in Table VII either Amytal or antimycin A alone inhibited the ATPase activity over 40% in the absence of added substrate. Addition of either succinate or β-hydroxybutyrate together with antimycin A or Amytal did not increase the degree of inhibition. However, if both β-hydroxybutyrate and succinate were added together with Amytal or antimycin A, the ATPase activity was maximally inhibited, as is also the case in

TABLE VII

ATPASE ACTIVITY AND THE OXIDATION–REDUCTION STATE OF THE CARRIERS

Added succinate and β-hydroxybutyrate concentrations were 10 mM, NAD^+ 0.2 mM, dinitrophenol, Amytal and cyanide were 0.5, 2.0 and 2.0 mM respectively. The antimycin A level was 2.0 μg/ml. Other conditions were those of Table VI.

Substrate	*Inhibitor*	*$-\Delta$ ATP (μmoles)*
—	—	0.08
—	Dinitrophenol	0.36
—	Dinitrophenol + Amytal	0.20
—	Dinitrophenol + antimycin A	0.21
β-Hydroxybutyrate + NAD^+ + succinate	Dinitrophenol + Amytal	0.05
β-Hydroxybutyrate + NAD^+ + succinate	Dinitrophenol + antimycin A	0.08
—	Dinitrophenol + cyanide	0.69
β-Hydroxybutyrate + NAD^+	Dinitrophenol + cyanide	0.55
Succinate	Dinitrophenol + cyanide	0.41
β-Hydroxybutyrate + NAD^+ + succinate	Dinitrophenol + cyanide	0.38

digitonin particles. On the other hand, cyanide greatly increased the dinitrophenol-stimulated ATPase of these particles, an effect quite contrary to the experiments with digitonin particles[41], in which ATPase activity is strongly inhibited by cyanide. The ATPase elicited by cyanide was reduced some 20 % on addition of β-hydroxybutyrate or 40 % by succinate but the combination of both substrates was only slightly more effective than succinate alone, in contrast to the synergistic action of the two substrates when Amytal or antimycin A were present. The stimulation of ATPase activity by cyanide in the presence of dinitrophenol suggests the possibility that the ATPase associated with Site 3 may be activated by the reduced state of the carriers at that level. This view is consistent with the results of lines 5, 6, 7, and 10 of Table VII. From these data it may be suggested that the ATPase associated with Sites 1 and 2 is almost completely inhibited by the action of both substrates in conjunction with any one of the three respiratory inhibitors. When both respiratory substrates are added to the system containing cyanide, they presumably cause inhibition of ATPase activity of Sites 1 and 2, but permit maximum stimulation of ATPase activity by the reduced state at Site 3. The data of Table VIII also suggest a marked difference in

TABLE VIII

EFFECT OF VARIOUS AGENTS ON THE ATP–P_i EXCHANGE

Each vessel contained 6 mM ATP (pH 6.5), 10 mM potassium phosphate (pH 6.5) containing about 10^6 counts/min ^{32}P, 1 mM $MgCl_2$, 0.4 % serum albumin, 1 mg particle protein, and water to a final volume of 1.0 ml. Incubated for 20 min at 25°.

Additions	*Concentration (mM)*	*$+\Delta$ [^{32}P] ATP (mμmoles)*
None	—	13.4
Dinitrophenol	0.10	6.2
Dinitrophenol	0.50	0
Oligomycin	1.0 μg/ml	0.8
Amytal	3.0	11.6
Antimycin A	1.0 μg/ml	11.2
$MgCl_2$ omitted	—	6.4

the contributions of the various coupling sites to the total ATPase activity of the sonic particles in comparison with the digitonin preparations. It appears also that a damaged or incomplete coupling site, *e.g.* Site 3 in the sonic particles, may make a substantial contribution to the ATPase activity and presumably to other partial reactions as well despite the fact that it is no longer capable of net phosphorylation of ADP. WADKINS AND LEHNINGER[41] have considered some other problems related to interpretation of rates of partial reactions as a function of oxidation state.

The ATP–P_i exchange reaction (Table VIII) was completely inhibited by uncoupling concentrations of dinitrophenol and oligomycin but only 15% or less by antimycin A or Amytal. The exchange rate was markedly reduced if Mg^{2+} was omitted.

TABLE IX

EFFECT OF VARIOUS AGENTS ON THE ATP–ADP EXCHANGE

Each vessel contained 10 mM ATP, 10 mM ADP labeled with ^{14}C, 1 mM $MgCl_2$, 1.2 mg particle protein and water to a final volume of 1.0 ml. The final pH was 6.8. Incubation for 10 min at 25°.

Expt.	*Additions*	*Concentration (mM)*	*+ Δ [^{14}C] ADP (mμmoles)*
1	None	—	342
	Oligomycin	3.0 μg/ml	362
	Pentachlorophenol	0.6	348
	Phosphate	1.0	381
	Phosphate	10.0	356
2	None	—	815
	Mg^{2+} omitted	—	240
	Dinitrophenol	0.5	450

The effect of various agents on the ATP–ADP exchange in the sonic particles is shown in Table IX. The exchange was unaffected by oligomycin, pentachlorophenol, or inorganic phosphate, as has been reported previously for the "loosely-coupled" digitonin particles prepared in this laboratory[42–44]. On the other hand, uncoupling concentrations of dinitrophenol caused about 50% inhibition of the ATP–ADP exchange, similar to that seen in digitonin particles[42]. As in the case of oxidative phosphorylation and the other partial reactions in these particles, the ATP–ADP exchange required Mg^{2+} for maximum activity.

ADDED COMMENTS

The particles described here vigorously oxidize a variety of substrates with P/2e ratios approaching 1.0 indicating a single functional phosphorylation site at the cytochrome *b*–cytochrome *c* level. This phosphorylation, which is completely uncoupled by a wide variety of uncoupling agents, shows remarkable stability to destruction during sonic disruption of the mitochondria and to freezing and thawing. The dinitrophenol-stimulated ATPase and the ATP–P_i and ATP–ADP exchange reactions occur in these particles, as in other submitochondrial preparations[3,4,44].

There, are however, striking differences in properties between intact mitochondria or digitonin particles and these sonic preparations. The very low activity of the

ATP–P_i exchange in the sonic particles may be compared to the much high rates in intact mitochondria. The stimulation of ATPase activity by cyanide in the sonic particles contrast to the inhibition of ATPase by this agent in the other preparations. The sonic particles also fail to show active uptake of Ca^{2+}, a process which has been shown to be closely related to oxidative phosphorylation and electron transport in intact mitochondria and in digitonin fragments[46].

Another significant difference in the behavior of the sonic particles and intact mitochrondria is the failure to demonstrate a requirement for activation of succinate oxidation in the particles after preincubation with uncoupling agents[20]. ERNSTER and coworkers[26] have concluded that succinate oxidation requires ATP, or high-energy intermediates in equilibrium with ATP, which are depleted by preincubation with uncoupling agents. This hypothesis[26] requires an intact coupling mechanism at Site 1 since added ATP must react with the respiratory carriers at this level through reversal of the mechanism by which ATP is formed. The inactivation of phosphorylation at this site would result in the inability to observe either succinate activation by ATP or succinate-linked reduction of either NAD^+ or acetoacetate since, according to ERNSTER[26], the same high-energy intermediate is involved in both reactions. Our failure to observe any of these reactions in the sonic particles is therefore consistent with the ERNSTER hypothesis[26] and with the other data suggesting that phosphorylation in the sonic particles occurs only at Site 2.

The properties exhibited by the sonic particles with respect to respiratory control relative to the digitonin particles or intact mitochondria are also markedly anomalous. These are described in a following report[13].

At present it is difficult to account for many of these properties in terms of currently favored mechanisms of oxidative phosphorylation[1-3]. It should be emphasized that the rates of the partial reactions in various types of mitochondrial preparations may represent a weighted average of the properties of the individual sites contributing to the total activity of oxidative phosphorylation and the partial reactions.

A mechanism based on these "averaged" properties may not, in fact actually reflect the mechanism of any individual site. As the contribution of the three sites to the weighted average is altered by procedures used to prepare submitochondrial particles, the properties of oxidative phosphorylation and the partial reactions may change drastically. Even if the coupling mechanism is the same at all sites, the individual sites may contribute unequally to the total activity of any given partial reaction. It is also probable that a coupling site may contribute to the total activity of a partial reaction such as the ATPase activity even though its capacity for net phosphorylation is lost. Further aberrations may be introduced into the coupling mechanism during the preparation of submitochondrial particles by "dislocation" or shunting of enzymes, as suggested by VIGNAIS *et al.*[47]. This will be discussed more fully in the following papers.

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REFERENCES

1 A. L. LEHNINGER, *Federation Proc.*, 19 (1960) 952.
2 E. RACKER, *Advan. Enzymol.*, 23 (1962) 323.
3 A. L. LEHNINGER AND C. L. WADKINS, *Ann. Rev. Biochem.*, 31 (1962) 47.
4 C. COOPER AND A. L. LEHNINGER, *J. Biol. Chem.*, 219 (1956) 489.
5 T. M. DEVLIN AN A. L. LEHNINGER, *J. Biol. Chem.*, 219 (1956) 507.
6 A. L. LEHNINGER, C. L. WADKINS, C. COOPER, T. M. DEVLIN AND J. L. GAMBLE, Jr., *Science*, 128 (1958) 450.
7 A. L. LEHNINGER AND C. T. GREGG, *Biochim. Biophys. Acta*, in the press.
8 W. W. KIELLEY AND J. R. BRONK, *J. Biol. Chem.*, 230 (1958) 521.
9 W. C. MCMURRAY, G. F. MALEY AND H. A. LARDY, *J. Biol. Chem.*, 230 (1958) 219.
10 A. W. LINNANE AND D. M. ZIEGLER, *Biochim. Biophys. Acta*, 29 (1958) 630.
11 M. E. PULLMAN, H. S. PENEFSKY, A. DATTA AND E. RACKER, *J. Biol. Chem.*, 235 (1960) 3322.
12 H. S. PENEFSKY, M. E. PULLMAN, A. DATTA AND E. RACKER, *J. Biol. Chem.*, 235 (1960) 3330.
13 C. T. GREGG AND A. L. LEHNINGER, *Biochim. Biophys. Acta*, in the press.
14 W. C. SCHNEIDER, in W. W. UMBREIT, R. BURRIS AND J. E. STAUFFER, *Manometric Techniques*, Burgess, Minneapolis, 1956, p. 189.
15 P. J. WALKER, *Biochem. J.*, 58 (1954) 699.
16 S. O. NIELSEN AND A. L. LEHNINGER, *J. Biol. Chem.*, 215 (1955) 555.
17 A. G. GORNALL, C. T. BARDAWILL AND M. M. DAVID, *J. Biol. Chem.*, 177 (1953) 574.
18 G. RENDINA AND T. P. SINGER, *J. Biol. Chem.*, 234 (1959) 1605.
19 T. KIMUURA, T. P. SINGER AND C. J. LUSTY, *Biochim. Biophys. Acta*, 44 (1960) 284.
20 G. F. AZZONE AND L. ERNSTER, *J. Biol. Chem.*, 236 (1961) 1518.
21 A. B. PARDEE AND V. R. POTTER, *J. Biol. Chem.*, 176 (1948) 1085.
22 D. B. TYLER, *J. Biol. Chem.*, 216 (1955) 395.
23 H. LÖW, H. KRUEGER AND D. M. ZIEGLER, *Biochem. Biophys. Res. Commun.*, 5 (1961) 231.
24 D. R. SANADI, A. L. FLUHARTY AND T. E. ANDREOLI, *Biochem. Biophys. Res. Commun.*, 8 (1962) 200.
25 F. A. HOMMES, *Biochem. Biophys. Res. Commun.*, 8 (1962) 248.
26 L. ERNSTER, *Symp. Intracellular Respiration: Phosphorylating and Non-phosphorylating Reactions, Proc. 5th Intern. Congr. Biochem., Moscow, 1961*, Pergamon Press Ltd., London, in the press.
27 S. ISHIKAWA AND A. L. LEHNINGER, *J. Biol. Chem.*, 237 (1962) 2401.
28 C. COOPER AND A. L. LEHNINGER, *J. Biol. Chem.*, 224 (1957) 547.
29 G. F. MALEY, *J. Biol. Chem.*, 224 (1957) 1029.
30 W. C. HÜLSMANN AND E. C. SLATER, *Nature*, 180 (1957) 372.
31 D. K. MYERS AND E. C. SLATER, *Biochem. J.*, 67 (1957) 558.
32 E. C. WEINBACH AND T. GARBUS, *J. Biol. Chem.*, 234 (1959) 412.
33 C. A. PRIVITERA, D. R. STRENGTH, M. ANGLIN AND H. PINKERTON, *J. Biol. Chem.*, 233 (1958) 524.
34 J. B. WISE AND A. L. LEHNINGER, *J. Biol. Chem.*, 237 (1962) 1363.
35 A. W. LINNANE AND D. M. ZIEGLER, *Biochim. Biophys. Acta*, 29 (1957) 630.
36 D. E. GREEN, R. L. LESTER AND D. M. ZIEGLER, *Biochim. Biophys. Acta*, 23 (1957) 516.
37 T. KIMURA, T. P. SINGER AND C. J. LUSTY, *Biochim. Biophys. Acta*, 44 (1960) 284.
38 J. R. BRONK, *Biochim. Biophys. Acta*, 37 (1960) 327.
39 J. H. PARK, B. P. MERIWEATHER AND C. R. PARK, *Biochim. Biophys. Acta*, 28 (1958) 662.
40 D. F. TAPLEY AND C. COOPER, *J. Biol. Chem.*, 242 (1956) 341.
41 C. L. WADKINS AND A. L. LEHNINGER, *J. Biol. Chem.*, 234 (1959) 681.
42 C. L. WADKINS AND A. L. LEHNINGER, *J. Biol. Chem.*, 233 (1958) 1589.
43 C. L. WADKINS AND A. L. LEHNINGER, *J. Biol. Chem.*, 238 (1963) 2555.
44 C. L. WADKINS, *Biochim. Biophys. Res. Commun.*, 7 (1962) 70.
45 J. R. BRONK AND W. W. KIELLEY, *Biochim. Biophys. Acta*, 29 (1958) 369.
46 F. D. VASINGTON, *J. Biol. Chem.*, 238 (1963) 1841.
47 P. V. VIGNAIS, P. M. VIGNAIS AND E. STANISLAS, *Biochim. Biophys. Acta*, 60 (1962) 284.