SOME BIOCHEMICAL PROPERTIES OF LYOPHILIZED MITOCHONDRIA*

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While studying the conversion of carbamyl aspartate to carbamyl β-alanine[1] we observed vigorous respiration when liver acetone powders were suspended and incubated with glutamate or other substrates. This prompted us to investigate in more detail the respiration and other properties of lyophilized mitochondria isolated from pigeon liver. It was found that dried mitochondria would catalyze the oxidation of a number of substrates. To the best of our knowledge the only recorded observations on respiration with dried preparations are those of KEILIN AND HARTREE[2] on the oxidation of succinate.

Dried mitochondria seem to offer a convenient system for respiration studies as discussed in this paper. We record here representative data on some of the properties of lyophilized mitochondrial preparations.

METHODS AND MATERIALS

O_2-uptake was measured at 38° in the Warburg apparatus using *ca.* 10 ml flasks and a reaction mixture volume of 1 ml.

Utilization of substrates during oxidation was confirmed occasionally by measurements of residual substrate, by the appearance of reaction products or both. Colorimetric methods were used[3] for these determinations.

Nitrogen determinations were made by the Kjeldahl method. Phosphate was determined by the method of GOMORI[4].

Adenosine triphosphatase measurements were carried out on aliquots of reaction mixtures containing 4 μmoles of adenosine triphosphate ATP***, 5 μmoles Mg^{++}, and 50 μmoles Tris buffer pH 7.4 and enzyme in a final volume of 1 ml. The mixtures were incubated at 38° for 15 min. The results are expressed in this paper as μmoles of inorganic phosphate liberated from ATP/mg dry powder under the described assay conditions.

Oxidative phosphorylation studies were conducted as previously described[5]. The preparation of acetone powders has also been described[6]. Glucose dehydrogenase was prepared by the method of STRECKER AND KORKES[7]. Other materials used were commercial products.

Preparation of lyophilized mitochondria

In order to prepare mitochondria in fairly large quantities, we have developed the following method:

After a preliminary passage of the tissue through a grater or meat grinder, the mince was suspended in one volume of 0.25 *M* sucrose. Homogenization was then carried out in a large

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*** The following abbreviations are used in this paper: ATP, adenosine triphosphate; Tris, Tris-(hydroxy)methyl aminomethane; A.P.S., A 10-h (20–25°) dialyzed water extract 1:10 (w/v) of rat liver acetone powder. ATP-ase, adenosine triphosphatase; DPN, TPN and DPNH, diphospho-, triphospho- and reduced diphosphopyridine nucleotide respectively. P_i, inorganic phosphate; w, water; g. dehydrogenase, glucose dehydrogenase.

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Potter-Elvehjem type tissue grinder consisting of a glass tube 26 mm. I.D. and 240 mm long with a teflon pestle attached to a stainless steel shaft. The pestle is driven by a ¼ h.p., 1750 r.p.m. motor through a 7-foot flexible steel shaft (Fig. 1). The motor should be placed on any convenient site several feet above the operator. In operating the tube is firmly clamped and immersed in an icewater bath. The flexible shaft permits easy raising and lowering of the pestle. Since the shearing force and therefore the rate and extent of tissue breakage is proportional to the rate of passage of the cells through the annular space between the pestle and tube, rapid up and down movement of the pestle gives the most efficient homogenization. Use of the ¼ h.p. motor rather than the usual 1/50 to 1/100 h.p. laboratory motor permits rapid raising and lowering of the pestle without stalling the motor. In addition the long hanging flexible shaft permits making the tube the stationary phase with the consequent lack of personal danger as for example with tube breakage.

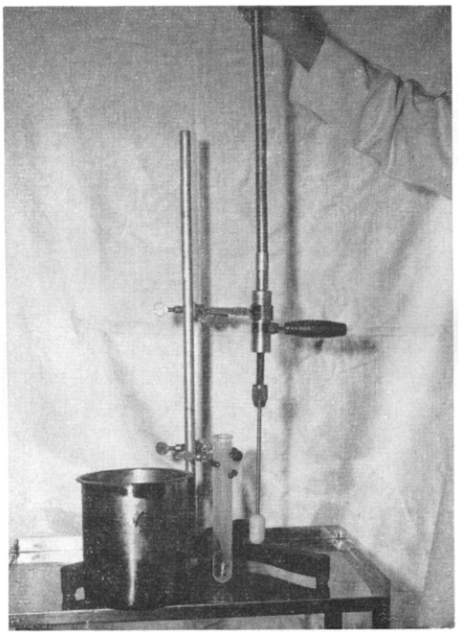

Fig. 1.

With the set-up described, aided by a foot pedal to start and stop the motor, one person can homogenize in 20 to 60 seconds over 50 g of tissue, even skeletal muscle. Presumably even larger homogenizing tubes could be used with correspondingly larger quantities of tissue*. The temperature of the homogenate remains at or near 0°C.

For isolation of mitochondria, the homogenate corresponding usually to 200 g wet tissue was diluted with 0.25 *M* sucrose to give a 1:10 (w/v) homogenate. Isolation of the mitochondria was then carried out as previously described[5]. The yield and activity of mitochondria isolated from homogenates prepared as described corresponds to that of mitochondria prepared by conventional methods. With glutamate as substrate, and conditions as described previously[5], P/O ratios of 2.2 to 2.8 were obtained.

For lyophilization the isolated mitochondrial pellet was washed once with twice its volume of 0.25 *M* sucrose, once with 0.154 *M* KCl and was finally suspended in two volumes of 0.154 *M* KCl. The KCl suspension was frozen and lyophilized. In some cases drying was carried out with a Rinco vacuum evaporator from sucrose suspension, in these cases the flask containing the suspension was kept at about 0° in an ice water bath in order to prevent ice-crystal formation.

* Recently, we have used a tube 35 mm I. D. and 240 mm long (capacity over 150 ml) with excellent results.

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RESULTS

Activity and stability of lyophilized mitochondria

The activity of different preparations varied considerably. For example with succinate as substrate and under equal conditions from 13 μA O_2/20 mg powder/h to 26 μA O_2/20 mg powder/h were measured with different preparations.

Variations were also observed with different preparations insofar as stability is concerned, for example one preparation which showed oxygen uptake of 21 μA O_2/10 mg powder/h, declined to 11 μA O_2/10 mg powder/h after one week storage at 0° in vacuum. On the other hand, preparations respiring at a rate of 20 μA O_2/20 mg powder/h lost no activity in 5 weeks storage[§].

Experiments with aqueous suspensions

Dried mitochondrial preparations are capable of oxidizing a number of the Krebs cycle intermediates. The greatest oxygen uptake occurs with succinate as substrate. Citrate, fumarate, and malate are also oxidized at appreciable rates. Some preparations oxidize α-ketoglutarate and oxalacetate at a slow rate but usually there is no oxygen uptake with these substrates. Representative data are given in Table I. No

TABLE I

THE INFLUENCE OF CO-FACTORS AND SUBSTRATES ON THE OXYGEN UPTAKE BY DRIED PIGEON LIVER MITOCHONDRIAL PREPARATIONS

Experiment	*Substrate*	*No ATP, Mg++, P_i μatoms O_2*	*Complete μatoms O_2*	*Complete + DPN* μatoms O_2*
1	Citrate	9.4	8.5	10.1
2	Citrate	12.0	—	—
1	α-Ketoglutarate	0.0	3.3	—
2	α-Ketoglutarate	—	3.8	—
1	Succinate	18.0	21.0	—
2	Succinate	—	23.0	—
3	Succinate	—	12.0	13.0
1	Fumarate	9.0	11.2	—
2	Fumarate	—	9.8	24.8
3	Fumarate	—	2.0	12.0
1	Malate	9.5	8.6	18.1
3	Malate	—	1.3	10.0
2	Oxaloacetate	—	5.0	—
2	Pyruvate	—	2.0	—
3	Glutamate	—	4.0	10.0
3	Aspartate	—	1.0	4.5
3	Glucose	—	1.0	9.0**
3	Glucose	—	—	0.0***

Complete system 20 mg mitochondrial powder (*ca.* 10 mg protein); 40 μmoles of substrate (100 for glucose) and when used 20 μmoles of phosphate buffer pH 7.4; 2 micromoles of ATP and 5 micromoles of $MgSO_4$. Final volume 1 ml 1 h at 38°.

* DPN and TPN were mixed and added in the proportion of 0.5 μmoles of each per vessel.

** glucose dehydrogenase (300 units[7]) was added for reduced nucleotide generation.

*** no glucose dehydrogenase added.

§ It is of interest that aqueous suspensions of lyophilized mitochondrial preparations retained their ability to oxidize succinate when stored at 2°C. For example after 1 to 4 days storage the retained activity varied from complete to 50%. The differences observed appear to be due to the concentration of the aqueous suspension (from 10 to 100 mg/ml), although this effect has not been studied in detail.

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absolute requirement for phosphate, ATP or $MgSO_4$ was found for the oxidation of any of the substrates, although lower oxygen uptakes were sometimes observed when these substances were omitted as shown in Table I. In general, linear rates of respiration were observed for the first 30 min. After this time the rates decreased considerably*.

The rate of oxidation of fumarate, malate, glutamate and aspartate was increased by the addition of a mixture containing 0.5 μM DPN and 0.5 μM TPN. Slight stimulation of succinate oxidation occurred apparently due to increased oxidation fumarate and malate. There is no effect on the oxidation of citrate on addition of these nucleotides. Examples of these results are tabulated in Table I. It is apparent also that DPNH can be oxidized by these preparations.

Maximum rates of oxidation are obtained at 40 μmoles for succinate and other substrates/ml of incubation mixture. Again as indicated above, increasing phosphate concentration effects oxidation little if at all. This is in contrast to the effects of phosphate on succinate oxidation shown by KEILIN AND HARTREE[2] with dried as well as with fresh heart preparations.

Attempts to measure oxidative phosphorylation with these preparations or with the modified preparations described below were unsuccessful. One of the difficulties preventing estimation of oxidative phosphorylative capability was that the mitochondrial powders showed intense ATP-ase activity. ATP was hydrolyzed at the rate of 5–10 μmoles/mg powder/15 min**.

We attempted to minimize the deleterious effects of freezing, particularly ice-crystal formation during lyophilization, with the use of the Rinco evaporator as mentioned above. These preparations oxidized glutamate vigorously (not oxidized by the freeze-dried preparations in the absence of added DPN). Attempts to estimate oxidative phosphorylation with these preparations were again unsuccessful.

Aqueous extracts of dry mitochondrial powders do not show oxygen uptake with any of the substrates tested. The aqueous residue will oxidize only succinate without supplementation although the oxygen consumption is decreased to half of that obtained with the unextracted powder.

The ATP-ase activity was not extracted by water, or KCl (up to 0.5 M) solutions. Treatment of the powder with 0.5 M urea at 0° for 15′ followed by centrifugation gave a residue with a slightly decreased ATP-ase activity while no activity could be detected in the urea supernatant fluid. It was found that exposure of the mitochondrial powders to 2.5 M urea – 0.5 M KCl markedly reduced the ATP-ase activity present in the residue obtained after centrifugation. The residue after such treatment had less than 1/10 of the ATP-ase activity of the whole powder. No phosphate was liberated from ATP by the urea–KCl supernatant fluid. Apparently the component or components responsible for ATP-ase activity was partially denatured.

The study of oxidation by preparations treated with 2.5 M urea and 0.5 M KCl was then undertaken. The powder was suspended in 2.5 M urea–0.5 M KCl (100 mg powder/40 ml solution) allowed to stand 15 min at 0°, centrifuged at 4000 $\times$ g for

* There is a considerable dilution effect with these preparations. In general, we have worked with concentrations giving best rates (10–20 mg powder/ml).

** Examination of these preparations for the presence of the ATP–P_i exchange enzyme[8] was carried out by Dr. G. W. E. PLAUT, who found that the total and specific activity of water extracts was of the same order as that of extracts of acetone powders[8]. We wish to thank Dr. PLAUT for his kindness in providing us with this unpublished information.

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TABLE II

THE EFFECT OF PHOSPHATE AND SUCCINATE CONCENTRATIONS ON OXYGEN UPTAKE BY DRIED PIGEON LIVER MITOCHONDRIA

Substrate added µmoles	*Phosphate added µmoles*	*Oxygen uptake µatoms*	*Keto acids µmoles*
10	20	9.1	4.7
20	20	15.4	5.6
40	20	19.0	1.6
80	20	18.8	1.0
100	20	17.6	1.5
40	10	21.3	1.8
40	20	21.6	2.6
40	40	17.6	0.8
40	80	17.9	1.6
20*	20	11.5	11.5

The data reported here were obtained under the standard conditions described in the text, except that the preparation used was suspended in distilled water before lyophilization (after the KCl-washing) and that 75 mg were used per test. Values in the table have been corrected for endogenous oxygen and keto acids blank.

* Succinate replaced by fumarate.

TABLE III

THE EFFECT OF CO-FACTORS ON OXYGEN UPTAKE BY LYOPHILIZED AND WASHED MITOCHONDRIAL PREPARATIONS

Expt.	*Preparation*	*Substrate*	*DPN-TPN*	*Boiled juice*	*A.P.S. ml*	*Oxygen uptake µatoms*
1	Powder	Succinate	—	—	—	18.8
1	Powder w. washed	Succinate	—	—	—	11.6
1	Powder	Fumarate	+	—	—	21.3
1	Powder w. washed	Fumarate	+	—	—	0.9
1	Powder w. washed	Succinate	—	+	—	7.8
1	Powder w. washed	Fumarate	+	+	—	4.8
2	Powder, urea–KCl washed	Succinate	—	—	—	3.0**
2	Powder, urea–KCl washed	Succinate	—	—	0.05*	11.8
2	Powder, urea–KCl washed	Fumarate	—	—	—	0.0
2	Powder, urea–KCl washed	Fumarate	—	—	0.05	0.0
2	Powder, urea–KCl washed	Fumarate	+	—	—	2.0
2	Powder, urea–KCl washed	Fumarate	+	—	0.05	7.2
3	Powder, urea–KCl washed	Malate	—	—	—	0.0
3	Powder, urea–KCl washed	Malate	—	—	0.05	0.6
3	Powder, urea–KCl washed	Malate	+	—	—	2.3
3	Powder, urea–KCl washed	Malate	+	—	0.05	7.3
4	Powder, urea–KCl washed	Citrate	—	—	0.05	0.0
4	Powder, urea–KCl washed	Citrate	+	—	0.05	0.5
4	Powder, urea–KCl washed	Citrate	+	—	—	0.0
4	Powder, urea–KCl washed	Citrate	—	—	0.05	0.0

The mitochondrial preparations were suspended in cold distilled water for 10′, centrifuged and the precipitate resuspended in water and used as such (Expt. 1). The supernatant fluid was boiled in water for 5′, centrifuged and used as such (Boiled juice). All other experiments represented here (2, 3 and 4) were conducted with the water suspended residues after urea–KCl extraction.

* From studies of concentration of added A.P.S., this amount appears to be optimum.

** Addition of *ca.* 500 γ of menadione did not increase appreciably the oxygen uptake.

TABLE IV

THE EFFECT OF SUPPLEMENTATION ON OXYGEN UPTAKE OF UREA–KCL TREATED DRIED MITOCHONDRIAL PREPARATIONS

Supplements					oxygen uptake
Succinate	*Glucose*	*DPN*	*Cytochrome c**	*g. dehydrogenase*	*μatoms*
+	—	—	—	—	6.9
+	—	—	+	—	14.0
—	+	+	—	+	2.0
—	+	+	+	+	7.0
—	+	—	—	+	0.0
—	+**	+**	+**	+**	0.0

The conditions, substrates and co-factors were as in Table I.

* Optimum oxygen uptake was obtained with 200 γ of cytochrome *c*, higher quantities up to 2 mg were equally effective.

** In the absence of the mitochondrial preparation.

10 min. and the supernatant fluid discarded. The residue was suspended in 40 ml of water and centrifuged. The water washing was repeated twice and the washed preparation was finally suspended in 2.5 ml of water.

Experiments with washed residues

Residues obtained from urea–KCl solutions retained the ability to oxidize succinate although at about ¼ to ½ the rate of the whole powder. None of the other substrates tested before were oxidized. When supplemented however with a dialyzed aqueous extract of a liver acetone powder which, *per se*, showed no respiration, succinate oxidation was increased approximately 50%, and the oxidation of malate and fumarate but not of citrate occurred when further supplemented with a DPN/TPN mixture. The oxygen uptake with these substrates, even when supplemented, was about half that of the whole powder. It is apparent then that the extraction procedure results in the solubilization and/or washing away of coenzymes as well as enzymes. Yet the electron-transfer mechanism, or a part thereof, is tightly bound to the insoluble residue, since succinate is oxidized. It was found that addition of 200 γ of cytochrome *c* to the urea–KCl residues restored the oxygen consumption with succinate to the original levels. The oxidation of fumarate and malate by the preparations was unaffected by cytochrome *c*. DPNH was also oxidized by the urea–KCl residue when supplemented with cytochrome *c*.

Studies on adenosine triphosphatase

An attempt was made to extract and fractionate the component(s) responsible for the ATP-ase activity. Although the ATP-ase is not easily extractable, it was found that dried butanol powders[9] of the dried mitochondria when extracted with 0.01 *M* sodium taurocholate resulted in a suspension of the component(s) showing ATP-ase activity which would withstand 25,000 × *g* for 20′. Fractionation with $(NH_4)_2SO_4$ of the taurocholate extract resulted in separation of the activity in a fraction which concentrated at the top after centrifugation when 75% saturation with $(NH_4)_2SO_4$ pH 7.4 was reached. Further purification could be accomplished by acid treatment (pH 4.1), however, attempts to repeat this fractionation with other lyophilized mitochondrial preparations have been inconsistent.

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DISCUSSION

It is known that physical alteration of respiratory particles results in profound changes in metabolic activity. The observations reported in this paper indicate clearly that drying of mitochondria results in the loss of phosphorylating ability without impairing markedly some of the oxidative reactions which have been studied. On the other hand, the oxidative systems are considerably altered since a number of substrates normally oxidized by fresh mitochondria are slowly oxidized or not at all, as for example α-ketoglutarate, while others such as succinate are oxidized remarkably well. Recently it has been shown, however, that the oxidative phosphorylating capability has been retained in mitochondrial fragments obtained by a number of procedures including digitonin treatment[10], sonic disintegration[11], alkali and solvent treatment[12] and by the action of a detergent[13].

There are two points which merit particular attention. First, the lyophilization procedure results in preparations with tightly bound succinoxidase. In fact it is remarkable that the preparations retained activity after the high concentration of urea–KCl extraction. HOPKINS *et al.*[14] have shown that extraction of pig-heart muscle preparations with urea led to the loss of succinoxidase activity even in the presence of added cytochrome *c*. Soluble succinic dehydrogenase is extracted from mitochondrial preparations by relatively mild procedures[15] although a particulate form of the enzyme has also been described[12]. Second, the insoluble preparations described here are not markedly affected by phosphate as is the case with the dry preparations of KEILIN AND HARTREE. Whether or not this represents an intrinsic difference in the susceptibility of tissues and species to drying [2], it is as yet too early to say.

The increase in adenosine triphosphatase activity upon lyophilization suggests that this enzyme must be structurally bound by two different kinds of bonds in the normal mitochondria. The first kind, easily broken, must be in the nature of relatively weak, perhaps hydrogen bonds, and are easily unmasked or ruptured by simply changing the physical state of the preparation. The second type of bonds must be stronger, possibly involving a lipid complex, since the enzyme activity is extractable (whether in particulate form or not) from the dry mitochondrial preparations only after butanol treatment.

It should be pointed out that MYERS AND SLATER[16] have shown the presence, as evidenced by pH-optima, of four different types of adenosine triphosphatase in mitochondria. No attempt has been made to differentiate the ATP-ase activity of these preparations although we have found that under conditions given, dinitrophenol does not stimulate the appearance of inorganic phosphate.

It is apparent that lyophilized mitochondrial preparations offer a convenient source of adenosintriphosphatase and perhaps a good starting material for purification of the enzyme(s). In addition it is clear that the lyophilized preparations offer a system for non-phosphorylating oxidation studies with the possibility of retaining the bulk of co-factors initially present in the preparation.

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SUMMARY

1. A rapid procedure for large scale preparation of mitochondria is presented.

2. Some biochemical properties of mitochondrial powders have been studied. It was found that these preparations, or modifications thereof, oxidized a number of substrates.

3. Measurements of oxidative phosphorylation, adenosine triphosphatase, and ATP–P_i exchange activities have been carried out with these preparations.

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ELASTOIDIN: A TWO-COMPONENT MEMBER OF THE COLLAGEN CLASS*

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Elastoidin, the large translucent fiber of the fins of selachian fish, is a member of the collagen class, as evidenced by its characteristic wide-angle X-ray diffraction pattern[1,2], the presence of a 600–800 Å period in the fibril[3,4], and its amino-acid distribution[4,5]. One unusual feature of its composition, however, is the presence of more than 6% tyrosine[4,5]. This amino acid rarely accounts for more than 1% of purified vertebrate collagen[6]. The fibers have a relatively high shrinkage temperature, 60–64°, and regain most of their length on cooling[4,5]. Elastoidin has been said to produce no gelatin on boiling, although it has been solubilized to the extent of 98% by autoclaving seven times at 120° [5].

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