Oxidative phosphorylation in sonic extracts of rat liver mitochondria

Recent reports from other laboratories[1-4] have described preparations of fragmented mammalian mitochondria exhibiting phosphorylation coupled to electron transport. In these reports, phosphorylation does not appear to have been successfully coupled to succinate oxidation and it has been emphasized that phosphorylation is effectively coupled to DPNH* oxidation only when the latter arises from particle-bound DPN and dehydrogenase. In this communication we wish to report on some of the elementary properties of a mitochondrial fragment preparation differing in some respects from those previously described. This preparation exhibits phosphorylation coupled to the oxidation of succinate or DPNH by oxygen with P:O ratios somewhat less than unity. This preparation also provides the first demonstration of a definite Mg requirement for the coupled phosphorylation.

Mitochondria were prepared from rat liver by the method of SCHNEIDER[5], modified as previously described[6]. After washing twice with sucrose, the mitochondria were washed once with a $0.025\,M$ KF – $0.050\,M$ KPO_4 (pH 7.0) solution and resuspended in the same medium. They were then treated in a 9 KC Raytheon sonic oscillator for one minute and the suspension was centrifuged at 25,000 × g for 20 minutes. The supernatant fluid was removed and recentrifuged at 80,000 × g for 30 minutes; the sedimented material was then resuspended in the fluoride-phosphate solution after pouring off the supernatant solution, recentrifuged at 80,000 × g and the washed pellet resuspended in the fluoride-phosphate solution. Oxygen uptakes as reported here were measured with the Clark oxygen electrode**. Phosphate uptake was measured by the ^{32}P incorporation procedure of NIELSEN AND LEHNINGER[7]. Conditions of incubation are given in Table I.

TABLE I

OXIDATIVE PHOSPHORYLATION IN MITOCHONDRIAL FRAGMENTS

Components	*Substrate*	*Δμatoms oxygen*	*Δμmoles P_i*	*P:O*
Complete system*	Succinate	0.470	0.188	0.40
Complete system*	DPNH	0.465	0.241	0.52
Complete system* + hexokinase	Succinate	0.456	0.241	0.53
Complete system* + hexokinase	DPNH	0.461	0.340	0.74
Complete system* — ADP	Succinate	0.465	0.012	0.03
Complete system* — ADP	DPNH	0.470	0.012	0.03
Complete system* — $MgCl_2$	Succinate	0.456	0.015	0.03
Complete system* — $MgCl_2$	DPNH	0.461	0.021	0.05
Complete system* + $2\cdot10^{-4}\,M$ dinitrophenol	Succinate	0.474	0.000	0.00
Complete system* + $2\cdot10^{-4}\,M$ dinitrophenol	DPNH	0.461	0.009	0.02
Complete system* + $4.5\cdot10^{-4}\,M$ DPN^+	β-OH butyrate	0.497	0.281	0.57
Complete system*	None	0.00	0.027	—

* Test system (1.9 ml) contained 5 μmoles ADP, 6 μmoles AMP, 50 μmoles glucose, 5 μmoles KF, 10 μmoles P_i, pH 6.5 ($6.85\cdot10^7$ c.p.m. ^{32}P), 10 μmoles $MgCl_2$ with succinate (20 μmoles) as substrate, and 5 μmoles $MgCl_2$ with DPNH (5 μmoles) or β-OH butyrate (DL) (20 μmoles) as substrate, enzyme suspension (0.186–0.372 mg N). Time, 3–5 min; temp. 24° C.

The results given in the table show that phosphorylation was associated with the oxidation of both succinate and DPNH, DPNH giving somewhat higher P:O ratios. In either case addition of hexokinase and glucose resulted in somewhat higher efficiencies. The products were identified chromatographically as ATP (primarily) or hexose-6-phosphate.

The table also shows that in absence of substrate and phosphate acceptor no significant amounts of labelled ATP were observed. AMP did not serve as an acceptor. Its purpose in the incubation medium was to suppress the small amount of adenylate kinase present in the enzyme preparation. Because of a potent ATP-$^{32}P_i$ exchange reaction in the preparation, any ATP arising from the ADP by this process (adenylate kinase) became labelled in the absence of hexokinase.

* The following abbreviations are used: ATP for adenosinetriphosphate; ADP for adenosinediphosphate; AMP for adenosine-5'-phosphate; DPN^+ for diphosphopyridine nucleotide; DPNH for reduced diphosphopyridine nucleotide; P_i for inorganic orthophosphate.

** Obtained from Yellowsprings Instrument Co., Yellowsprings, Ohio.

The phosphorylation accompanying oxidation of either substrate was sensitive to dinitrophenol as shown in the table. As in the work of TAPLEY *et al.*[8] on fragments obtained by digitonin treatment of mitochondria, our material was not sensitive to thyroxin.

The final suspension of the enzyme may be made in water rather than in the fluoride-phosphate solution indicated above. Under these circumstances the preparation exhibits a very marked dependence on phosphate concentration. The table indicates that Mg was an obligate component for the coupled phosphorylation. While Mg has generally been assumed to be essential we believe this demonstration to be the first definite evidence for it. Different levels of Mg were employed for the two substrates, however, the requirement was found to be the same in the two cases. In the absence of Mg, no labelling of any sort occurred.

Although our preparations have β-hydroxybutyric dehydrogenase, no oxidation of β-hydroxybutyrate occurs without added DPN^+, in which case the P:O ratios are similar to those obtained with DPNH (see Table I).

Despite all attempts to improve the efficiency of the preparations, P:O ratios higher than 1 have never been observed. This suggests that only one stage of phosphorylation may have been operating. However, in extensive studies of the two processes of electron transfer, from substrate to cytochrome *c* (in presence of KCN) and from ascorbate-cytochrome *c* to oxygen, no phosphorylation was observed in either reaction. The number of functioning phosphorylation stages is therefore indeterminate at this time. Since added cytochrome *c* increased oxygen uptake in the over-all reaction without associated phosphate uptake it is possible that in the stage of cytochrome *c* reduction, free cytochrome *c* may have been reacting out of sequence—by-passing the coupled process at this level.

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Received November 9th, 1956

The enzymic synthesis of chitin by extracts of *Neurospora crassa*

A particulate enzyme from *Neurospora crassa*, wild strain 5297A, prepared by homogenization of frozen mycelia, in tris(hydroxymethyl)aminomethane buffer (Tris), pH 7.5, with a Ten Broeck glass tissue homogenizer, followed by collection of the material sedimented between 2000 × *g* and 140,000 × *g*, has been found to catalyze the incorporation of the N-acetylglucosamine moiety (AG) of uridine diphospho-N-acetylglucosamine (UDPAG) into an insoluble polysaccharide fraction. Neither N-acetylglucosamine-6-phosphate (AG-6-P), a mixture of N-acetylglucosamine-1-phosphate (AG-1-P) and AG-6-P, nor free AG will replace UDPAG in this reaction (Table I).

The structural similarity of the product to chitin is indicated by the following observations. (a) When radioactive polysaccharide is prepared enzymically from ^{14}C glucosamine-labelled UDPAG and then is acid-hydrolyzed, all of the radioactivity is found in the glucosamine isolated by chromatography on Dowex-50 by the procedure of GARDELL[1]. (b) AG from UDPAG is incorporated as a unit into the insoluble polysaccharide, as shown by the data in Table I where the same amount of ^{14}C was incorporated whether the UDPAG was labelled only in the glucosamine chain or only in the acetyl group. (c) When the insoluble ^{14}C-labelled polysaccharide is subjected to partial acid hydrolysis followed by removal of the acid and deionization with a mixed-bed resin (to remove any deacetylated oligosaccharides), a series of radioactive, acetylated oligosaccharides remains in solution. These substances can be separated by paper chromatography and are found to include AG, N, N'-diacetylchitobiose (using as a reference the crystalline disaccharide prepared from chitin by the method of ZILLIKEN *et al.*[2]), and higher molecular weight