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Permeability of Housefly Flight-Muscle Mitochondria for Succinate

By A. TULP (introduced by K. VAN DAM). (*Laboratory of Biochemistry, University of Amsterdam, Amsterdam, The Netherlands*)

It has been proposed that mitochondria from the flight muscle of the housefly (Van den Bergh & Slater, 1962) and blowfly (Sacktor & Childress, 1967) are impermeable to intermediates of the tricarboxylic acid cycle. We have found, however, that a very rapid oxidation of added succinate occurs in the absence of phosphate (about 450 ng-atoms of O/min per mg of protein). This result suggests that, in the absence of phosphate, succinate can permeate the mitochondrial membrane. The properties of this movement of succinate will be discussed.

The oxidation of succinate is loosely coupled, since no stimulation of oxidation by 2,4-dinitrophenol is observed in the absence of phosphate acceptor.

The oxidation of succinate is strongly inhibited by small amounts of phosphate (e.g. 86% inhibition by 3 mM-phosphate in the presence of 60 mM-succinate). It was found that the phosphate concentration inside the mitochondrion was lower when succinate oxidation was rapid than when it was inhibited.

The inhibition by phosphate could be overcome by mercurials (Tyler, 1968; Fonyo, 1968) such as mersalyl. This effect was dependent on the concentration of mersalyl. Low concentrations caused a temporary release of the phosphate-inhibited oxidation. A second addition of mersalyl restored the rapid oxidation after the inhibition had set in. Higher concentrations (>60 nmol/mg of protein) caused an irreversible inhibition after a temporary stimulation.

Since mersalyl inhibits, not only the movement of phosphate across the mitochondrial membrane (Fonyo, 1968; Tyler, 1968), but also the dicarboxylate-phosphate exchange (Meijer & Tager, 1969), it is suggested that the mercurial is able to overcome the phosphate inhibition of succinate oxidation by enhancing the net movement of dicarboxylate ions (succinate, fumarate and malate) across the mitochondrial membrane.

A Substoichiometric Assay for the Determination of Free Adenosine Triphosphate in the Presence of Reversibly Bound Adenosine Triphosphate

By E. A. NEWSHOLME and KAY TAYLOR. (*Agricultural Research Council Unit for Insect Physiology, Department of Zoology, University of Oxford, Oxford OX1 3PS, U.K.*)

The usual methods for determining ATP concentration suffer from the disadvantage that they measure total ATP and do not distinguish between free and reversibly bound ATP. A possible method for providing such a distinction was suggested from the enzymic-isotope-dilution principle developed by Newsholme & Taylor (1968). Assays based on this principle are substoichiometric: an enzyme converts only a small fraction (10–20%) of radioactive substrate into product and, as this is sufficient for determination of the substrate concentration, the conversion may not seriously disturb an equilibrium between free and bound substrate.

Glycerol kinase was selected for the assay of ATP by such a method because it obeys the enzymic-isotope-dilution principle for glycerol (Newsholme & Taylor, 1968). The assay medium was as described previously except it contained 0.9 mM-glycerol, 0.25 M-sucrose, 3.3 mM-Mg²⁺ and 0.13 mM-[¹⁴C]ATP (specific radioactivity 15.6 μ Ci/ μ mol) as final concentrations. A sample (100 μ l) of this was incubated with 20 μ l (5 μ g) of glycerol kinase for 15 s at 0°C; the reaction was stopped by addition of 100 μ l of ethanol containing 5 mM-ATP, 5 mM-ADP, 10 mM-EDTA and 10 mM-ethanedioxybis(ethylamine)tetra-acetate. T.l.c. on silica-gel-coated plastic sheets was used to separate ADP from ATP; the separated spots were cut out and radioactivity was measured as described previously.

Addition of 6, 12, 18, 24 and 36 nmol of unlabelled ATP (S) to the above system caused a decrease in the incorporation of radioactivity into ADP so that C_0/C_s ratios were 1.20, 1.40, 1.64, 1.85 and 2.30 for the above concentrations respectively. A plot of C_0/C_s against [S] is linear and C_0/C_s is unity when [S] is zero; these results are in agreement with the enzymic-isotope-dilution principle.

The C_0/C_s ratio was less if unlabelled ATP was added to the incubation together with creatine phosphokinase. From this decrease in ratio the bound concentration of ATP could be calculated; 1mg of creatine phosphokinase was found to bind 9.8nmol of ATP. By equilibrium dialysis it was shown that 1mg of creatine phosphokinase under similar conditions bound 10.0nmol of ATP. Thus this method can distinguish between free and bound ATP.

The method may have a number of applications, but in particular it may provide the basis for distinguishing between mitochondrial and cytoplasmic ATP concentrations in living tissues.

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Manganese in Chloroplasts

By ROSALIND JENKINS and D. E. GRIFFITHS. (*School of Molecular Sciences, University of Warwick, Coventry CV4 7AL, U.K.*)

Spinach chloroplasts (Mn/chlorophyll ratio 1:80) when heated at 40°C in 0.02M-phosphate buffer, pH 6.6, lost most of the manganese; further heating could not remove the residual manganese (Mn/chlorophyll ratio 1:400). Various experiments showed that this residual manganese was not of mitochondrial origin or due to an insoluble manganese salt, but was associated with chloroplast membranes. This suggested that there were two kinds of manganese in chloroplasts bound in different ways, a conclusion also reached by Cheniae & Martin (1968) by different methods.

Manganese was bound most strongly to the chloroplast at pH 6–6.8 and in this pH range chelating agents at 0°C were shown to have little effect in removing bound manganese (Cheniae & Martin, 1966). A combination of heat treatment (40°C) and chelating agents was more effective than heat treatment alone. Under these conditions (40°C, 0.02M-phosphate buffer, pH 6.6), 0.1mM-*o*-phenanthroline, 0.1mM-8-hydroxyquinoline and 1mM-EDTA removed all the labile manganese within 15min. On further incubation 1mM-EDTA, unlike the other chelating agents, also removed up to 80% of the tightly bound manganese from spinach chloroplasts. However, similar treatment with 1mM-EDTA had no effect on the tightly bound manganese of pea chloroplasts.

Treatment of pea chloroplasts with 0.8M-tris buffer, pH 8.0, for 10min at 4°C caused the loss of nearly all manganese present (Mn/chlorophyll ratio 1:1160), whereas treatment with tricine under similar conditions resulted in a 138:3 Mn/chlorophyll ratio. Tris at this concentration is known to inhibit electron transport between water and PSII

(Yamashita & Butler, 1968) and the inhibition may be due to removal of manganese from PSII.

Triton particles prepared from spinach chloroplasts by the method of Vernon, Shaw & Ke (1966) show Mn/chlorophyll ratios dependent on size. Large particles (PL) had a 1:51 Mn/chlorophyll ratio and small particles (PD10) a 1:129 ratio, which corresponds to a partial separation of photochemical systems, enrichment of manganese being associated with the heavier particles enriched in PSII. The rapid way in which Triton inhibits photochemical activity in PSII particles has been suggested by some authors to be due to it releasing manganese (Hind & Olson, 1968). However, the enriched ratios obtained on Triton fractionation were similar to those of digitonin-particles that were still photochemically active, so inhibition of PSII activity by Triton is not due to displacement of manganese.

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Subcellular Fractionation of Aerobically Grown *Saccharomyces carlsbergensis* by Zonal Centrifugation

By T. G. CARTLEDGE, LYNDIA HOWELLS and D. LLOYD. (*Department of Microbiology, University College, Cathays Park, Cardiff CF1 3NR, U.K.*)

Studies were carried out to investigate the distribution of marker enzymes in homogenates prepared from *Saccharomyces carlsbergensis*. Sphaeroplasts were prepared [after growth of cells aerobically in a medium containing glucose (0.8%)] by the two-stage method of Duell, Inoue & Utter (1964) and disrupted in a Teflon hand-operated homogenizer (clearance 0.004in).

Samples homogenized in 0.25M-sucrose–2mM-EDTA–10mM-tris buffer, pH 7.4, were centrifuged at 2000g for 5min to remove whole cells, loaded on to a sucrose density gradient in 2mM-EDTA–10mM-tris buffer, pH 7.4, in a B XIV rotor and centrifuged at 35000rev/min for 2.75h ($\approx 6 \times 10^6$ g-min at the sample zone).

The following results were obtained for percentage sedimentabilities and median buoyant densities (g/ml in parentheses) of membrane-bound activities: