PRELIMINARY NOTES

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Effect of anions on oxidative phosphorylation in submitochondrial particles

A recent note entitled "Effect of monovalent cations on oxidative phosphorylation in submitochondrial particles"[1] has prompted us to publish results obtained some time ago, which in some respects are similar to those reported[1] but have led us to a different interpretation. Experiments with various submitochondrial particles at different stages of resolution have shown that the rate of respiration can be influenced to a variable degree by certain cations, anions and uncouplers. The first observation was that when *N,N'*-dicyclohexylcarbodiimide (DCCD) was added to highly resolved mitochondrial particles[2] the rate of NADH oxidation was inhibited by 75 % and was restored to the original rate by addition of carbonylcyanide *p*-trifluoromethoxy phenylhydrazone (FCCP)[3]. This observation which was confirmed by BEYER *et al.*[4], corroborates the data of LEE AND ERNSTER[5] who first recorded an oligomycin-induced "respiratory control" in submitochondrial particles. The second observation was that addition of 5 mM Ca^{2+} stimulated NADH oxidation 2–4-fold in submitochondrial particles, particularly those prepared from light layer mitochondria[6], whereas FCCP stimulated only slightly. The third observation (unpublished experiments) was that the oxidation of NADH in freshly prepared electron transfer particles from beef heart heavy mitochondria[7] was stimulated 4–6-fold by FCCP. VALLIN[8] also observed a stimulation of respiration in submitochondrial particles by addition of FCCP.

The three observations had two features in common: (a) stimulation of respiration was pronounced with NADH but was slight or absent with succinate as substrate; (b) several fold stimulation was observed only when relatively low concentrations (10–25 mM) of potassium Tricine or glycylglycine buffer were used. In the presence of Tris sulfate respiration increased with increasing buffer concentrations (from 25 to 100 mM) thus obscuring any effects of FCCP or Ca^{2+}.

When the difference between Tris and Tricine buffer was first noted, we thought that we were dealing with a specific uncoupling effect of Tris similar to that described in chloroplasts[9,10]. Further studies revealed, however, that the effect was not specific for Tris sulfate since other sulfate salts also stimulated the oxidation of NADH. A variety of Tris and potassium salts were then tested and found to stimulate respiration and inhibit phosphorylation. The data in Table I show that the depression of the P:O ratio was most pronounced with Tris nitrate and least pronounced with Tris acetate (Expt. A). Similar results were obtained when K^+ was used as the cation (Expt. B). An interpretation of the marked decrease in P:O ratio in the presence of the salts was complicated by the fact that a stimulation of respiration may by itself cause a depression of the P:O ratio[12]. To avoid this phenomenon the effect of various

Abbreviations: DCCD, *N,N'*-dicyclohexylcarbodiimide; FCCP, carbonylcyanide *p*-trifluoromethoxy phenylhydrazone.

salts on the P:O ratio with succinate was analyzed. It can be seen from Expt. C in Table I that nitrate and formate salts did not stimulate oxidation of succinate yet inhibited the accompanying phosphorylation very strongly; sulfate and chloride salts inhibited moderately and acetate not at all. These effects were similar to those observed with NADH as substrate but not as pronounced, probably because the dehydrogenation of succinate was rate-limiting. As will be shown below, a decreased sensitivity was also noted when the rate of NAD^+ reduction was made rate-limiting with β-hydroxybutyrate as substrate.

It should be pointed out that these findings are actually not in conflict with the data reported in Table I (ref. 1) which show that chloride salts of K^+, NH_4^+, Li^+

TABLE I

EFFECT OF ANIONS ON OXIDATIVE PHOSPHORYLATION IN SUBMITOCHONDRIAL PARTICLES

In a final volume of 1.1 ml, the following components were added to the polarograph chamber: 2.2 μmoles $MgSO_4$, 0.55 μmole EDTA (pH 7.4), 5.5 μmoles potassium Tricine (pH 7.4), 35.2 μmoles glucose, 1.1 μmoles ATP (pH 7.4), 16.5 units dialysed hexokinase, 2.2 mg dialysed bovine serum albumin, and 20 μmoles $K^{32}PO_4$, pH 7.4 ($1.2 \cdot 10^5$ counts/min per μmole). Further additions to this basic mixture in experiments A and B included 10 μmoles ethanol, 0.5 μmole dithiothreitol, 40 μg purified alcohol dehydrogenase and 110 μg purified aldehyde dehydrogenase[6]. After addition of buffer or salt as indicated in the table and electron transfer particles from beef heart heavy mitochondria (240 μg in experiment A and 348 μg in experiment B), respiration was initiated by the addition of 0.6 μmole NAD^+. Further additions to the basic system in experiment C included the potassium salts as indicated in the table and 348 μg electron transfer particles from beef heart heavy mitochondria. Respiration was initiated by the addition of 10 μmoles potassium succinate, pH 7.4. In experiment A 75 μmoles of each Tris salt were used, and in experiments B and C 75 μmoles of each potassium salt were used except in the case of K_2SO_4 where 37.5 μmoles were added. Respiration was terminated by adding the entire sample to 0.1 ml 50% trichloroacetic acid and chilling. Glucose-6-^{32}P (ref. 11) and protein[12] were assayed as described previously.

Additions to complete system	*Oxygen uptake (μatoms/min per mg)*	*Glucose-6-^{32}P formation (μmoles/min per mg)*	*P:O*
A. *Effect of various Tris salts with NADH as substrate*			
None	0.18	0.31	1.74
Tris acetate	0.46	0.46	1.00
Tris chloride	0.43	0.24	0.56
Tris sulfate	0.36	0.17	0.46
Tris formate	0.25	0.08	0.30
Tris nitrate	0.48	0.06	0.13
B. *Effect of various potassium salts with NADH as substrate*			
None	0.18	0.27	1.50
Potassium acetate	0.38	0.33	0.87
Potassium chloride	0.56	0.29	0.52
Potassium sulfate	0.64	0.20	0.32
Potassium formate	0.23	0.063	0.27
Potassium nitrate	0.60	0.072	0.12
C. *Effect of various potassium salts with succinate as substrate*			
None	0.16	0.16	0.96
Potassium acetate	0.21	0.21	1.01
Potassium chloride	0.16	0.13	0.79
Potassium sulfate	0.18	0.13	0.73
Potassium formate	0.12	0.052	0.43
Potassium nitrate	0.12	0.032	0.25

and $Tris^+$ were about equally effective. However, we are drawn to the conclusion that the inhibitory effect is primarily due to the anions rather than the cations. This would allow for a simple explanation of the finding[1] that the inhibition by KCl was competitive with ADP since at these high salt concentrations a relatively non-specific anion competition may take place. Indeed we have found (Fig. 1A) that the inhibition of phosphorylation associated with NADH oxidation is also competitive with P_i. Experiments with a rate-limiting NADH regenerating system (β-hydroxybutyrate) gave essentially the same results (Fig. 1B). However, as in the case of succinate oxidation there was no stimulation of respiration and higher concentrations of salt were required for a comparable inhibition of phosphorylation.

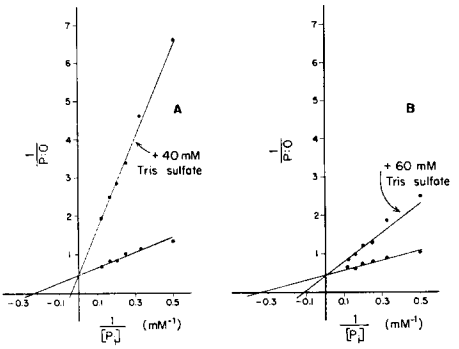

Fig. 1. Lineweaver–Burk plots of P:O ratio *versus* the concentration of phosphate in the presence and absence of Tris sulfate. A. The complete system was as described in the legend of Table I for experiment A except that the amounts of P_i and Tris were varied as indicated in the figure. The specific radioactivity of $^{32}P_i$ in the assay was kept constant. B. The complete system was as for A except that the NADH regenerating system was replaced by 10 μmoles of potassium β-hydroxyhydrate *plus* 1 μmole of NAD^+.

In contrast to these salt effects, the uncoupling by valinomycin *plus* K^+ (ref. 1) most probably represents a specific effect of valinomycin on K^+ translocation[13]. It can be seen from Fig. 1 (ref. 1) that the inhibition was only apparent at very high concentrations of K^+ (50–200 mM). This is in contrast to the findings of MONTAL *et al.*[14] and of COCKRELL[15] who observed uncoupling of submitochondrial particles at low K^+ (5 mM) provided both valinomycin and nigericin were added. The most plausible explanation for this uncoupling effect is as follows: according to MITCHELL[16] coupled electron transport in submitochondrial particles obtained after sonication of mitochondria initiates an inward movement of protons. In the presence of nigericin which facilitates a H^+/K^+ exchange (*cf.* ref. 17), the particles take up K^+ (ref. 14) which is extruded again in the presence of valinomycin. This energy-dependent cycling of K^+ is thus responsible for the uncoupling. If this explanation is adopted for the findings of PAPA *et al.*[1] it stands to reason that in the absence of nigericin very high K^+ concentrations were required to maintain a sufficient influx of K^+. In line with this view is the observation of COCKRELL[15] that valinomycin uncouples submitochondrial particles at low concentrations of NH_4Cl in the absence of nigericin. This can be readily explained in terms of the high permeability of membranes to NH_3 (ref. 18) and the subsequent formation of NH_4^+ from NH_3 and H^+ inside the particles.

Submitochondrial particles have in general been considered to be less susceptible

to ionic influences (*e.g.* Ca^{2+}) and to some uncoupling agents (*e.g.* valinomycin) than intact mitochondria. It is apparent from the findings that the type and concentration of externally added cations and anions may profoundly influence the phosphorylating efficiency of submitochondrial particles and their affinity for ADP and P_i.

Section of Biochemistry and Molecular Biology, Cornell University, Ithaca, N.Y. 14850 (U.S.A.)

R. O. CHRISTIANSEN
A. LOYTER
E. RACKER

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The effect of some local anesthetic compounds on sarcotubular calcium transport

Local anesthetics have been shown to have varied effects on the contractility and calcium fluxes of skeletal muscle. FEINSTEIN[1] showed that the caffeine-induced contracture of frog skeletal muscle, along with the accompanying calcium efflux, was blocked by procaine and tetracaine. KUPERMAN *et al.*[2] observed that higher concentrations of the same compounds caused a release of calcium from frog skeletal muscle. BIANCHI AND BOLTON[3] and WEISS[4] demonstrated that if the intracellular pH was elevated the local anesthetics themselves were able to induce contracture, thus suggesting that the specific effect of these compounds was a function of the relative concentrations of the charged and uncharged forms.