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The Effects of Colloids on the Appearance and Substrate Permeability of Rat Liver Mitochondria

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Abstract

The electron microscopic appearance of rat liver mitochondria fixed in glutaraldehyde is altered if certain colloids (serum albumin, dextran or Ficoll) are present in the medium at about 3%. To compare behaviour in control and albumin-supplemented media, the rate of stimulated respiration was measured with various substrates. It was found that the rate of respiration was reduced with succinate or pyruvate and was almost abolished with oxoglutarate, while malate oxidation (in presence of glutamate) was unaffected. The rate of oxoglutarate oxidation could be restored by causing mitochondrial swelling. It is suggested that the effects are due to the presence of endogenous colloids in the particles whose effects on water activity have to be balanced by external colloid. In the absence of external colloid, swelling of the internal colloid-containing compartments may give rise to an enhanced permeability of the membrane so that reactions occur *in vitro* which do not take place rapidly if at all *in vivo*.

Introduction

The cytosol in which mitochondria are situated *in vivo* contains about 0·3 osmolar salts and appreciable concentrations of large molecules such as proteins and polysaccharides. When isolated mitochondria are suspended in 0·3 osmolar sucrose they seem to be swollen as compared with their state when visualized in the intact cell. Exposure to 0·3 osmolar saline media leads to still more pronounced swelling.[1] Sucrose solution at 0·8 osmolar was found by early workers[2] to produce an appearance more like that of the intracellular mitochondria. However, use of such high concentrations gave little or no capacity to respire or phosphorylate in response to ADP + phosphate. To explain this, Lehninger[3] suggested that sucrose is an inhibitor of the processes.

The outer membrane is rapidly penetrated by sucrose and the inner membrane admits this solute more slowly[4, 5] with a concomitant swelling. These observations suggest that molecules larger than sucrose may be required to balance the osmotic contributions from the internal colloids.

Although serum albumin applied at concentrations about 0·2% has been used to bind fatty acids, it is only recently[6] that high concentrations (1·5% or more) have been applied. Allmann *et al.* found that the P/O ratio obtained with adrenal cortex mitochondria was increased by serum albumin, and also that either albumin or Mg^{2+} or Mn^{2+} alter the appearance of the particles when they are observed under the electron microscope.

This report is concerned with the results obtained with serum albumin and some other colloids on the appearance and respiratory behaviour of rat liver mitochondria with a range of substrates. The oxidation of oxoglutarate is inhibited by serum albumin; some experiments in which oxoglutarate distributions were measured with and without serum albumin are also described.

Materials and Methods

Rat liver mitochondria were prepared by initial homogenization in 0·25 M sucrose, 0·5 mM EGTA, 0·1% BSA. The medium used for the three washes and resuspensions was 0·3 M sucrose, 5 mM KCl, 0·5 mM EGTA, 0·1% BSA. The final suspension was in 0·3 M sucrose. The BSA (Fraction V, Sigma Chemical Co., London) was purified as follows. An approximately 10% (w/v) solution of the albumin in distilled water was

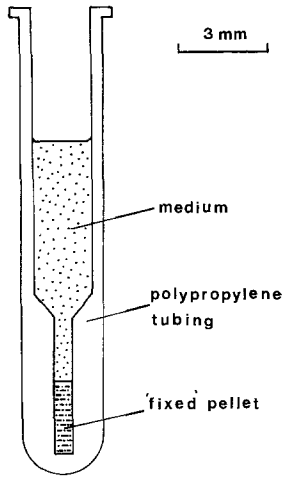

Figure 1. Sketch of plastic centrifuge tube used to collect a mitochondrial pellet by centrifuging the suspension through a volume of medium containing glutaraldehyde which is preloaded into the tube.

prepared by slow stirring of the mixture at 4°. The resultant solution was adjusted to pH 4, then treated with activated charcoal granules for 2–3 h to remove fatty acids.[7] After this, the mixture was filtered, the filtrate adjusted to pH 7 and the solution passed successively down a "Dowex 50" and a "Biodemineralit" (Permutit Ltd., London, W.3) column to remove free anions and cations, notably Ca^{2+} and substrate anions. The pH of the solution was readjusted to 7 and, after dialysis for 12 h against 1 mM EDTA, the concentration was adjusted to 5% BSA (w/v). Similar treatment rendered human serum albumin fit for use in the preparative procedures. Untreated recrystallized BSA (Sigma) was also used.

Samples for electron microscopy were prepared using modified centrifuge tubes suitable to fit a Coleman 8K-115 microfuge (see Fig. 1). Glutaraldehyde, to a final concentration of 0·8%, was added to 0·2 ml medium at the bottom of the tube. A 0·2 ml sample of mitochondrial suspension was added to the top and spun on a Coleman microfuge for 45 sec. The resultant pellet is formed on passing through the glutaraldehyde into the capillary at the bottom of the tube. After 5–10 min, a nylon tube was inserted into the pellet and the latter was extracted from the centrifuge tube. The

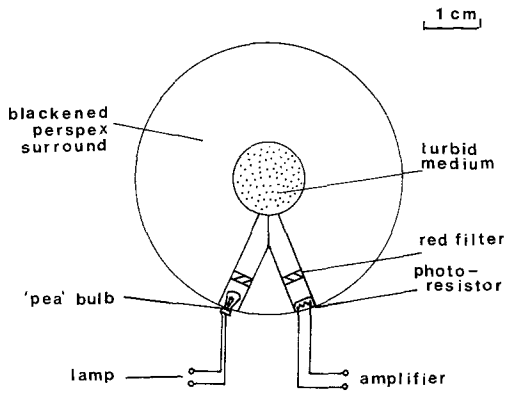

Figure 2. Diagrammatic sketch of arrangement of light source and photoelement added to oxygen polarograph to obtain a record of reflectance.

sample was ejected from the nylon tube, by means of air pressure from a syringe, into 1% osmic acid in "veronal" buffer. After 1 h the sample was transferred through different alcohol/water mixtures until dehydration was complete. The sample was then equilibrated with propylene oxide for 30 min, followed by treatment with propylene

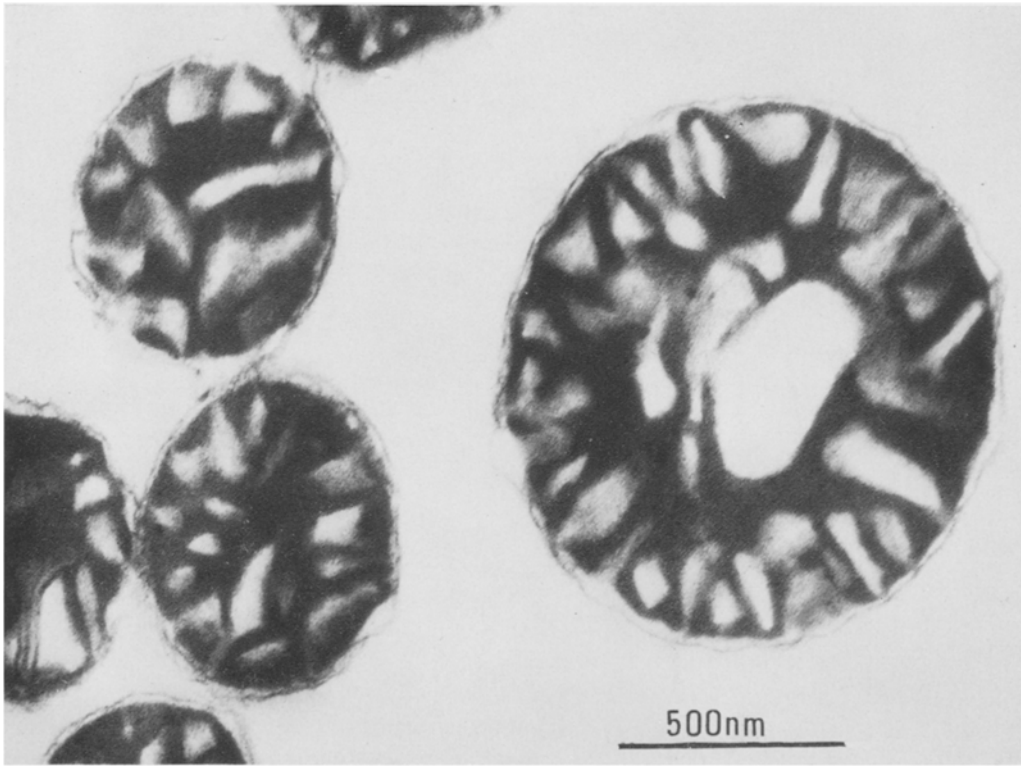

Plate 1. Rat liver mitochondria fixed with glutaraldehyde as described in the text. No colloid was added to the suspension medium (KCl 150 mM; mannitol 60 mM; tris chloride 20 mM.)

oxide/Araldite (1:1), followed by Araldite alone for 24 h. After heating in an oven for 36 h, the Araldite block was sectioned. The sample was finally stained on the grid with saturated uranyl acetate in alcohol/water (1:1), followed by lead citrate.

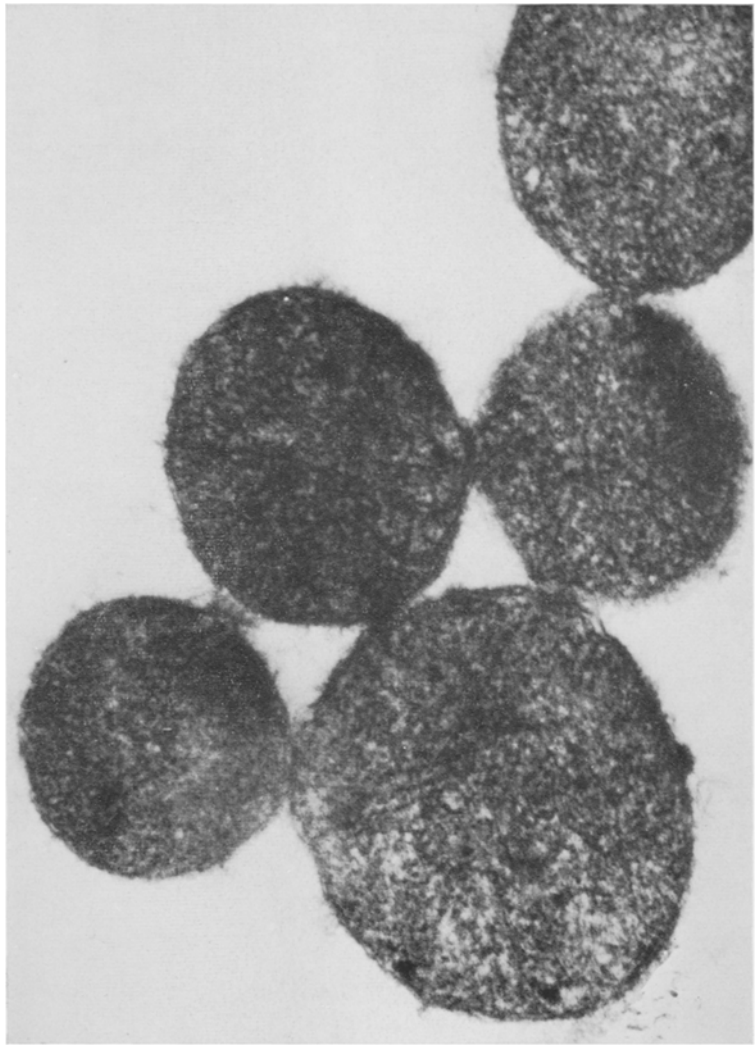

Plate 2. A similar preparation to that used for Plate 1 but fixed with 3% serum albumin in the medium. Magnification as in Plate 1.

Respiratory rates were measured on a Clarke-type oxygen polarograph (Rank Bros. Bottisham, Cambridge, England). Light scattering (at 30°) was measured with the special apparatus shown in Fig. 2. This consisted of a cylindrical block of blackened perspex, machined to fit the base of the polarograph detailed above. The photoconductive element was a simple photoresistor.

Results

Effects of albumin and other colloids on electron microscopic appearance. The plates 1–4 show comparisons made between particles taken from the control (colloid-free) medium and media including either albumin 2·5%, Ficoll 3% or dextran 3%. The average molecular weights are 68,000, 400,000 and 70,000. Plate 5 shows the albumin supplemented material at lower magnification.

The effect of the colloid is to lessen the proportion of unstained internal space and to

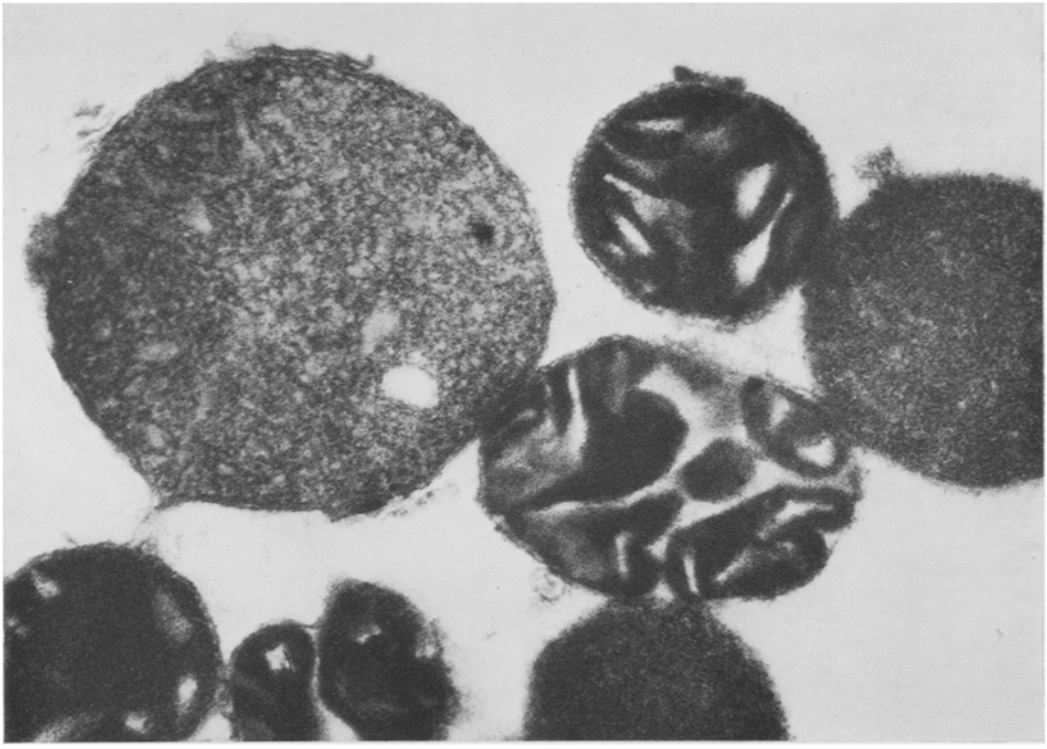

Plate 3. A similar preparation to that used for Plate 1 but fixed with 3% dextran in the medium. Magnification as in Plate 1.

spread out the stained material more uniformly within the volume of the particles; staining is less intense than in absence of colloid.

The effect of colloid on the ADP-stimulation rates of mitochondria with different substrates. Rates of phosphorylation for liver mitochondria in the presence of different substrates were measured polarographically in the medium described in Table I. Aliquots of 1 μmole ADP were added to 3 ml medium to obtain stimulated rates. Table I lists the effect of increasing quantities of BSA on both the phosphorylation rate and the ADP:O ratio observed with liver mitochondria. With all the substrates tried (excepting glutamate/malate), the ADP-stimulated rate is reduced by increasing the amount of BSA present while the P/O ratio remains unchanged. Ficoll has a similar effect on oxoglutarate and α-glycerol phosphate oxidation.

That isocitrate entry is impeded by BSA was shown by taking samples from incubations with isocitrate in presence of arsenite to inhibit further oxidation of the oxoglutarate formed. Assays were made for internal and external isocitrate, oxoglutarate and citrate; the latter is formed by the aconitase reaction occurring on the internal isocitrate. Fig. 3 shows that when albumin is present isocitrate is only slowly removed from the medium even after adding ADP. In the absence of albumin there is a comparatively rapid removal of isocitrate, with the formation of oxoglutarate and citrate. When albumin is present a higher internal content of oxoglutarate is reached after ADP addition, despite the

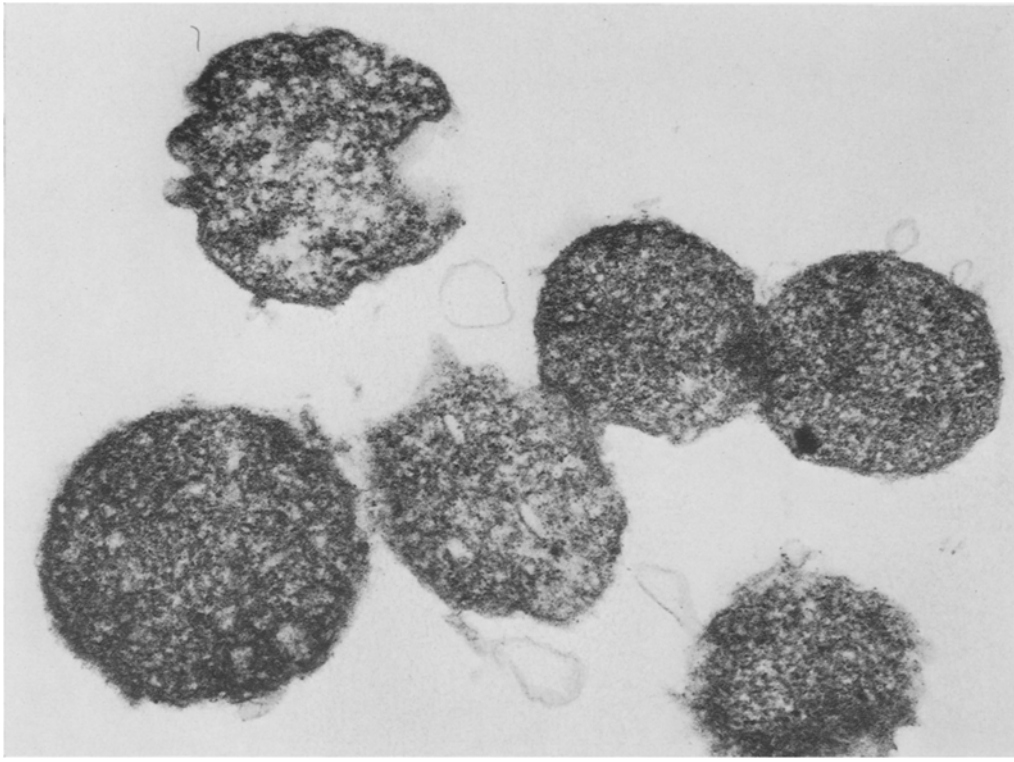

Plate 4. A similar preparation to that used for Plate 1 but fixed with 3% Ficoll in the medium. Magnification as in Plate 1.

lower external oxoglutarate concentration, this shows that the outward permeability to oxoglutarate is less than in the absence of albumin.

The inhibitory effect of albumin on oxoglutarate penetration was removed if swelling was induced. Figure 4 shows oxygen polarograph records of mitochondria in presence of albumin with oxoglutarate (+malate) as substrate with ADP additions to stimulate respiration. The oxygen consumption rate is low even after ADP addition until swelling has been induced, in one example a low concentration of valinomycin and in the other with glutathione. A similar result to the latter was obtained with ascorbate. Both latter agents led to loss of respiratory control, doubtless because of peroxidation of membrane lipids.

The lessened reactivity of external oxoglutarate caused by the albumin could also be

shown by observing the rate of oxidation of endogenous pyridine nucleotides by a combination of ammonia (added as ammonium chloride) and oxoglutarate. Figures 5 and 6 compare the oxidation rates of the pyridine nucleotides without (A) and with (B) albumin at 2%. In Fig. 5 ammonium chloride at 1 mM was present and small additions of oxoglutarate were made; in Fig. 6 oxoglutarate at 0·1 mM was present and additions of ammonium chloride were made. In each case the oxidation of the nucleotides was slowed by albumin though their effect could be due to reduced permeability to either or both reagents.

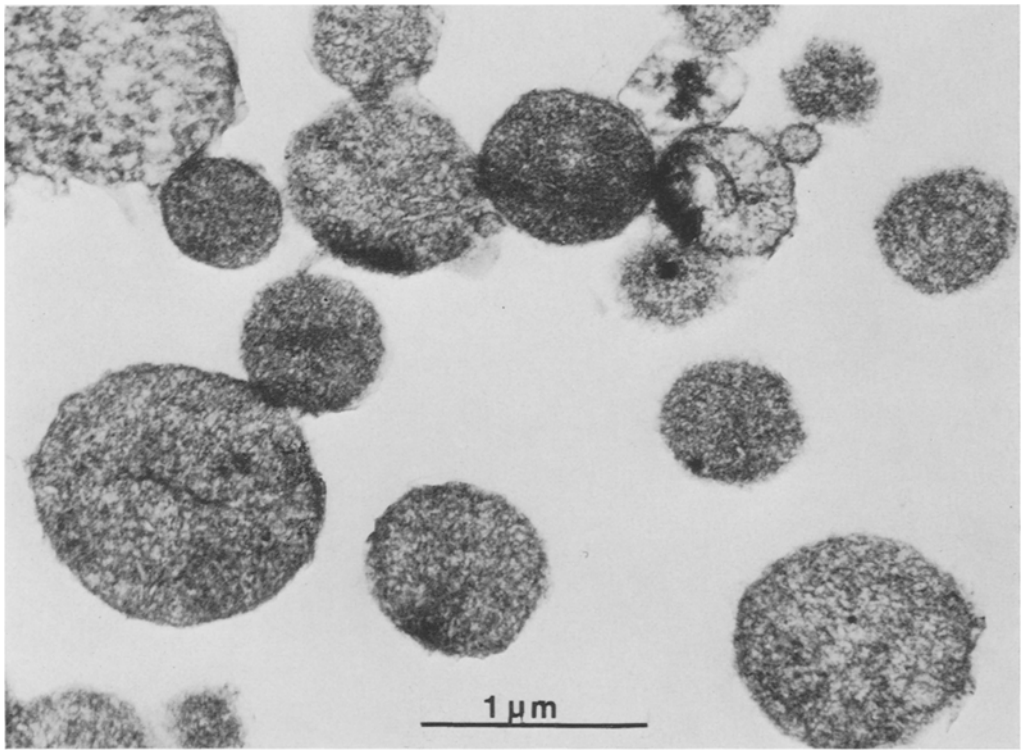

Plate 5. A similar preparation to that used for Plate 1, fixed with 3% albumin in the medium. Lower magnification than Plates 1–4.

Discussion

The appearance in the electron microscope show that the presence of colloid at the time of glutaraldehyde fixation alters the state of condensation of the mitochondrial interior. This result can come about either (1) because there is a real pre-existing difference, or (2) because the fixative (glutaraldehyde) penetration is changed by the colloid. For the present case it does not matter which alternative is correct: we can state that either the presence of colloid visibly alters the conformation of the internal stainable material or that it alters the permeability of the membrane. Allmann *et al.*[6] have stated that the colloid maintains the interior in what they call the aggregated form, rather than the

TABLE I. The effect of serum albumin, dextran and Ficoll on the ADP-stimulated respiration rates observed with rat liver mitochondria. The medium was 150 mM KCl, 20 mM tris chloride, 60 mM mannitol, 6 mM phosphate pH 7·4. The mitochondrial protein was 1 mg/ml and the temperature 20°

Substrates and additions	Respiratory rate natom O per mg per min		ADP/O ratio
	Resting	ADP-stimulated	
2·5 mM malate + 0·5 mM glutamate	13	67	2·7
+1·5% BSA	12	67	2·6
+3·0% BSA	12	67	2·4
+4·5% BSA	13	65	2·5
10 mM DL-hydroxybutyrate	7·0 ± 2·1	16·5 ± 3·7	2·3 ± 0·3
+1·7% BSA	4·3 ± 2·1	11·0 ± 2·4	2·4 ± 0·2
+2·3% BSA	4·3 ± 0·4	7·1 ± 1·2	—
+3·4% BSA	5·0 ± 2·0	3·1 ± 1·2	—
10 mM DL-isocitrate + 0·5 mM malate	9	28	1·4
+1·7% BSA	9	22	1·4
+3·0% BSA	8	8	—
5 mM α-glycerol phosphate	13	22	2·0
+3·0% Dextran	10	10	—
+3·0% BSA	9	9	—
15 mM proline	7·5	24	2·0
+3·0% BSA	3·5	3·5	—
2·5 mM glutamate	20	100	1·8
+3·0% BSA	11	11	—
+3·0% Ficoll	10	40	1·4
3·0 mM pyruvate	3·0	14	2·3
+3·0% BSA	3·0	4·0	—
3·0 mM pyruvate + 2·0 mM malate	7·1 ± 2·4	19·2 ± 3·2	2·7 ± 0·3
+3·0% BSA	4·6 ± 2·5	14·7 ± 3·2	3·0 ± 0·2
+5·0% BSA	7·0 ± 1·7	7·0 ± 1·5	—
3·0 mM succinate + 10 μg rotenone	25 ± 1·2	116 ± 1·2	1·4 ± 0·3
+1·5% BSA	24 ± 4·9	53 ± 6·6	1·7 ± 0·1
+3·0% BSA	21 ± 2·7	24 ± 4·1	—
10 mM oxoglutarate + 1 mM malonate	6	50	3·5
+1·5% BSA	6	6	—
+2% Ficoll	6	41	3·8
+3% Ficoll	6	33	3·8
+5% Ficoll	6	20	3·8

"orthodox" form; these appearances seem to correspond to our with colloid and without colloid appearances.

When colloids are present the permeability of the internal membrane to malate does not appear to be affected. Hydroxybutyrate, isocitrate, α-glycerol phosphate, proline, glutamate, pyruvate and succinate penetration rates as inferred from the ADP-stimulated respiratory rates are all reduced, while 1·5% BSA suffices to inhibit oxidation of oxoglutarate even in the presence of malonate and phosphate. That the oxoglutarate oxidase complex is not poisoned was shown by the observation that addition of valinomycin would initiate respiration and permit phosphorylation. The effect

seems to be one of preventing entry of oxoglutarate and this explanation is borne out by measurements of the internal content of the substance under similar conditions. The reduction in permeability to oxoglutarate seems to be directly correlated to the

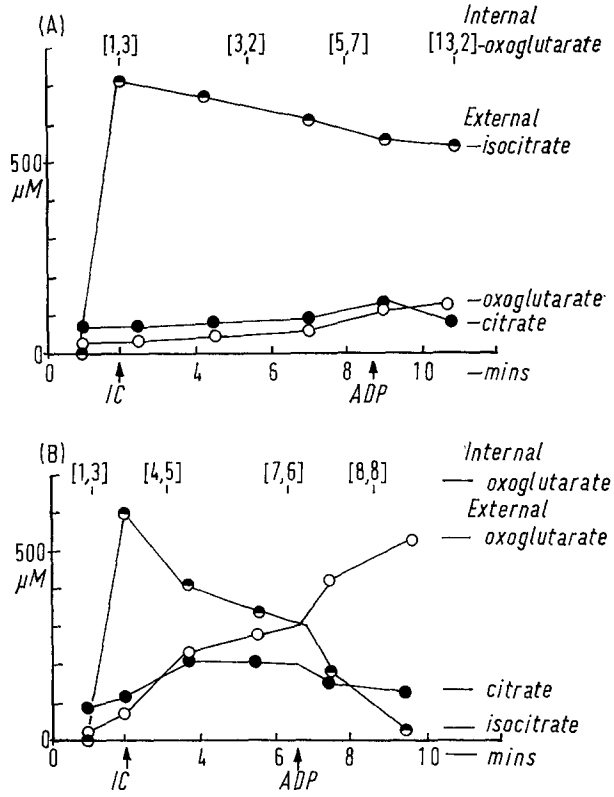

Figure 3. External concentrations of metabolites and internal contents of oxoglutarate (in square brackets) mitochondrial suspension oxidizing isocitrate. At "I.C." isocitrate to about 700 μM was added. At "ADP", ADP to 1·0 mM was added. The media contained:

A (top)	KCl	150 mM
	Mannitol	60 mM
	Tris phosphate	2 mM
	Tris chloride pH 7·4	20 mM
	Na arsenite	1 mM
	Albumin	2%

B (bottom) as A but without albumin

Note that in (B) there is more conversion to citrate and oxidation to oxoglutarate than in (A). In (A) the final oxoglutarate content of the mitochondria after ADP addition is higher than in (B).

condensed appearance of the mitochondrial interior and suggests that intracellular mitochondria may have a very low permeability to this substrate and perhaps others such as glutamate and pyruvate. It also raises the paradox that "good" mitochondria may not metabolize exogenous oxoglutarate.

The changed appearance of the internal structures obtained with external BSA or

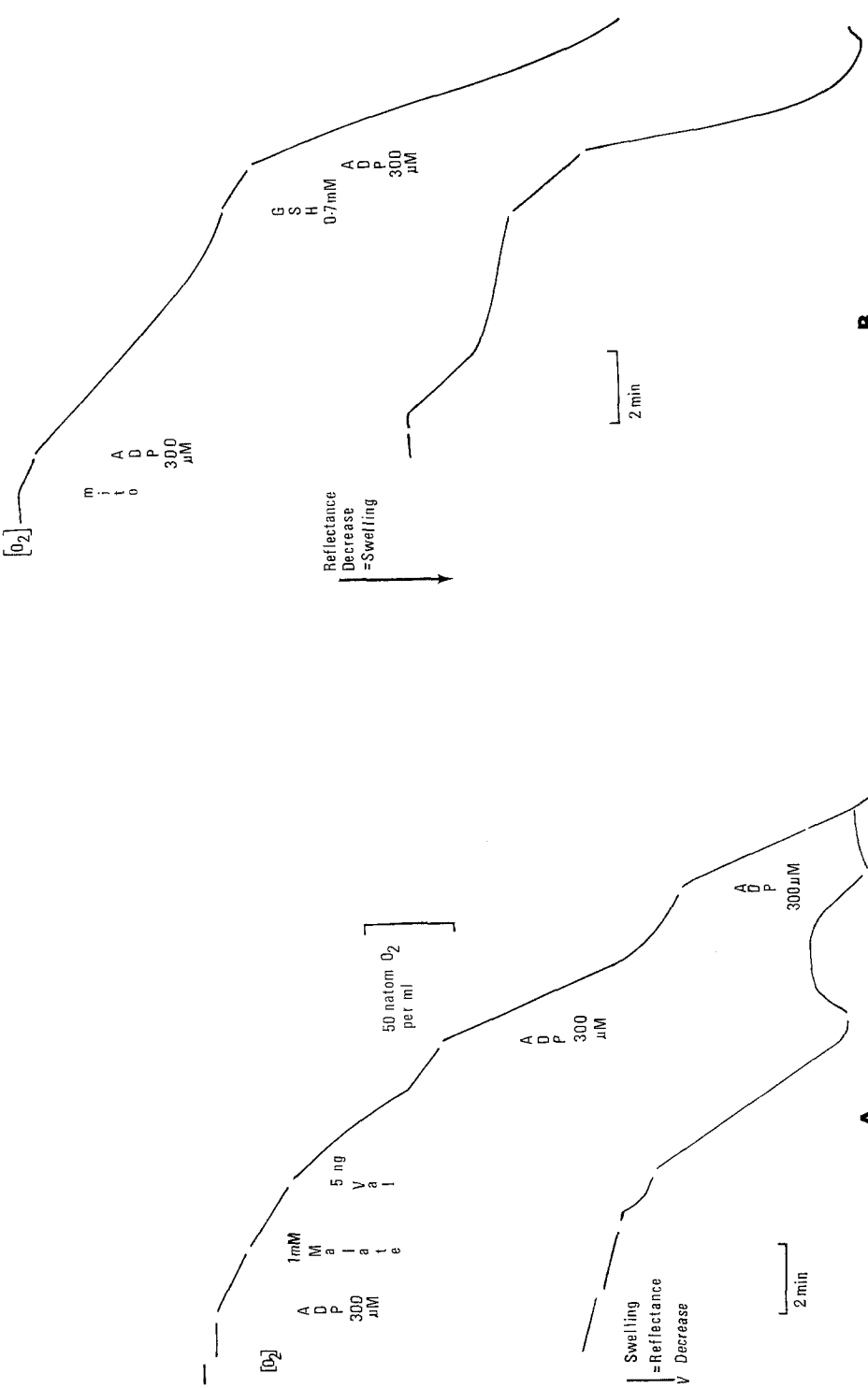

Figure 4. Traces from simultaneous recordings of oxygen tension and light reflection (30° to the incident beam) obtained with 5 mM oxoglutarate as substrate, ADP as respiratory stimulant and swelling agents added after the first trial of ADP. Note that before adding the swelling agent there is little response to ADP (even when malate was also added, in A). After swelling by (A) valinomycin 5 ng per mg protein or (B) by 0.7 mM reduced glutathione the respiratory rate is stimulated. The medium contained 250 mM sucrose, 5 mM KCl, 20 mM tris chloride pH 7·4, 3% serum albumin, protein 0·7 mg/ml at a temperature of 20°. The light reflectance scale is arbitrary and the record is slightly displaced in time with respect to the polarographic trace.

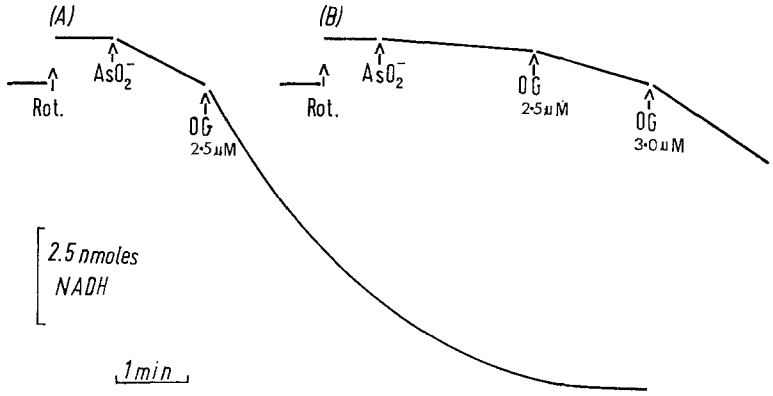

Figure 5. To show the slower oxidation of endogenous pyridine nucleotides by the reaction between ammonia and oxoglutarate. When 2% albumin is present (in B). In this pair of experiments ammonium chloride had been added to 0·75 mM and additions were of oxoglutarate. The medium contained KCl 150 mM, mannitol 60 mM, tris chloride pH 7·4 20 mM. Rotenone was added to 2·5 μg/ml and sodium arsenite to 0·75 mM. Mitochondrial protein 1·1 mg/ml.

other colloid reflects the effect of the internal colloids held between the outer and inner membranes on the water distribution. Those solutes which can pass through the outer membrane cannot, by their distribution, balance the reduction of water activity due to internal non-penetrants. The situation resembles that described by Wilbrandt[9] in relation to the maintenance of the red cell. In absence of metabolic energy being available to maintain an asymmetric ion distribution, red cells, like mitochondria, swell unless osmotic effect of the internal colloid is balanced by a sufficiently large solute added externally. The maintenance of a "condensed" form, requiring energy and Mg^{2+} has recently been illustrated by Dow, Walton and Fleischer.[10] This must be distinguished

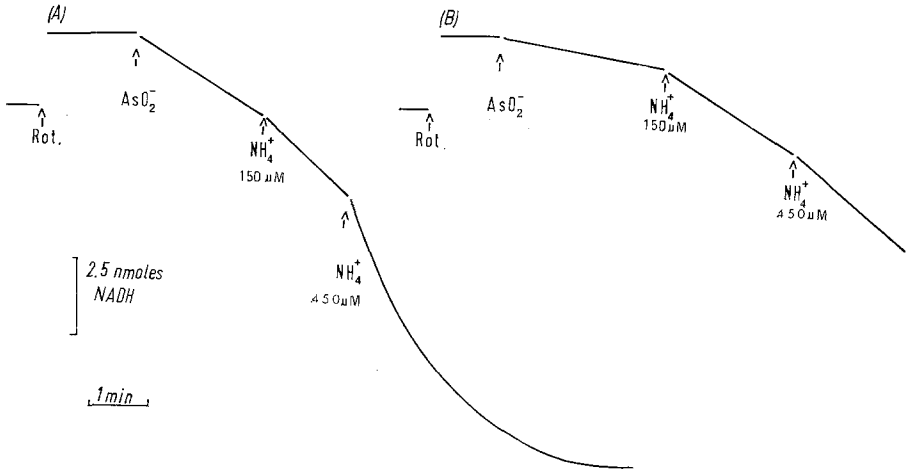

Figure 6. A similar demonstration of the slowing effect of albumin (present at 2% in B) on the ammonia-oxoglutarate reaction but in this pair of experiments oxoglutarate was present at 1·5 mM and additions of ammonium chloride were made. Other details as for Fig. 5. (Mitochondrial protein 1·6 mg/ml.)

from the energized swelling associated with the gaining of ions, e.g., with alkali acetate in the absence of Mg^{2+}.

Similar differences in appearances have been described for potato mitochondria fixed in media with and without colloids.[11] These authors commented upon the need for investigation of the metabolic effects induced by the colloids. There has also been a report[12] that chloramphenicol given *in vivo* leads to a changed electron microscopic appearance of mitochondria. Taken together it seems to be fair to conclude that the electron microscopic appearance depends more on factors such as colloids than on a special change related to phosphorylation.

Acknowledgements

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