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Effect of High Sucrose Concentrations on Mitochondria: Analysis of Mitochondrial Populations by Density-Gradient Centrifugation after Fixation with Glutaraldehyde

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Abstract

It has been shown previously that intact rat liver mitochondria can be separated into two populations (designated B2 and B3) with mean buoyant densities of 1·184 and 1·216 respectively, by isopycnic sucrose density gradient centrifugation. A comparison has been made of some properties of these mitochondrial fractions from density gradients with non-fractionated mitochondria. Use was made of density gradient centrifugation for analysis of preparations fixed with appropriate concentrations of glutaraldehyde. The permeability of the membranes of non-fractionated mitochondria to sucrose was increased by exposure to hypoosmotic sucrose solutions. The B3 mitochondria differed from the non-fractionated mitochondria in their response to changes in osmotic pressure of the suspending medium while the B2 mitochondria showed essentially identical behaviour with the controls. However, under conditions of energized swelling the B2 mitochondria were markedly different to the controls. This difference, which is attributed to reduced permeability of the mitochondrial membranes to metabolites brought about by exposure to the high concentrations of sucrose encountered in the density gradient, was reversed by incubation in hypo-osmotic sucrose solutions in the presence of oxidizable substrate and permeant ions.

Introduction

The structure and other properties of isolated mitochondria are determined in part by the composition of the medium in which they are suspended.[1] One of the properties of mitochondria which has received a great deal of attention is their response to changes in the osmotic pressure of the medium: it has been shown that they behave as simple osmometers undergoing rapid and apparently reversible changes in volume in response to changes in the external osmotic pressure, but with an osmotic dead space of some 40–60% of the volume of the mitochondria. Werkheiser and Bartley[2] found that about 60% of the volume of mitochondria isolated from rat liver in 0·25 M sucrose solutions was penetrated by sucrose and it has been shown that several other solutes of low molecular weight (including NaCl, KCl,[3] AMP, ATP and NAD^+,[4,5] CoA, acetyl-CoA,

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butyryl-CoA, L-carnitine and acetyl-L-carnitine,[6] and pyruvate, succinate and citrate[7, 8]) permeate a volume of rat liver mitochondria roughly equal to that accessible to sucrose. Werkheiser and Bartley[2] proposed that either there are two sorts of mitochondria, one completely permeable to sucrose and the other impermeable, or that mitochondria contain two internal aqueous phases the concentration of sucrose in the more accessible of these compartments being the same as that in the external medium. The second proposal has received considerable support and has been related to the known structure of mitochondria; the matrix space has been equated with the sucrose-inaccessible space and the inner membrane with the barrier to sucrose and other low molecular weight solutes.[9, 10] The two proposals are not mutually exclusive however and intact rat liver mitochondria isolated and suspended in 0·25 M sucrose, in which they became spherical, with enlarged intracristal spaces and a contracted matrix space, can be separated by density gradient centrifugation into two populations (with mean buoyant densities of 1·184 and 1·216) which differ only in the extent to which their inner membranes are permeable to sucrose.[11] The permeability of the inner membranes normally impermeable to sucrose can be increased by incubation of the mitochondria with 10 mM sodium succinate and 10 mM sodium phosphate pH 7·0 which also restores the appearance of the mitochondria to one more closely resembling that of mitochondria fixed *in situ*.

In contrast to the spherical form of rat liver mitochondria isolated in 0·25 M sucrose solutions, mitochondria in 0·44 M and higher concentrations of sucrose retain the rod-like form seen *in vivo*,[12] however the intracristal spaces are somewhat enlarged and the matrix space somewhat decreased compared to that of mitochondria fixed *in situ*. It has been proposed that mitochondria in hypertonic solutions are altered in some way with regard to function, thus Atsman and Davis[13] have shown that unusually high concentrations of sucrose and electrolytes inhibit respiration and energy-linked pyridine nucleotide reduction, and lower the respiratory control index. The preparation of sub-cellular fractions by sucrose density gradient techniques which employ high concentrations of sucrose may therefore be expected to influence the properties of some osmotically sensitive organelles[14] but this technique could be used to advantage with mitochondria fixed with 0·8% glutaraldehyde since the volume and light scattering of these have been shown to be unaltered by subsequent alteration of the osmotic environment.[15]

In the present study the properties of the mitochondrial fractions which can be obtained by density gradient centrifugation have been compared with those of the non-fractionated mitochondria. Emphasis has been placed on examining the influence of high sucrose concentrations on the permeability of the mitochondria to substrates and other anions, particular use being made of the technique of density gradient centrifugation to analyse preparations stabilized by fixation with glutaraldehyde.

Materials and Methods

(a) *Materials*

Glutaraldehyde was obtained from Polysciences Inc., Warrington, Pennsylvania, as an 8% aqueous solution at neutral pH sealed in ampoules under nitrogen, or cruder commercial material was redistilled. Other compounds were of Analytical Reagent quality.

(b) *Preparation of Mitochondria*

Liver mitochondria were isolated from rats starved for 16 h and were washed twice; the medium consisted of 0·25 M or 0·44 M sucrose containing 1 mM ethylenediaminetetracetate (EDTA), pH 7·4. Mitochondrial protein was determined by the modification by Miller[16] of the method of Lowry, Rosebrough, Farr and Randall,[17] crystalline bovine plasma albumin (Armour Pharmaceutical Co., Eastbourne, Sussex) being used as a standard.

(c) *Isopycnic Density-gradient Centrifugation*

Sucrose-density gradients were prepared as described by Pollak and Munn.[11] Sucrose solutions, of 11 concentrations from 1·9 M to 0·9 M were successively layered in 0·4 ml portions in 5 ml centrifuge tubes. The gradients were allowed to stand overnight at 2°C to smooth out discontinuities, and a 1 ml sample of mitochondrial suspension was then placed on the top of each. The tubes were centrifuged at 132,000 *g*(av.) for 90 minutes in the 59589 swing out rotor of the MSE Superspeed 65 centrifuge (Measuring and Scientific Equipment Ltd., London). The position of the bands, and hence the buoyant density of the mitochondria in them, was determined by fixing the tubes in a transparent plastic holder attached to a movement, with vernier scale, taken from a micromanipulator (W. R. Prior & Co., Bishop's Stortford, Herts). To obtain samples from the gradients, the tubes were cut by means of a tube-cutter[18] and the fractions were removed by Pasteur pipettes.

(d) *Measurement of Mitochondrial Respiration and Light-scattering*

Mitochondria were incubated in a glass cell of square cross-section (13 × 13 mm internally) mounted in a black perspex box through which water at 22°C was circulated. Oxygen uptake, 90° light scattering and optical extinction were recorded simultaneously on a Rikadenki three-channel recorder. The oxygen uptake was measured polarographically with a Clark Electrode (Yellow Springs Instrument Co., Ohio, U.S.A.). The use of a diode logarithmic converter[19] in conjunction with a Pye D.C. amplifier (Pye Unicam Ltd., Cambridge) enabled extinction to be recorded on a linear scale. The incident light, from a metal-filament lamp, was passed through a filter which transmitted wave lengths above 650 nm. For some purposes, the extinction of mitochondrial suspensions was determined in cells of 1 cm lightpath in a Unicam SP500 spectrophotometer, the distance between the cells and the photocell being about 8 cm.

Conditions for inducing mitochondrial oscillations were in general those described by Packer, Utsumi and Mustafa.[20] Mitochondria were fixed with glutaraldehyde while in suspension.[21, 22]

Results

(a) *Fixation of Mitochondria*

The criteria taken as showing adequate fixation of the mitochondria by glutaraldehyde were their resistance to osmotic- and triton-induced swelling and lysis in mitochondria not treated with fixative. Mitochondria isolated in 0·44 M sucrose medium were suspended for 5 min at a final concentration of 1 mg protein/ml in 0·44 M sucrose, 1 mM

EDTA, containing various concentrations of glutaraldehyde. The suspensions were then diluted 10-fold with water or sucrose solutions and their apparent optical densities determined at 546 nm (Fig. 1). Triton was then added to each preparation and the E_{546nm} redetermined. Mitochondrial suspensions treated with 1 mM and 5 mM glutaraldehyde were not fixed according to these criteria, but suspensions treated with 10 mM or 40 mM glutaraldehyde were fixed, i.e. they showed no significant change in E_{546nm} with change in sucrose concentration or on addition of triton. Mitochondria treated with 5 mM glutaraldehyde were resistant to osmotic volume changes but showed a rather

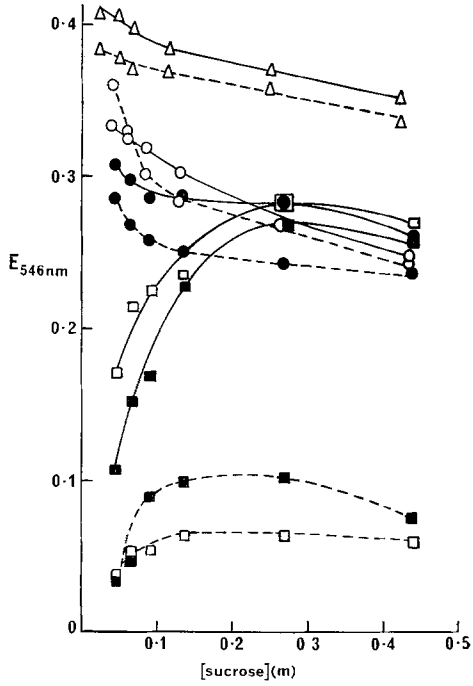

Figure 1. Test for fixation of mitochondria by glutaraldehyde. Mitochondria isolated in 0·44 M sucrose, 0·5 mM EDTA were suspended in 0·44 M sucrose, 0·5 mM EDTA containing various concentrations of glutaraldehyde at a final concentration of 1 mg mitochondrial protein/ml. After 5 min aliquots were diluted 10-fold in sucrose solutions to give the final molarities indicated in the figure. After 10 min at room temperature the absorbancy at 546 nm was recorded. Then triton was added to each tube to a final concentration of 0·017% and after another 10 minutes the readings at 546 nm were taken again. —□—, control; --□--, control plus triton; —■—, 1 mM glutaraldehyde; --■--, 1 mM glutaraldehyde plus triton; —●—, 5 mM glutaraldehyde; --●--, 5 mM glutaraldehyde plus triton; —o—, 10 mM glutaraldehyde; --o--, 10 mM glutaraldehyde plus triton; —△—, 40 mM glutaraldehyde; --△--, 40 mM glutaraldehyde plus triton.

larger extinction change when Triton was added than the mitochondria clearly fixed according to both criteria.

The osmotic and energized swelling properties of freshly isolated rat liver mitochondria (control mitochondria) were compared with those of subfractions obtained by centrifugation of the mitochondria through sucrose density gradients.

(b) *Control Mitochondria*

(i) *Osmotic Swelling*

Mitochondria isolated from rat liver in 0·25 M sucrose, EDTA solution were resuspended in 0·05, 0·25 and 0·50 M sucrose solutions to give 2·5 mg mitochondrial protein/ml, then fixed with glutaraldehyde and placed on sucrose density gradients. Following centrifugation at 132,000 × g (40,000 rpm) for 90 min, the patterns shown in Fig. 2 were obtained. The upper band, corresponding to B1 of Pollak and Munn,[11] was a

consistently observed but very minor component. The main band containing approximately 2 mg of protein in each case was located at a different position in the gradient depending upon the molarity of the sucrose in which the mitochondria had been sus-

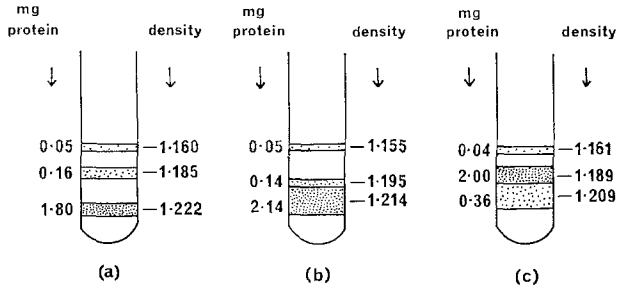

Figure 2. Effect of sucrose concentration at the time of fixation on the separation of mitochondrial populations by sucrose density gradient centrifugation. Mitochondria were distributed in tubes containing final concentrations of (a) 0·05, (b) ;·25 and (c) 0·5 M sucrose. After several minutes, the suspensions were treated with 10 mM glutaraldehyde. After 10 min, 1 ml of each suspension (2·5 mg protein/ml) was layered on a density gradient and centrifuged for 90 min at 40,000 rpm.

pended. The main band from osmotically swollen preparations, i.e. those which had been suspended in 0·05 M sucrose had a greater equilibrium sucrose density (1·222) than that from mitochondria which had been suspended in 0·25 M sucrose. Similarly the mitochondria in 0·25 M sucrose had a greater equilibrium sucrose density (1·214) than the mitochondria suspended in 0·5 M sucrose (density 1·189).

(ii) *Energized Swelling*

For energized swelling the control mitochondria were suspended in 100 mM sucrose, 0·5 mM EDTA at pH 7·8. Under these conditions the addition of succinate plus phosphate leads to rapid respiration and extensive uptake of ions associated with a decrease in light scattering by the mitochondria.[20] The mitochondria underwent a damped series of oscillations which terminated when the energy source was interrupted when the system went anaerobic (Fig. 3). In separate experiments at various points before, during and after swelling, glutaraldehyde was rapidly introduced into the reaction vessel to fix the various structural states of the mitochondria; the $E_{546\ nm}$ values obtained after addition of the fixative are shown by the dotted lines in the figure. Samples were collected and then centrifuged on sucrose density gradients for 90 min at 40,000 rpm. The mean equilibrium sucrose density of the main band on each gradient is shown in the figure. The mitochondria "swollen" as a result of energized ion accumulation had a significantly higher equilibrium density than those fixed just after initiation of swelling. The equilibrium density is virtually unchanged for the mitochondria before and following ion accumulation, although the apparent optical density of the mitochondria in the anaerobic state after ion accumulation is not completely restored to that of the original mitochondria.

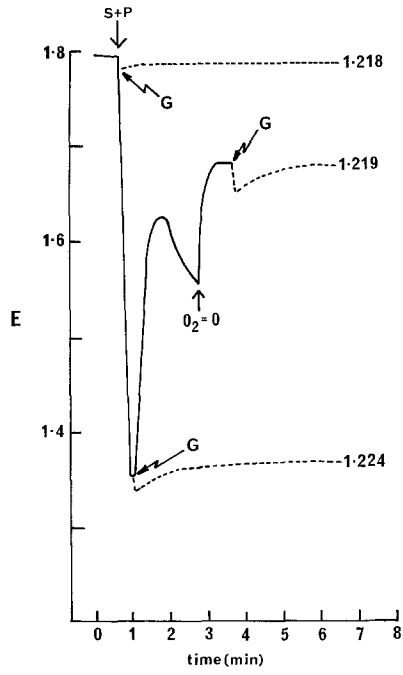

Figure 3. Absorbancy changes shown by control rat liver mitochondria under oscillatory-state conditions during the energized accumulation of ions. Mitochondria were isolated in 0·25 M sucrose –0·5 mM EDTA medium (pH 7·8) and resuspended in this medium for these experiments. The reaction mixture (4.0 ml) contained 100 mM sucrose, 0·5 mM EDTA at pH 7·8, and mitochondria at 1·0 mg protein/ml. Where indicated (S + P), 5 mM succinate plus 25 mM sodium phosphate at pH 7·8 were added. In three separate experiments glutaraldehyde was added, at the points indicated (*G*), to a final concentration of 40 mM to fix the mitochondria. The absorbancy of the samples after addition of glutaraldehyde is shown by the dotted lines. Aliquots were placed on density gradients and centrifuged at 40,000 rpm for 90 min; the mean equilibrium density of each sample thus determined is shown on the right.

(c) *Density-gradient Fractionated Mitochondria*

The three fractions (B1, B2 and B3) which can be obtained by density gradient centrifugation of non-fixed mitochondria as described by Pollak and Munn[11] were isolated and tested for osmotic and/or energized swelling properties.

(i) *Osmotic Swelling*

Aliquots of B1, B2, B3 and the control mitochondria were suspended in sucrose solutions at final concentrations of 0·025, 0·050, 0·075, 0·125, 0·250 and 0·50 M and their absorbancy at 546 nm was determined. Fraction B2 and the control mitochondria behaved virtually identically. On the other hand, fraction B3 had an initial absorbancy considerably less at the same protein concentration than fraction B2 or the non-fractionated mitochondria although a dependence on osmolarity was also evident (Fig. 4). Fraction B1 seemed to lack osmotic properties, as was to be expected since it is known to be composed primarily of damaged mitochondrial membranes.[11]

(ii) *Energized Swelling*

The energized swelling of mitochondria from fraction B2 diluted to about 1 mg protein/ml at a final concentration of 100 mM sucrose, 0·5 mM EDTA was examined under identical conditions to that of control mitochondria. The pattern for the accumulation of anions by the B2 fraction (Fig. 5) was very different from that of the control mitochondria (Fig. 3). First, addition of sodium succinate and phosphate resulted in an initial transient increase in transmission. This was rapidly followed by a decrease and then a steady state was approached. After 2 to 3 min had elapsed, the mitochondria manifested

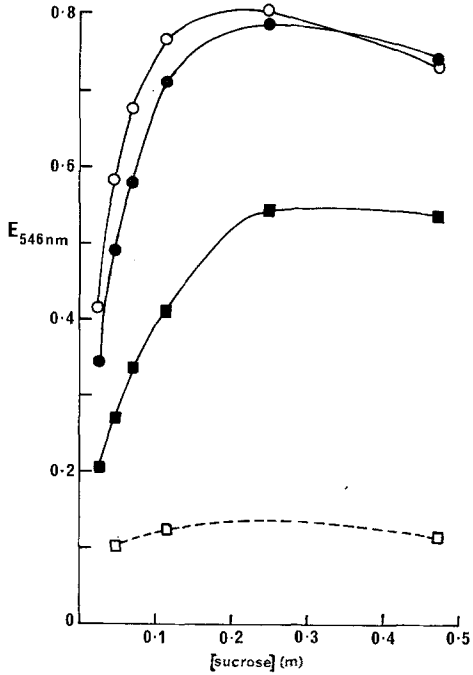

Figure 4. Apparent optical densities of control and fractionated mitochondria populations under varying osmotic conditions. Control mitochondria isolated in 0·25 M sucrose, 1 mM EDTA or the various fractions obtained therefrom by density gradient centrifugation were suspended in sucrose solutions of the concentrations indicated at 0·3 mg protein/ml (except for fraction B1 where the protein concentration was 0·09 mg/ml). The results for the B1 fraction were normalized on the basis of 0·3 mg protein/ml. —●—, control mitochondria; --□--, fraction B1 mitochondria; —○—, fraction B2 mitochondria; —■—, fraction B3 mitochondria.

a further decrease in transmission which again reached a steady state. Finally, when the energy source was removed, either by depleting the oxygen of the reaction mixture (Fig. 5), or in other experiments by interrupting electron flow by adding antimycin A, or if the electron flow was uncoupled by adding dinitrophenol (DNP), a partial reversal

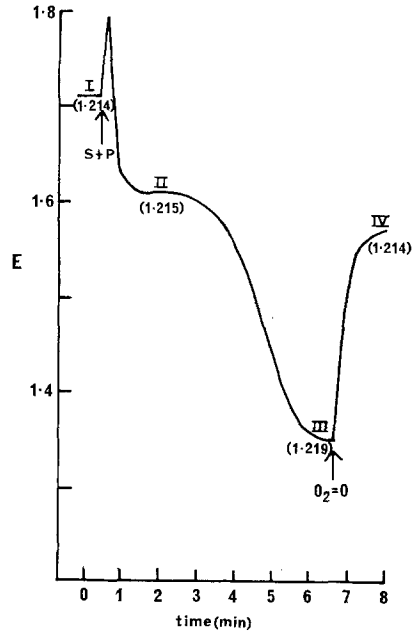

Figure 5. Test for the accumulation of ions by the B2 mitochondrial fraction. The B2 fraction was collected from gradients (by tube slicing) in the 1·4 M sucrose equilibrium density region. For experiments, the B2 fraction was diluted to 1 mg mitochondrial protein/ml and at a final concentration of 100 mM sucrose-0·5 mM EDTA at pH 7·8. Other conditions as in Fig. 3. I–IV, in separate experiments the mitochondria were fixed at these points and the mean equilibrium densities (shown in parentheses) were determined by density gradient centrifugation

of the transmission change to a new steady state level occurred. Apparently the test substances are taken up much more slowly than in the corresponding control experiment performed under identical conditions.

The equilibrium densities of these mitochondria fixed at intervals during the sequence (Fig. 5) were consistently lower than the corresponding control samples and did not show such a large increment associated with the minimum of absorbancy.

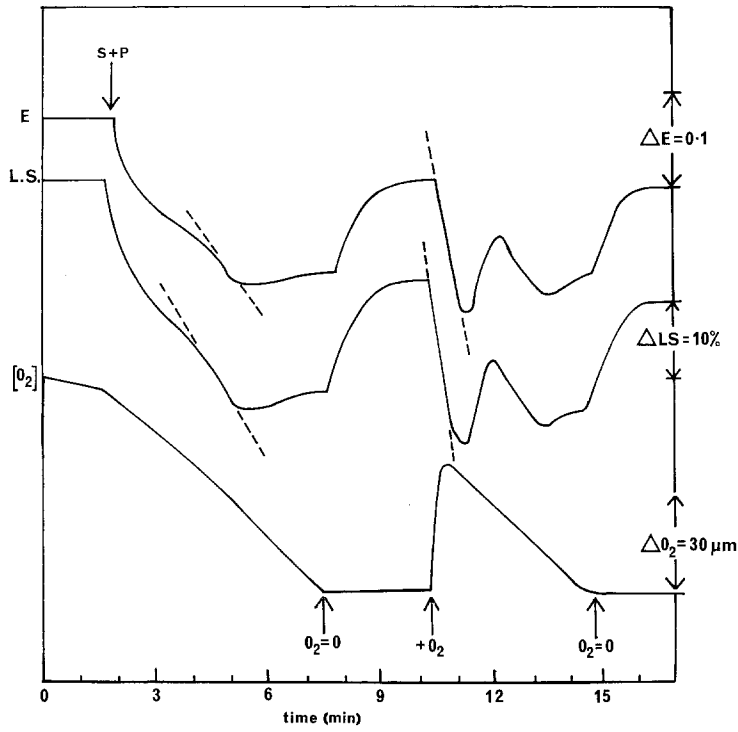

Figure 6. Transformation of high-sucrose-treated rat liver mitochondria into the uninhabited state. The reaction mixture (4·0 ml) contained: 210 mM sucrose, 0·5 mM EDTA, and rat liver mitochondria (0·77 mg protein/ml), and other additions as indicated at pH 7·8. The mitochondria were isolated in 0·44 M sucrose (plus EDTA) and resuspended in 1·4 M sucrose for 1 h prior to dilution into the test medium. The initial light-scattering level was arbitrarily taken as 100% for an initial absorbance of 1·38.

When DNP was added to the reaction mixture prior to the addition of succinate and phosphate then the second decrease in transmission which is interpreted as a secondary accumulation of ions was not observed. These experiments indicate that there are two types of ion uptake; the first is energy-independent and the second is energy-dependent.

The comparatively low permeability to substrate and anions of mitochondria treated with high concentrations of sucrose could be restored to normal levels by incubation with succinate and phosphate until the oxygen in the reaction mixture became exhausted and then reoxygenating the system (Fig. 6). Within a few seconds of mixing the mitochondria, in this instance treated with 1·4 M sucrose for 1 hr, into the reaction medium

(at a final concentration of 210 mM sucrose) a stable level measured by either light scattering or transmission was observed. This is in agreement with the presence of osmotic properties. Addition of oxidizable substreate plus permeate ion caused an initial increase in absorbance or scattering (osmotic response) followed by a rapid decrease until equilibrium occurred. Then a further energy dependent decrease commenced as substrate oxidation (shown by O_2 uptake) drove ion accumulation (shown by a further slow decrease of scattering and transmission) until a new steady state was reached. When the oxygen in the reaction mixture was exhausted the scattering and transmission changes were partially reversed. Reoxygenation of the reaction medium by a vigorous stream of oxygen over the surface of the reaction mixture restarted substrate respiration, but this time the initial rate of uptake (measured from scattering and transmission changes) was about 5-fold faster than before. Moreover, an oscillatory state was observed which is characteristic of the uninhibited control mitochondria (compare Fig. 3). Hence, following the slow expansion of the mitochondria during the initial cycle of ion uptake and accumulation the inhibitory effect of high concentrations of sucrose on permeability is overcome.

Discussion

(a) *Analysis of Mitochondrial Populations*

In the present study density gradient centrifugation has been used both as a preparative and as an analytical technique. For purposes of analysis use has been made of the fact that glutaraldehyde at appropriate concentrations (Fig. 1, and Packer and Greville[23]) will fix the osmotic state of mitochondria[15] apparently without altering the permeability of the inner membrane to sucrose.[11] The physiological and biochemical properties of the mitochondria are unfortunately not sufficiently well preserved by this reagent to justify its use during preparative density gradient centrifugation. Pollak and Munn[11] have demonstrated that intact rat liver mitochondria can be separated into two fractions, designated B2 and B3, which differ in that the inner membranes of mitochondria in fraction B2 are impermeable to sucrose whilst those of mitochondria in fraction B3 are permeable to sucrose. In partial agreement with the earlier observations of Beaufay and Berthey[24] and Beaufay *et al.*, [14] it was concluded that the act of centrifugation of the mitochondria through a gradient containing relatively high concentrations of sucrose altered the permeability of the inner membranes of some potential B2-type mitochondria so that they became B3-type. It is somewhat surprising, therefore, to find that the mitochondria exposed to 0·05 M sucrose, which would cause hypotonic swelling, have also been rendered more permeable to sucrose (Fig. 2). Conversely, all the mitochondria exposed to 0·5 M sucrose have inner membranes essentially impermeable to sucrose (Fig. 2). It appears that the permeability of the inner membrane to sucrose can be altered in more than one way (see section (c) below).

(b) *Comparison of Sub-fractions from Density Gradients with Non-fractionated mitochondria*

Of the mitochondria in the two fractions, B2 and B3, only those in B2 showed osmotic properties closely similar to the control mitochondria (Fig. 4). The B2 mitochondria, however, showed a marked difference in behaviour to the controls when their energized swelling was compared (Figs. 3 and 5). It appeared that the mitochondria in the B2

fraction had a decreased permeability to ions. We interpret the changes in extinction as follows: the initial transient increase in extinction could possibly be due to the osmotic effect resulting from the addition of 5 mM succinate and 25 mM phosphate (although it was not observed in the controls), as the substrates were taken up by the mitochondria the extinction fell to a steady state (II, figure 5); after the substances had equilibrated between the responding mitochondrial compartments energy made available from substrate oxidation led to an energized accumulation of ions shown by a fall in the extinction to approach a new steady state (III, figure 5).

The equilibrium density (1·218–1·219, Fig. 3) of control mitochondria suspended in sucrose medium prior to, and subsequent to energized swelling, is that expected of mitochondria suspended in 0·10 M sucrose (compare Fig. 2). In both control and B2 fraction mitochondria the occurrence of a minimum in the extinction curve corresponded with the development of the maximum equilibrium density by the mitochondria. We cannot at present account for the disparity between the equilibrium densities of the control and the B2 fraction mitochondria but the development of maximum equilibrium density at this time is in accord with observations on the change in equilibrium density of rat liver mitochondria associated with change in structure as revealed by electron microscopy.[11] It has been shown[20] that, whilst rat liver mitochondria under oscillatory conditions initially have enlarged intracristal spaces and a decreased matrix space, at the time of minimum extinction induced about one minute after the addition of succinate and phosphate the matrix space is enlarged and the intracristal spaces are correspondingly reduced. Just such a structural change, occurring over a much greater period of time, was shown to be associated with the conversion of B2-type mitochondria to B3-type mitochondria.

(c) *Effect of High Concentrations of Sucrose on Mitochondria*

An inhibitory action of some kind by high sucrose concentrations upon mitochondrial function has been generally recognized since Johnson and Lardy[25] reported that substrate respiration of rat liver mitochondria was severely depressed by raising the molarity of sucrose from 75 mM to 480 mM. It was suggested that this effect was a consequence of decreased permeability to substrates. Subsequently, Atsman and Davis [13] in a detailed study showed that varying the osmolarity of sucrose and electrolytes up to about 1600 mosM progressively inhibits respiration, the respiratory control index, and energy-linked pyridine nucleotide reduction. The use of an electron feeder system ascorbate plus tetramethyl-*p*-phenylenediamine (TMPD) was not as susceptible to such inhibition. Further studies[23] established that respiration of dicarboxylic acid substrates is more severely inhibited by high sucrose than is electron transport from ascorbate-TMPD or certain aldehyde substrates, such as glutaraldehyde at very low concentrations, indicating that mitochondria may be more permeable to uncharged and positively charged substrates than to weak acid anion substrates. The general conclusion from these studies was that the inhibitory effects of sucrose may be explained on the basis of decreased substrate permeability.

The occurrence of different populations of mitochondria separated on the basis of equilibrium density observed in the present and previous studies[26] may require another explanation because the increased equilibrium density of mitochondria after treatment with high sucrose concentration is indicative of an increased sucrose permeable space.

Dehydration of mitochondria by high concentrations of sucrose could cause changes in the structure of lipids, proteins, and lipoprotein structures and in the ordered structure of water in the membrane and matrix. Such changes could cause membrane damage permitting increased entry of sucrose into the inner compartment.

This explanation seems reasonable because the action of sucrose is unspecific. Thus, extreme dehydration arising from the treatment of osmotically sensitive mitochondria with very hyper-osmotic concentrations of non-penetrating electrolytes and non-electrolytes could cause changes in membrane structure and permeability. However, although certain mitochondria are more susceptible than others to this hyperosmotic effect, as seen here and in the studies of Pollak and Munn.[11] It has been established that these alterations in membrane structures are reversible. Reversibility is strikingly evident by the slow transformation of B2 mitochondria, or mitochondria treated with correspondingly high concentrations of sucrose, which are functionally inactive, into an active preparation showing normal permeability properties by incubation in the presence of hypotonic concentrations of sucrose at 25° (cf Fig. 7).

It is not entirely clear why the permeability of mitochondria treated with very hypertonic solutions of sucrose is initially inhibited when they are first tested at hypotonic concentrations. Perhaps dehydration resulting from prolonged treatment with sucrose may cause aggregation and cohesion of matrix material to itself and to the inner membranes imposing a mechanical force preventing the rapid expansion of the inner membrane system, hence impeding ion transport. In agreement with this suggestion it has been found by J. M. Wrigglesworth and L. Packer (unpublished work) that treatment with high concentrations of sucrose causes molecular conformational changes indicative of aggregation as judged by redshifts and decreased magnitude of ORD signals of mitochondria fixed with glutaraldehyde at various sucrose concentrations. Dehydration of the membrane system is also indicated by increased intensity of fluorescence of the molecular probe 8-anilino-1-naphthalene-sulfonic acid (ANS) which increases its fluorescence as a result of increasing hydrophobicity of the environment in which it is bound. Once the hydrophobic bonds are broken during the slow swelling of the high sucrose-treated mitochondria, it appears that the functional properties characteristic of control mitochondria are restored.

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