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MITOCHONDRIAL RESPIRATION UNDER CONDITIONS OF VARYING OSMOLARITY

ABRAHAM ATSMON* AND ROBERT P. DAVIS

Department of Medicine and The Unit for Research in Aging, Albert Einstein College of Medicine and the Bronx Municipal Hospital Center, New York, N.Y. (U.S.A.)

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SUMMARY

Inhibition of mitochondrial respiration with NAD-linked substrates and with succinate occurs in hyperosmolar media containing sucrose, mannitol, NaCl or KCl as the principal osmotic agent. Mitochondrial NADH levels have been found to be lower in hyperosmolar than in normoosmolar media and uncoupling agents do not release the respiratory inhibition. Therefore, a block in energy transfer does not appear to be the main cause of the respiratory inhibition. On the other hand, under appropriate conditions the reduction of NAD^+ is not inhibited by hyperosmolarity as shown in studies of reversal of electron flow utilizing tetramethyl-*p*-phenylenediamine –ascorbate as substrate. The respiratory block in hyperosmolar media appears therefore to be situated before NAD with NAD-linked substrates and before cytochrome *b* with succinate as substrate. The elimination of this inhibition after destroying the integrity of the mitochondrial membranes by freezing and thawing suggests that there may be an impediment to entry of substrates or a decreased accessibility of substrate to appropriate dehydrogenases under conditions of hyperosmolarity and that this impediment is the principal cause of the respiratory inhibition.

INTRODUCTION

The uptake of oxygen by mitochondria is decreased in media containing high concentrations of sucrose[1,2]. As was shown by JOHNSON AND LARDY[3] 2,4-dinitrophenol does not release this inhibition, which, however, is completely reversed when the mitochondria are resuspended in a medium of lower sucrose concentration. PACKER[4] has demonstrated that high sucrose concentrations decrease mitochondrial respiration with succinate as substrate, lower the steady-state level of NADH with mitochondria and decrease mitochondrial swelling. JOHNSON AND LARDY[3] have found that under conditions of hyperosmolarity different substrates show dissimilar degree of inhibition of respiration. These authors have suggested that this variation could be caused by changes in selective permeability of the mitochondria under conditions of high sucrose concentration.

Abbreviation: TMPD, tetramethyl-*p*-phenylenediamine.

* Present address: The Weizmann Institute, Rehovoth, Israel.

LEHNINGER[5,6] investigated extensively the inhibitory action of high concentrations of sucrose and other polyhydroxylic compounds on respiration, dinitrophenol-stimulated ATPase, ATP–$^{32}P_i$ exchange and contraction and swelling of mitochondria and mitochondrial fragments; following these investigations LEHNINGER proposed that an enzymatic reaction common to the above processes is inhibited by high concentrations of polyols.

We have attempted to elucidate further the mechanism of the inhibibion of respiration under conditions of high osmolarity and to ascertain whether the inhibition is due to a specific effect of sucrose and other polyols or to high osmolarity as such.

METHODS

Mitochondria were prepared from kidney or liver of Sprague–Dawley rats killed by decapitation. Homogenization of tissue was carried out with Dounce glass homogenizers and the homogenates were subjected to repeated differential centrifugation in a Sorvall RC-2 refrigerated centrifuge. All operations were carried out at 0°. The homogenizing solution was that of CHANCE AND HAGIHARA[7] except that the concentration of ethylenediaminetetraacetic acid was generally 1 mM instead of 0.05 mM. This medium contains mannitol and sucrose in concentrations of 0.23 M and 0.07 M respectively. The mitochondrial pellets from the final centrifugation were resuspended in homogenizing medium such that the mitochondrial protein concentration was about 10 mg/ml. All reaction media contained a constant concentration of electrolytes according to HAGIHARA[8], except again that the concentration of EDTA was 1 mM. Mitochondrial respiration, P:O ratio and respiratory control ratio were unaffected by this concentration of EDTA. In addition, sucrose or other test substances were added in varying concentrations as indicated below. All media were adjusted to pH 7.2.

Mitochondrial protein was measured according to LOWRY *et al.*[9]. Oxygen uptake was measured polarographically with a vibrating platinum electrode, the applied voltage being 0.65 V. The electrode was coated with collodion such that the sensitivity was about 25–30 % of that of the uncoated electrode[8]. The current was measured as the potential drop over a fixed resistance by means of a Keithley Model 610A electrometer and recorded by a Texas Rectiriter recorder. During measurements of oxygen uptake the temperature of the reaction vessel was kept constant at 26° by means of a water-jacket through which circulation was maintained by means of a Haake Type F circulating bath.

NADH was measured fluorimetrically in an Aminco-Bowman spectrofluorimeter at 25°. The excitation wavelength was 340 mμ and measurement was made of emitted light at 445 mμ. Corning filters 7-54 and 3-73 were used in the path of the excitation and emission beams respectively. Since we were interested primarily in relative fluorescence intensity, readings of photocurrent were not corrected to "true" fluorescence intensity except for determination of difference spectra. The low fluorescence readings of the blanks (basic reaction media containing the osmolarity-raising test substance such as sucrose) were subtracted from all subsequent readings. Both in the measurements of oxygen uptake and in the fluorimetric recordings, the mitochondria from the basic stock suspensions were diluted 10–20 fold.

The various substrates (succinate, glutamate, glutamate–malate, β-hydroxy-

butyrate and others) were added in final concentrations of 4 mM each, ADP was added in final concentration of 0.2 mM. Final concentration of 2,4-dinitrophenol was $2.5 \cdot 10^{-5}$ M, dicoumarol 2–4 μM, oligomycin 1 μg/ml, antimycin A 1 μg/ml, sodium amytal 1 mM for NAD-linked substrates and 20 mM for succinate, TMPD 0.1 mM, ascorbate 15 mM. The ascorbate was made up in water which had been deoxygenated by prolonged passage of nitrogen, prepared free of oxygen according to MEITES AND MEITES[10]. The pH of the ascorbate solution was adjusted to 5.6. Like the homogenization and basic reaction media, the substrates and ADP were adjusted to pH 7.2. All water was prepared by distillation and two passages over a mixed-bed ion-exchange resin column. All glassware was cleaned in a hot sulfuric–nitric acid bath.

P:O ratio and respiratory control ratio were measured according to CHANCE AND WILLIAMS[11]. Substrates, ADP and inhibitors were added serially to each preparation, which served as its own control. The chemicals were obtained from the following sources: succinate, pyruvate, DL-β-hydroxybutyrate and α-ketoglutarate from California Corporation for Biochemical Research; ADP from Sigma Chemical Co.; mannitol and sucrose from Fisher Scientific Corporation; L-glutamic acid from Nutritional Biochemical Corporation; dicoumarol from Mann Research Laboratories; antimycin A and oligomycin from Wisconsin Alumni Research Foundation. Sodium amytal was generously provided by Gane's Chemical Works Inc. Inorganic reagents were of analytical grade.

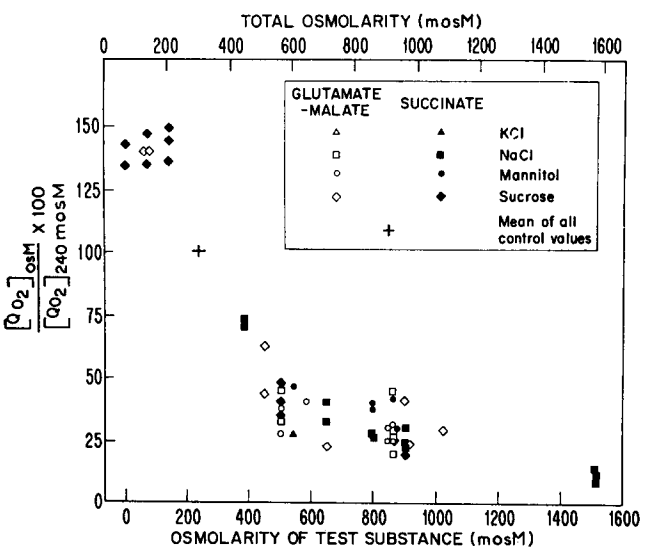

Fig. 1. Effect of osmolarity on O_2 consumption by kidney mitochondria in State 3. The basic reaction medium contained KCl 10 mM, $MgCl_2$ 5 mM, Tris buffer 10 mM (pH 7.2), potassium phosphate buffer 5 mM (pH 7.2) and EDTA 1 mM. In addition either one of the following test substances, NaCl, KCl, mannitol or sucrose, was added to raise the osmolarity of the complete reaction medium. All values are expressed as percentages of the O_2 consumption in a control reaction medium containing the appropriate test substance in a final concentration of 240 mosM. Substrate was either succinate (closed symbols) or glutamate–malate (open symbols) in final concentrations of 4 mM. O_2 uptake was measured polarographically at 26° after addition of substrate and ADP in a final concentration of 0.2 mM. The average mitochondrial protein concentration in the reaction medium was 0.5 mg/ml.

RESULTS

Fig. 1 shows the uptake of oxygen in State 3 (see ref. 11) by kidney mitochondria in the presence of varying concentrations of the test substances NaCl, KCl, sucrose or mannitol. All values are expressed as percentage of the uptake of oxygen in a reaction medium containing the basic electrolytes and the test substance in a final concentration of 240 mosM ("control" or "normoosmolar" media). At this latter concentration respiration averaged 100–200 μatoms per mg protein per min and no differences were noted between the different test substances. All four test substances caused essentially the same respiratory inhibition when present in hyperosmolar concentration. When the concentration of the test substances was reduced to 150 mosM respiration increased markedly but remained approximately constant at concentrations between 150 and 0 mosM. Without any test substance added, *i.e.*, in an electrolyte solution of about 70 mosM, the mitochondria were almost completely uncoupled. The effect of media of different osmolarity on the State-3 respiratory rate relative to that at 240 mosM, was similar with either succinate or glutamate–malate as substrate, though the absolute respiratory rates were higher with succinate than with glutamate–malate.

A few experiments with other substrates did not differ from those in Fig. 1. Mitochondria obtained from liver showed similar respiratory responses to hyperosmolar media, as did rat-kidney cortical, medullary and papillary mitochondria prepared separately. In order to ascertain that these results are not peculiar to Sprague–Dawley rats a few experiments were performed on mitochondria obtained from rats of the Holtzman strain (originally derived from the Sprague–Dawley strain) and from two mongrel dogs. The results in the dogs (Table I) and in the Holtzman rats did not differ from those obtained with mitochondria from Sprague–Dawley rats.

The action of electron-transport-blocking agents such as amytal and antimycin A was not influenced by the osmolarity of the medium. At 240 mosM, the quantitative effects of oligomycin and dinitrophenol were conventional: oligomycin (1 μg/ml) reduced the respiration of mitochondria in State 3 to 10–25 % of the State-3 value.

TABLE I

THE EFFECT OF HYPEROSMOLARITY ON THE O_2 CONSUMPTION OF RENAL CORTICAL AND MEDULLARY MITOCHONDRIA OF THE DOG

O_2 consumption was measured in State 3 and State 4. All values are expressed as percentages of those obtained in control reaction media containing the test substances NaCl or mannitol in a concentration of 240 mosM. The concentration of the test substances in the hyperosmolar media was 868 mosM. Substrate, glutamate–malate, 4 mM each; ADP, 0.2 mM. All values represent the average of two separate experiments performed on mitochondria from two dogs; maximal difference between two similar experiments was 4.8 % for the O_2 consumption and 5.4 % for the respiratory control.

	O_2 consumption (%)		*Respiratory control (%)*	
	NaCl	*Mannitol*	*NaCl*	*Mannitol*
Cortex	19.8	25.4	19.3	25.2
Medulla	28.6	28.2	28.8	27.7

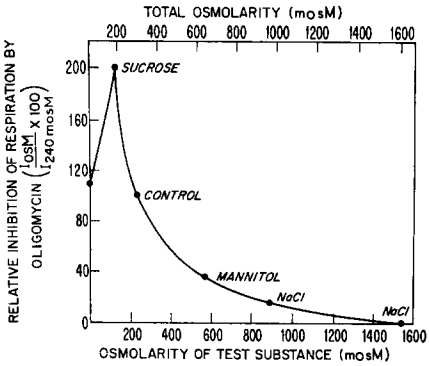

Fig. 2. The effect of osmolarity on the inhibition of kidney mitochondrial respiration in State 3 by oligomycin. Inhibition is calculated as the difference in O_2 uptake in State 3 and that obtained after addition of oligomycin 1 μg/ml. The inhibition in hyper- and hypoosmolar media is expressed as per cent of the inhibition in control media containing the test substances in a concentration of 240 mosM. Test substances used were NaCl, mannitol and sucrose. Substrate was succinate, 4 mM. The maximal difference between similar experiments with different test substances was 10%. Further details in legend of Fig. 1. While State-3 respiration at 125 mosM was considerably higher than that at 240 mosM, the absolute decrease in respiration produced by oligomycin at 125 mosM was two times the decrease produced by oligomycin at 240 mosM. This corresponds with the high respiratory control ratios observed at 125 mosM.

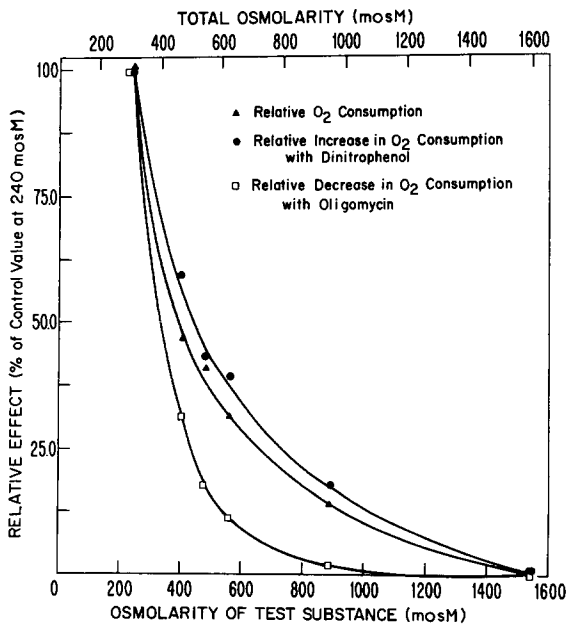

Fig. 3. The effect of hyperosmolarity on mitochondrial respiration in State 3, after addition of oligomycin, 1 μg/ml, and after addition of dinitrophenol, $2 \cdot 10^{-5}$ M. The values obtained are expressed as per cent of those in control media containing the test substances in a concentration of 240 mosM. The inhibition of respiration due to oligomycin is calculated similarly to Fig. 2. The test substances were sucrose, mannitol, NaCl and KCl. The substrate was succinate. A few experiments with dicoumarol gave results similar to those with 2,4-dinitrophenol. When glutamate–malate was used as substrate, the absolute values of O_2 consumption were lower, but the per cent changes in respiration after addition of oligomycin and 2,4-dinitrophenol were similar to those with succinate.

Subsequent addition of dinitrophenol restored respiration nearly to the State-3 rate with glutamate–malate and pyruvate–malate as substrates and to slightly above State-3 levels with succinate as substrate. On the other hand the inhibition of respiration in State 3 by oligomycin was inversely related to the osmolarity of the test substances commencing from 125 mosM and up to 1540 mosM. Below 125 mosM the inhibition became smaller due to increasing degrees of uncoupling of oxidative phosphorylation (Fig. 2). Neither dinitrophenol nor dicoumarol released the inhibition of respiration caused by hyperosmolar media, though they released the oligomycin-induced decrease in respiration in State 3 (Fig. 3). In hypoosmolar media in which mitochondria still showed good respiratory control, *i.e.* between 100 mosM and 240 mosM, uncoupling by 2,4-dinitrophenol or dicoumarol caused a marked increase in respiration, up to 30 % above that in State 3, with all substrates examined.

Hyperosmolarity caused an essentially similar, though much less marked inhibition of State-4 respiration (excess substrate, no ADP). Since respiration in State 4 was found to be lower in hyperosmolar media than in normoosmolar media, the rate of respiration in State 3 was compared to that in State 4 with the various test substances at different concentrations. Fig. 4 shows the increase in respiration going from State 4 to State 3 under various osmotic conditions. The results are again expressed as percentages of the increase seen in the control reaction media (*i.e.* test substance 240 mosM). The similarity of this curve to that in Fig. 1 shows that respiration in State 4 is also inhibited, like that in State 3. However, at osmolarities above 400 mosM State-4 respiration is somewhat less inhibited than State-3 respiration. The respiratory control ratio, therefore, is affected at the higher osmolarities where

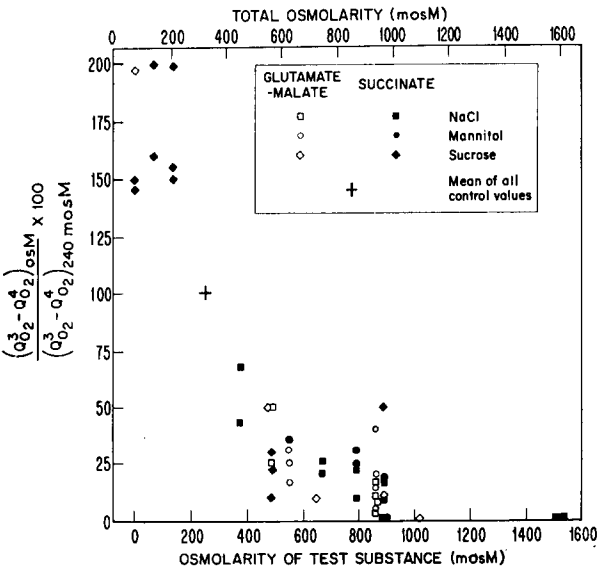

Fig. 4. Effect of osmolarity on the increase in O_2 consumption by kidney mitochondria upon changing from State 4 to State 3. All values are expressed as percentage of the increase in O_2 consumption in a control reaction medium containing the appropriate test substance in a final concentration of 240 mosM. Since this figure is based upon numerical differences between the various respiratory rates and since both States 3 and 4 respirations decline under conditions of hyperosmolarity this figure does not represent the respiratory control ratio, but it does reflect the phenomenon of respiratory control.

it declines towards one (Fig. 5). At 240 mosM the respiratory control ratio was 6–10 with NAD-linked substrates and 3–6 with succinate. This decline in respiratory control with increasing osmolarity is not due to partial uncoupling, *i.e* a relative increase in State-4 respiration, but to inhibition of State-3 respiration. Actually, the relative respiration in State 4 at varying osmolarities is essentially similar to that shown in Figs. 1 and 4. However, due to the slow respiration in State 4 in the control reaction medium the difference between respiration in this medium and in hyperosmolar media is much less marked.

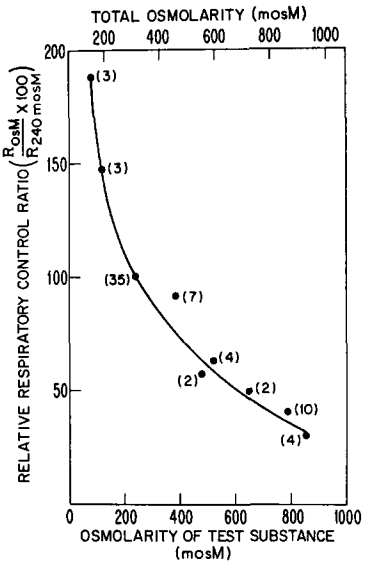

Fig. 5. Effect of osmolarity on the respiratory control of kidney mitochondria. All values are expressed as percentage of the respiratory control ratio in a control reaction medium containing the appropriate test substance in a final concentration of 240 mosM. For further details see legend to Fig. 1. The number of separate experiments is indicated beside each point. Each of these experiments, in turn, is the mean of several observations. The decline in respiratory control in hyperosmolar media is not due to uncoupling, *i.e.* increase in State-4 respiration but to inhibition of State-3 respiration. Respiratory control ratio was calculated from the rate of O_2 consumption in State 3 and the rate in the presence of substrate before addition of ADP. Experiments in which the mitochondria showed a markedly active ATPase or were partially uncoupled, *i.e.* the O_2 consumption after the ADP had been converted to ATP was markedly higher (>10%) than that before addition of ADP, or a high O_2 consumption was found even before addition of ADP, were discarded. At osmolarities beyond 865 mosM the respiratory control ratio approaches, or is equal to, one.

The reversibility of the inhibition of respiration under conditions of high osmolarity due to sucrose was shown as follows: mitochondria (kidney) were prepared in the usual homogenization medium. O_2 uptake, P:O ratio and respiratory control (according to CHANCE AND WILLIAMS[11]) were measured in the control reaction medium containing sucrose in a concentration of 240 mosM, with glutamate–malate as substrate. Following this, mitochondria were suspended in the basic reaction medium containing sucrose in a final concentration of 960 mosM. The above measurements were repeated in half of this suspension. In this preparation, the rate of State-3 respiration had fallen to 18.5% of the control value; the P:O ratio had fallen from

2.5 to zero and respiratory control (ratio in control medium was 7.0) was absent. As usual at this high sucrose concentration there was practically no increase in respiration after addition of either ADP or dicoumarol. The remaining mitochondria were centrifuged for 10 min at 10000 × *g*, the surface of the pellet was carefully washed with control reaction medium and the pellet was resuspended in the control reaction medium with sucrose 240 mosM, such that the total volume was identical to the volume before centrifugation. Respiration was restored in this preparation to 87 % of the control value, P:O ratio to 2.25 and respiratory control ratio to 6.0.

Disruption of the mitochondria by thrice-repeated freezing and thawing predictably showed complete uncoupling of oxidative phosphorylation, both ADP and 2,4-dinitrophenol being without effect on respiration. Moreover, O_2 uptake per mg of mitochondrial protein was faster than in intact mitochondria. Doubling the osmolarity of the medium with sucrose had no effect on O_2 uptake.

TABLE II

THE EFFECT OF HYPEROSMOLARITY ON KIDNEY MITOCHONDRIAL RESPIRATION

The test substance was sucrose. Other components were TMPD 0.1 mM, ascorbate 15 mM, ADP 0.2 mM, dicoumarol 0.002 mM. All respiratory rates are expressed as percentage of the respiratory rate in State 4 at 240 mosM sucrose. For further details of the reaction medium see legend to Fig. 1.

Osmolarity (mosM)	*Per cent respiration State 4*	*Per cent respiration State 3*	*Respiratory control ratio*	*P:O ratio*	*Per cent respiration with dicoumarol*
240	100	117	1.17	1.3	126
860	136	136	—	—	150
1000	92	92	—	-	131

Measurements of O_2 uptake with TMPD and ascorbate showed no inhibition of State-4 respiration in media with a high concentration of sucrose (Table II). There was an increase in respiration upon addition of ADP to the control reaction medium but this increase in respiration was absent in hyperosmolar media with sucrose as the osmolar reagent. This lack of increased respiration upon addition of ADP in State 4 in hyperosmolar media precludes measurements of P:O ratios (by the platinum-electrode technique) and "respiratory control" is absent. The respiratory control at 240 mosM was rather low in the particular experiment of Table II. The P:O ratio of 1.3 is in accord with the finding of HOWLAND[12] at a TMPD concentration of 0.1 mM. Dicoumarol slightly increased State-4 respiration in both normal and in hyperosmolar media. A few incomplete experiments with mannitol, NaCl and KCl as test substances gave essentially similar results.

Next, the level of NADH under various conditions of osmolarity and in the different metabolic states was examined fluorimetrically. The results are expressed as the ratio of the NADH fluorescence, read as photocurrent, observed under the specific experimental conditions, to the NADH fluorescence of the same mitochondrial suspension prior to any additions, *i.e.* in State 1.

The results showed that the initial fluorescence intensity in the starved mito-

chondrial suspension, State 1, became slightly lower as the osmolarity of the media was raised. Addition of glutamate–malate to mitochondria in the control reaction medium caused the expected rise in NADH level amounting to an average increase of fluorescence to nearly four times the baseline level, *i.e.* the level in State 1 (Fig. 6). Addition of either ADP or dicoumarol resulted in a decrease in NADH levels to slightly above the baseline level. When oligomycin was added in State 3 or the suspension reached anaerobiosis the NADH level became again similar to that in State 4.

In hyperosmolar media the increase in fluorescence intensity after addition of substrate was considerably less, both when calculated according to the baseline in the control reaction medium and to that in the relevant hyperosmolar medium. The difference fluorescence spectrum (Fig. 7) between substrate-treated mitochondria in hyperosmolar solutions and in control medium is characteristic of decreased NADH fluorescence in hyperosmolar media. This decreased NADH fluorescence is not affected by a doubling of the substrate concentration. Addition of ADP caused NADH levels to drop, but the decline in NADH fluorescence was markedly less in hyperosmolar

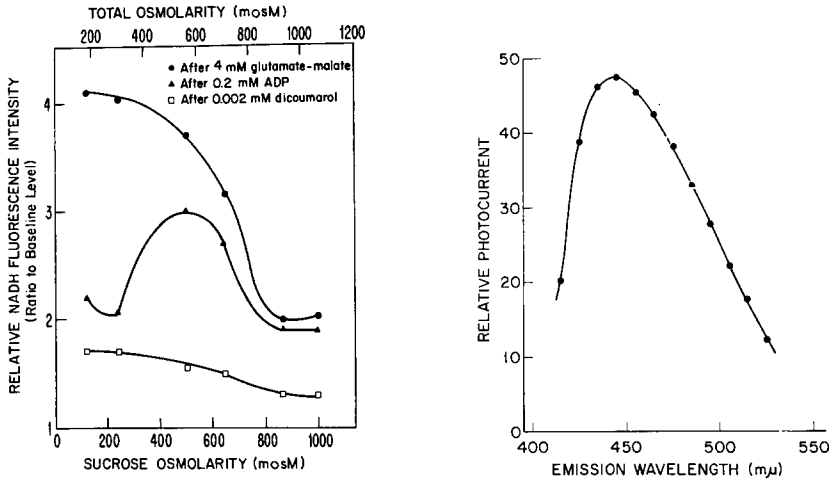

Fig. 6. The effect of hyperosmolarity on kidney mitochondrial NADH fluorescence with glutamate–malate as substrate. A fixed amount of mitochondria in a final concentration of approx. 0.5 mg mitochondrial protein per ml was suspended in the basic reaction medium and sucrose was added as the test substrate. Fluorescence was measured with the Aminco-Bowman spectrofluorimeter at 25° (for further details see METHODS). The fluorescence was measured as photocurrent and all calculations are based upon changes in photocurrent. The low fluorescence of the reaction medium without mitochondria was deducted from all subsequent readings. Reaction media with high sucrose concentrations had a higher blank reading than those with a normoosmolar sucrose concentration. In each experiment mitochondrial integrity was checked by simultaneous respiratory measurements. The fluorescence of the mitochondria in the reaction medium, State 1, is designated as the "baseline level" for that particular sucrose concentration. Changes in photocurrent after addition of substrate, ADP or dicoumarol are expressed as the ratio of the new reading to the "baseline level". The baseline level of fluorescence in hyperosmolar media was slightly lower than that in the control reaction medium.

Fig. 7. Mitochondrial NADH fluorescence in hyperosmolar and normoosmolar solutions. Oxygenated rat-kidney mitochondria were treated with substrate, 4 mM each of glutamate and malate, in one case in control medium of 240 mosM, in the other case in the same medium containing 1000 mosM added sucrose. The relative fluorescence spectrum was determined for the mitochondria in each medium with an excitation wavelength of 335 mμ. The "true" difference fluorescence spectrum calculated from these spectra is shown here and is characteristic of mitochondrial NADH fluorescence. Results were identical with β-hydroxybutyrate as substrate.

media than in the control reaction medium. Addition of dicoumarol caused a further drop in NADH fluorescence to slightly lower levels than that reached in the control reaction medium (Fig. 6). Similar results were obtained with succinate as substrate. After addition of amytal or of antimycin A, which resulted in high NADH levels, neither ADP nor dicoumarol influenced the NADH fluorescence at all levels of osmolarity.

TABLE III

THE EFFECT OF HYPEROSMOLARITY ON KIDNEY MITOCHONDRIAL NADH FLUORESCENCE WITH TMPD–ASCORBATE AS SUBSTRATE

For details see legend to Fig. 6.

Osmolarity	*Sucrose 240 mosM*		*Sucrose 880 mosM*	
	Photocurrent	*Per cent of State-1 level*	*Photocurrent*	*Per cent of State-1 level*
State 1	20	100	14	100
State 4 (TMPD-ascorbate)	86	430	71	507
State 3 (ADP)	33	165	66	471
Dicoumarol	31	155	27	193

In control reaction media TMPD–ascorbate caused a marked increase in fluorescence to more than 4 times the level in State 1 (Table III). ADP reduced the NADH level by more than 50% and dicoumarol caused a further slight reduction. Under conditions of hyperosmolarity the increase in fluorescence upon addition of TMPD–ascorbate was similar to that in the control reaction media but ADP decreased this fluorescence by a small amount only. The further addition of dicoumarol caused an abrupt decline in the NADH levels which then became similar to that in normoosmolar media. The fluorescence experiments were carried out with sucrose as the osmolar test substance. A few experiments with NaCl gave similar results.

DISCUSSION

Our results confirm again the observation that the uptake of oxygen by the kidney and liver mitochondria with succinate and NAD-linked substrates is depressed by hyperosmolarity due to sucrose and mannitol. It has previously been noted by various workers that the inhibitory effect of hyperosmolarity on mitochondrial respiration is not specific for sucrose and other polyhydroxylic compounds but that high concentrations of NaCl have similar effect[13]. In this investigation it was shown that high concentrations of KCl also depress mitochondrial respiration. Since both NaCl and KCl in high concentration depress respiration to the same extent as do equiosmolar sucrose and mannitol concentrations, the inference seems warranted that it is the hyperosmolarity as such which exerts this effect when succinate and NADH-linked substrates are used, and not some specific effect of polyols. Among the many studies of the effect of high concentrations of sucrose and other polyols on oxidative phosphorylation, COOPER AND LEHNINGER[14,15] have demonstrated that the final steps

in energy transfer are inhibited under conditions of hyperosmolarity. Whether this applies also to high concentrations of other substances has not been known; however, our limited studies of TMPD–ascorbate oxidation in the presence of mannitol, NaCl or KCl as osmolarity-raising substances (see below) suggest that it is so for these agents as well as for the polyols. The block in energy transfer, however, does not appear to be the major cause of the respiratory inhibition (if current views of the mechanism of oxidative phosphorylation are correct) since uncoupling agents do not release this inhibition, neither when sucrose is the osmolarity-raising substance[1] nor with mannitol, NaCl or KCl. Moreover, if the respiratory block were due to inhibition of oxidative phosphorylation the level of NADH in State 3 would be expected to be high, similar to the findings in mitochondria in normoosmolar media with substrate only (State 4) or in mitochondria treated with substrate, ADP and oligomycin. Under conditions of hyperosmolarity, however, the NADH level was found to be low in all states of mitochondrial metabolism. It is most unlikely that the low fluorescence found under these conditions is due to leakage of the pyridine nucleotides from the mitochondria. This phenomenon is known to occur during swelling of mitochondria[16] whereas hyperosmolar media cause shrinkage of mitochondria[17–19]. Thus the respiratory inhibition cannot be due mainly to the block in the phosphorylation process.

The observation that the NADH fluorescence of uncoupled mitochondria in hyperosmolar media is slightly lower than that of mitochondria in the control reaction medium is remarkable in view of the fact that the rate of oxygen consumption by these mitochondria is very much reduced in the hyperosmolar media. The level of NADH at any time is a dynamic equilibrium between the number of reducing units converting NAD^+ to NADH and the activity of the enzymes oxidizing NADH to NAD^+ per unit of time. Since the absolute number of reducing units flowing from NADH into the electron-transfer chain must be very small in hyperosmolar media in view of the reduced oxygen consumption, this finding of low NADH levels in uncoupled mitochondria in hyperosmolar media would imply that the absolute number of reducing units reaching NAD must be small as well. This finding, then, would imply a block in electron flow at a site prior to NAD.

Studies with TMPD–ascorbate showed that when electrons are donated directly to cytochrome *c* hyperosmolarity does not inhibit respiration in State 4. The finding that under conditions of hyperosmolarity the uncoupling of oxidative phosphorylation increases the respiratory rate of TMPD–ascorbate-treated mitochondria, whereas State-4 respiration is not increased by the addition of ADP indicates that under these condtions with TMPD as electron donor the block in the phosphorylation process may indeed be the respiratory rate-limiting factor in State 3.

This interpretation is in accordance with the results of our measurements of mitochondrial NADH levels in normo- and in hyperosmolar media with TMPD–ascorbate as substrate. It has been shown that reversal of electron flow and NAD^+ reduction in State 4 are not inhibited by hyperosmolarity. In normoosmolar media addition of ADP causes a marked reduction in NADH levels followed by a further small reduction upon addition of dicoumarol. In hyperosmolar media the lowering of NADH fluorescence after ADP addition is very slight only, showing again the effects of the phosphorylation block. The subsequent addition of dicoumarol lowered the NADH fluorescence to the levels observed under normoosmolar conditions, showing that uncoupling does take place and is unaffected by hyperosmolarity.

It is remarkable that reversal of electron flow in State 4 with TMPD–ascorbate as substrate did take place since reversal involves the reactions of oxidative phosphorylation, which is inhibited under these conditions of hyperosmolarity.

Respiration with succinate as substrate is inhibited by hyperosmolarity similarly to that with NAD-linked substrates. Measurements of NADH fluorescence with succinate show a low NADH level in hyperosmolar media, both in State 3 and in State 4, in spite of the fact that reversal of electron flow is not inhibited as shown above.

These findings place the main cause of the inhibition of respiration at some point before NAD with NAD-linked substrates and before cytochrome *b* with succinate as substrate. In order to ascertain whether the dehydrogenases are inhibited or whether the entry of substrates is inhibited by hyperosmolarity, mitochondrial integrity was destroyed by repeated freezing and thawing. This causes complete uncoupling of oxidative phosphorylation and a markedly higher respiratory rate than that of normal mitochondria uncoupled by 2,4-dinitrophenol or dicoumarol. Since the respiratory rate of these fragmented mitochondria was not influenced by the osmolarity of the medium the most likely explanation of the inhibited respiration of coupled mitochondria in hyperosmolar media is that there is an impediment to the access of substrates to the substrate dehydrogenase system. This may result from impaired entry of substrate into mitochondria, as suggested also by the work of KLEIN AND NEFF[20]. The increase in mitochondrial respiration under conditions of hypoosmolarity can accordingly be explained as being caused by facilitated or increased entry into or access of substrates to the mitochondria and the mitochondrial substrate dehydrogenases. These functional changes may be reflections of the finding that media of different osmolarities have a marked effect on the mitochondrial double-membrane configuration, as shown by the experiments of TEDESCHI[17,18], MALAMED[21] and others[20]. Major structural alterations, however, seem excluded by the reversibility of the hyperosmolar effects. Moreover the inhibition of respiration under conditions of hyperosmolarity must perforce lead to decreased formation of high-energy compounds serving as energy sources for physiological function.

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