

RESPIRATION AND MITOCHONDRIAL CONTENT IN SINGLE NEURONS OF THE SUPRAOPTIC NUCLEUS

A Correlative Study in Osmotic Stress

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ABSTRACT

The study was undertaken to investigate the possible correlation of total volume of mitochondria per cell with the rate of succinate oxidation in isolated nerve cell bodies, after various functional stresses in the experimental animals. Significant cytological effects were found in the nerve cells of the supraoptic nucleus in rats which had been thirsting for 4–12 days or had been given 2% sodium chloride solution as a substitute for drinking water for a few weeks. Quantitation of mitochondria was done from electron micrographs. The cell volumes were calculated from sections of Epon-embedded tissue under phase-contrast microscopy. Succinate oxidation was measured on groups of 10 nerve cells with the microdiver technique. As a result of either thirst or sodium chloride load, the volume of mitochondria per nerve cell more than doubled. The rate of succinate oxidation was not changed after the rats had been thirsting but was enhanced by over 100% after they had drunk sodium chloride. A linear relationship was found for the amount of mitochondria versus respiration in the supraoptic neurons for all experimental groups except the thirsting animals. The mitochondria in the supraoptic neurons from thirsting animals were of the same size or smaller than those in controls, whereas in animals given sodium chloride solution the mitochondria were considerably enlarged. The observed effects were specific for the supraoptic nucleus.

INTRODUCTION

In the study of cell function in the nervous system, morphological criteria such as the volumes of the cell, nucleus, and nucleolus are applied. The amounts and/or activities of cytoplasmic organelles can be estimated by histochemical techniques, although changes in concentration of organelles may give misleading impressions for the evaluation of the total amount per cell (Hamberger and Sjöstrand, 1966). The application of a quantitative micromethod to determine the ribo-

nucleoprotein content in isolated cells (Edström and Eichner, 1958; Hydén, 1961) has demonstrated that an elevation in the amount of total RNA follows increased function in mammalian neurons.

In view of the reactive ability of the mitochondrial population in organs more homogeneous than brain (Miller, 1953; Gabler, 1961; Laguens et al., 1966; Gustafsson et al., 1965; Hudson et al., 1961; Braun et al., 1963; Roodyn

et al., 1965) and the previous studies on oxidative ability of single cells in the nervous system (Hydén and Pigon, 1960; Hamberger and Sjöstrand, 1966; Watson, 1966; Epstein and O'Connor, 1966), we have attempted to quantitate the cellular mitochondrial response in neurons. Two different techniques have been applied: light and electron microscopy for quantitation of total mitochondrial volume of the cell, and manometric microanalysis of the rate of cell respiration.

Cells from the supraoptic nucleus (SON) of the rat brain were chosen as material for a number of reasons. The nucleus is well-delineated, and the neuron to glia ratio is very high. The neurons have relatively few dendrites and are easily dissected free. Study of single cells had to be considered since the neuron to glia ratio varies as a result of functional changes, and the mitochondria of the glial cells give the impression of functional alterations. Also, the glial cells have a higher respiratory enzyme activity than have the nerve cells as demonstrated by histochemical technique (Iijima et al., 1967). Finally, the SON is well investigated in many respects and offers the possibility of being stimulated specifically as a result of different functional stresses on the experimental animal.

High plasma osmolarity achieved by salt load or water deprivation increases the antidiuretic hormone (ADH) activity (vasopressin level) of the blood (see Sawyer, 1963; Sawyer and Mills, 1966) and of the urine (for references see Rodeck, 1964). Analysis of the octapeptide hormone content in the hypothalamus (see Lederis, 1962) and investigations using radioautographic techniques (Wells, 1961; Léonardelli, 1964) indicate that the SON neurons are the chief manufacturers of vasopressin.

There is a depletion of stored octapeptide hormones, mostly vasopressin, from the posterior pituitary lobe after dehydration or salt load has occurred (see Lederis, 1962; Sloper and King, 1963). The ADH activity of the SON was decreased in dogs thirsting for 8–14 days (Hild and Zetler, 1953). Salt load for 3 wk produced a considerable decrease in ADH activity of the hypothalamus in rats (Stutinsky, 1957).

Experimentally induced osmoregulatory activity produces a variety of morphological changes in the SON neurons. The volumes of cells, nuclei, and nucleoli increase, the granules stainable with chrome alum hematoxylin phloxine and

paraldehyde fuchsin are initially depleted, the Nissl substance is redistributed, and vacuolization of the cells and degenerative changes may occur after prolonged stress (Hillarp, 1949; Ortmann, 1950, 1951; Laqueur, 1952; Leveque, 1953; Eichner, 1953; Kovács et al., 1954; Bachrach, 1957; De Groot, 1957; Stutinsky, 1957; Edström and Eichner, 1958, 1960; Campanacci, 1960; Raiha, 1960; Sloper and King, 1963). An antagonism between the renal effects of adrenocortical and neurohypophyseal hormones has been suggested (Silvette and Britton, 1938; for references see Kennedy, 1960). Sawyer and Roth (1953) found an increase of stored hormones in the neurohypophysis after adrenalectomy and proposed that this could represent a decrease in the rate of inactivation of the neurohypophyseal hormones rather than a hypersecretion. By means of histochemical techniques the SON nerve cell bodies have been shown to give a strong reaction for glucose-6-phosphate dehydrogenase, 6-phosphogluconate dehydrogenase, lactate dehydrogenase (cf. Friede, 1966), acid phosphatase (Eränkö, 1951; Rinne and Kivalo, 1958) and esterases (Pepler and Pearse, 1957; Kivalo et al., 1958), but low activities for the citric acid cycle enzymes, especially succinic dehydrogenase (Eichner, 1958; Kivalo et al., 1959; Arvy, 1962; further ref. in Rinne, 1966) and cytochrome oxidase (Arvy, 1962). Somewhat conflicting results exist concerning the effect on respiratory enzyme activities during osmotic stress (Kivalo et al., 1959; Ifft et al., 1964). The ribonucleic acid content of the SON cells is strikingly increased as a result of thirst or salt load (Edström and Eichner, 1958).

Few electron microscopic studies have been published on the SON in osmotically stressed adult rats (Nemetschek-Gansler, 1965; Zambrano and De Robertis, 1966). There is evidence for depletion of elementary granules and increased amounts of granular endoplasmic reticulum and free ribosomes.

MATERIALS AND METHODS

Experimental Material

In the main series, 53 rats of the Sprague-Dawley strain were used. The animals had standard food ad libitum, were regularly weighed, and urine volumes were measured. The humidity and temperature of the air were kept constant. Osmotic stress was produced by deprivation of drinking water for various periods or by supplying the animals with drinking

water containing NaCl of different concentrations (Table I). Bilateral adrenalectomy was performed in four animals, and the results were checked at autopsy. All animals were sacrificed by decapitation without anesthesia at about 10 a.m. The brains were removed within 2 min. A frontal section at the level of the optic chiasma was laid with a razor blade through the center of the SON. The rostral part was taken for microscopy and the caudal part for cell dissection and respiration analyses.

Light and Electron Microscopy

The rostral part of the brain section, about 1 mm thick, was immediately prefixed for 1 hr in ice-cold 6.25% glutaraldehyde in 0.2 M NaCl buffered with 0.067 M cacodylate, pH 7.4. Under the binocular microscope, 1 mm² pieces were dissected out of the

Quantitative Analysis of Mitochondria on Electron Micrographs

27 animals were analyzed according to Chalkley's method (1943) as adapted by Bellairs (1959) for electron micrographs. A piece of cellophane with a grid of definite dimensions formed by perpendicular lines was projected over the negative. The points of intersection of the grid lines were used for the sampling. The grid had 234 points. The magnifications used on the negatives were 7,000 or 9,100 and on the projection screen were 140,000 or 180,000. The number of sampling points falling on mitochondria and cytoplasm were counted. Care was taken to analyze equally the central and peripheral areas of the cells. The number of points falling on mitochondria was then expressed as the percentage of the total

TABLE I
Percentage of Points Falling on Mitochondria, According to Adaptation of Chalkley's Method

Animals	Mean	SE	No. animals	Total No. points
	%			
Controls	9.3	0.63	6	12,834
Thirst				
4 days	11.0	1.47	2	2373
7 days	10.4	0.65	4	7405
12 days	7.6	0.16	2	5949
Adrenalectomy + thirst 4-5 days	11.4	0.19	3	10,599
2% NaCl 17-25 days	11.4	0.42	3	9204
2% NaCl 12 days + 1.5% NaCl 27-33 days	11.1	0.84	4	6361
2% NaCl 15 days + H ₂ O 3-14 days	10.1	0.34	3	7445
Total			27	62,170

SON. After fixation for 2-2½ hr in ice-cold 1% osmium tetroxide in Michaelis buffer, pH 7.4, the tissue was washed in physiologic saline and dehydrated in graded acetone in the cold. The pieces were cleared in propyleneoxide, immersed in equal parts of propyleneoxide and Epon 812, and left in Epon 812 overnight. The final embedding and polymerization were performed according to Luft (1961). 1.5-2.5- μ sections were prepared for phase-contrast microscopy, and thin sections were prepared for electron microscopy. Some of the thick sections were stained with toluidine blue, paraldehyde fuchsin, and alcian blue for light microscopy.

The thin sections were mounted on copper grids without supporting film and examined in a Philips EM 200 electron microscope operated at 40 kv with double condenser and anticontamination stage. Agfa-Gevaert Scientia 23D50 plates were used for photography.

number of points, excluding nuclei, cell membranes, and extraneuronal objects. The percentage for mitochondria was calculated for each animal, and the mean for each group of animals was given. The total number of points thus analyzed was 62,170 from 384 electron micrographs. The percentage of sampling points will be representative for the percentage of whole cytoplasm occupied by mitochondria.

Estimation of volumes of the SON nerve cells was achieved by measuring the largest diameter (D) and the diameter (d) perpendicular to this one at the level of the nucleus and nucleolus. Epon sections photographed in phase contrast at 400 times primary magnification were used. The measurements were then performed on prints enlarged three times.

This technique was also applied for estimations of the volumes of nuclei. The area was calculated according to the formula $\pi Dd/4$, the volume of the

prolate ellipsoid = $\pi Dd^2/6$, and for spherical nuclei, $\pi D^3/6$.

Calculation of volume of mitochondria per cell was worked out from the volume of cytoplasm (cell volume minus nuclear volume) and mitochondrial concentration: (volume of cytoplasm \times percentage of mitochondria) / 100.

Statistics

Means and standard errors were calculated according to common methods (see Snedecor, 1946). The homogeneity in the control groups was examined by analysis of variance. All tests were carried out on the 5% significance level.

The standard error of M , i. e. the mean volume of mitochondria per cell, was estimated as follows. If C denotes the volume of cytoplasm and M_p the per-

centage of mitochondria per cell, the standard error of M is calculated from

$$M \pm SEM = (C \pm SEC) (M_p \pm SEM_p)$$

where SE are standard errors. This gives:

$$SEM \simeq C \times SEM_p + M_p \times SEC$$

which is the standard error of the mean volume of mitochondria per cell.

Determination of Succinate Oxidation in Cells Isolated from the SON

The SON was identified, and a small piece was cut out and put into a cooled glass cup containing a drop of 0.25 M sucrose. By gentle manipulation of the

RESULTS

Animals

Thirst induced a rapid loss of weight in the rats, from a 25% weight loss after 4 days to

TABLE II
Oxidation of Succinate in Isolated Nerve Cells from SON
Oxygen consumption expressed as $\mu\text{l O}_2 \times 10^{-4}$ per hour. Analyses made on groups of 10 cells.

Animals	Mean	SE	No. animals	P*
Controls	0.31	0.05	10	—
Thirst				
4 days	0.32	(0.31; 0.32)	2	—
7 days	0.29	0.04	10	—
12 days	0.11	(0.17; 0.05)	2	—
Adrenalectomy + thirst 4-5 days	0.53	0.1	3	<0.001
2% NaCl 17-25 days	0.72	0.14	4	0.005
2% NaCl 12 days + 1.5% NaCl 27-33 days	0.55	0.12	5	0.005
2% NaCl 15 days + H ₂ O 3-14 days	0.59	0.05	4	0.01

* P = probability of a significant difference from controls.

almost 50% loss after 12 days. Food intake was decreased to one-third. After 12 days the mortality was about 50%. The mortality was high in adrenalectomized animals as early as after 5 days of thirst. With 2% NaCl solution substituted for drinking water, the weight reduction proceeded relatively slowly, maximally about 30%. The longest time period endured by this strain of rats was about 3 wk. However, food intake, weight, and well-being increased when the NaCl concentration was lowered to 1.5%. Rehydration invariably brought about a rapid increase in body weight. The urine volumes fell strikingly to a few milliliters per day during thirsting. The adrenalectomized animals had a less dramatic decrease in urine volumes which were at a higher

level during 4 days of thirst. 2% NaCl in the drinking water caused an increase in the urine volumes of up to 500% after about 1 wk. The urine volumes were lowered promptly by a decrease in NaCl concentration to 1.5%, but they were still much higher than those for the controls.

Phase-Contrast Microscopy

THIRST: Whereas the cell volumes (Table III) increased markedly up to 7 days of thirst, they were somewhat lower at 12 days than at 7 days. After adrenalectomy the increase in cell volume was two-thirds of that of unoperated animals thirsting equally long. The expanded

Ultrastructure of Mitochondria

The mitochondria in the SON of control animals were fairly homogeneous in size and form, about 1 μ long and 0.2 μ wide. The cristae and matrix appeared relatively dense (Fig. 1). No matrix granules were seen. Most cristae were perpendicular to the long axis of the mitochondrion. In thirsting animals the mitochondria appeared somewhat smaller, and the structure of the mitochondrial membrane systems was rather loose and disorganized (Fig. 2); the density of the matrix decreased. In animals adrenalectomized prior to the thirsting period, however, the mitochondria were considerably larger (Fig. 3). In

TABLE III
Volumes of Cytoplasm and Total Mitochondria in SON Neurons from Control and Experimental Groups

Animals	Volume of cytoplasm	Volume of mitochondria per cell	Changes in volume of mitochondria from controls		Specific activity of mitochondria
			%	μ l O ₂ × 10 ⁻⁷ /hr/ μ ³	
Controls	2517.5 (±198.4)	234.1 (±34.3)			1.3
Thirst					
4 days	4840.7 (±347.7)	532.5 (±109.4)	+127.5		0.6
7 days	6859.4 (±432.3)	713.4 (±89.5)	+204.7		0.4
12 days	5905.7 (±466.5)	448.8 (±44.9)	+89.6		0.2
Adrenalectomy + thirst 4-5 days	3522.4 (±365.8)	401.6 (±48.4)	+71.6		1.3
2% NaCl 17-25 days	5840.1 (±285.4)	665.8 (±57.1)	+184.4		1.1
2% NaCl 12 days + 1.5% 27-33 days	3425.0 (±251.4)	380.2 (±56.7)	+62.4		1.4
2% NaCl 15 days + H ₂ O 3-14 days	3623.7 (±289.6)	366.0 (±41.6)	+56.3		1.6

* Mean ± SE.

cell volume led to a reduction of the spacing between the neurons which often lie close together. The distance between capillaries and nerve cells was markedly less than that in controls. Glial cells were compressed.

SALT LOAD: After the animals had been given 2% NaCl solution for 17-25 days, the SON neurons increased in volume to a value midway between the values for 4 and 7 days of thirst. A lowering of the NaCl concentration to 1.5% or the introduction of fresh water reduced the increase in cell volume. The glial compression and the spaces between neurons and capillaries appeared as after thirst.

Calculated cell volumes in the *Nuclei arcuata* from thirsted animals did not differ appreciably from those in the *Nn. arcuata* from controls.

the SON from the 2% NaCl group the size of the mitochondria was more heterogeneous; many very large mitochondria were seen. After 2-1.5% NaCl solution had been substituted for drinking water, large mitochondria were frequent (Fig. 4). They were often very long and exhibited a regular crista structure. During early rehydration the mitochondria were still large, but after 10-14 days they looked similar to control mitochondria.

Quantitation of Mitochondria from Electron Micrographs

The results (Table I) show that during the first week of thirst there was a 15% average increase in the ratio of mitochondria to total cytoplasm in the SON nerve cell bodies. Adrenal-

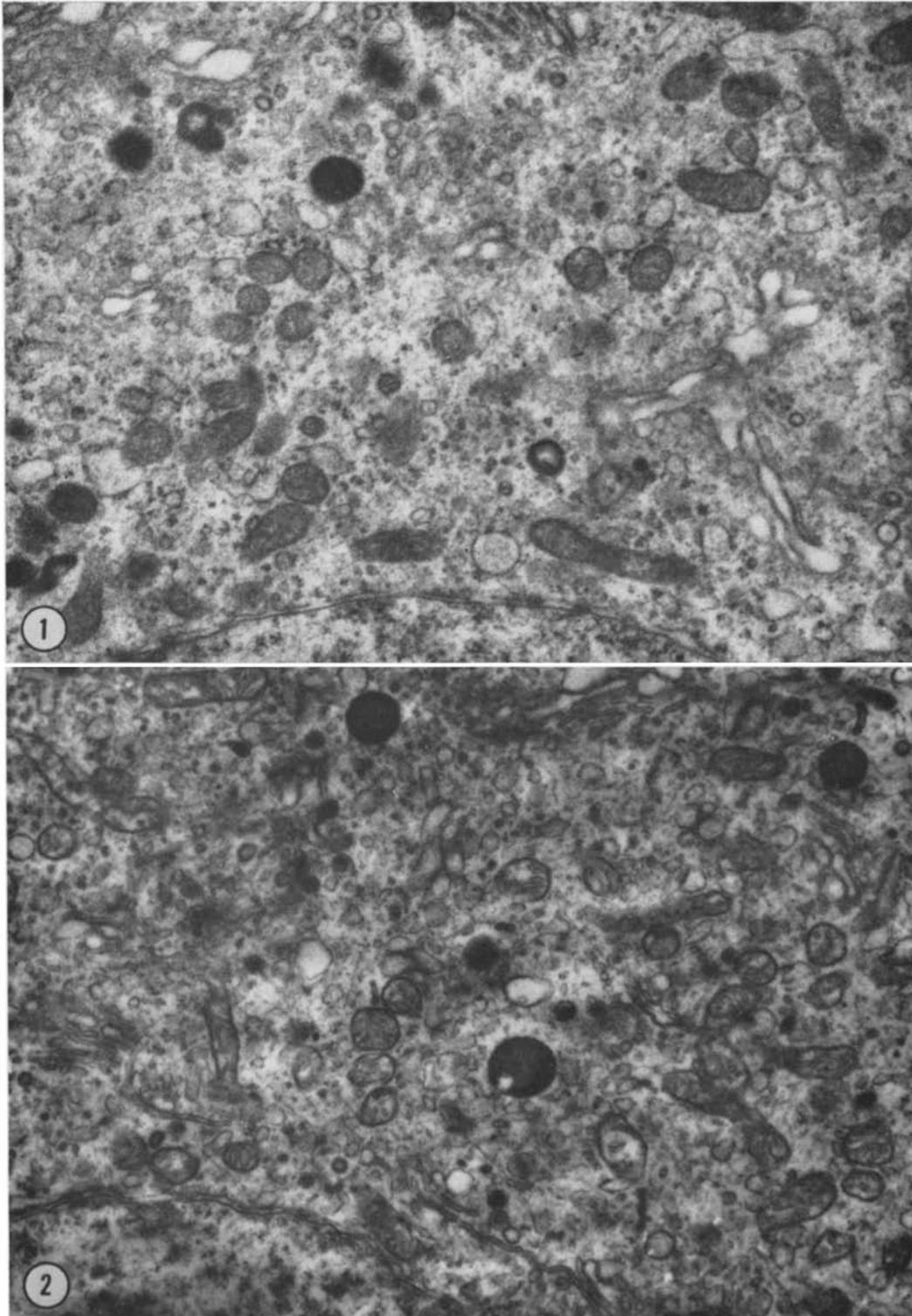


FIGURE 1 SON nerve cell from control rat. The mitochondria appear relatively dense. Unstained. $\times 23,500$.

FIGURE 2 SON nerve cell from rat thirsting for 7 days. The mitochondria are similar in size to those of controls but appear less dense and have a more irregular crista structure. Unstained. $\times 23,500$.

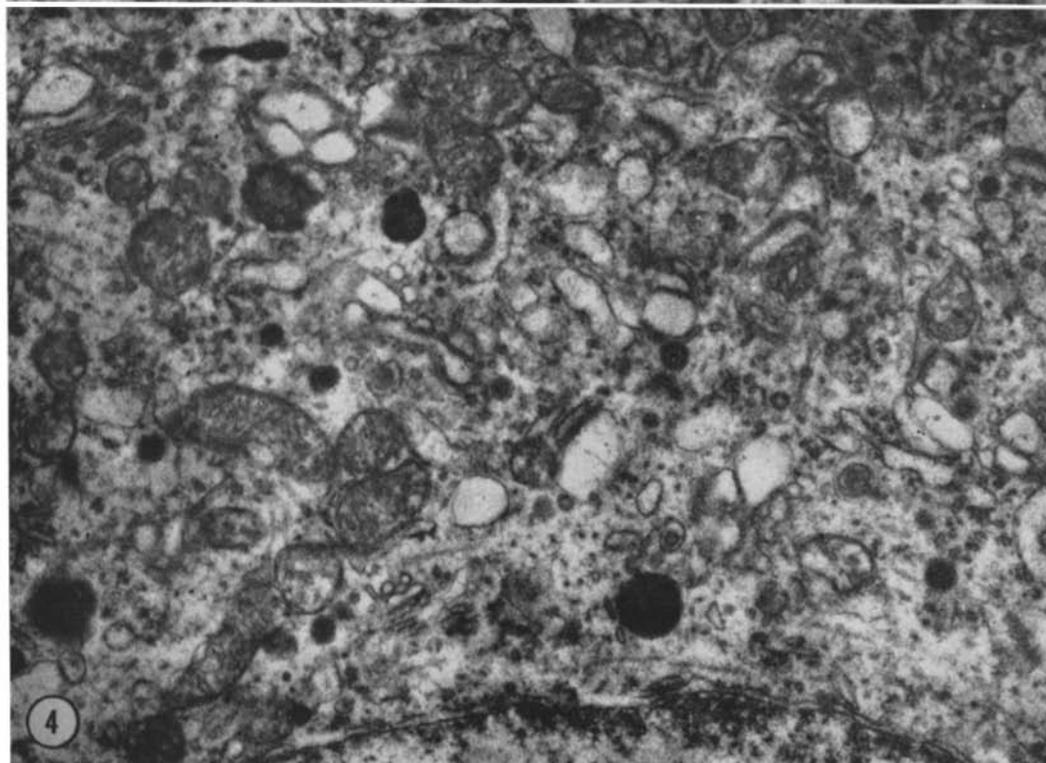
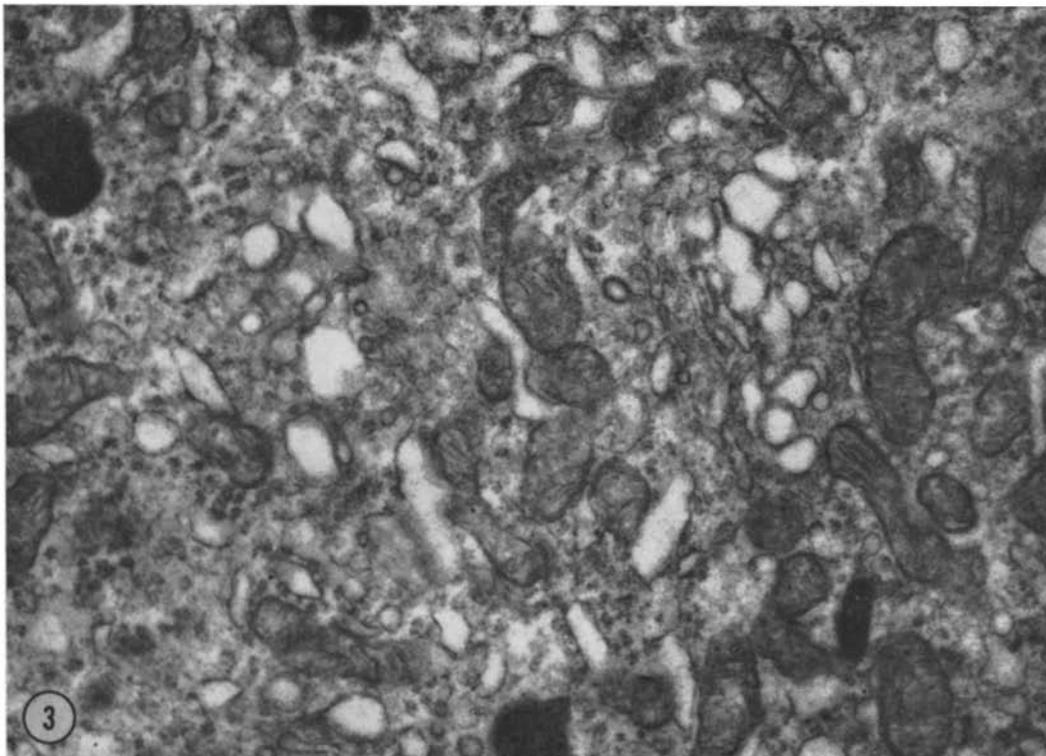


FIGURE 3 SON nerve cell from adrenalectomized rat thirsting for 4 days. The mitochondria are considerably enlarged but still fairly dense. Unstained. $\times 23,500$.

FIGURE 4 SON nerve cell from salt-loaded rat. Relatively large mitochondria. Unstained. $\times 23,500$.

ectomy prior to thirst did not change the percentage. This increase changed to a definite decrease in the more prolonged thirst period. Also, in the different groups on NaCl solutions there were increases in the range of 10–15% in the volume occupied by mitochondria. The total volume of mitochondria per nerve cell was calculated from the percentage of cytoplasmic volume occupied by mitochondria and the total cytoplasmic volume. Table III shows that there is a considerable increase in volume of mitochondria per cell in all the experimental groups, particularly after 7 days of thirst or drinking of 2% NaCl solution. It is of interest to note that adrenalectomy seems to interfere with this aspect of cell response. All the results on mitochondrial volume per cell are quite proportional to those on cell volumes, since the variations in mitochondrial concentration are relatively small.

Oxidation of Succinate by Nerve Cell

Bodies from SON

Thirsting for 4 or 7 days had no appreciable effect on the succinate oxidation of the nerve cells, but after 12 days a depressed respiration was noted (Table II). In contrast, thirst in adrenalectomized animals stimulated the oxidative activity. The three groups receiving NaCl solutions at various concentrations and periods were all stimulated in terms of succinate oxidation, and rehydration did not cause restitution towards control values until after 2 wk. In the group drinking 2% NaCl for 17–25 days, the oxidative activity increased linearly with time. The correlation of the rate of succinate oxidation and the total mitochondrial volume per cell is shown in Fig. 5. There is a linear relation for the controls, salt-loaded, and adrenalectomized animals. The linearity was proved for the regression line calculated from the results for 15 animals within these five groups for which both respiration and mitochondrial quantitation had been performed. The SON neurons from simply thirsting animals, however, differed extremely from those of the other groups, since their respiration did not match their volume of mitochondria.

In order to ascertain that the observed effects on cell respiration were specific for neurons from the SON, material was also taken from other parts of the brain (N. Deiters). No measurable changes were observed after thirsting or salt loading.

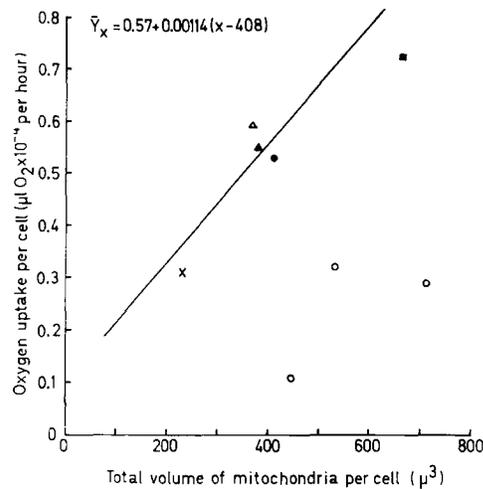


FIGURE 5 Regression of oxygen uptake on total volume of mitochondria per cell. Controls, X; 2–1.5% NaCl, ▲; 2–0% NaCl, △; adrenalectomy and thirst, ●; 2% NaCl, ○; thirst (4–7–12 days), ■. The regression line was calculated from results of 15 animals in the first five groups where both oxygen uptake and volume of mitochondria were measured. The linearity was proved.

DISCUSSION

The purpose of the present investigation was to test the correlation of mitochondrial function in isolated nerve cells with the total volume of mitochondria per cell.

The techniques for quantitative electron microscopy of mitochondria may be liable to some methodologic errors since the plasticity of the mitochondria can cause variations in the intracellular distribution of these organelles. We have tried to minimize the errors by using a preparative technique which gives a very low shrinkage and by sampling equally from peripheral and central parts of the cells from a large material. Earlier studies on the quantitation of mitochondria in the nervous system demonstrated the possibility of obtaining results in which the variations are within a desirable range (Hartmann, 1948, 1954; Hudson et al., 1961; Nafstad and Blackstad, 1966).

The analysis of respiration of single cells, when the rate of oxidation of succinate is used as an indicator of mitochondrial protein, involves methodologic difficulties, for some of which it may be hard to test in a microsystem. We have used a hypotonic incubation medium in order to

minimize permeability factors and to increase enzyme accessibility. When succinate concentration versus rate of oxidation was plotted, no significant difference was found between a single cell system and a mitochondrial preparation (Hamberger, 1963). Analyses on single cells did not reveal any measurable increase in the rate of oxygen uptake when an adenosine diphosphate regenerating system was included in the medium.

The percentage of SON neuronal cytoplasm which was occupied by mitochondria was 9.3 for the control material. In none of the experimental groups did the comparable value deviate more than 20% from this value. In all groups except one, however, this value showed a slight increase, but it may be pretentious to press those results too far. The changes we see do not approach such striking changes as those reported for nerve cell bodies from the hypoglossal nucleus where the mitochondrial concentration increased by 70% 10 days after nerve transection (Hudson et al., 1961). The present value of 9.3% agrees well with the value of 9% found by Hudson et al. (1961), for the hypoglossal nucleus. This value is in accordance with earlier reports on a "normal" mitochondrial amount in the SON neurons (Romieu et al., 1953) and with our own data on succinate oxidation relative to other types of nerve cells; but it is challenged by the low activity of oxidative enzymes demonstrated with histochemical techniques (Eichner, 1958; Arvy, 1962). The differences in mitochondrial concentrations were of minor importance for the calculation of the total mitochondrial volume per cell, since the cell volumes in all the experimental groups increased from 40 to 70% in agreement with earlier reports (see Introduction). Thus the volume of mitochondria per SON nerve cell body was more than doubled in animals thirsting for 4 or 7 days or given 2% sodium chloride solution for 3 wk; the mitochondrial volume per cell was augmented by at least 50% in the other groups.

The SON nerve cells from salt-loaded animals oxidize succinate at a rate about 100% above the controls. Thirst, on the other hand, induces no increase in succinate oxidation whatsoever, except in adrenalectomized animals. The correlation between the total mitochondrial volume per cell and the rate of succinate oxidation (Fig. 5) is good for all the experimental groups except the simply thirsting animals. Accordingly, the values for the "specific activities" (oxidation rate per

volume, Table III) of the mitochondria are within a very close range for controls, salt-loaded, and adrenalectomized groups. In contrast, for thirsting animals the specific activities are only 30% of this value. To accept these specific activities for mitochondria according to the conventional definition (i.e. per milligram), one has to assume that the estimated total mitochondrial volume corresponds to the total mitochondrial protein of the cell. This is reasonable since few mitochondria in the total samples appeared swollen. In particular, since the mitochondria in the thirsting groups were conspicuously small, it seems improbable that an overestimation of the real mass of mitochondria per cell due to swelling should account for the low specific activities in these groups. The results thus suggest that there may be a deficiency in or an inhibition of certain important enzymes in the mitochondria of the SON neurons during prolonged thirst. These mitochondria had a low density relative to mitochondria in the other groups. The mitochondrial composition, function, and rate of synthesis are known to be largely variable in mammals, as well as in lower organisms, and to be dependent upon the availability of essential nutrients, etc. (Luck, 1963; Lee and Miller, 1966).

The qualitative difference in the mitochondria in the SON nerve cells between controls and salt-loaded animals on one hand and thirsting animals on the other hand may be due to the much more devastating effect of prolonged thirst on the whole animal (body weight, food intake, urine volumes, behaviour, etc.). There is also a difference in time required to reach sufficient stimulation; thirst requires 7 days, whereas salt load takes three or four times as long. The production of new mitochondria in the SON will have to be increased considerably more in the thirsting animals than in the others. It is of interest to note that the SON mitochondria in adrenalectomized and thirsting animals are similar to the SON mitochondria in salt-loaded rats.

Ifft et al., (1964), in agreement with us, could not detect any increase in succinic dehydrogenase per DNA in SON of animals after thirsting. We consider it important to stress that the SON nerve cells in all the groups which showed an increased respiration had a correspondingly higher mitochondrial volume, which indicates that these mitochondria, with respect to respiration, do not differ qualitatively from control

mitochondria. It is improbable that enzyme activation, changed substrate permeability, or enzyme availability would play an important role. Thus the results indicate that nerve cells have the ability to adjust the mitochondrial pool according to the requirements of the physiological activity.

Adaptive changes of microsomal and mitochondrial enzymes have been reported to occur in various organs following the administration of active drugs. The sensitivity of such changes to agents which inhibit protein synthesis indicate an enhancement of organelle protein per cell (Zenker et al., 1968). The present results demonstrate

that different types of osmotic stress induce very similar adaptive changes vis-à-vis cytoplasmic and mitochondrial volumes. In spite of these similar changes, there are differences in the rate of succinate oxidation between thirsting and salt-loaded animals. These circumstances stress the necessity of a multifactorial analysis even when the characterization of adaptive changes is limited to the mitochondria.

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