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Differential Metabolism of Various Brain Regions

BIOCHEMICAL HETEROGENEITY OF MITOCHONDRIA

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Brain is an organ characterized by a high oxidative metabolism, the oxygen consumption on the basis of DNA content being the highest of any organ in the rat (Mandel, Bieth & Weill, 1957). Several reports have appeared on the distribution of the respiratory enzymes in the brain which broadly run parallel to the q_0 values of these areas. According to Meyer (1958), the cortical vulnerability to hypoxia is related to the concentration of cytochrome oxidase. Hess & Pope (1960), employing quantitative histochemical techniques, have shown that the highest activity of cytochrome oxidase is in layers II, III and IV of human cerebral cortex. The enzyme thus would be principally located in the cell bodies, in dendrites of the neurons and in axon terminals. As in other tissues this enzyme is almost exclusively mitochondrial (Brody, Wang & Bain, 1952; Friede & Pax, 1961). The activity of cytochrome oxidase varies in different areas of the brain; thus that in corpus callosum, populated with only non-neuronal glial cells, is significantly lower than the activity in cortical areas (Tolani & Talwar, 1961). These results are in line with the electron-micrographic observations indicating higher densities of mitochondria in most neurons and their larger processes than in oligodendroglia or astrocytes (Windle, 1958). Allen (1957) also observed low cytochrome-oxidase activity in well differentiated human gliomas. However, Hyden & Pigon (1960),

employing microchemical techniques and working on isolated neuronal and oligodendroglial cells, found that the cytochrome-oxidase activity in oligodendroglia was twice as high as in an equal volume of nerve cells. The methods of assay employed by various workers have differed in some respects, which can introduce extraneous factors in the measurement of enzyme activity. For example, the addition of exogenous cytochrome *c* to the medium can raise the cytochrome-oxidase activity of rat brain, an observation that led Elliott & Greig (1938) to postulate that the concentration of cytochrome *c* is the rate-limiting factor in rat brain. The human-cortex enzyme, on the other hand, does not show any such limitation (Hess & Pope, 1960). By taking into consideration these factors and employing an optimally constituted system, it was observed that the cytochrome-oxidase activity/mg. of mitochondrial protein is not the same in different brain regions. This could imply (a) a heterogeneity of the physical composition of the mitochondrial pellet obtained from different regions under the standard experimental conditions, (b) that the amount of enzyme in analogous particles is different, indicating a constitutional heterogeneity of the mitochondria, or (c) that the nature of the enzyme protein could be qualitatively different.

The present paper describes a few experiments to inquire into these points.

MATERIALS AND METHODS

Animals. Rhesus monkeys (*Macacus multatus*) were used as experimental animals. They usually weighed 1.5–2 kg. and were fed *ad libitum*.

Removal of tissues. The brain was exposed under hypothermia. The monkey was kept under a blanket of ice till the rectal temperature dropped to 22°. Under these conditions the animal remains conscious but feels no pain. The use of anaesthesia was avoided, as narcotics are known to inhibit selectively the activity of certain regions of brain (Quastel, 1955). The head was shaved and a trephine hole drilled in the skull with an electric burr. The skull bones were removed, and the cerebral coverings cleared. The brain was quickly dissected from the base and rinsed in cold 0.9% NaCl, and the tissues of different regions were taken for study.

Preparation of mitochondria. The method employed was essentially that of Aldridge (1957). The tissues were quickly weighed, and homogenized in 9 vol. of chilled (2–4°) 0.3M-sucrose, pH 7.4, containing EDTA (1 mM). The homogenization was done in a Potter–Elvehjem-type homogenizer (Potter, 1955) fitted with a Teflon pestle. The nuclei and cellular debris were removed by centrifuging in an International refrigerated centrifuge at 1000g for 10 min. (2500 rev./min.; head no. 269). The supernatant was then centrifuged at 10 000g for 15 min. in a Spinco model L preparative ultracentrifuge (12 500 rev./min.; rotor no. 40). The pellet, together with the fluffy layer, was washed once with sucrose solution and resedimented at the same speed. The mitochondrial pellets obtained were suspended in sucrose solution so that 0.5 ml. of the suspension contained approx. 4 mg. of mitochondrial protein (approx. 5 ml./g. of original tissue) and used within 10 min.

Density-gradient centrifuging. The mitochondrial pellet obtained by the method of Aldridge (1957) was further purified as described by Whittaker (1959) by layering over 0.8M- and 1.2M-sucrose successively.

Treatment of mitochondria. Preliminary assays indicated that digitonin treatment enhanced the enzyme activity of the particulate fraction. The digitonin treatment was superior in this respect to freezing-and-thawing, or to treatment with deoxycholate or Tween 20. Optimum activation was obtained by using 5 ml. of 1% (w/v) digitonin adjusted to pH 7.4 (with dilute HCl) for mitochondria obtained from every 500 mg. of brain tissue, higher concentrations of digitonin being inhibitory. The mitochondrial suspension was stirred with digitonin solution for 5 min. at 3° and then centrifuged in an International refrigerated centrifuge (2000 rev./min.; head no. 269) for 5 min. The sediment was discarded, the supernatant centrifuged in a Spinco model L ultracentrifuge (34 000 rev./min.; rotor no. 40) and the sediment taken up in 5 ml. of 0.3M-sucrose for further assay.

Assay of cytochrome-oxidase activity. The enzyme assay was done manometrically. Each Warburg flask contained: 0.2 ml. of 0.2M-phosphate buffer, pH 7.4; 0.3 ml. of 0.3 mM-cytochrome *c* dissolved in water; 0.5 ml. of mitochondrial suspension; 1.7 ml. of water. After temperature equilibration for 10 min. at 38°, 0.3 ml. of 0.2M-*p*-phenylenediamine hydrochloride was tipped from the side arm into the flask. Readings were taken every 5 min. for 30–40 min. Activities are expressed as μ l. of O₂ consumed/hr./

mg. of mitochondrial protein (q_{O_2}). The V_{max} values were deduced from Lineweaver–Burk plots of the reciprocal of the cytochrome *c* concentration and the reciprocal of the enzyme reaction rate (q_{O_2}), and are expressed as μ l. of O₂ consumed/hr./mg. of mitochondrial protein for infinite concentration of cytochrome *c*.

Determination of mitochondrial protein. A sample of the mitochondrial suspension was analysed for protein content by a turbidity method. Suspension (0.05 ml.) was diluted to 1.5 ml. with water, to which was added 3 ml. of the reagent containing 1% (w/v) of (NH₄)₂SO₄ and 2.5% (w/v) of trichloroacetic acid. The turbidity produced after standing for 10 min. was read in a Klett–Summerson photometer fitted with a green filter (540 m μ). The standard curve was obtained with purified bovine plasma albumin (fraction V). The results obtained by this method were proportional to the total nitrogen content as measured by a micro-Kjeldahl procedure.

Reagents. Cytochrome *c* (type III, from horse heart), digitonin and tris were purchased from the Sigma Chemical Co., *p*-phenylenediamine hydrochloride from E. Merck, EDTA from British Drug Houses Ltd., bovine plasma albumin (fraction V) from L. Light and Co. Ltd., deoxycholate (sodium salt) from C. H. Boehringer und Soehne, and Tween 20 from Biddle Sawyer and Co. (India) Ltd. All-glass-double-distilled water was used throughout.

RESULTS

Specific activity of cytochrome oxidase in mitochondria from cerebral cortex, cerebellum, thalamus and corpus callosum. With the mitochondrial fractions prepared as described by Aldridge (1957), the cytochrome oxidase was determined by using increasing concentrations of cytochrome *c*. A Lineweaver–Burk plot of the results permitted calculation of the enzyme activity for infinite concentration of cytochrome *c* (Table 1). The V_{max} /mg. of mitochondrial protein is 23.9 for corpus callosum, 95.2 for thalamus, 175.4 for cerebral cortex and 188.7 for cerebellum. Digitonin treatment increases the values for cerebellum and cerebral-cortex mitochondria. The activity of mitochondria from these areas appears to be about ten times as high as that of corpus-callosum mitochondria.

The specific activity of the enzyme isolated from corpus callosum is significantly lower than that from cerebral cortex or cerebellum, the enzymes from the latter two regions having approximately the same values. The presence of nerve end-plates and myelin sheaths as contaminants of the mitochondrial pellet so obtained has been shown by de Robertis, Pellegrino de Iraldi, Rodriguez de Lores Aramaiz & Salgani Coff (1962). Experiments were therefore performed with purified pellets obtained by layering the mitochondrial fraction over 0.8M- and 1.2M-sucrose successively, as recommended by Whittaker (1959). The pellet in such cases has been reported to be free from much

non-mitochondrial material by Whittaker (1959) and this procedure has been employed by other workers (Marks & Lajtha, 1962) to obtain mitochondria reasonably free from myelin fragments, nuclei and other particulate impurities. The pellet in our experiments was examined in some cases under the light-microscope after staining with Janus Green and methylene blue. The non-mitochondrial contamination is low and does not seem to exceed 5% of the total mass. Table 2 summarizes the results obtained with corpus-callosum and cerebral-cortex preparations. The $V_{\max}$ /mg. of mitochondrial protein was 62.5 for corpus callosum and 285 for cerebral cortex. The mitochondria from cerebral cortex thus are about four times richer in this enzyme than the corresponding particles in corpus callosum.

Characteristics of the enzyme. The K_m (for cytochrome *c*) was calculated from these experiments and the values obtained are given in Table 3. Since the enzyme activity was measured with particulate preparations, these values are only apparent K_m values. Digitonin treatment of the mitochondria does not significantly alter these values. The apparent K_m of the enzyme for cytochrome *c* is about the same in thalamus, cerebral cortex and cerebellum, but is lower for the corpus-callosum preparation.

In the assay procedure, the enzyme catalyses the transfer of electrons from cytochrome *c* to molecular oxygen, which thus is its immediate substrate. The cytochrome *c* is maintained continuously in the reduced form by *p*-phenylenediamine. Thus, before any conclusion is reached about any differences in the enzyme from different areas, it is necessary to exclude the possible restrictions imposed on the velocity of the reaction by: (a) transfer of electrons from *p*-phenylenediamine to cytochrome *c*, and (b) the passage of exogenous cytochrome *c* to the enzyme site. The same concentration of *p*-phenylenediamine was needed for maximal enzyme activity (Table 4). The *p*-phenylenediamine concentration is thus effective equally in the three cases and is not likely to be the limiting factor in the oxidation rate of cytochrome *c* by the cytochrome oxidase.

The possible difference in the transfer rate of added cytochrome *c* to the particulate enzyme site, resulting ultimately in apparent alteration of the affinity of the enzyme for cytochrome *c* in the corpus callosum, remains an open issue. Attempts have been made to simplify the particulate structure and expose the enzyme to best advantage. Digitonin treatment gives higher activities of the enzyme than freezing-and-thawing, or treatment with Tween 20 or deoxycholate. The K_m values of the enzyme for cytochrome *c* in digitonin-treated preparations from these areas show a twofold

difference between the corpus-callosum enzyme and that of other areas.

Slater (1949) had reported the inhibitory effect of phosphate ions at concentrations greater than

Table 1. *Cytochrome-oxidase activity of the mitochondrial fractions from different areas of brain*

Experimental details are given in the text.

Region	$V_{\max}$ (μ l. of O_2 /hr./mg. of protein)	
	Untreated mitochondria	Digitonin-treated mitochondria
Corpus callosum	23.9	22.0
Thalamus	95.2	93.5
Cerebral cortex	175.4	204.1
Cerebellum	188.7	256.4

Table 2. *Cytochrome-oxidase activity of mitochondrial fractions purified by density-gradient centrifuging from corpus callosum and cerebral cortex*

Experimental details are given in the text.

Region	$V_{\max}$ (μ l. of O_2 /hr./mg. of protein)	Apparent K_m (μ M)
Corpus callosum	62.5	3.7
Cerebral cortex	285.0	9.0

Table 3. *Apparent K_m values (for cytochrome *c*) of cytochrome oxidase from different areas of brain*

Experimental details are given in the text.

Region	Apparent K_m (μ M)			
	Intact mitochondria	Digitonin-treated mitochondria		
		Expt. 1	Expt. 2	Mean
Corpus callosum	4.9	6.3	5.0	5.6
Thalamus	9.8	8.9	9.2	9.0
Cerebral cortex	9.4	9.4	13.3	11.3
Cerebellum	8.3	10.0	13.7	11.8

Table 4. *Effect of the concentration of p-phenylenediamine on cytochrome-oxidase activity of various areas of brain*

Experimental details are given in the text.

	Concn. of <i>p</i> -phenylenediamine (M)			
	0.01	0.02	0.04	0.06
	Cytochrome-oxidase activity (μ l. of O_2 /hr./mg. of protein)			
Corpus callosum	12.7	18.9	21.4	24.4
Cerebral cortex	83.4	142.4	161.0	164.9
Cerebellum	165.0	180.6	213.2	226.0

Fig. 1. Inhibition by phosphate of cytochrome-oxidase activity in different areas of brain: ●, cerebellum; Δ, cerebral cortex; ▲, thalamus; ○, corpus callosum. Each reaction flask contained, in 3 ml.: cytochrome *c* (30 μM), *p*-phenylenediamine (20 mM), 0.5 ml. of mitochondrial preparation and various concentrations of phosphate buffer, pH 7.4.

0.1M on the cytochrome-oxidase activity. To investigate further the question of identity or otherwise of the enzyme from different areas, the effect of phosphate ions on the cytochrome-oxidase activities of the mitochondria obtained from the four regions was determined. Fig. 1 shows that the inhibition by phosphate is similar for all the areas.

The effect of pH on the enzyme activity in the mitochondria from the cerebellum, cerebral cortex, thalamus and corpus callosum is again similar (Fig. 2), the optimum pH being 7.5 in all cases.

DISCUSSION

Corpus callosum is an area of the brain where the cellular density is nearly as high as in cerebral cortex (Elliott & Heller, 1957). It is, however, characterized by a virtual absence of neurons, the cell population being entirely composed of glial elements. This region of the brain is therefore a good experimental material for investigation of the properties of non-neuronal tissue. Since neuroglial cells outnumber the neuronal cells eightfold or tenfold in the brain taken as a whole (Elliott & Heller, 1957), this study is relevant.

The present studies point to a low activity of the cytochrome oxidase, and possibly of the electron-transport-chain enzymes in general, in the neuroglial cells as compared with the neuron-containing areas. Further, the possibility that the intramitochondrial concentration of cytochrome *c* might be

Fig. 2. Effect of pH on cytochrome-oxidase activity of various areas of brain: ○, corpus callosum; ▲, thalamus; ●, cerebellum; Δ, cerebral cortex. The buffers employed were acetic acid-sodium acetate for pH 5, Na₂HPO₄-NaH₂PO₄ for pH 6, tris-HCl for pH 7.5 and 8, and glycine-NaOH for pH 9. The concentration in each case was 13 mM. Each reaction flask contained, in 3 ml.: cytochrome *c* (30 μM), *p*-phenylenediamine (20 mM), appropriate buffer (13 mM) and 0.5 ml. of mitochondrial preparation.

the limiting factor responsible for this low activity has been eliminated.

It is possible that the fraction sedimented under standard centrifugal force from corpus callosum may be composed to a greater degree of particles other than 'mitochondria'. The existence of lysosome-like particles has been indicated in rat-brain tissue which possess similar sedimentation properties to mitochondria, but contain a different set of enzymes (Beaufay, Berleur & Doyen, 1957). Lysosomes, however, have lower rates of sedimentation and are themselves enzymically inhomogeneous (Beaufay, Bendall, Baudhuin, Wattiaux & de Duve, 1959). In our studies the mitochondrial pellet has been obtained by more than one method including density-gradient centrifuging, which according to Whittaker (1959) and de Robertis *et al.* (1962) gives biochemically and morphologically pure mitochondrial fractions from rat-brain tissue. The results have, however, remained similar. It is thus unlikely that physical contamination of the mitochondrial preparation with non-mitochondrial matter (which did not exceed 5% after density-gradient centrifuging) is an explanation for the low specific activity of the enzyme in corpus callosum.

The variation in apparent K_m values (for cytochrome *c*) of the enzyme from corpus callosum and cerebral cortex or cerebellum is significant. These observations might point to qualitative differences in the nature of the enzymes or associated protein structures or both. Because of the particulate nature of the material studied in those experiments this interpretation is uncertain. One can conceive of the constitutional heterogeneity of the mitochondria at several levels. The mitochondrial enzyme equipment could be quantitatively identical, differences residing in the composition and nature of the membrane or associated structures. The alteration of permeability characteristics, in turn, could affect the expression of enzyme activity. The quantitative distribution of particular enzymes could be dissimilar in different mitochondrial particles, especially those from different tissues. Palade (1952) has in fact observed a variability in the number of cristae in mitochondria from different cell types. A correlation has been found between the number of cristae and the oxidative rate of the tissue (Green & Hatefi, 1961).

The question of biochemical heterogeneity of the mitochondria has been raised by other workers. Kuff & Schneider (1954), from an analysis of the chemical composition and enzymic equipment of the fractions obtained by gradient centrifuging of liver mitochondria, hinted at a marked heterogeneity of the particulate mass. Most of these studies have been conducted with an organ tissue and not with the single-cell type of suspensions. It is therefore difficult to discern whether the heterogeneity observed was caused by the presence of more than one type of tissue or by cells containing more than one type of particle having different functions. The general acceptance of lysosomes as an entity apart from mitochondria is a pointer to the possible existence of other types of particles in the same size range but with different enzymic equipment. It could also be possible that several such clones may exist in a tissue in varying proportions. Further, a differentiation on tissue level needs consideration.

Weiner (1960) has studied the distribution in brain of two particulate enzymes, monoamine oxidase and succinate dehydrogenase, both present in mitochondria and in similar proportions. They observed that succinate-oxidase activity is particularly high in the cerebellar and cerebral cortex, whereas monoamine-oxidase activity is higher in the thalamic and hypothalamic regions. The mitochondrial fractions from various regions of the brain thus seem to be heterogeneous. The present studies suggest a difference in the mitochondria from the neuroglial tissue of the corpus callosum as compared with similar particles obtainable from cerebellum or cerebral cortex.

SUMMARY

1. Cytochrome-oxidase activity has been determined in mitochondrial fractions from cerebral cortex, cerebellum, thalamus and corpus callosum of monkey brain.

2. The enzyme activity in corpus callosum/mg. of mitochondrial protein is significantly lower than the specific activity of the enzyme in cerebellum or cerebral cortex, the values in thalamus being intermediate. This is also true when the mitochondrial fraction has been further purified by density-gradient fractionation. The apparent K_m values of the enzyme for cytochrome *c* also differ.

3. The enzymes in these areas have similar pH optima and are inhibited in a parallel manner with phosphate ions.

4. The mitochondrial fraction from the neuroglial tissue of the corpus callosum appears to be constitutionally different from that of cerebellum or cerebral cortex.

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Injection of eggs. The eggs were candled and a small pencil mark was made over the region where well-developed membranes appeared, but away from any large chorio-