The Subcellular Distribution of Cerebral Phosphoproteins

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1. The subcellular distribution of the phosphoprotein fraction of grey matter from guinea-pig brain has been determined. Phosphoproteins occurred in all fractions of the tissue, the mitochondrial fraction containing 29%, supernatant 26%, microsomal fraction 23% and nuclear fraction 15%. The quantity of phosphoproteins per unit quantity of protein was highest in the microsomal fraction and lowest (by a factor of nearly 50%) in the mitochondrial fraction. 2. Observations are reported on the nature of the nuclear subfraction believed from earlier work to contain a phosphoprotein sensitive to the effects of electrical pulses applied to cerebral slices *in vitro*. With respect to its content of phosphoprotein, cholesterol, ribonucleic acid and succinate-dehydrogenase activity, the subfraction resembles the nerve-ending fraction of the mitochondrial fraction, from which it appears to be derived by cross-contamination. Electron microscopy tended to confirm this impression. Reasons are adduced for discounting the earlier claim that the sensitive phosphoprotein is contained in a new subcellular structure distinct from nuclei, mitochondria or microsomes.

Electrical pulses, applied *in vitro* to slices of cerebral cortex respiring in the presence of [³²P]orthophosphate, increase the specific radioactivity of the tissue's phosphoprotein fraction (Heald, 1957), an observation that has led to the suggestion that a phosphoprotein is involved in mechanisms that maintain and restore ionic gradients across the neuronal cell membrane (Heald, 1960, 1962). Such a function implies the participation of the phosphorylated protein in reactions consuming phosphate-stored energy in the cell, rather than in synthetic reactions directed to the restoration of the reserves of phosphocreatine depleted by excitation. Though the available evidence on this point (see Heald, 1957) on the whole favours a role in an energy-consuming reaction, it does not completely exclude the alternative explanation. For a more definitive interpretation localization of the sensitive phosphoprotein within the cell is required. For example, a mitochondrial location would suggest a role in oxidative phosphorylation, an energy-yielding process, whereas association with the membrane fraction would point to participation in some transport phenomena, among which that concerning

cations is an important energy-consuming reaction in cerebral tissue.

In a previous study of this problem it was found that the increase in phosphoprotein radioactivity after stimulation occurred in a particulate fraction of the tissue rather than in the fraction containing the soluble proteins (Heald, 1959). This particulate material appeared to be part of the nuclear fraction, and from the results of subfractionation studies a more precise location was proposed in a structure, probably of membranous origin, and distinct from the nuclei or contaminating mitochondria and microsomes (Heald, 1961a). The primary aim of the present work has been to examine further the chemical and morphological characteristics of the fraction held to contain the phosphoprotein-rich structure. We also report the results of a study of the general subcellular distribution of cerebral phosphoproteins, which served as a basis for the main part of the investigation. A preliminary report of this work has already appeared (Trevor, Rodnight & Schwartz, 1963).

EXPERIMENTAL

Preparation and dispersion of tissue

Guinea pigs were stunned by a blow on the neck and exsanguinated, and the cerebral hemispheres removed and rapidly dissected to give 1–1.2 g. of grey matter from each animal. The tissue was dispersed in 9 vol. of ice-cold

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0.32M-sucrose containing EDTA (0.5mM) (brought to pH 7 with KOH) in a homogenizer with a Teflon pestle of clearance 0.005–0.007 in. between the bore of the tube and the pestle diameter. The dispersing procedure was standardized and consisted of ten passages of the pestle, rotating at approx. 1800 rev./min., up and down through the dispersion over a period of about 30 sec., with intermediate periods of cooling in ice.

Fractionation of dispersions

Primary fractions. In the initial studies tissue dispersions were fractionated by differential centrifugation into five primary fractions as follows: nuclear (800g for 10 min.); mitochondrial I (10⁴g for 10 min.); mitochondrial II (1.3 × 10⁴g for 15 min.); microsomal (1.2 × 10⁵g for 60 min.). The nuclear and mitochondrial fractions were prepared in an MSE Medium refrigerated centrifuge (Measuring and Scientific Equipment Ltd., Birmingham), and the microsomal fraction in the Spinco model L machine. The nuclear fraction was washed twice by resuspending it in 0.32M-sucrose and centrifuging; both the mitochondrial fractions were washed once in the same manner.

Subfractions. In certain experiments primary nuclear and mitochondrial fractions, prepared as described above or under conditions indicated in the text, were further subfractionated, after resuspension in 0.32M-sucrose to approx. 2mg. of protein/ml., by means of discontinuous gradients of sucrose solutions. After being prepared, the gradients were allowed to equilibrate for 15 hr. at 2°. The procedure was essentially the same as that followed by Heald (1959) and was carried out in either the SW 25 or SW 39 head of the Spinco model L centrifuge. The nuclear fraction was first layered on 1M-sucrose and centrifuged at 10⁵g for 45 min. The sedimented particulate matter was resuspended in 0.32M-sucrose, layered on a gradient of 1M-, 1.2M- and 1.4M-sucrose and centrifuged at 10⁵g for 75 min. when using the SW 39 head and at 5.3 × 10⁴g for 1.5 hr. when using the SW 25 head, giving in both cases an integrated field of 7.5 × 10⁶g-min. The mitochondrial fraction was treated identically except that the preliminary spin over 1M-sucrose was omitted. After centrifuging the subfractions were separated by means of a tube cutter (Beckman Instruments Inc., Palo Alto, Calif., U.S.A.) for analysis and morphological examination.

Analytical methods

Protein. This was determined by the biuret procedure of Gornall, Bardawill & David (1949), with crystallized bovine serum albumin as standard.

Succinate dehydrogenase. Enzyme activity was measured by the manometric method of Aldridge & Johnson (1959), as modified by Schwartz, Bachelard & McIlwain (1962).

Phosphoprotein. This was determined by measuring the alkali-labile P in the insoluble residue remaining after extraction of the tissue with 10% (w/v) trichloroacetic acid, ethanol and chloroform-methanol (2:1, v/v) (McIlwain & Rodnight, 1962). Phosphorus was determined by the extraction procedure of Martin & Doty (1949) scaled down to cover the range 1–10 μg. of P in a volume of 4 ml.

Ribonucleic acid. During the phosphoprotein determination an approximate measure of RNA was obtained as follows. The alkaline digest (1 ml.) was acidified with 1.5 ml. of 1.33N-perchloric acid and the mixture centrifuged. A portion (0.5 ml.) of the supernatant was withdrawn and diluted to 3 ml. with a mixture (2:3, v/v) of N-NaOH and 1.33N-perchloric acid. The extinction of this solution at 266 mμ was taken as a measure of RNA. Yeast RNA, purified by repeated solution in alkali and reprecipitation with acid, was used as standard. The remaining 2 ml. of the acidified digest was taken for estimation of inorganic phosphate released from phosphoproteins.

Cholesterol. A digitonin procedure given by McIlwain & Rodnight (1962) was used.

Electron microscopy. This was kindly undertaken by Dr P. L. Williams. The fractions were fixed with osmium tetroxide and dehydrated by the method of Gray & Whitaker (1962). The pellets were embedded in Araldite.

RESULTS

Primary fractions prepared by differential centrifugation. Phosphoproteins, determined as alkali-labile P, were detected in all parts of the cell (Table 1). The largest amount was found in the supernatant fraction, but the microsomes contained the highest amount of protein phosphorus in terms of unit quantity of protein. It is noteworthy that the amount of the microsomal phosphoprotein/mg. of

Table 1. *Subcellular distribution of cerebral phosphoproteins*

Subcellular fractions were prepared from freshly excised guinea-pig grey matter as described in the Experimental section. Protein was determined by a biuret procedure and phosphoprotein P by the measurement of alkali-labile P (see the text). The results are the means of seven experiments; means for the amounts/mg. of protein are followed by their standard deviation.

Fraction and centrifugal force used (g-min.)	Phosphoprotein P		
	(μmoles/g. of tissue)	(percentage recovered)	(μmoles/mg. of protein)
Original dispersion	1010	—	9.7 ± 0.2
Nuclear (8 × 10 ³)	151	15	10.7 ± 0.3
Mitochondrial I (10 ⁵)	223	22	6.0 ± 1.8
Mitochondrial II (2 × 10 ⁵)	72	7	7.2 ± 1.0
Microsomal (7 × 10 ⁶)	235	23	12.3 ± 2.2
Supernatant	266	26	10.1 ± 1.4

Total recovery 93%

protein was twice that of the first mitochondrial fraction although the latter contained 22% of the total.

Subfractions prepared by density-gradient fractionation. The conclusions reached by Heald (1959, 1961a) were based on analysis of a nuclear fraction isolated by the procedure of Brody & Bain (1952), which employs a centrifugal field for the nuclear fraction of 15×10^3 g.-min., compared with one of about 8×10^3 g.-min. later found to be more suitable for cerebral tissues (see McIlwain & Rodnight, 1962). In these experiments, however, where a direct comparison with the subfractionation procedure used by Heald (1959, 1961a) was considered important, we have used the higher centrifugal force. The primary nuclear fraction was washed once and subfractionated in sucrose density gradients as described by Heald (1959). Similar subfractions were also isolated from the crude mitochondrial fraction by essentially the same procedure. Comparison between the subfractions isolated from both primary fractions was considered important because the high centrifugal force used to isolate the primary nuclear fraction resulted in a considerable cross-contamination of this fraction with structures from the mitochondrial fraction. From each crude fraction, therefore, four subfractions, designated A, B, C and D, were obtained and analysed for phosphoprotein, RNA, cholesterol and succinate dehydrogenase (Table 2).

The nuclear subfraction considered by Heald (1959, 1961a) to be enriched in the phosphoprotein sensitive to electrical pulses corresponds in these fractions to subfraction B, sedimenting in the gradient to a position between 1M- and 1.2M-sucrose. In terms of protein this fraction contains more phosphoprotein and cholesterol and less RNA and succinate dehydrogenase than subfraction C, although for phosphoprotein and RNA the differences are not very marked. A similar trend in the analytical results can be seen in subfractions B and C from the mitochondrial fraction, which correspond respectively to the subfractions rich in nerve endings and mitochondria obtained by Whittaker (1959). In subfractions A and D from both primary fractions the values for cholesterol and succinate dehydrogenase are similar, but those for phosphoprotein and RNA show considerable differences. Thus the nuclear subfraction A was the highest of all the subfractions in RNA and phosphoprotein, whereas the corresponding mitochondrial subfraction was relatively low in these constituents. The reason for the high phosphoprotein and RNA in nuclear subfraction A is unclear, but since relatively little protein was recovered in this fraction the results here are subject to considerable error.

The similarity in composition between the corresponding subfractions from the two primary frac-

Table 2. *Distribution of phosphoprotein, RNA, cholesterol and succinate dehydrogenase in subfractions of the nuclear and mitochondrial fractions*

The primary nuclear and mitochondrial fractions were prepared from dispersions of fresh guinea-pig cerebral cortex in 0.32M-sucrose at the centrifugal forces stated. The subfractions were prepared by centrifuging the primary fractions, resuspended in 0.32M-sucrose, for 7.5×10^3 g.-min. in a discontinuous sucrose gradient as described in the text. The subfractions were designated as follows: A, material not sedimented through 1M-sucrose; B, material not sedimented through 1.2M-sucrose; C, material not sedimented through 1.4M-sucrose; D, material sedimented through 1.4M-sucrose. Where standard deviations are given the results are the means of five experiments; in other cases the means are derived from three experiments.

Primary fraction (g.-min.)	Subfraction	Protein (mg/g. of cortex)	Phosphoprotein P (mμmoles/mg. of protein)	RNA (μg./mg. of protein)	Cholesterol (μg./mg. of protein)	Succinate-dehydrogenase activity (μmoles of succinate/mg. of protein/hr.)
Nuclear (15×10^3)	A	0.9	14.9	26.2	224	0
	B	2.6 ± 1.1	9.5 ± 1.1	12.1 ± 3.3	118 ± 20	5.5
	C	2.6 ± 0.6	7.2 ± 2.1	13.3 ± 3.3	85 ± 27	10.4
	D	2.3	9.2	19.6	80	3.4
Mitochondrial (2×10^5)	A	14.6	9.3	13.1	158	0.7
	B	13.3	9.7	17.9	108	6.7
	C	9.2	6.6	22.8	77	12.5
	D	3.2	7.6	17.0	52	0

Table 3. *Protein content of subfractions prepared by density-gradient centrifugation of washed nuclear fractions*

Three primary nuclear fractions were prepared by centrifuging dispersions of fresh guinea-pig cerebral cortex in 0.32 M-sucrose for 15×10^3 g-min. Each fraction was washed one, two or three times by resuspension in fresh 0.32 M-sucrose and centrifuging again for 15×10^3 g-min. The washed fractions were then submitted to density-gradient fractionation as described in the Experimental section and Table 2.

Subfraction	Protein (mg./g. of tissue)		
	Washed once	Washed twice	Washed three times
A	0.90	0.27	0
B	1.62	0.78	0.28
C	2.22	0.80	0.28
D	2.50	2.24	2.03

tions suggested that cross-contamination of the fractions was occurring through inadequate washing. This conclusion was confirmed by dividing a crude nuclear fraction into three parts and washing the parts one, two or three times by resuspension in fresh sucrose solution and centrifuging for 15×10^3 g-min. The washed fractions were then subjected to the density-gradient procedure and the subfractions analysed for protein (Table 3). The protein content of subfractions A, B and C was markedly decreased after two washings, and after a third wash no protein was detectable in subfraction A and only a small amount in subfractions B and C. By contrast, subfraction D changed little in protein content after three washes, suggesting that the material comprising this fraction normally sediments with larger particles such as nuclei and whole cells. It therefore appears that only subfraction D can correctly be described as a component of the nuclear fraction; the other subfractions, including the crucial phosphoprotein-rich subfraction B, represent particles mainly sedimented at the centrifugal forces used to separate the mitochondrial fraction.

Morphological observations. Electron microscopy confirmed the presence of common structures in the B subfractions, but indicated that the nuclear subfraction was more heterogeneous than the chemical analyses suggested. The mitochondrial subfraction B contained many nerve endings and membrane-derived fragments, together with a few mitochondria. In the corresponding nuclear subfraction nerve endings, membrane fragments and occasional mitochondria were also seen, but in addition there occurred a considerable quantity of amorphous material, possibly derived from disintegrated nuclei (Gray & Whittaker, 1962).

DISCUSSION

Primary fractions. In considering the observations on the general distribution of phosphoproteins in the primary fractions, it is necessary to comment first on the specificity of the procedure for determining phosphoprotein phosphorus in mammalian

tissues by hydrolysis with alkali. It is well known that this procedure, which is the only quantitative method available, is based on an early observation that casein and similar phosphoproteins release inorganic phosphate into solution on treatment with mild alkali (Plimmer & Bayliss, 1906). Some years later the tissue phosphoprotein fraction became operationally defined by the quantity of inorganic phosphate liberated from the acid-washed lipid-free tissue residue by treatment with N-alkali for 18 hr. at 37° (Schmidt & Thannhauser, 1945). As nucleic acids do not give rise to inorganic phosphate on incubation with mild alkali, their previous separation is not necessary; however, even after complete removal of nucleic acids, the alkaline digest contains a considerable quantity of organically combined phosphorus, which is only partly precipitated with acid (Logan, Mannell & Rossiter, 1952; Heald, 1960). The precise origin and nature of this residual organic phosphorus has not been determined, although in part it appears to consist of inositol phosphates derived from protein-bound inositides (Rossiter, 1962). The possibility has never been excluded, therefore, that part of the inorganic phosphate determined in the digest is derived from the partial or complete hydrolysis of organically combined phosphorus other than phosphorus bound as phosphorylserine in protein.

Assuming that the alkali-labile phosphorus does represent, in the main, phosphorylserine residues in protein, the distribution and function of these groups in the tissue proteins are only partly understood. The value for phosphoprotein phosphorus obtained in the present work of approx. $10 \text{ m}\mu\text{moles of P/mg. of protein}$ indicates that one phosphorus residue is available/protein of particle weight 100 000. Although this consideration indicates that a wide distribution of phosphorus in the many different species of tissue protein is possible, such a deduction seems unlikely to be true in view of the number of proteins already recognized as not containing phosphorus. Tissue phosphoproteins that have been isolated and described so far comprise certain enzymes. Those, such as phosphoglucosomutase, that

are involved in phosphate transfer and become phosphorylated at a single serine group in the presence of their substrates, probably only constitute 1–2% of the cerebral protein phosphorus (Heald, 1960). A different class of phosphorylated enzyme is typified by the glycogen phosphorylases of muscle and liver (Krebs & Fischer, 1962); muscle phosphorylase α , for example, is a tetramer with four phosphoryl-serine groups/molecule apparently not directly involved in the mechanism of actions of the enzyme. This class of molecule shows that phosphorylation of enzymes, presumably resulting in conformational changes influencing activity, may occur at sites other than those intimately concerned with catalysis, and suggests a possible role for phosphorus in other enzymes not necessarily involved in phosphate transfer. Another, and possibly major, origin for tissue protein phosphorus that has often been proposed is from phosphoproteins lacking enzymic function and analogous in their structure to casein and phosvitin, which contain many phosphoryl-serine groups/molecule. There is a considerable amount of indirect evidence for the existence of this category of phosphoprotein in tissues, but a successful characterization has yet to be accomplished. However, suggestive evidence has been given for the occurrence of polyphosphorylserine peptides in cerebral proteins with similar electrophoretic properties to phosphopeptides derived from phosvitin (Heald, 1961b); also, enzymes phosphorylating (Rodnight & Lavin, 1964) and dephosphorylating (Rose & Heald, 1961; Rose, 1962) phosvitin and casein have been described in brain.

In view of the above considerations it is perhaps not surprising that there is no particularly striking variation in the subcellular distribution of protein phosphorus in cerebral-cortex tissue. The relatively low value for the mitochondrial phosphoproteins is noteworthy, however, especially when compared with the value for the microsomal fraction, which is approximately twice as high. The mitochondrial fraction from brain contains some 20% of the phosphoprotein phosphatase of the tissue (Rose, 1962), but the satisfactory recovery of protein phosphorus from the fractions makes it unlikely that the low value is due to the action of this enzyme. The microsomal fraction used in the present study was isolated by a centrifugal force of 6×10^6 g-min. and therefore contained both membrane structures and ribosomal granules (Toschi, 1959); thus the extent to which these components contribute to the phosphoprotein of the fraction cannot be judged from the values in Table 1. However, determinations made later on a membrane-rich fraction isolated by the procedure of Schwartz *et al.* (1962) have resulted in similar high values (K. Kothary & R. Rodnight, unpublished work). It appears likely therefore that membrane structures are

slightly richer in phosphoproteins than are other parts of the cell.

Observations on subfractions. The primary aim of the present work has been to examine the nature of a subfraction of the nuclear fraction, prepared from dispersions of cerebral cortex, and considered by Heald (1959, 1961a) to be involved in the response of the tissue *in vitro* to electrical excitation. The evidence for this conclusion rested mainly on the direct observation that, on fractionation and analysis of dispersions of electrically stimulated slices of cortex, the major change in phosphoprotein radioactivity, compared with control unstimulated slices, occurred in a nuclear fraction rather than in a composite fraction said to contain the mitochondria, microsomes and soluble proteins. Further localization within a subfraction of the nuclear fraction was apparently based solely on the finding that, of the several subfractions obtained after separation of the nuclei, one (named R_2 by Heald and B in the present work) was richest in metabolically active phosphoprotein and relatively low in succinate-dehydrogenase activity. In a subsequent study (Heald, 1961a), the same conclusion was reached with fresh tissue and by determining phosphoprotein by digestion with alkali, rather than by measurement of incorporated radioactivity. These observations led to the concept of a specific phosphoprotein-rich structure, probably derived from the neuronal membrane, and distinct from nuclei, mitochondria and endoplasmic reticulum (Heald, 1962).

The present work confirms the previous observations that the R_2 or B subfraction, derived from fresh tissue, contains relatively more phosphoprotein than the other subfractions, the enrichment in terms of protein or nitrogen over the mitochondria-rich subfraction C being some 30% in our work and in that of Heald (1961a). When compared with the distribution of phosphoproteins in the primary fractions, this finding suggests that the subfraction is rich in membrane material; the relatively high content of cholesterol in the subfraction also supports this conclusion, since cerebral microsomal fractions contain more cholesterol in terms of protein than do other subcellular fractions (Trevor, 1963). However, there seems no reason now to explain this observation in terms of a special phosphoprotein-rich structure. The material in this fraction is clearly derived from lighter fractions by cross-contamination since it is readily removed by repeated washing; and it is no richer in protein phosphorus as determined by the alkali-labile P method than are the membrane-rich fractions from other sources, such as the nerve-ending fraction or the microsomes.

The finding of nerve-ending structures in a nuclear subfraction led us at one stage of the investigation to consider the possibility that the phosphoprotein

sensitive to electrical excitation was contained in these structures. Subsequent studies, however, have excluded this possibility and have shown that the main increase in phosphoprotein radioactivity on stimulation occurs in the microsomal fraction, which, unless certain precautions are taken, is mostly sedimented from dispersions of incubated tissue with the nuclear and mitochondrial fractions (Trevor & Rodnight, 1965).

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