3. Seven other Gram-negative bacteria contain ubiquinone but not vitamin K.

4. None of the Gram-positive organisms examined contains ubiquinone. Vitamin K₂ was found in five types, but was not in *Lactobacillus casei*, *Clostridium sporogenes*, anaerobically cultured *Staphylococcus albus* or in spores of *Bacillus subtilis*.

5. No long-chain polyisoprenoid alcohols were detected in *Escherichia coli*, *Proteus vulgaris* or *Pasteurella pseudotuberculosis*. The last-named, however, yielded a compound tentatively identified as a long-chain saturated alcohol.

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The Localization of Cerebral Phosphoprotein Phosphatase

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The partial purification of a phosphoprotein phosphatase from ox brain has been reported by Rose & Heald (1960, 1961). The enzyme was labile, magnesium-activated, and extremely specific, acting only on certain phosphoproteins, e.g. casein, phosvitin and brain phosphoprotein, and partially hydrolysed phosphopeptides prepared from α -casein. The enzyme differed from other mammalian phosphoprotein phosphatases (Hofman, 1958;

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Glomset & Porath, 1960; Revel & Racker, 1960) in that it liberated only a small portion of the phosphoprotein phosphorus before ceasing to act. This released phosphorus included all the radioactivity incorporated into phosvitin during phosphorylation with radioactive phosphate in a mitochondrial preparation (Burnett & Kennedy, 1954; Rabinowitz & Lipmann, 1960).

Such distinctive properties made desirable a knowledge of the distribution and intracellular localization of the enzyme. In this paper some data

relating to the distribution of phosphoprotein phosphatase in different regions of ox brain, and to the localization of the enzyme in various subcellular fractions, are presented, and are compared with similar values for a variety of cerebral constituents.

A preliminary report of part of this work has been given (Rose, 1961).

EXPERIMENTAL

Materials

Ox brain. Ox brain was obtained from the Metropolitan Abattoir, Islington, as soon as possible after the death of the animal, and was packed in a polythene bag, surrounded by ice, during transport to the laboratory. Before dissection, which was performed in the cold room, strands of pia and adhering blood clots were removed. Sections of approx. 1 g. were cut from various regions of the brain, rapidly weighed on a torsion balance, and dispersed in 9 vol. of ice-cold water with a Potter-Elvehjem-type Teflon-glass homogenizer. Between removal of the brain from the animal and the commencement of dissection approx. 45 min. elapsed; dissection and dispersion of the sections occupied a further 40 min.

Guinea-pig brain. Guinea-pig brain was used for studies on the intracellular localization of the enzyme. Whole brain, or the pooled slices of cerebral cortex from two brains (cut with a razor and guide lubricated with 0.32M-sucrose and rapidly weighed on a torsion balance), was dispersed with 9 vol. of ice-cold 0.32M-sucrose containing Mg^{2+} ions as $MgCl_2$ (1.0 mM) and buffered with sodium acetate (1.0 mM), pH 6.8 (Hanzon & Toschi, 1960), in a Teflon-glass homogenizer with a clearance of 0.007 in. (Aldridge & Johnson, 1959). Ice was packed round the homogenizer during use. After a brief (30 sec.) homogenization, the dispersion was passed through a Model 10 Emanuel-Chaikoff hydraulic tissue press (Microchemical Specialities Co., U.S.A.), with an orifice diameter of 25μ (Potter, 1955; Wolfe & McIlwain, 1961). The press was chilled in the refrigerator for at least 20 min. before use.

The dispersion was centrifuged at low speeds in nylon tubes in the high-speed attachment of the M.S.E. Major refrigerated centrifuge at 0° . For the light-mitochondrial and microsomal fractions the no. 40 head of the Spinco Model L ultracentrifuge was used. For density-gradient centrifuging the swing-out SW 25 and SW 39 heads of the Spinco were used.

The procedure initially adopted for subcellular fractionation was that of Aldridge & Johnson (1959). Thus the subcellular fractions were collected after centrifuging at the following forces and times (g values refer to the bottom of the centrifuge tube): nuclear fraction, 1000g for 10 min. (1N10); mitochondrial fraction, 7000g for 15 min. (7M15); light-mitochondrial fraction, 13 000g for 15 min. (13L15); microsomal fraction, 145 000g for 60 min. (145P60).

Each fraction, except the microsomal, was washed once with 5-8 ml. of sucrose and centrifuged again at the same force and for the same time, the wash supernatant being combined with that first collected before proceeding with the next stage of the fractionation. The supernatant remaining after collection of the microsomal fraction

formed the soluble fraction, S. The entire procedure was completed within 3 hr. Fractions were stored in stoppered tubes packed in ice and were assayed as soon as possible after fractionation was completed.

For density-gradient centrifuging of the nuclear and mitochondrial fractions, the procedure adopted was based on those described by Kuff & Schneider (1954), by Heald (1959) and by Hanzon & Toschi (1960).

For nuclei, the method was that of Heald (1959), except that the initial nuclear fraction was collected at 800 and not 1500g, and was washed three times with 0.32M-sucrose before layering. It was then resuspended in 0.32M-sucrose, and portions were layered directly on 15 ml. of 1.0M-sucrose in the tubes of the SW 25 head of the Spinco. The tubes were then centrifuged for 1 hr. at 90 000g, and the rotor was then allowed to run down unbraked.

The once-washed mitochondrial fraction from guinea-pig cortex, separated from the nuclei at 800g and from the post-mitochondrial supernatant at 7000g, was taken up in 0.88M-sucrose to form a concentrated suspension (1 ml./g. of cortex). Portions (1 ml.) of this suspension were layered over gradients made up of 1.6M-sucrose (0.8 ml.), 1.4M-sucrose (1.0 ml.), 1.2M-sucrose (1.0 ml.) and 1.0M-sucrose (1.0 ml.), in the 5 ml. tubes of the Spinco SW 39 head. The tubes containing these gradients were stored, packed in ice, sometimes overnight (Hanzon & Toschi, 1960), but for at least 30 min. before use.

The tubes were centrifuged for 1 hr. at 173 000g and the centrifuge was allowed to run down unbraked. The layers were separated by mounting each tube firmly in a stand and piercing the bottom with a fine hypodermic needle; the sucrose layers were then collected in turn in graduated tubes. For small tubes with many layers, this method of collection was found preferable either to removal with fine-drawn Pasteur pipettes or to slicing the tube with the tube-cutting device described by Phelps & Roodyn (1957). Negligible material was lost during the collection, nitrogen recoveries being in the range 87-103%. This procedure was only possible, however, where, as with the mitochondria, no material passed through the lowest layer (of 1.6M-sucrose) to form a pellet at the bottom of the tube.

Methods

Phosphoprotein-phosphatase activity was determined in the various fractions with an α -casein substrate and in the absence of added activators. The assay method, and the definition of units of activity and specific activity, were as described by Rose & Heald (1961). Succinate dehydrogenase was determined spectrophotometrically by the method of Slater & Bonner (1952). With this method, the distribution of succinate-dehydrogenase activity between the various subcellular fractions, prepared with the centrifugal forces and times of Aldridge & Johnson (1959), was as follows: nuclear fraction, 7-9%; mitochondrial fraction, 75-80%; light-mitochondrial fraction, 3-7%; supernatant, 10-15%. These figures show a higher proportion of the succinate-dehydrogenase activity in the mitochondria than is revealed by the manometric method quoted by Bonner (1955).

Phosphoprotein was estimated, after precipitation with trichloroacetic acid, by the method described by Heald (1958, 1961).

Inorganic phosphate was estimated by the method of Martin & Doty (1949) as modified by Rose & Heald (1961).

Nitrogen was estimated after micro-Kjeldahl digestion by the method of Chibnall, Rees & Williams (1943) by distillation in the apparatus of Markham (1942).

RESULTS

Distribution of the enzyme in ox brain

The following sections were chosen for examination: cerebral (parietal) and cerebellar cortex, caudate nucleus, thalamus, putamen, globus pallidus, corpus callosum, floor of the IVth ventricle and medulla. As the dissection of sections from all of these regions from a single brain took about 40 min., during which time some autolysis and loss of enzymic activity might occur, the first and last sections cut from each brain were of parietal cortex, from opposite hemispheres, and the activity of these two sections was compared. In five experiments the activity of the second sample of cortex did not differ from that of the first by more than $\pm 8\%$.

In eight experiments the activity of ox-brain parietal cortex varied from 16.0 to 22.0 (average 19.0) μ moles of α -casein phosphorus released/g. wet wt./hr. Table 1 shows the phosphoprotein-phosphatase activity of various regions of brain expressed as a percentage of that of the parietal cortex of the same brain (first-cut sample). Each result quoted is the mean of at least six experiments. To check whether the differences were expressions of different specific activities in the various tissue sections or could be attributed to extraneous factors, e.g. variability in water content, in one experiment the nitrogen content of the samples was measured. The results (Table 1) were expressed as specific activity of the sample as a percentage of that of parietal cortex, and agree with the mean values calculated/g. wet wt. Hence the measured variations represented real differences in the enzyme activity of the chosen brain regions.

The results of Table 1 show that the regions fall into three groups. The cerebellar cortex, parietal cortex and caudate nucleus all showed similar

activity (approx. 20 μ moles of phosphorus released/g. wet wt./hr.). All these tissues consist predominantly of grey matter.

The medulla, the floor of the IVth ventricle and the corpus callosum showed activities of 42–47% of that of the parietal cortex. These tissues are composed largely of white matter.

Intermediate between these two groups, with activities of 61–70% of the cortex, were the thalamus, putamen and globus pallidus. These regions are composed of mixed grey matter and white matter.

For comparison, data (assembled from a number of references) relating to the activities in different areas of the brain of a variety of enzymes are set out in Table 2. These data are discussed below.

Table 1. *Phosphoprotein-phosphatase activity in various brain regions*

Sections of fresh ox brain were dissected out and dispersed with 9 vol. of cold water in the cold room. A section of parietal cortex was the first cut, and a section from the second hemisphere was cut as the last section taken. The enzymic activity in the dispersions was measured as described in the Experimental section, in the absence of added activators. Activity is expressed as the mean of not less than six experiments, and as a percentage of the activity found in the first-cut section of parietal cortex (19.0 ± 0.7 μ moles of α -casein phosphorus/g. wet wt./hr.). Percentages are based on phosphorus release/g. wet wt., and, in one experiment on specific activity, as defined by Rose & Heald (1961). The numbers of experiments are given in parentheses.

Region	Activity (%)	Specific activity
Parietal cortex (1st cut)	100	100
Caudate nucleus	107 $\pm$ 11 (6)	107
Cerebellar cortex	87 $\pm$ 7 (7)	91
Thalamus	61 $\pm$ 2 (8)	58
Putamen	64 $\pm$ 7 (6)	60
Globus pallidus	70 $\pm$ 6 (8)	81
Floor of IVth ventricle	47 $\pm$ 10 (7)	40
Medulla	46 $\pm$ 11 (8)	55
Corpus callosum	42 $\pm$ 12 (6)	29
Parietal cortex (2nd cut)	103 $\pm$ 5 (5)	—

Table 2. *Distribution of some enzymes in various brain regions*

Values are expressed as percentages of the concentration in cerebral (parietal) cortex, and are calculated from the data of Gordon (1953) (alkaline phosphatase, acid phosphatase and pyrophosphatase), Himwich & Fazekas (1939) (oxygen uptake), Chesler & Himwich (1944) (glycolysis), Feldberg & Vogt (1948) (choline acetylase) and Burgen & Chipman (1951) (cholinesterase).

Species	System	Cerebral cortex	Cerebellar cortex	Caudate nucleus	Thalamus	Medulla	Corpus callosum
Ox	Phosphoprotein phosphatase	100	87	107	61	46	42
Sheep	Alkaline phosphatase	100	—	74	74	76	31
Sheep	Acid phosphatase	100	—	86	56	60	23
Sheep	Pyrophosphatase	100	—	88	87	91	96
Dog	O ₂ uptake	100	121	117	87	59	—
Dog	Glycolysis	100	33	108	85	13	—
Dog	Choline acetylase	100	12.5	186	147	18	38
Dog	Cholinesterase	100	760	2460	310	50	10

Intracellular localization of the enzyme

In these experiments the procedure initially adopted was that of Aldridge & Johnson (1959) and is described in the Experimental section.

Table 3 shows the distribution of activity in subcellular fractions of preparations of whole brain and of slices of cerebral cortex. In four experiments, 31–35 % of the enzymic activity was present in the nuclear and mitochondrial fractions, and 56–60 % in the soluble supernatant. Only very small

Table 3. *Distribution of activity in subcellular fractions of guinea-pig whole brain and cortex*

Guinea-pig whole brain, or the pooled cortex slices of two brains, was homogenized in 10 vol. of ice-cold sucrose (0.32 M), buffered at pH 6.8 with sodium acetate (1.0 mM) and containing magnesium chloride (1.0 mM), with a Teflon-glass homogenizer; the homogenate was passed through an Emanuel-Chaikoff press and fractionated by differential centrifuging as described in the text. Low-speed centrifuging was performed on the M.S.E. 'Major' refrigerated centrifuge with the superspeed attachment; for high speeds the no. 40 head of the Spinco Model L centrifuge was used. Centrifugal forces are as described by Aldridge & Johnson (1959). Each fraction, except for the microsomes, was washed once, and the wash supernatants were combined with the material for the next centrifuging. Phosphoprotein phosphatase was determined in the fractions as described in the Experimental section.

Fraction	Percentage of initial activity recovered	
	Whole brain	Cortex
Homogenate	100	100
1 N 10	12	12
7 M 15	19	20
13 L 15	8	6
145 P 60	0	3
Supernatant	60	56
	99	97

Table 4. *Distribution of phosphoprotein phosphatase between particulate and soluble fractions under a variety of conditions*

A guinea-pig brain was trisected and the portions were dispersed with 9 vol. of 0.32 M-sucrose, 0.88 M-sucrose and 0.33 M-sodium chloride respectively. The dispersions were centrifuged for 60 min. at 145 000g in the no. 40 head of the Spinco Model L centrifuge and washed once with their respective extractants for the same time and at the same force. Activity was determined in the separated fractions as described in the Experimental section.

Extractant	Percentage of recovered activity	
	Particulate fraction	Soluble fraction
0.32 M-Sucrose	44	56
0.88 M-Sucrose	48	52
0.33 M-Sodium chloride	51	49

amounts were present in the light-mitochondrial and microsomal fractions.

To test whether the 60 % of the phosphatase activity appearing in the supernatant represented soluble enzyme leached out from the particulate fractions during purification, a guinea-pig brain was divided into three portions (of approximately equal weight) which were dispersed in 9 vol. of 0.32 M-sucrose, 0.88 M-sucrose and 0.33 M-sodium chloride respectively. The dispersions were centrifuged for 60 min. at 145 000g, washed once with their respective extractants and recentrifuged. The resuspended residues, and their respective combined supernatants, were assayed for phosphoprotein-phosphatase activity. The results (Table 4) show that there was little difference in enzyme distribution between particulate and soluble fractions with the three extractants. Were the activity in the soluble fraction the result of solubilization of particulate enzyme during separation, it is unlikely that the percentage dissolved in the three different extractants would be similar. Homogenization for 1 min. or longer instead of 30 sec. did not alter the distribution, nor did leaving the homogenate, in ice, for up to 1 hr. before fractionation.

Partition between nuclear and mitochondrial fractions. The enzymic activity associated with the particulate fraction appeared to be distributed between the nuclear and mitochondrial fractions. Examination under the phase-contrast microscope showed considerable contamination of the nuclear fraction with mitochondria-like particles, an observation that was confirmed by staining the samples with Janus Green. The mitochondrial fraction appeared to be relatively free of observable contaminating nuclei. Different authors quote different centrifugal forces for the isolation of the nuclear fraction in brain. Thus Brody & Bain (1952) and Heald (1959) centrifuged at 1500g for 10 min., Aldridge & Johnson (1959) at 1000g for 10 min., and Wolfe & McIlwain (1961) at only 800g for 10 min. It might be expected that the degree of mitochondrial contamination of the nuclear fraction would vary according to the centrifugal force.

Accordingly, whole guinea-pig brain was suspended in 0.32 M-sucrose and the nuclear fraction collected by centrifuging at 800, 1000 or 1500g for 10 min., with one wash at the same centrifugal force. In each case the heavy-mitochondrial fraction was collected by centrifuging at 7000g for 15 min., and washed once by centrifuging at the same force. The distribution of enzyme between nuclear, mitochondrial and post-mitochondrial-supernatant fractions was measured for each of these procedures (Table 5). The percentage of recovered activity in the nuclear fraction depended on the centrifugal force at which the nuclear fraction was collected. Between 800 and

Table 5. *Distribution of phosphoprotein phosphatase and nitrogen in nuclear and mitochondrial fractions of guinea-pig brain at different centrifugal forces*

Guinea-pig brain was dispersed in 0.32M-sucrose (pH 6.8, 1.0 mM with respect to magnesium chloride) and samples of the homogenate were centrifuged at 800, 1000 and 1500g respectively for 10 min. and washed once by centrifuging at the same force. The mitochondrial fractions were all collected by centrifuging at 7000g for 15 min. and washed once. Centrifuging was carried out in the M.S.E. 'Major' refrigerated centrifuge. Phosphoprotein phosphatase and nitrogen were determined in the separated fractions as described in the Experimental section. In Expts. A, B and C, the nuclear fraction was collected by centrifuging at 0.8 N 10, 1.0 N 10 and 1.5 N 10 respectively.

Fraction	Expt. A		Expt. B		Expt. C	
	Activity (%)	Nitrogen (%)	Activity (%)	Nitrogen (%)	Activity (%)	Nitrogen (%)
Nuclear	3	14	7	20	15	27
Mitochondrial	33	40	28	37	18	31
Post-mitochondrial supernatant	64	46	65	43	67	42

Fig. 1. Phosphoprotein phosphatase, succinate dehydrogenase and nitrogen in submitochondrial fractions. Sub-mitochondrial fractions of guinea-pig cortex were prepared as described in the text: a once-washed mitochondrial fraction in 0.88M-sucrose (1 ml.) was layered over a gradient composed of 1.0, 1.2, 1.4 and 1.6M-sucrose and centrifuged for 1 hr. at 173 000g in the SW 39 head of the Spinco. The fractions were separated by piercing the bottom of the tubes with a fine hypodermic needle and collecting the sucrose layers in graduated tubes surrounded with ice. Phosphoprotein phosphatase (■), succinate dehydrogenase (▨) and nitrogen (□) were determined in the sub-fractions as described in the Experimental section.

1500g the percentage of nitrogen and of phosphoprotein-phosphatase activity in the nuclear fraction increased with increasing centrifugal force of collection. Examination of the suspensions under the phase-contrast microscope showed that the nuclear fractions collected at the greater forces appeared, qualitatively, to be considerably more contaminated with mitochondria than were those obtained at the lower forces.

Density-gradient centrifuging of nuclear and mitochondrial fractions. The distribution of the enzyme within the nuclear and mitochondrial fractions was further examined by density-gradient centrifuging, as described in the Experimental section, and phosphoprotein-phosphatase, succinate dehydrogenase and nitrogen concentrations were determined on the separated subfractions (Fig. 1). In Fig. 1, the

Fig. 2. Phosphoprotein phosphatase and nitrogen in sub-fractions of a preparation of guinea-pig cortex nuclei. A thrice-washed nuclear preparation of guinea-pig cortex, made by centrifuging at 800g and resuspended in 0.32M-sucrose, was layered over 15 ml. portions of 1.0M-sucrose and centrifuged for 1 hr. at 90 000g in the SW 25 head of the Spinco. Phosphoprotein phosphatase (■) and nitrogen (□) were determined in the fractions passing and failing to pass through 1.0M-sucrose as described in the Experimental section.

percentages are calculated on the basis of total recovered activity, and they are represented for phosphoprotein phosphatase, 92–98 %, for succinate dehydrogenase, 75–85 %, and for nitrogen, 87–103 %, of the original mitochondrial activity. Little separation of the three measured components was observed.

Subfractionation of the nuclear fraction. For the cortical nuclear fraction, the procedure adopted was that of Heald (1959), and is described in the Experimental section.

Two fractions were collected from this preparation. That fraction not passing through the 1.0M-sucrose interface, opalescent in appearance, appeared under the phase-contrast microscope to

consist mainly of nuclei, with some mitochondrial contamination. That passing through 1.0M-sucrose collected as a pellet at the bottom of the tube, and contained red blood corpuscles, cell debris, and a sizable number of mitochondria-like particles. The mitochondria in both fractions could be stained with Janus Green.

Determination of phosphoprotein-phosphatase

Fig. 3. Further subfractionation of nuclear fraction passing through 1.0M-sucrose. The nuclear subfraction passing through 1.0M-sucrose was resuspended in 0.88M-sucrose, layered over a gradient of 1.0, 1.2, 1.4 and 1.6M-sucrose and centrifuged for 1 hr. at 173 000g in the SW 39 head of the Spinco. Phosphoprotein phosphatase (■) and nitrogen (□) were determined in the separated subfractions as described in the Experimental section.

Fig. 4. Distribution of phosphoprotein phosphatase, phosphoprotein and nitrogen in subcellular fractions of guinea-pig cortex. Phosphoprotein phosphatase (▨) and nitrogen (□) concentrations were determined in subcellular fractions, prepared as described in Table 4 and in the text, by the methods described in the Experimental section. Phosphoprotein (■), determined as described in the Experimental section, was estimated in fractions prepared with sucrose-EDTA, under identical conditions of centrifuging; results are the mean of five experiments performed by Dr A. Schwartz. The quoted figures for phosphoprotein represent a recovery of 80–85% of that initially present; other recoveries were 94–106%.

activity in these two fractions revealed (Fig. 2) that 85% of the activity passed through 1.0M-sucrose, though only about half the nitrogen did so.

The fraction passing through 1.0M-sucrose was further subfractionated by layering it, suspended in 0.88M-sucrose, over 1.0, 1.2, 1.4 and 1.6M-sucrose in the tubes of the SW 39 head of the Spinco as described for the mitochondrial subfractionation, and centrifuging for 1 hr. at 173 000g. The distribution of phosphoprotein phosphatase and of nitrogen in these subfractions is shown in Fig. 3. Nearly half the recovered enzymic activity, representing 44% of the activity initially present in the nuclear fraction, failed to pass through 1.2M-sucrose; this fraction, when viewed under the phase-contrast microscope, consisted mainly of mitochondria-like particles. The denser fractions contained quantities of cell debris and red blood corpuscles, some passing through 1.6M-sucrose.

Comparison of phosphoprotein and of phosphatase distribution. It was not possible to measure both phosphatase and phosphoprotein directly in the same samples, because under conditions which preserve the phosphatase considerable degradation of its substrate occurs during the fractionation. The Mg^{2+} ions present in the sucrose (Hanson & Toschi, 1960) act an activator for phosphoprotein phosphatase (Rose & Heald, 1961). In one experiment in which the phosphoprotein was measured in subcellular fractions prepared under these conditions, only 30% of the phosphoprotein present in the original cortex dispersion could subsequently be recovered. These difficulties led Heald (1959) to add EDTA to the sucrose used for extraction. EDTA functions as an inhibitor of the phosphatase (Rose & Heald, 1961), and under these conditions 60% or more of the original phosphoprotein could be recovered in the subcellular preparations. More recently similar methods have yielded recoveries of up to 85% (A. Schwartz & R. Rodnight, unpublished work).

As the two concentrations could not be compared directly, the figures obtained for the phosphatase have been compared (Fig. 4) with those found by Dr Schwartz for phosphoprotein in fractions isolated under identical centrifugal conditions, but in the absence of Mg^{2+} ions and in the presence of EDTA (0.5–5 mM) as an enzymic inhibitor (A. Schwartz & R. Rodnight, unpublished work).

DISCUSSION

Table 1 shows clearly that phosphoprotein-phosphatase activity was present in grey matter of ox brain in concentrations more than twice that found in white matter. The disparity between the activities of the cerebral and cerebellar cortex and caudate nucleus on the one hand, and the medulla,

floor of the IVth ventricle and the corpus callosum on the other, was confirmed by the finding that areas composed of both grey matter and white matter, namely the thalamus, putamen and globus pallidus, showed enzymic activities midway between those of the other two groups.

Few major cerebral components except for RNA are significantly more concentrated in grey than in white matter (Folch, Ascoli, Lees, Meath & LeBaron, 1951; Rossiter, 1955; McIlwain, 1959). Many enzymes, however, differ widely in activity between different areas of brain (Table 2). Species variation and discrepancies in the treatment of tissues in the references quoted make direct comparisons difficult to draw, but the pattern of distribution shown by the respiratory enzymes (Himwich & Fazekas, 1939), for acid and alkaline phosphatase (Gordon, 1953) and for phosphoprotein phosphatase are not dissimilar, though very different distributions characterize choline acetylase (Feldberg & Vogt, 1948), cholinesterase (Burgen & Chipman, 1951) and pyrophosphatase (Gordon, 1953).

This parallel distribution cannot be regarded as so meaningful as the results obtained by the more refined techniques of single-cell analysis (Strominger & Lowry, 1955; Lowry, Roberts & Chang, 1956), or the 'chemoarchitectonic' examination of series of frozen cortex slices for concentrations of enzymic activity (Scharrer & Sinden, 1949; Robins, Smith & Edyt, 1956; Pope, 1960; Hess & Pope, 1959, 1960), but it is indicative of a general relationship; phosphoprotein phosphatase is concentrated in those areas of the brain where respiratory, and hence overall metabolic activity, is at its maximum.

More significant are the findings with respect to the intracellular localization of the enzyme. The results given in Tables 2-5 and Figs. 1-3 enable the following conclusions to be drawn: (a) The distribution of phosphoprotein phosphatase between subcellular fractions of whole brain and of cerebral cortex was not significantly different (Table 3). (b) In both whole brain and in cortex some 60% of the activity was localized in the supernatant which remained after centrifuging for 9×10^6 g min., and 32% was present in the mitochondrial and nuclear fractions (Table 3). (c) The distribution between soluble and particulate fractions was almost completely independent of the method or duration of the extractive procedures (Table 4). (d) The distribution of enzyme between mitochondrial and nuclear fractions depended on the centrifugal force at which the nuclear fraction was collected (Table 5). (e) Density-gradient centrifuging revealed that only 10% of the activity initially associated with the nuclear fraction could not be separated from the nuclei themselves, and that even the purer nuclear fraction obtained by

density-gradient centrifuging contained some mitochondria visible under the phase-contrast microscope and capable of being stained with Janus Green (Fig. 2). (f) Further layering of the nuclear subfraction resulted in a concentration of activity in the mitochondria-rich fraction not passing through 1.2 M-sucrose (Fig. 3). (g) Within the mitochondrial fraction, density-gradient centrifuging could not separate phosphoprotein-phosphatase and succinate-dehydrogenase activities (Fig. 1).

In view of the uncertainty associated with assay methods for succinate dehydrogenase (Johnson, 1960; A. Schwartz, personal communication), caution must be expressed with the conclusions reached in (g). In the absence of contrary evidence, and in view of the convincing separation of phosphatase activity from the nuclei and its subsequent appearance in a mitochondria-containing fraction, there seems no reason to doubt it. However, the absence of succinate-dehydrogenase activity in some of his subfractions led Heald (1959) to question the allocation of phosphoprotein to the mitochondria, and this issue cannot be taken as conclusively settled.

However, the evidence presented above points to a bimodal distribution for the enzyme of approximately 60% in the post-microsomal supernatant and 40% in the mitochondria. During enzyme purification (Rose & Heald, 1961) these two fractions behave identically, and preliminary results suggest that no distinction in enzymic properties can be found.

Paigen & Griffiths (1959) found that, with mouse-liver phosphoprotein phosphatase, 70% was present in the lysosomal fraction and 30% in the soluble supernatant, and that the particulate fraction differed from the soluble in having a higher specific activity and in being inhibited by molybdate. The particulate enzyme behaved in a typically lysosomal manner. The differences found for the cerebral enzyme in this respect may be taken as yet another factor distinguishing it from the spleen and liver phosphoprotein phosphatases of Paigen & Griffiths (1959), Singer & Fruton (1957), Hofman (1958) and Revel & Racker (1960). These distinctions have been discussed in greater detail by Rose & Heald (1961).

The question may be asked: how far does the distribution found for the phosphatase resemble that of its substrate phosphoprotein? Phosphoprotein is uniformly distributed between grey matter and white matter, in a concentration of 2-3 mg. of phosphoprotein phosphorus/100 g. of tissue (Rossiter, 1955; McIlwain, 1959). However, 'phosphoprotein' is an omnibus term used to describe all that phosphorus-containing material isolated by the fractionation procedures of Schmidt & Thannhauser (1945) or of Schneider (1946) that is

not otherwise named. Certainly it contains more than one protein (Heald, 1959, 1961). The studies which led to the formulation of the 'phosphoprotein cycle' [see Heald (1960) for references] were performed with cortical material, and the extent to which phosphoprotein turnover occurs in the white matter of brain has not been similarly examined. It may well be that the phosphoprotein which undergoes cyclical phosphorylation and dephosphorylation (Heald, 1960; Rabinowitz & Lipmann, 1960) represents a comparatively small proportion of the total cellular phosphoprotein and that the remainder is metabolically more inert. This active fraction could be unequally distributed between grey matter and white matter.

A comparison of the concentrations of phosphoprotein and of phosphatase in different subcellular fractions, however, reveals a considerable similarity of distribution, despite the unavoidable losses that occur during fractionation of the phosphoprotein (Fig. 4). Phosphoprotein, like the phosphatase, shows a bimodal distribution between soluble-supernatant and (mitochondrial + nuclear) fractions. Heald (1959) was able further to separate phosphoprotein from the nuclei by density-gradient centrifuging, and subfractionation led to a concentration of the phosphoprotein amongst those particles failing to pass through 1.2 M-sucrose. However, the fact that Heald was unable to measure any succinate-dehydrogenase activity in this fraction led him to postulate that the phosphoprotein was present in a class of particles distinct both from the nuclei and from the mitochondria. The deficiencies in the method of assay of succinate dehydrogenase, to which reference has been made above, which render its use as a mitochondrial marker uncertain, must mean that this conclusion is at best tentative. At the least, Heald's subfractionation, despite the fact that he obtained his nuclear fraction by centrifuging at 1500 rather than at 800g, and that severe losses of material occurred during the centrifuging, led to a concentration of phosphoprotein in the same subfraction in which the phosphatase has been found (Figs. 2 and 3).

It is suggested that the evidence presented here points to a similarity of gross distribution between phosphoprotein phosphatase and the enzymes of respiration, and to a close relationship between cortical phosphoprotein and its phosphatase. This relationship, taken in conjunction with the evidence put forward by Rose & Heald (1961) that phosphoprotein phosphatase requires a stabilizing material of a protein or lipoprotein nature, suggests that *in vivo* the phosphoprotein substrate of the enzyme might fulfil this function, and indicates that the metabolic role of the enzyme and its substrate are intimately related.

SUMMARY

1. The distribution of phosphoprotein phosphatase in various regions of ox brain has been measured. Activity was high in the parietal and cerebellar cortex and in the caudate nucleus, low in the corpus callosum, the floor of the IVth ventricle and the medulla, and intermediate in the thalamus, putamen and globus pallidus. This distribution has been compared with that found by other authors for a variety of cerebral enzymes.

2. The subcellular localization of phosphoprotein phosphatase in guinea-pig cortex and whole brain has been examined. The enzyme showed a bimodal distribution, 56–60 % of the activity being present in the soluble supernatant and 20–30 % in the mitochondrial fraction. The activity present in the nuclear fraction (10 %) could be separated from the nuclei by density-gradient centrifuging and concentrated in a mitochondria-rich subfraction. Small amounts of activity in the light-mitochondrial and microsomal fractions were attributed to contamination with mitochondria or with supernatant. No separation of phosphoprotein-phosphatase and succinate-dehydrogenase activities within the mitochondrial fraction was obtained by density-gradient centrifuging.

3. The similarity of distribution of phosphatase and of phosphoprotein suggests a close relationship of enzyme and substrate *in vivo*.

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. Interaction of some Disaccharides with the Carrier System for Aldoses in Erythrocytes

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At low temperature (0°), when the transport of sugars into erythrocytes is markedly suppressed, exchange transfer of aldoses can take place (Lacko, Burger, Hejmová & Rejnková, 1960). The mechanism of the exchange reaction is visualized as $\text{sugar}_1\text{-carrier} + \text{sugar}_2 = \text{sugar}_2\text{-carrier} + \text{sugar}_1$. Because it operates at 0° it is thought to involve lower activation energy than the reaction $\text{sugar} + \text{carrier} = \text{sugar-carrier}$ (Burger, 1960). Lacko & Burger (1961) used the exchange transfer to study in detail the requirements of the common carrier for aldoses in human erythrocytes with respect to the configuration of transported sugars.

In the present paper we have used exchange transfer to study the interaction with the carrier of some disaccharides which are assumed not to cross the erythrocyte membrane (Tschau-Tjiän Yang, 1950).

MATERIALS AND METHODS

Experimental procedure. Human blood (4 vol.) was diluted with 1 vol. of a solution of 35 g. of glucose, 11 g. of trisodium citrate and 4 g. of citric acid in 1000 ml. of water, and, after 30 min. at room temperature, erythrocytes were separated by centrifuging; in this way erythrocytes were obtained containing 4–5 mg. of glucose/ml. A portion (1 ml.) of these erythrocytes was then suspended in 5 ml. of incubation medium containing 25 mg. of galactose in (unless stated otherwise) 83 mm solution of disaccharide. Iso-osmoticity of the incubation medium was preserved by adding the appropriate amount of sodium chloride. The temperature of incubation was 0°. After 1, 7, 13 and 20 min. erythrocytes were separated from the medium and analysed for glucose and galactose.

Separation of erythrocytes from the incubation medium. Samples (1 ml.) of the erythrocyte suspension were filtered through a layer of Hyflo Super Cel placed on a sintered-glass filter. After the incubation medium was removed by