the absence of added alkali. With more dilute solutions and with solutions of the hydrochloride it is necessary to add alkali. For purposes of estimation by the colorimetric method of Reissig *et al.* (1955) for *N*-acetylglucosamine, the borate buffer employed for chromogen formation is sufficiently alkaline. If present in molar excess, relative to acetic anhydride, sodium hydroxide prevents acetylation by competition with the amino group.

Acetylation of the amino group in glucosamine with acetic anhydride in aqueous solution seems to be almost instantaneous. In their method for the estimation of hexosamine, based on this reaction, Roseman & Daffner (1956) employ sodium bicarbonate as the catalyst. After 10 min. at room temperature, excess of acetic anhydride is destroyed by heating to 100°. Sodium carbonate is then added for the determination of *N*-acetylglucosamine by the procedure of Aminoff *et al.* (1952).

SUMMARY

1. Glucosamine and galactosamine can be determined by the method of Reissig, *et al.* (1955) for the *N*-acetylated hexosamines by preliminary treatment in aqueous solution with acetic anhydride. The borate buffer employed for chromogen formation catalyses the *N*-acetylation of the hexosamines.

2. *N*-Acetylglucosamine can be prepared from glucosamine base by treatment of aqueous solu-

tions with acetic anhydride. If glucosamine hydrochloride is employed alkali must also be added.

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Studies on the Phosphoproteins of Brain: the Intracellular Localization in Brain of a Phosphoprotein Involved in the Metabolic Response of Cortical Slices to Electrical Stimulation

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The application of electrical pulses for 10 sec. to slices of cerebral cortex which had metabolized radioactive phosphate for a few minutes resulted in marked changes in the distribution of radioactivity in phosphocreatine, adenosine triphosphate and in the phosphoprotein fraction (Heald, 1956, 1957). Under these conditions, the specific radioactivity of phosphocreatine and the γ -phosphorus of adenosine triphosphate decreased (Heald, 1956), whereas that of the phosphoprotein phosphorus increased (Heald, 1957). Subsequently it

was found that acid hydrolysis of the radioactive phosphoprotein fraction yielded only radioactive phosphorylserine (Heald, 1958).

Sequences illustrating ways in which such changes in phosphates may be induced by electrical pulses have been outlined by McIlwain & Gore (1953) and Heald (1956). Briefly, pulses are considered to alter structures involved in the transport of ions from one membrane surface to another, and the accompanying changes induced in the phosphates are considered to reflect the increased

energy expenditure necessary to restore the disturbed ionic balance. Present evidence indicates that phosphoprotein is more closely associated with the structures changed by pulses than is a phosphate such as adenosine triphosphate.

In such sequences phosphoprotein is assigned a role either similar to that of a phosphotransferase or as an intermediate in the transfer of energy-rich phosphorus, and not that of a simple acceptor of phosphate in a system in which the activity of the enzymic phosphorylating mechanisms has been increased by the experimental procedure. This view is supported by the finding that there is no change in the total quantity of phosphoprotein phosphorus accompanying the increase in specific radioactivity resulting from the application of pulses (Heald, 1957).

Enzymes known to yield phosphorylserine on acid hydrolysis, and to become phosphorylated during their action, are yeast hexokinase (Agren & Engstrom, 1956) and liver phosphorylase (Rall, Sutherland & Wosilait, 1956; Wosilait, 1958). Other phosphorylated proteins which are enzymes include pepsin (see Neurath, 1957) and phosphoglucomutase (Sibbury & Najjar, 1957). Suggestions that the phosphoproteins of brain may be of this type have been made by Engelhardt & Lissovskaya (1953) and Rossiter (1955).

On the other hand, the phosphoprotein increasing in radioactivity as a result of application of pulses to cerebral tissues may be non-enzymic and occupy a position as a source of energy-rich phosphorus in the brain. In this respect it could be analogous to phosvitin, which has been shown to be capable of phosphorylating adenosine diphosphate to the triphosphate in the presence of a yeast phosphokinase, and of being phosphorylated in the reverse reaction (Rabinowitz & Lipmann, 1958). This is considered to be a more probable role of phosphoprotein in brain, for the quantities of phosphoprotein phosphorus contributed by enzymes such as those described above, assuming a similarity with brain enzymes, can be calculated to be not more than 1–2% of the total quantity of phosphoprotein phosphorus found.

In either case the knowledge that the phosphoprotein fraction exhibits an increased turnover of phosphorus in response to a very brief application of pulses to the tissue makes it of interest to examine its nature and metabolism. At the start of this work no evidence was available to indicate that the phosphorus of the phosphoprotein fraction was derived from other than a single substance. As a preliminary to the attempted isolation of the phosphoprotein it was therefore considered desirable to investigate this point, as far as possible, and to discover the subcellular fraction containing the phosphoprotein with a view to obtaining a starting material reasonably free from other contaminants.

The results described below show that brain contains at least two phosphoprotein fractions, in only one of which the phosphorus increases in specific radioactivity when electrical pulses are applied to the intact tissue.

MATERIALS AND METHODS

Tissues and media. Slices of guinea-pig cerebral cortex were prepared as described by McIlwain (1951) and incubated in medium buffered with 2-amino-2-hydroxy-methylpropane-1:3-diol, from which added phosphate was absent (Heald, 1956). Slices of 100–150 mg. wet wt. were incubated in 3.5 ml. of medium in conventional Warburg manometric flasks under an atmosphere of oxygen at 37.5°, in the presence of 10–20 μ C of carrier-free radioactive orthophosphate (The Radiochemical Centre, Amersham, Bucks). Incubations were usually for 60–90 min., at the end of which the contents of the manometric flasks were either tipped into an open dish and the slices rapidly transferred to 10 vol. of ice-cold sucrose solution, or, more frequently, tipped into a large tube cooled in ice, and briefly centrifuged and the tissue residue was dispersed in 10 vol. of sucrose solution. The usual medium for preparing the dispersions was sucrose (0.25M) containing ethylenediaminetetra-acetate (EDTA) (0.5 mM), adjusted to pH 7.4 with NaOH solution. In experiments *in vivo* the radioactive phosphate was injected intracisternally by a technique similar to that of Strickland (1952). The animals were lightly anaesthetized with ether before puncture of the cisterna magna; about 0.2 ml. of fluid was withdrawn and was replaced by a solution of radioactive phosphate injected through the same needle. After 3 hr. the animal was killed, the brain removed and the cortex, together with some underlying white matter, was weighed and dispersed in 10 vol. of sucrose-EDTA. All subsequent operations with sucrose suspensions were at 0–2°.

For short-term experiments, in which electrical pulses were applied for brief periods, the apparatus and techniques have been described (Heald, 1956). In experiments *in vivo* in which animals were frozen in liquid nitrogen before removal of the brain, the procedure was similar to that described by Dawson & Richter (1950) and Kratzing & Narayanaswami (1953).

Dispersion of the tissue. Since the biochemical integrity of subcellular particles is affected by the method of producing the initial dispersions of the tissue (Aldridge, 1957), in the present experiments all dispersions were made with a polythene pestle constructed according to the method of Kamphausen & Morton (1956). Such pestles fitted the tubes tightly and produced excellent dispersions, in which few intact cells were visible when viewed under phase contrast but in which the nuclei were maintained largely intact. This was taken to indicate that little general mechanical disruption of the particles had taken place during the initial stage of dispersion of the tissue.

Fractionation of dispersions. The sucrose dispersions were initially fractionated by differential centrifuging mainly according to the procedure of Brody & Bain (1952). Dispersions were first centrifuged for 10 min. at 1500 *g* to obtain the 'nuclear fraction', which was washed by resuspending it in sucrose-EDTA and recentrifuging for 10 min. at 1500 *g*. The supernatants were centrifuged either at 104 000 *g* for 30 min., to obtain a composite

fraction of mitochondria and microsomes, or else centrifuged first at 12 000 *g* for 25 min., to obtain the mitochondrial fraction, and then at 104 000 *g* for 30 min. to yield the microsomal fraction and a clear supernatant. The nuclear fraction was further subfractionated by centrifuging in various sucrose density gradients as indicated in the figures and text. This procedure was carried out in the S.W. 39 head of the Spinco ultracentrifuge. Other centrifugings were performed in the no. 40 head of this instrument. All fractions were treated with trichloroacetic acid to a concentration of 10% (w/v) before obtaining the phosphoprotein.

Succinic dehydrogenase. This was determined in various subcellular fractions by the method of Schneider & Potter (1943).

Total nitrogen. This was determined after digestion of the fraction by the semi-micro procedure and catalyst of Chibnall, Rees & Williams (1943). The final distillation was carried out in the Markham (1942) still, with boric acid solution (Conway, 1947) as the trapping agent.

Protein. This was estimated by the method of Lowry, Rosebrough, Farr & Randall (1951) with amorphous albumin fraction IV (L. Light and Co. Ltd.) as a standard.

Phosphoprotein. The quantitative determination of phosphoprotein as inorganic phosphorus and determinations of its specific radioactivity were performed as described previously (Heald, 1957), by the method for preparing the phosphoprotein fraction described in that paper. Extensive use has also been made of the finding (Heald, 1958) that the phosphoprotein fraction sought yielded phosphorylserine upon acid hydrolysis. Preparations of crude phosphoprotein obtained from tissue slices which had been allowed to metabolize radioactive phosphate were hydrolysed for 8–10 hr. at 100° in 2*N*-HCl. The hydrolysates were freeze-dried and the residues, in 0.05*N*-HCl, were fractionated upon a column of Dowex-50 as described by Schaffer, May & Summerson (1953). Inactive phosphorylserine was added as a carrier. Since the position of phosphorylserine in eluates from such a column was highly reproducible, and it appeared to be the only major radioactive peak other than inorganic phosphate, counts due to the presence of radioactive phosphorylserine could be readily detected. In any one experiment where the distribution of the phosphoprotein in different fractions was being investigated, all hydrolyses were performed at the same time in the same heating bath. Under these conditions it was assumed that the partial hydrolysis of phosphorylserine would be the same in all fractions. Within such an experiment the results obtained are thus comparable with one another. Comparison between different experiments was made on a percentage basis, since in different experiments differing tissue weights and quantities of radioactive phosphate were used.

Acid and alkaline phosphatase activity. These were determined by the method of King & Armstrong, as described by Harrison (1947), disodium phenylphosphate being used as substrate.

RESULTS

In initial experiments tissues were dispersed in sucrose-EDTA solutions, the dispersions were centrifuged to yield the supernatant and particulate fractions and the radioactivity of the phos-

Table 1. *Distribution of phosphoprotein between the particulate and supernatant fractions of guinea-pig-brain dispersions in vitro and in vivo*

Tissues were dispersed in 0.25*M*-sucrose-0.5 *mm*-EDTA. Phosphoprotein was determined as radioactive phosphorylserine. For details see Materials and Methods section. Values are given $\pm$ s.d. Figures in parentheses are the number of determinations.

Tissue	Fraction	Nitrogen		Protein		Phosphoprotein (%)		Phosphoprotein (%)	
		(mg./g. wet wt.)	(% of total)	(mg./g. wet wt.)	(% of total)	(% of total)	(% of total)	Nitrogen (%)	Protein (%)
Slices incubated <i>in vitro</i>	Insoluble	13.87 $\pm$ 1 (4)	64.6 $\pm$ 1.5	57.8 $\pm$ 4 (4)	78.1 $\pm$ 5.1	87.2 $\pm$ 2.7 (10)	1.35	1.12	
	Soluble	7.2 $\pm$ 2 (4)	35.4 $\pm$ 5.6	21.9 $\pm$ 5 (3)	21.9 $\pm$ 2.20	12.8 $\pm$ 2.4 (10)	0.36	0.58	
Cortex, <i>in vivo</i>	Insoluble	12.39 $\pm$ 0.2 (3)	62.1 $\pm$ 1.6	49.3 $\pm$ 8.5 (6)	60.1 $\pm$ 11.2	44.0 $\pm$ 5.6 (6)	0.66	0.68	
	Soluble	7.80 $\pm$ 1.4 (3)	37.9 $\pm$ 3.8	27.2 $\pm$ 4.6 (6)	39.2 $\pm$ 9.0	56.0 $\pm$ 5.5 (6)	1.56	1.48	

phorylserine derived from the phosphoprotein fraction was determined. Experiments were performed both with slices of tissue which had been incubated in medium containing radioactive phosphorus and with brain cortex from guinea pigs which had been injected, *in vivo*, intracisternally with radioactive phosphorus some 2–3 hr. before removal of the brain. The experiments *in vivo* were originally included as a check upon the distribution of phosphoprotein found in slices *in vitro*, for it was thought that incubation of tissues in medium might have caused changes in the slices, sufficient to alter the distribution of the phosphoprotein. The results are presented in Table 1.

With slices most of the radioactivity arising from the phosphoprotein was present in the particulate fraction, being concentrated in terms of nitrogen or protein some three to four times that in the soluble fraction. *In vivo* the reverse situation obtained, the radioactivity from phosphoprotein being highest in the supernatant fraction, where it was concentrated in terms of nitrogen or protein some two times that in the insoluble fraction. During experiments *in vivo*, removal and weighing of the brain occupied some 4–5 min. before it was dispersed, as opposed

to 10 sec. for a similar transfer of slices from the incubation medium to the sucrose solution. It was therefore considered possible that some rapid enzymic action after death might be releasing phosphoprotein from a more firmly bound form, which was later reformed during metabolism *in vitro*. Experiments were carried out in which the brain was frozen *in vivo* in liquid nitrogen before being chiselled out and fractionated. This procedure had little effect upon the distribution of radioactivity (Table 2). It was also found that dispersion of tissues, from experiments *in vivo*, in sucrose solution containing 0.1 M-KCl, did not alter the distribution of radioactivity sufficiently to suggest that the presence of medium, carried with the slices during transfer from the incubation medium to sucrose solution, caused enough agglutination of the soluble phosphoprotein to account for the distribution found with slices incubated *in vitro*.

Conversely, with brain slices, freezing the tissue before dispersion, or applying brief electrical pulses to simulate the stimulus to the brain upon death, was without appreciable effect upon the distribution of radioactive phosphoprotein (Table 3). The

Table 2. *Distribution of phosphoprotein between the soluble and particulate fractions of dispersions of guinea-pig cerebral cortex obtained in vivo*

Animals were injected intracisternally with radioactive phosphorus 2–3 hr. before death. Tissues were dispersed in 0.25 M-sucrose with additions detailed below. Phosphoprotein was determined as radioactive phosphorylserine.

Expt. no.	Treatment of tissue	Phosphoprotein (counts/min.)		Phosphoprotein (% of total)	
		Residue	Supernatant	Residue	Supernatant
1	Brain removed and dispersed in 0.25 M-sucrose containing EDTA (5 mM)	2900	4232	41.2	58.8
2	Brain removed and dispersed:				
	(a) in 0.25 M-sucrose	2197	4183	34.3	65.7
	(b) in 0.25 M-sucrose + 0.1 M-KCl	1260	960	56.5	43.5
3	Brains frozen <i>in vivo</i> in liquid nitrogen	810	1640	33.0	67.0
		1430	3097	33.2	66.8
	Dispersed in 0.25 M-sucrose	460	611	43.0	57.0

Table 3. *Effect of different treatment of slices of guinea-pig-brain cortex upon the distribution of phosphoprotein between the soluble and particulate fractions*

Slices were incubated in medium containing radioactive orthophosphate and were dispersed in 0.25 M-sucrose containing additions as stated. Where electrical pulses were passed these were condenser pulses, 15 v peak potential, duration 0.4–0.5 msec. Phosphoprotein was determined as radioactive phosphorylserine.

Expt. no.	Treatment of tissue	Radioactivity in phosphoprotein (counts/min.)		Radioactivity in phosphoprotein (% of total)	
		Residue	Supernatant	Residue	Supernatant
1	Dispersed in sucrose	7757	1109	87.5	12.5
2	Dispersed in sucrose and EDTA (50 mM)	3877	623	85.0	15.0
3	Dispersed in water	1458	1516	49.0	51.0
4	Electrical pulses applied for 10 sec. before dispersion	3549	320	92.0	8.0
5	Slices frozen in solid CO ₂ for 5 min. before dispersion	2260	657	78.0	22.0

Table 4. *Effect of incubating guinea-pig-brain slices in medium upon the quantities of phosphoprotein present in the soluble and particulate fractions of the tissue*

Slices were incubated in medium for 30 min. before being removed and dispersed in 0.25M-sucrose-0.5 mM-EDTA. Analyses were as described in the Materials and Methods section. Values for phosphoprotein are in μ moles of P/g. wet wt. of tissue. Values for protein are in mg./g. wet wt. of tissue. Figures in parentheses indicate the number of experiments. Protein values are averages of two determinations.

Fraction	Before incubation		After incubation		Change	
	Phosphoprotein	Protein	Phosphoprotein	Protein	Phosphoprotein	Protein
Particulate	0.57 $\pm$ 0.1 (4)	91	0.48 $\pm$ 0.07 (4)	99	-0.09 $\pm$ 0.01 (4)	+8
Soluble	0.40 $\pm$ 0.01 (4)	42.1	0.22 $\pm$ 0.01 (4)	25.6	-0.18 $\pm$ 0.01 (4)	-16.5
Medium	0.0	0.0	0.17 $\pm$ 0.008 (4)	21.5	+0.17 $\pm$ 0.008 (4)	+21.5
Total	0.97 $\pm$ 0.12 (4)	133.1	0.90 $\pm$ 0.10 (4)	146.1	-0.12 $\pm$ 0.07 (4)	+13

inclusion of EDTA (50 mM) in the sucrose solution likewise did not result in more of the radioactivity passing to the solution, indicating again that the greater concentration of radioactive phosphoprotein in the particulate fraction was not due simply to a precipitation by salts carried from the incubation medium. Even when the tissues were dispersed in water no more than 50 % of the total radioactive phosphoprotein of the slice was found in the supernatant fraction.

These results suggested that during incubation there was either a loss of phosphoprotein from the slice to the medium, or that the incorporation of radioactivity into the supernatant phosphoprotein was poor. Phosphoprotein and total protein were estimated in the supernatant and particulate fractions of cortical slices cut and weighed before incubation and in slices from the same brain after incubation. Similar determinations were also performed upon the incubation medium. There was a loss of some 50 % of the phosphoprotein phosphorus and of protein from the supernatant fraction to the medium during incubation, whereas little change occurred in the quantity of these substances in the particulate fraction (Table 4). This last result confirmed that incubation in medium did not result in an increased quantity of phosphoprotein from the supernatant being precipitated to form part of the particulate fraction. To determine whether the two fractions incorporated phosphate at different rates, slices of brain cortex were incubated with radioactive phosphate for different times and the specific radioactivities of the phosphoprotein present in the two fractions were determined. The result (Table 5) showed that the specific radioactivity of the phosphoprotein in the supernatant fraction was several times lower than that in the particulate fraction. Thus the phosphoprotein in the supernatant fraction incorporated radioactive phosphorus to a lesser degree than that in the particulate fraction. The differences in the distribution of radioactive phosphoprotein in brain tissues *in vivo* and slices *in vitro* is therefore partly

Table 5. *Incorporation of radioactive orthophosphate into the phosphoproteins of the soluble and particulate fractions of guinea-pig-brain slices*

Slices were incubated under oxygen, in medium containing carrier-free radioactive orthophosphate (10 μ Ci/flask). After 15 or 30 min. the slices were removed, dispersed in 0.25M-sucrose-0.5 mM-EDTA and the dispersion was centrifuged for 20 min. at 104 000 g. The residue and supernatant were treated to obtain the phosphoproteins as described in the Materials and Methods section.

Expt. no.	Period of incubation (min.)	Specific radioactivity (counts/min./ μ g. of phosphoprotein P)	
		Residue	Supernatant
1	15	958	218
	30	1700	245
2	15	716	143
	30	1748	505

attributable to a loss of part of the fraction soluble in aqueous sucrose to the medium during incubation of the slices and to a lowered rate of incorporation of phosphate into that remaining in the slices.

Fractionation of the tissue into subcellular fractions. In view of the results described above, all further work was carried out with tissue slices incubated in medium containing radioactive phosphate. After incubation the slices were centrifuged from the medium and dispersed in aqueous sucrose. In each fraction, phosphoprotein was determined as radioactive phosphorylserine. Of the total radioactivity 66 % was present in the nuclear fraction, where its concentration was some two times that of other fractions (Fig. 1). The amount present in the mitochondrial fraction probably represented the degree of contamination with particles from the nuclear fraction.

To determine whether the nuclear fraction actually contained the phosphoprotein sought, tissues were incubated for a brief period of 3 min. in the presence of radioactive orthophosphate, and washed and reincubated in fresh medium for 2 min.

and then subjected to electrical pulses for 10 sec. This procedure is identical with that previously used to demonstrate the participation of phosphoproteins in the increased metabolic response of brain tissue to an electrical stimulus. After stimulation the tissues were rapidly transferred to ice-cold sucrose-EDTA solution, and dispersed, and the nuclear fraction separated. The major increase in specific radioactivity occurred in the phosphoprotein present in the nuclear fraction (Table 6), whereas a small and variable change took place in the phosphoprotein of the supernatant, which also contained the mitochondria and microsomes. It was clear that the phosphoprotein involved in the metabolic response to pulses resided largely in the nuclear fraction.

Fig. 1. Distribution of phosphoprotein radioactivity and nitrogen in subcellular fractions from guinea-pig-brain slices, after incubation in medium. The 'nuclear' and 'mitochondrial and microsomal' fractions were obtained by centrifuging dispersions, made in 0.25M-sucrose-0.5 mM-EDTA, in integrated fields of 15×10 g min. and 9×10 g min. respectively. Radioactivity in phosphoprotein was determined as described in the Materials and Methods section. ▨, Phosphoprotein; □, nitrogen.

The nuclear fraction was subfractionated by equilibrium centrifuging in a sucrose density gradient (cf. Kuff & Schneider, 1954) in two stages. In the first sub-fractionation the nuclear fraction in 0.25M-sucrose-EDTA was layered over M-sucrose and centrifuged to yield two main fractions. The top layer not entering M-sucrose consisted largely of nuclei and platelets together with some very small particles. The residue consisted of blood vessels, some platelets, myelin fragments and cell debris together with a large number of particles resembling mitochondria in size. This residue contained the bulk of the nitrogen [78.8 ± 2.2 (9) %] and phosphoprotein radioactivity [87.4 ± 3.3 (6) %] of the nuclear fraction, the ratio of the percentage of radioactivity to nitrogen being 1.10. The top layer contained 21.7 ± 1.8 (10) % of nitrogen and 13.0 ± 2.7 (6) % of the phosphoprotein, the ratio of phosphoprotein radioactivity to nitrogen being 0.6. Recoveries of both nitrogen and radioactive phosphoprotein were 100 %, showing that no loss had occurred during this stage of the fractionation procedure. The residue was resuspended in 0.25M-sucrose and layered over a gradient consisting of three successive 1 ml. portions of 1.0M-, 1.2M- and 1.4M-sucrose. The tubes were centrifuged and the various layers removed carefully with a fine Pasteur pipette. This procedure is presented in Fig. 2, together with the distribution of phosphoprotein radioactivity and nitrogen in the fractions obtained. Fraction R_2 contained the highest concentration of phosphoprotein and nitrogen, the ratio of the percentages of these two being 1.05; all the other fractions contained more nitrogen than phosphoprotein. The recovery of nitrogen in this step was 109 %, whereas the recovery of phosphoprotein was only 70 %, which suggests that some metabolism had occurred during the long periods of centrifuging.

In the above-described experiments measurement of the quantities of phosphoprotein had been made on the basis of the radioactivity of the phosphorylserine derived from the phosphoprotein

Table 6. *Effect of electrical pulses on the incorporation of radioactive phosphorus into the phosphoproteins of brain fractions*

Slices of guinea-pig-brain cortex were incubated and exposed to pulses under the conditions described by Heald (1957). Pulses were condenser pulses, 15 v peak potential, 0.4-0.5 msec. duration, applied for 10 sec. The nuclear fraction was separated from dispersions made in 0.25M-sucrose-0.5 mM-EDTA by centrifuging (Brody & Bain, 1952). Phosphoprotein was determined as described in the Materials and Methods section.

Expt. no.	Radioactivity of the phosphoprotein (counts/min./μg. of P)					
	With pulses		Without pulses		Change with pulses	
	Nuclear	Supernatant	Nuclear	Supernatant	Nuclear	Supernatant
1	1770	417	922	445	+748	-45
2	2720	480	1580	362	+1140	+118

present. Since each fraction might have contained phosphoproteins of differing specific radioactivities, measurements of the total radioactivity could not strictly be taken as a measurement of quantity. The validity of this procedure was therefore examined by measuring the specific radioactivity of phosphoprotein phosphorus in the various fractions. Slices of cerebral cortex were incubated with radioactive inorganic phosphorus for different times before fractionation, since it was thought that such a procedure would emphasize whether

the phosphoprotein in any one fraction incorporated radioactive phosphorus to a markedly greater or lesser extent than the others. The results are presented in Table 7. Comparison of specific radioactivities of the different fractions was made by relating them to that of fraction R_2 . Although there was sometimes marked variation in any one experiment the averages of the sums of the ratios for each fraction did not differ significantly. Similar results were obtained if the comparison was made by relating the specific radioactivities to those of fraction R_3 . The results are considered to show that the incorporation of radioactive phosphorus into the phosphoprotein of the individual particulate fractions proceeded to essentially the same extent, and thus the use of radioactivity as a measure of the quantity of phosphoprotein was a valid one. When viewed by phase-contrast microscopy, fraction R_1 consisted of extremely small particles together with some nuclei and platelets; R_2 consisted of mitochondria-like particles and was not homogeneous (particles of many different shapes being visible); R_3 was similar to R_2 except that the particles appeared to be larger, and R_4 contained cellular debris, myelin fragments and blood vessels together with a few small particles. Fraction R_2 was diffused on either side of the boundary between 1.0M- and 1.2M-sucrose layers, which suggested the possibility of further sub-fractionation. This was achieved by resuspension in 0.25M-sucrose and layering of the suspension over 1 ml. portions of 1.0M-, 1.1M- and 1.2M-sucrose. After centrifuging, the fractions were removed and analysed for phosphoprotein and nitrogen. The results, presented in Fig. 3, show that the fraction $R_{2(b)}$ contained the highest concentration of phos-

Fig. 2. Subfractionation, in a sucrose density gradient, of the 'nuclear' fraction (Fig. 1) obtained from guinea-pig-brain slices. (a) Details of concentrations of sucrose and centrifugal fields used; (b) the distribution of phosphoprotein radioactivity and nitrogen amongst the main fractions obtained. ▨, Phosphoprotein; □, nitrogen.

Table 7. Comparison of the relative specific radioactivities of phosphoprotein in different subcellular fractions from cerebral cortex

Slices were incubated in medium with different amounts of radioactive orthophosphate for varying times and were disintegrated and fractionated as described in the Materials and Methods section and in the text. Specific radioactivities were estimated after alkaline degradation of the phosphoprotein to inorganic phosphate as described in the text, with the exception of Expt. 7, in which fractions were acid-hydrolysed to yield phosphorylserine, which was separated on Dowex-50 and wet-ashed to yield inorganic phosphate.

Expt. no.	Period of incubation (min.)	Specific radioactivities of the phosphoprotein in the various fractions (counts/min./μg. of P)				Ratio			
						Specific radioactivity of fraction			
		Nuclei and R_1	R_2	R_3	R_4	Nuclei and R_1	R_2	R_3	R_4
1	3	—	75.5	75.3	65.5	—	1.0	1.0	0.86
2	3	—	1010	667	900	—	1.0	0.67	0.89
3	3	—	1080	904	815	—	1.0	0.84	0.75
4	10	103	200	246	Lost	0.52	1.0	1.17	—
5	10	87.5	72	66	Contaminated	1.22	1.0	0.62	—
6	20	241	475	345	Contaminated	0.51	1.0	0.73	—
7	30	515	422	396	Lost	1.21	1.0	0.94	—
Averages ± S.D.M.	—	—	—	—	—	0.86 ± 0.38	1.0	0.85 ± 0.19	0.83 ± 0.075

phoprotein with the fraction $R_{2(e)}$ containing a somewhat lower concentration. Here also, recoveries of nitrogen were 108 %, whereas the recovery of phosphoprotein was only 80 %. With phase-contrast microscopy, recognition of individual differences between the fractions was difficult; each fraction, however, appeared to be much more homogeneous in the type of particle present and was clearly different from the parent fraction R_2 .

In Table 8 is presented a summary of the recoveries and distribution of phosphoprotein and

nitrogen of the various fractions, compiled from the tables and figures previously given. The overall recovery of nitrogen was 119 %, whereas the recovery of radioactive phosphorylserine was only 55 %. These different recoveries are reflected in the low ratios of phosphorylserine to nitrogen in the last column of Table 8. However, since the degree of metabolism of phosphoprotein by the individual fractions is not known, no corrections can be made for such losses.

Succinic dehydrogenase and phosphatases in fractions R_1 – R_4 . Fractionation of dispersions of brain by the method of Brody & Bain (1952) is known to include some mitochondria in the nuclear fraction. This inclusion is increased when dispersions are made from slices of brain which have been previously incubated in medium. Thus the percentages of nitrogen of the dispersion found in the nuclear and mitochondrial fractions from brain dispersed immediately after removal from the animal were 27.4 and 34.6 % respectively, and the percentages of succinic dehydrogenase in the fractions were 13 and 69 % respectively. Similar fractions from slices incubated *in vitro* gave nitrogen distributions of 47.6 and 16.9 % and a succinic dehydrogenase distribution of 52 and 34 % respectively. It was therefore considered possible that the particles isolated could consist largely of mitochondria. The distribution of succinic dehydrogenase in the fractions R_1 – R_4 is presented in Fig. 4 together with the corresponding figures for the distribution of phosphoprotein. The greatest activity was found in fractions R_3 and R_4 , which contain the least phosphoprotein, whereas in R_1 no succinic dehydrogenase was found in any of three experiments. When a sample of mitochondria, prepared from guinea-pig brain immediately after

Fig. 3. Refractionation of fraction R_2 (Fig. 2) in a sucrose density gradient. (a) Details of the concentrations of sucrose and centrifugal fields used; (b) distribution of phosphoprotein radioactivity and nitrogen in the various fractions. ▨, Phosphoprotein; □, nitrogen.

Table 8. *Distribution of phosphoprotein in various sub-fractions from the 'nuclear' fraction of guinea-pig-brain cerebral slices*

Fractionation procedure was as described in Figs. 1–3. Phosphoprotein was determined as radioactive phosphorylserine as described in the Materials and Methods section. Values are the percentage distribution $\pm$ s.e.m. of nitrogen and phosphoprotein calculated as a percentage of the quantities taken for each stage in the fractionation.

Stage	Fraction	Nitrogen	Counts/min. in	Counts in
		(% of whole original fraction)	phosphorylserine (% of whole original fraction)	phosphorylserine (%) Nitrogen (%)
I	Nuclei	21.7 $\pm$ 1.8 (10)	13.0 $\pm$ 2.7 (6)	0.60
	Residue	78.8 $\pm$ 2.2 (9)	87.4 $\pm$ 3.3 (6)	1.10
	Sum of fractions	100.5	100.4	1.00
II	R_1	17.3 $\pm$ 1.5 (4)	11.1 $\pm$ 3.5 (3)	0.64
	R_2	45.2 $\pm$ 5.1 (4)	42.6 $\pm$ 0.6 (3)	0.92
	R_3	23.8 $\pm$ 5.4 (4)	9.6 $\pm$ 0.6 (3)	0.40
	R_4	23.6 $\pm$ 1.4 (4)	6.6 $\pm$ 2.4 (3)	0.28
	Sum of fractions	109.9	69.9	0.64
III	$R_2(a)$	23.1 $\pm$ 2.6 (3)	11.4 $\pm$ 3.4 (3)	0.49
	$R_2(b)$	23.7 $\pm$ 3.0 (3)	31.2 $\pm$ 1.3 (3)	1.32
	$R_2(c)$	25.8 $\pm$ 1.7 (3)	24.8 $\pm$ 7.6 (3)	0.96
	$R_2(d)$	36.9 $\pm$ 6.7 (3)	11.6 $\pm$ 4.5 (3)	0.76
	Sum of fractions	109.5	79.0	—

removal from the animal, was fractionated in a similar sucrose density gradient, 75 % of the total succinic dehydrogenase recovered was found in the fraction corresponding to R_3 and 25 % in the fraction corresponding to R_2 . Hebb & Whittaker (1958) similarly found that when brain mitochondria were fractionated in a sucrose density gradient, 75 % of the total succinic dehydrogenase recovered was in particles which passed through a layer of 1.2M-sucrose. Values for alkaline and acid phosphatase activity in the fractions are also presented in Fig. 4. Whereas fractions R_1 – R_4 contained acid phosphatase, only fractions R_2 and R_4 contained alkaline phosphatase. The high alkaline phosphatase activity of R_4 probably reflects the quantities of cell debris and small blood vessels present, which are generally found to contain the highest alkaline phosphatase activity in the brain (Feigin & Wolf, 1955).

DISCUSSION

The work described above has shown that in brain there are at least two phosphoprotein fractions of differing metabolic activities as judged by the incorporation of radioactive phosphorus. The fraction most active *in vivo* is soluble in 0.25M-sucrose solution and is partly lost to the medium when tissues are incubated under conditions normal for metabolic studies. The second fraction, least active *in vivo*, is associated with particulate material and undergoes little or no change in quantity when slices of brain are incubated in medium. Under

these conditions this latter phosphoprotein incorporates radioactive phosphorus to a greater degree than does the phosphoprotein soluble in aqueous sucrose. Furthermore, the extent of the incorporation is markedly increased when the slices, from which it is later derived, are briefly stimulated with electrical pulses. Previous work, which had shown that the phosphorus metabolism of a phosphoprotein of brain is closely connected with the response evoked by applied electrical stimulus, was performed with tissue slices *in vitro*, and so far it has been considered desirable to pay attention to the phosphoproteins exhibiting such properties under these conditions rather than to the others, soluble in 0.25M-sucrose solution, for which a similar role has not yet been described.

The fractionation described above has shown that particles containing a high concentration of the phosphoprotein showing an increased incorporation of ^{32}P in response to electrical pulses can be obtained from slices of brain cortex incubated in suitable media under oxygen. The particles do not appear to be mitochondria, for not only are the mitochondria, when isolated as such both *in vivo* and *in vitro*, extremely poor in phosphoprotein, but in the fractions isolated there is no correlation between the content of succinic dehydrogenase, generally regarded as indicative of the presence of mitochondria, and the phosphoprotein content. Indeed, one fraction which contains phosphoprotein does not contain succinic dehydrogenase. It is well recognized that the mitochondrial fraction from brain is heterogeneous, and it has recently been shown to contain a variety of particles, some of which exhibit little or no succinic dehydrogenase activity (Hebb & Whittaker, 1958). Nevertheless, the fact that preparations of mitochondria containing such particles contain little of the phosphoprotein, together with the evidence of the distribution of succinic dehydrogenase presented above, argues against the particles containing the phosphoprotein arising from this source.

The problems of dealing with the quantitative intracellular localization of a metabolite by means of a property dependent upon its metabolism are considerable, not the least being the possibility of rapid degradation during the fractionation procedure. In this respect the phosphoprotein fraction studied seemed to be reasonably stable, a total loss of 50 % occurring during a fractionation occupying some 4–5 hr. This rate is of the order found by Livanova (1957) for the loss of phosphoproteins in liver homogenates at 0°; some 20 % was lost in 2–3 hr. and about 30 % was lost in 5 hr. There is at present no evidence to suggest that any one group of particles metabolizes the endogenous phosphoprotein more rapidly than another. Indeed the distribution of the acid phosphatase, operative at

Fig. 4. Distribution of succinic dehydrogenase, phosphoprotein and acid and alkaline phosphatases in subcellular fractions from guinea-pig-brain cortex. ■, Succinic dehydrogenase; □, phosphoprotein; ▨, alkaline phosphatase; ▩, acid phosphatase. Values for acid and alkaline phosphatase in whole cortical dispersions were 2.4 and 11.4 mg. of phenol liberated/g. wet wt. of tissue under the conditions of the test.

a pH near the optimum for tissue phosphoprotein phosphatases (Livanova, 1957; Sundararajan & Sarma, 1957), might suggest the opposite.

In isolation studies of this type, and particularly with brain, where the content of the phospholipids and lipoproteins is high, the possibility that some small particles may be artifacts, produced by dispersion of the tissue, is always present. For a full assessment of this an examination of the particles by different microscopical techniques and comparison with data obtained by examination of whole tissues would be necessary.

SUMMARY

1. A study has been made of the intracellular localization of the phosphoproteins of brain, and in particular of the phosphoproteins whose incorporation of radioactive phosphate is greatly increased when the intact tissue is excited by electrical pulses.

2. When dispersed in isotonic sucrose solution cerebral tissues have been found to contain two major phosphoprotein fractions, one soluble in sucrose solution, the other insoluble. *In vivo*, the incorporation of intracisternally-injected radioactive phosphate was greatest into the soluble phosphoprotein fraction. With slices of brain *in vitro*, incorporation of radioactive phosphate was greatest into the insoluble phosphoprotein fraction.

3. These differences are due to a loss of most of the soluble phosphoprotein from the slice to the incubating medium and to a decreased rate of incorporation of phosphate into the remainder. Incubation in medium had little or no effect upon the total quantity of the insoluble phosphoprotein.

4. After incubation in medium containing radioactive phosphate, tissue slices have been dispersed in isosmotic sucrose and fractionated by differential centrifuging. The bulk of the phosphoprotein was present in the nuclear fraction, the mitochondrial, microsomal and supernatant fractions containing relatively small amounts. Application of electrical pulses to the tissue slices before fractionation showed that the increased incorporation of radioactive phosphate took place largely in the phosphoprotein of the nuclear fraction.

5. The nuclear fraction has been further sub-fractionated by centrifuging in two sucrose density gradients. The phosphoprotein was concentrated in particles which possess little or no succinic dehydrogenase activity and are not considered to be mitochondria. The acid and alkaline phosphatase activity of the particles has been measured.

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