Studies on the Phosphoproteins of Brain

2. PARTIAL PURIFICATION OF A PHOSPHOPROTEIN ATTACHED TO SUBCELLULAR PARTICLES*

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It has been shown that cerebral tissues contain at least two types of phosphoproteins, one attached to particulate matter separable in low centrifugal fields from dispersions of brain made in iso-osmotic sucrose, and the other remaining in the particulate-free supernatant solution (Heald, 1959). The first type is of particular interest, since the incorporation of radioactive phosphate into this phosphoprotein is greatly increased by the brief passage of electrical pulses through slices of cerebral tissue which are actively metabolizing phosphate in the presence of glucose.

To investigate further the systems involved in the phosphorylation and dephosphorylation of this phosphoprotein, it seemed desirable to have available a more purified fraction than was represented by cerebral particles. This paper describes the extraction and partial purification, from such particles, of a lipophosphoprotein. When incubated in a suitable phosphorylating system the purified fraction acts as a phosphate acceptor.

MATERIALS AND METHODS

Tissues. Slices of guinea-pig cerebral cortex were cut and incubated under conditions and in media described by Heald (1959). Ox brains were obtained, as fresh as possible, from the Metropolitan Cattle Market and were packed in a plastic bag surrounded by ice during transportation to the laboratory.

Extraction of phosphoproteins and preparation of a partially purified fraction. Small-scale experiments were performed with samples of guinea-pig brain. Slices were cut and incubated with radioactive phosphate (20–30 μ C/slice) for 30–60 min., and were then removed and dispersed with two whole guinea-pig cerebral cortices in 10 vol. of iso-osmotic sucrose (0.25 M), containing 0.5 mm-ethylenediaminetetra-acetate (EDTA), pH 7.4, with a homogenizer similar to that described by Kamphausen & Morton (1956). The dispersion was centrifuged at 1500 g for 10 min. and the residue washed twice in the centrifuge with fresh sucrose–EDTA under the same conditions. This residue was then used for testing various extraction procedures. Unincubated cortex was included both to provide sufficient material for separation and in an attempt to

dilute any adverse effect of incubation in the medium upon the sedimentation properties of the particles isolated. When a large-scale preparation was made, several such small preparations were stored at -20° and combined to provide sufficient radioactive phosphoprotein to be used as a 'marker' (see below).

Preparations from ox brain. Ox brain from which the medulla, cerebellum and most of the accessible white matter had been removed was freed from meninges and large surface blood vessels, and dispersed, in batches of 36 g., in 10 vol. of iso-osmotic sucrose–EDTA at 2° . Each batch was centrifuged at 1500 g for 10 min.; the residue was resuspended in fresh sucrose–EDTA and again centrifuged. The washed residue in iso-osmotic sucrose was stored at 2° . Successive batches were processed until 150 g. wet wt. of tissue had been treated. The combined residues were then mixed with the stored marker fractions (total volume 500 ml.) and to the suspension Teepol L (Shell Chemical Co., Ltd., a mixture of secondary-alkyl sulphates) was added [0.2 ml. (30 mg. of solids)/g. wet wt. of tissue]. After 15–20 min. at room temperature, the lysed suspension was centrifuged at 21 000 rev./min. for 1 hr. in the no. 21 head of the Spinco Model L. The pale-brown supernatant (fraction II) was carefully decanted or pipetted from the white viscid mass at the bottom of the tube. At this point the pH was 7.2. To the supernatant (volume 400 ml.) was added sodium phosphate buffer (M, pH 7.2), 2.0 ml. for each 100 g. wet wt. of tissue taken, and, with the solution cooled in ice and vigorously stirred, the pH was carefully adjusted to 6.8 with dilute HCl. The supernatant was then precipitated at -5° by adding acetone to 35% (v/v), and as much precipitate (fraction III) as possible was removed by centrifuging at 5650 g in the MSE angle head (no. 69167) for 1 hr. Acetone was then added to the cloudy supernatant to a concentration of 45% (v/v) and the precipitate (fraction IV), which settled rapidly, was centrifuged. It was resuspended in water (120 ml.) either by hand or with a Vibromix (Shandon Scientific Co. Ltd., 63 Southern Row, London, W. 10), and the water suspension cooled in ice and stirred while being adjusted to pH 9.5–9.6 with 0.1 N-NaOH. After being stirred for 20–30 min. in ice, the solution was centrifuged for 30 min. at 90 000 g, and the clear supernatant removed (fraction V). The pH was adjusted to 7.4 with dilute HCl and an equal volume (usually 100 ml.) of 2 M-NaCl was slowly added. The pH of the solution sharply decreased by 0.5–1.0 unit on addition of the first few millilitres of NaCl and was brought back by the addition of alkali. Thereafter no further decrease occurred on the addition of NaCl to a final molar concentration. The solution was stirred at 2°

* Part 1: Heald (1959).

for 30 min. and centrifuged at 90 000*g* for 30 min. to yield a residue (fraction VI). This was dissolved in ice-cold water with the aid of dilute NaOH, care being taken to keep the pH below 9.5. After 30 min. stirring, solution was usually complete, the pH was adjusted to 7.4 and the whole solution was dialysed against distilled water (20–40 vol.) overnight at 2°. The solution was then centrifuged for 20 min. at 90 000*g* to remove traces of undissolved or precipitated material and the clear supernatant was freeze-dried to yield a pale-straw-coloured powder, yield 0.5–0.6 g./180 g. fresh wt. of brain. After freeze-drying, the material suffered some loss of solubility in water, the solutions being faintly turbid.

Lysolecithin. This was given by Dr C. Long.

Radioactive phosphorus. This was obtained as carrier-free orthophosphoric acid from The Radiochemical Centre, Amersham, Bucks.

Analyses

Total nitrogen. This was determined by distillation, after digestion as described by Chibnall, Rees & Williams (1943).

Total phosphorus. This was determined as orthophosphate after wet-ashing suitable samples of the material with perchloric acid (60%, w/v), as described by Long (1943).

Lipid phosphorus. Fractions in solution were first precipitated with trichloroacetic acid (to a concentration of 10%, w/v), and the residues extracted with 20 vol. of CHCl₃-methanol (2:1, v/v). The extracts were made up to volume and samples were taken for the determination of total phosphorus. Freeze-dried fractions were extracted for 2 hr. with ether-ethanol (1:3, v/v), and the extract was made to volume before sampling.

Cerebrosides. These were determined by a method of C. Long & D. A. Staples (unpublished work) with galactose as a standard.

Glycerol esters. These were determined by the method of Shapiro (1953) with tristearin as a standard.

Neuraminic acid. This was determined by the method of Long & Staples (1959) with synthetic *N*-acetylneuraminic acid (given by Dr C. Long, a gift to him from Miss P. Carroll) as a standard.

Nucleic acids. These were determined by the methods of Logan, Mannel & Rossiter (1952) in samples of the fractions after precipitation with trichloroacetic acid. Results are expressed as μ moles of P, with the extinction coefficient ϵ_p 9830.

Lipids. These were determined as the dry weight, at 110°, of lipid extracts made in CHCl₃-methanol (2:1, v/v) or in ethanol-ether (3:1, v/v).

Phosphoproteins. Phosphoprotein phosphorus and the radioactivity of phosphorylserine derived from acid hydrolysates of phosphoprotein were determined in fractions precipitated with trichloroacetic acid (10%, w/v) by methods described by Heald (1958). In freeze-dried preparations, phosphoprotein phosphorus was estimated by digesting samples in 2 ml. of *N*-NaOH at 37.5° overnight. The alkaline samples were acidified with 0.4 ml. of 10*N*-H₂SO₄ and after 30 min. in the refrigerator the residues were centrifuged. The entire supernatants were taken for the determination of orthophosphate.

Phosphorylation of phosphoprotein fractions. The phosphorylation of phosphoprotein fractions was examined in two systems: the liver mitochondrial system described by Burnett & Kennedy (1954) and a dispersion of whole guinea-pig cerebral cortex. Cortical tissue was dispersed in 5 vol. of 0.25*M*-sucrose and incubated in a medium containing, as final concentrations in 3.0 ml.: adenosine triphosphate, 2.5 mM; MgCl₂, 8 mM; sodium pyruvate, 13 mM; sodium fumarate, 1.6 mM; KCl, 50 mM; diphosphopyridine nucleotide, 0.5 mM; cytochrome *c*, 8.3 μ M; sodium phosphate buffer, pH 7.4, 0.01*M*; 2-amino-2-hydroxymethylpropane-1:3-diol (tris)-HCl buffer, pH 7.4, 50 mM; together with radioactive phosphate and the fraction dissolved in water. Incubations were at 37.5° for 30 min. in air.

RESULTS

Distribution of phosphoprotein in dispersions of guinea-pig brain. Previous work demonstrating the presence of a phosphoprotein attached to particulate matter had been carried out largely with tissue slices incubated in saline media (Heald, 1959). Under these conditions the greater part of such phosphoprotein was attached to particles sedimented at 1500*g* for 10 min., i.e. in the 'nuclear' fraction. This distribution also holds for particles centrifuged from dispersions of guinea-pig cerebral cortex prepared immediately after removal of the brain from the animal. Table 1 shows that the nuclear fraction contains the greater quantity

Table 1. *Distribution of phosphoproteins in subcellular fractions from guinea-pig brain*

Tissues were fractionated by centrifuging dispersions made in 0.25*M*-sucrose (containing 0.5 mM-EDTA), according to the method of Brody & Bain (1952). Total phosphoprotein and the radioactivity of phosphorylserine released from phosphoprotein upon acid hydrolysis were determined as described in the text.

	Fraction		
	Nuclear	Mitochondria + microsomes	Supernatant
Phosphoprotein phosphorus { (μ moles of P/g. wet wt.) (% of total)	0.41 27.0	0.21 13.8	0.90 59.2
Nitrogen (mg./g.)	6.7	8.3	10.2
Phosphoprotein phosphorus { (μ moles of P/g. wet wt.) (% of total)	0.37 25.0	0.20 13.6	0.90 61.0
Nitrogen (mg./g.)	6.1	8.9	10.7
Radioactivity of phosphorylserine { (counts/min.) (% of total)	1312 31.4	438 10.5	2432 58.1

of particulate phosphoprotein, and that it is present in a higher concentration, in terms of nitrogen, than in the mitochondrial fraction. Also in Table 1 are presented the results of an experiment in which the phosphoproteins were made radioactive by injecting radioactive orthophosphate intracisternally into a guinea pig. After 3 hr. the brain was removed and the particulate fractions were isolated. The distribution of radioactivity in phosphorylserine derived from the phosphoprotein closely paralleled that of phosphoprotein phosphorus determined by direct analysis. The larger quantity of phosphoprotein in the supernatant fraction of such dispersions is similar to that found previously (Heald, 1959). This fraction has been shown to be metabolically distinct from phosphoproteins attached to particles. Since the greatest concentration of phosphoprotein attached to particulate matter was found in the nuclear fraction, this fraction was chosen as the starting material.

A characteristic feature of the phosphoprotein sought is the ability to yield phosphorylserine on acid hydrolysis. To enable this to be detected more readily, the phosphorus of the phosphoprotein was labelled with radioactive phosphate by incubating cerebral slices with radioactive phosphorus in the presence of glucose. Particulate fractions, prepared from these slices, were added to those prepared from ox brain. After hydrolysis of samples of the appropriate fractions, phosphorylserine was separated chromatographically and the total radioactivity determined. A similar use of radioactive phosphorylserine has been made by Glomset (1956, 1957) during the fractionation of phosphoproteins from the cytoplasm of rat liver. As the number of fractions increased, the radioactivity decayed or was reduced by loss to an extent where such measurements were no longer reliable, and accordingly measurements of phosphoprotein were also made in terms of alkali-labile phosphorus.

Solubilization of phosphoproteins from particles. Particulate fractions were extracted in a variety of ways, some of which are listed in Table 2. Of those tested, only Teepol L was effective in solubilizing the phosphoprotein without loss. The use of saline media at pH 8.0 (Expts. 1 and 2), conditions found to be optimum for the extraction of cerebral proteins (LeBaron & Folch, 1959), was ineffective, as was the use of butan-1-ol (Morton, 1955). Other conditions tested but found either to result in a total loss of radioactivity or to fail to extract phosphoprotein were: extraction with aqueous buffers (sodium acetate, 0.2M, pH 5.0, and tris-hydrochloric acid, 0.5M, pH 8.6), either of the fresh particulate material or after dehydrating in 10 vol. of acetone; extraction of the acetone-dried material with butan-1-ol either by the one- or two-

Table 2. *Effects of various extraction conditions upon the solubilization of phosphoprotein from particulate fractions of brain*

Extractant	Extraction		Particles before extraction		Extract		Residue	
	Time (hr.)	Temp.	Phosphoryl-serine (counts/min.)	N or protein (mg.)	Phosphoryl-serine (counts/min.)	N or protein (mg.)	Phosphoryl-serine (counts/min.)	N or protein (mg.)
1. NaCl (1.25 in 0.1M-tris-HCl, pH 8.0)	2	2°	10 200	252*	1177	69.0*	3260	174*
2. MgSO ₄ (1.6 in 0.1M-tris-HCl, pH 8.0)	2	2	5 500	268*	734	154.0*	4762	114.0*
3. Butan-1-ol, 1 phase (pH 7.0) (Morton, 1955)	1.5	2	3 805	15.0	209	1.9	—	—
4. Sodium deoxycholate (1%, w/v)	0.5	17-20	1 301	2.26	10	1.44	338	0.80
5. Lyssolecithin (4 mM)	0.5	17-20	600	3.4	137	2.14	0.0	1.33
6. Teepol L (0.2 ml./g. of tissue)	0.5	17-20	4 291	3.3	4293	2.06	0.0	0.8
7. Teepol L (0.2 ml./g. of tissue)	0.5	17-20	1 054	2.7	1046	1.4	0.0	1.32

* Protein estimated by the method of Lowry, Rosebrough, Farr & Randall (1951).

phase procedures at pH 7.4–8.6 (with tris-hydrochloric acid, 0.03 M, as buffer). Although extraction with magnesium sulphate (Table 2) yielded a residue containing a greater amount of radioactive phosphoprotein (in terms of nitrogen) than the original particles, the residual material was not solubilized by Teepol L. Cetavlon (cetyltrimethylammonium bromide) yielded results similar to those obtained with lysolecithin.

Preparation of partially purified phosphoprotein. The conditions chosen to yield a partially purified preparation still retaining radioactivity in phosphorylserine are described in detail in the Materials and Methods section. Steps in the procedure are given in Table 3 showing a typical preparation. In terms of total phosphorus the preparation was purified some 15 times over the original starting material with a yield of about 25% as judged by radioactivity remaining in the phosphorylserine. This figure agrees with recoveries of alkali-labile phosphorus. Thus in two other preparations from 180 g. of ox brain the quantities of alkali-labile phosphorus were: fraction I, 43 and 44 μ moles of P; fraction VII, 12.0 and 14.3 μ moles of P. Purification in terms of nitrogen was fourfold.

Certain points may be noted in connexion with the individual steps. The nuclear fraction used as the starting material contains small amounts of a phosphoprotein phosphatase (P. J. Heald, unpublished work) the pH optimum of which is 5.5–6.0. Little activity is shown above pH 7.0. A simple and complete inhibitor for this enzyme has not yet been found. The enzyme is considered to be responsible for the loss of phosphoprotein in the stages before acetone precipitation. Since no precipitate could be obtained with acetone (to 50%, v/v) above pH 7.0, precipitations were performed rapidly at pH 6.8. No phosphatase was detectable in the precipitates. The phosphoprotein is not stable in dilute alkali. Thus in one experiment after incubation of fraction VII at 37.5° for 30 min. at different pH values the recoveries of radioactive phosphorylserine derived from the phosphoprotein were: pH 5.6, 73.5%; pH 7.7, 27%; pH 9.2, 10%. The lability is thus greater than that of the radioactive phosphorus in phosphorylated casein (Sundararajan, Kumar & Sarma, 1960). For these reasons it was necessary to carry out the fractionations as rapidly as possible without exposing the extracts to strongly alkaline conditions for too long.

Precipitation of fraction VII from fraction V was best achieved with m-NaCl, pH 7.4, which removed considerable quantities of nucleic acid, phosphorus and nitrogen. In addition to the results in Table 3 three other experiments showed that only 3–9% of the nucleic acid present in fraction V was found in the precipitate. Ammonium

Table 3. Steps in the isolation of a phosphoprotein fraction from cerebral particles

Fraction	Vol. (ml.)	Nitrogen (mg.)	Total P (μ moles)	Lipid P (μ moles)	Nucleic acid P (μ moles)	Radioactivity in phosphoryl- serine from phosphoprotein (counts/min.)	For details see Materials and Methods section.	
							Counts/min./ mg. of N	Counts/min./ μ mole of P
I. Total particles	355	935	9200	3700	246	10 000	11.2	1.1
II. Teepol extract	225	415	1480	745	105	6 350	15.3	4.3
III. 0.36% Acetone	33	46	320	—	11.5	680	14.8	2.1
IV. 36–45% Acetone	150	242	890	508	60.0	4 700	19.5	5.3
V. 36–45% Acetone (alkali-sol.)	138	152	610	250	44.0	4 700	31.0	7.7
VI. 36–45% Acetone (alkali-insol.)	40	—	280	—	—	450	—	—
VII. NaCl insol.	95	69	186	140	11.6	2 740	40.0	14.8

sulphate ($I\ 0.1$, pH 7.4) or precipitation at pH 5.0 failed to yield any purification, the entire material being precipitated. Attempts to remove lipids by treatment with excess of butan-1-ol (cf. Sundararajan *et al.* 1960) over a pH range 6.5–8.6 were unsuccessful since the entire radioactive protein collected as a denatured layer at the interface. Virtually no protein passed into solution. Fraction V was not absorbed on carboxymethylcellulose, pH 5.0, but was firmly retained by diethylaminoethylcellulose at pH 7.4. From this material it could be eluted only by alkaline buffers and 0.1N-sodium hydroxide. No radioactive phosphorylserine was obtained from any fraction, probably owing to the alkali lability of the phosphorus.

The gross composition of three preparations is given in Table 4. As will be seen, the quantity of lipids in preparation 1 was higher than in the other preparations and the total quantity of phosphorus in the lipid-free residues was lower. Of this phosphorus 50–80% was labile in alkali. Lipids were extracted by shaking with ethanol-ether (3:1, v/v) for 2 hr. at 2°, after which time no further quantities were removed. The amount removed was similar to that extracted from moistened samples with chloroform-methanol (2:1, v/v) and was not increased by extracting with acidified ethanol-ether. No material was extracted by dry chloroform-methanol, indicating that free lipid was absent. The Lieberman-Burchard reaction was negative. Glycerol esters in the lipid extracts, determined by the method of Shapiro (1953) with tristearin as a standard, yielded values calculated as tristearin almost equivalent to the dry weight of the lipids. Since the extracts contain little cerebroside and about 25–30% of phospholipids ($P \times 25$) it is considered that the remaining lipids estimated by the hydroxamate method consist largely of triglycerides. The lipid-free residue contained 11.2–12.0% of nitrogen, a value similar to that found by Folch & Lees (1951) for the protein of ox-brain proteolipids. However, in contrast with the latter material, the percentage of nitrogen in the phosphoprotein was not increased on boiling with chloroform-methanol (2:1, v/v) for 6 hr. Hexosamines were not detectable and neuraminic acid was absent. Examination for carbohydrates released on acid hydrolysis in N-hydrochloric acid for 3 hr. (Heald, 1951) was negative. Qualitative examination of the amino acids revealed a preponderance of acidic amino acids (Table 5) together with appreciable quantities of serine. In the absence of a quantitative estimation of the individual acids present it was not possible to decide whether the low nitrogen values were in fact due to raised quantities of glutamic and aspartic acid, or indicated lipid material firmly bound to the protein. After removal of the lipids

Table 4. *Composition of phosphoprotein fractions isolated from cerebral particles*

For preparation and analytical methods see text.

Prep. no.	Total P (%)	Total N (%)	Lipids (% by wt.)	Lipid-free residue				Lipid extract				
				N (%)	P (%)	Alkali-labile P (%)	Hexos-amine (%)	N (%)	P (%)	'Galactose' (%)	Neuraminic acid (%)	Glycerol esters calculated as tristearin (%)
1	0.31	5.0	66.0	11.2	0.1	0.05	0	—	—	—	—	—
2	0.65	8.6	43.0	11.6	0.21	0.10	0	2.0	0.96	2.60	—	85.0
3	0.44	8.1	38.0	12.0	0.24	0.20	0	1.16	1.17	1.18	0.31	108.0

by ether-ethanol, the residues were insoluble in water but dissolved slowly in alkali.

Phosphorylation of the fractions. A primary purpose of the study described above was to obtain preparations for use in the study of phosphorylation and dephosphorylation systems. The results are presented in Table 6. In Expts. 2 and 3, phosphoprotein phosphorylation was detected by the increased amount of radioactive phosphorylserine found in the hydrolysed phosphoprotein at the end of the experiments. In Expt. 1, the phosphoprotein fraction, after phosphorylation, was isolated as described by Burnett & Kennedy (1954) and counted directly. In all three experiments it was clear that the fractions

obtained were capable of accepting radioactive phosphate, which appeared as phosphorylserine on acid hydrolysis of the phosphoprotein.

Attempts at further fractionation of fraction VII. A preliminary examination of fraction V in the analytical ultracentrifuge was made by Dr K. V. Shooter, using an aqueous 0.5% solution at pH 8.0-9.0. Only one peak was observed, the peak being asymmetric ($S\ 0.39 \times 10^{-13}$). However, the area under the peak was small, suggesting that some material could have been present as large aggregates easily spun down. Since no boundary was seen during acceleration any such aggregates must have been very polydisperse in size. Examination by electrophoresis on paper or in a starch gel showed that the bulk of the material remained firmly attached to the starting line on the paper and did not enter the gel. Precipitation curves obtained in sodium chloride solutions (Fig. 1)

Table 5. *Amino acids in phosphoprotein fractions isolated from brain*

* Protein was hydrolysed for 12 hr. in 6N-HCl. After removal of the HCl under vacuum the hydrolysate, in water, was spotted on paper and run first in butanol-acetic acid-water (120:30:50, by vol.) and secondly in phenol-water (160:40, w/w).

Amino acid	Relative intensity
Alanine	++++
Arginine	++
Aspartic acid	++
Cysteine	+(weak)
Glutamic acid	++++
Glycine	++
Histidine	+
Lysine	++
Phenylalanine	+
Proline	+
Serine	+++
Threonine	++
Tyrosine	+
Valine	++
(Leucine) (isoLeucine)	+++

Fig. 1. Precipitation of components of crude brain phosphoprotein by increasing quantities of NaCl. Buffer: tris, pH 7.4, $I\ 0.05$. *a*, Protein concentration 3.35 mg./ml.; *b*, protein concentration 1.0 mg./ml. Equilibrium 18 hr. at 2°. All solutions were centrifuged for 7 min. at 100 000 *g* before $E_{280\ m\mu}$ was read.

Table 6. *Phosphorylation of brain phosphoprotein fractions*

In Expt. 1 the mitochondrial system of Burnett & Kennedy (1954) was used. In Expts. 2 and 3 a fortified cerebral dispersion was used (see text). In Expt. 1 the counts were obtained from the total phosphorus present in the protein residues prepared as described by Burnett & Kennedy (1954). In Expts. 2 and 3 the counts are counts in phosphorylserine separated after acid hydrolysis of the fractions.

Expt. no.	System	Counts/min. in system	O ₂ uptake (μl.)	³² P added (μc)
1	Mitochondria	8 500	44.0	10.0
	Mitochondria + phosphoprotein	20 800	47.0	
	Phosphoprotein	260	0.0	
2	Dispersion	321	—	2.0
	Dispersion + phosphoprotein	480	—	
	Phosphoprotein	0	—	
3	Dispersion	216	248.0	2.0
	Dispersion + phosphoprotein	594	290.0	
	Phosphoprotein	0	0.0	

Table 7. *Analysis of phosphoprotein fractions prepared by subfractionation of fraction VII in sodium chloride solutions*

Conditions are as in Fig. 1 (a)

Fraction	Lipid-free residue						Lipid extract				
	Total P (%)	Total N (%)	Lipids (%)	N (%)	P (%)	Alkali-labile P (%)	N (%)	P (%)	Galactose (%)	Neuraminic acid (%)	Glycerol esters calculated as tristearin (%)
(A)	0.50	7.75	40.5	10.9	0.18	0.1	1.20	1.15	0.96	0.38	115.0
(B)	0.20	6.8	31.0	12.0	0.15	0.05	3.30	0.45	1.70	—	85.0

revealed the presence of at least three protein-containing substances. Quantities of the two major fractions were prepared under the conditions described for *a* in Fig. 1 and were analysed. The results are given in Table 7. Apart from the differences in solubility, the composition of the lipid extracts were also different. No quantitatively detectable differences were noted in the amino acid pattern from hydrolysed samples of the lipid-free residues, which were similar to that shown in Table 5.

DISCUSSION

As stated at the outset an object of this work was to obtain from a preparation of brain particulate material a soluble fraction containing phosphoprotein for use as a substrate in further studies upon phosphorylation and dephosphorylation by cerebral enzymes. It has been found possible to prepare such a fraction by extraction of the subcellular particles with a detergent. The fraction is a lipophosphoprotein complex and has been purified 10–14 times in terms of phosphorus and 4–5 times in terms of nitrogen. The preparation obtained cannot be considered to be a single entity, for examination of the precipitation curves obtained with increasing concentrations of sodium chloride showed that at least three components were present, the major ones differing in gross composition. Whether these differences do indeed indicate the presence of two or more phosphoproteins or whether they represent a similar protein combined to a differing extent with different lipids cannot be assessed from the present data and requires further study by other techniques. The high proportion of glycerol esters (possibly triglyceride in nature) and the absence of cholesterol are of considerable interest since all glycerol-containing lipids so far characterized in brain are phospholipids (LeBaron & Folch, 1957). The precise nature of these esters remains to be established. The absence of cholesterol could conceivably be due to a dissociation of a lipoprotein-cholesterol complex by the anionic detergent used in the extraction (Rideal & Taylor, 1957). However, it has been

found (P. J. Heald & M. H. Cohen, unpublished work), in a more extended study of the chemical and morphological characteristics of the subcellular fractions containing the phosphoprotein, that the fraction richest in phosphoprotein is amongst the poorest in cholesterol. The material is therefore not associated with myelin. Since it has been shown that the phosphoprotein is also not associated with nuclei, mitochondria or microsomes (Heald, 1959) it seems possible that the material is a constituent of the cell walls, in which triglycerides have been considered to be essential components (see Burgen, 1957).

There is little previous work upon the nature of phosphoproteins of animal tissues, other than enzymes listed previously (Heald, 1959). Glomset (1957) showed that the separation of rat-liver cytoplasmic proteins, obtained by dispersing liver in aqueous sucrose solutions, upon columns of diethylaminoethylcellulose, yielded several fractions containing phosphorylserine but the identity of these was not further investigated. More recently, Sundararajan *et al.* (1960) obtained a soluble fraction, containing phosphoprotein, from rabbit mammary gland by extraction with dilute alkali at pH 9.7 and removal of lipids with butan-1-ol. It was not clear whether this preparation was contaminated with casein. Both these preparations differ from that now described, which is not readily eluted from diethylaminoethylcellulose and is degraded by treatment with dilute alkali or butan-1-ol. Though the quantities of alkali-labile phosphorus are somewhat lower than those found in phosphoprotein fractions obtained from milk (Perlman, 1955), egg yolk (Fevold, 1951; Joubert & Cook, 1958) or the plasma of the laying hen (McIndoe, 1959), this is considered to be due to dephosphorylation occurring during the initial stages of purification. Cerebral tissues contain an active phosphoprotein phosphatase (Feinstein & Volk, 1949), which probably contributes to the major loss of phosphate which occurred during the preparation.

A significant feature of the fractions obtained is their retention of the ability to become phosphoryl-

ated when incubated in suitable phosphorylating systems. It has been convincingly demonstrated (Rabinowitz & Lipmann, 1958, 1960; Burnett & Kennedy, 1954) that of several purified proteins containing phosphorus only a few, including casein and phosvitin, or peptones derived from these substrates, are capable of acting as phosphate acceptors when incubated with mitochondria or with a purified phosphokinase. Such a specificity is likely to be due to the possession of a specific configuration which, with casein and phosvitin, can be destroyed by prolonged treatment with dilute alkali (Burnett & Kennedy, 1954; Rabinowitz & Lipmann, 1960). Thus the finding that cerebral phosphoprotein is also capable of accepting phosphate in a similar phosphorylating system indicates that this degree of structural specificity has been retained throughout the isolation procedure. As with casein (Burnett & Kennedy, 1954, see also Table 6), only radioactive phosphorylserine could be detected in hydrolysates of the phosphoprotein fraction. It has been well established that serine itself is not phosphorylated by liver mitochondria or by purified phosphokinase, and discussions of the manner in which serine in phosphoproteins becomes radioactive are commonly made in terms of the structures surrounding the active serine residue in trypsin and chymotrypsin (cf. Sundararajan *et al.* 1960). However, a general reactivity of a serine site in an enzyme does not make it capable of accepting phosphate via a protein phosphokinase reaction, for trypsin, chymotrypsin, pepsin, hexokinase, ribonuclease and muscle phosphorylase *b* are not phosphorylated by liver mitochondria (Burnett & Kennedy, 1954) or by purified yeast protein phosphokinase (Rabinowitz & Lipmann, 1960). On this basis the phosphoprotein fraction from brain might be expected to possess some structural similarities to casein and phosvitin, in which the sequences of linked phosphorylserine residues found may be considered as possible sites for phosphorylation (Williams & Sanger, 1959).

SUMMARY

1. A lipophosphoprotein fraction has been prepared from easily sedimenting particles of ox brain. The preparation contains 8% of nitrogen, 0.3–0.6% of P and 40–66% of lipids. Calculated in terms of the total phosphorus content of the particles, the preparation has been purified 10–14 times. Calculated in terms of the total nitrogen content of the particles it has been purified 4–5 times. The recovery is 25% of the starting material. The preparations are not pure and can be separated into at least two major components by salt fractionation.

2. The lipid-free residue contains 11.2–12.0%

of nitrogen. This figure is not increased by exhaustive extraction of the residue. It contains 0.1–0.24% of phosphorus of which 50–75% is labile in alkali. Qualitative analysis revealed an amino acid pattern suggestive of an acidic protein.

3. The fraction does not contain cholesterol. Neuraminic acid and galactose from cerebroside are present only in small amounts. The lipids contain a high proportion of glycerol esters thought to be triglycerides.

4. When incubated either with a fortified cerebral dispersion or with rat-liver mitochondria in the presence of radioactive phosphate, the preparations are phosphorylated, radioactive phosphorylserine being detected in hydrolysates of the phosphoprotein at the end of incubation.

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Studies on Pituitary Adrenocorticotrophin

3. IDENTIFICATION OF THE OXIDATION-REDUCTION CENTRE*

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The oxidation-reduction behaviour of corticotrophin A_1 was studied in detail by Dedman, Farmer & Morris (1957). The hormone is oxidized by hydrogen peroxide under appropriate conditions to give a biologically inactive product which can be distinguished chromatographically from the untreated corticotrophin (Dixon & Stack-Dunne, 1955; Farmer & Morris, 1956). Reduction of the oxidized hormone with thiol compounds completely restores the biological activity, and the reduction product is chromatographically identical with the original untreated hormone. The oxidation-reduction process is thus readily reversible.

Dedman *et al.* (1957) examined the potentially oxidizable amino acid residues in corticotrophin A_1 and concluded that it was difficult to explain the experimental results on the basis of the known structure. In particular, methionine was excluded because Raney-nickel-treated corticotrophin, which was apparently sulphur-free, exhibited the characteristic oxidation-reduction behaviour. The subsequent inability to isolate α -aminobutyric acid from Raney-nickel-treated corticotrophin A_1 has prompted a re-examination of this material. Improved analytical methods with hydrolysis by leucine aminopeptidase show that it contains methionine, which was destroyed by the earlier method of acid hydrolysis. The evidence excluding this amino acid from participation in the oxidation-reduction centre is thus no longer valid. This paper presents evidence that the oxidation-reduction centre of corticotrophin is in fact the thioether grouping of methionine.

* Part 2: Dedman, Farmer & Morris (1957).

EXPERIMENTAL

Bioassay methods. The biological activities of all preparations were measured by the adrenal-ascorbic acid-depletion method of Sayers, Sayers & Woodbury (1948) with minor modifications (Morris, 1951).

Preparation of corticotrophin A_1 . The hormone was isolated from pig anterior-pituitary glands by the method of Dedman *et al.* (1957), or by the method of Farmer (1959). The oxidized hormone was prepared by H_2O_2 oxidation as described by the former authors.

Preparation of leucine aminopeptidase. The enzyme was prepared according to the procedure of Hill & Smith (1957). Enzymic activity was measured by the digestion of leucyltyrosine. The enzyme was stored at 2° as a solution in 2-amino-2-hydroxymethylpropane-1:3-diol (tris) buffer, pH 8.0, which was 0.05M with respect to $MgCl_2$. The enzyme was activated before use by heating for 30 min. at 40° at pH 8.5 (Hill & Smith, 1957).

Preparation of the tryptic-digestion products of corticotrophin A_1 and oxidized corticotrophin A_1 . (a) Digestion. Corticotrophin A_1 (9.8 mg.) was dissolved in an aqueous 0.025% solution of trypsin (L. Light and Co. Ltd., 50 Northrop units/mg.) in 0.01M-sodium phosphate buffer, pH 8.0 (1.0 ml.). The mixture was maintained at 30° for 30 min. 1M-Pyridine-acetate buffer (pyridine, 16 ml.; 2N-acetic acid, 100 ml.), pH 4.4 (0.11 ml.), was added and the mixture was heated at 90° for 2 min. to inactivate the trypsin. Oxidized corticotrophin A_1 (8.8 mg.) was also digested by this procedure.

(b) Chromatography. Carboxymethylcellulose (0.6 mequiv./g.; 2 g.) was suspended in aqueous 0.5% pyridine and gently dispersed in an all-glass Potter-Elvehjem homogenizer. After the greater part of the material had sedimented, the supernatant liquid and very fine material were removed. The carboxymethylcellulose was washed several times with 0.067M-pyridine-acetate buffer, pH 4.4 (redistilled pyridine, 5.30 ml.; 2N-acetic acid, 66.7 ml.; diluted to 1 l. with water). A column 62 cm. $\times$ 0.5 cm. was packed