unit involves a chelate mechanism and a thio ester intermediate (Hele, 1960*a*), along the lines suggested by Ingraham & Green (1958) for the activation of acetate.

SUMMARY

1. The pyrophosphate-exchange reactions which are catalysed by rat-liver preparations and depend upon leucine or isoleucine are profoundly modified by 'soluble' ribonucleic acid and by changes in magnesium concentration. The preponderant influence is exerted by the terminal nucleotide sequence of the 'soluble' ribonucleic acid. Lysine-dependent pyrophosphate exchange occurs only at relatively high magnesium concentrations and is not greatly influenced by 'soluble' ribonucleic acid. The transfer of [¹⁴C]lysine to 'soluble' ribonucleic acid is catalysed more rapidly by 'recombined' systems than by pH 5.0 fractions and may be related to the inability of the enzyme to react with low concentrations of magnesium, and to the presence of an inhibitor.

2. The terminal nucleotide sequence of 'soluble' ribonucleic acid may be concerned in the protection of a labile, essential site associated with the activating enzyme. The leucine- and lysine-activating enzymes show highly individual responses to activators and inhibitors of sulphydryl groups. The possible role of the labile site in the reaction mechanism is discussed.

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A Phosphoprotein Phosphatase from Ox Brain

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It has been shown [see Heald (1959) for references] that the phosphorus of a phosphoprotein fraction of brain is exchanged rapidly when the tissue is stimulated *in vitro* by electrical pulses.

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During attempts to isolate this material (Heald, 1961*a*) it became apparent that brain contains an enzyme capable of splitting phosphate from phosphoprotein. Knowledge of such enzymes is limited and, apart from brief reports by Feinstein & Volk (1949) and Norberg (1950) that dispersions of rat

brain liberated phosphorus from unfractionated casein, no information was available about the existence of a cerebral phosphoprotein phosphatase. We have therefore extracted and partially purified a phosphoprotein phosphatase from ox brain and studied some of its properties. An account of part of this work was reported by Rose & Heald (1960).

MATERIALS AND METHODS

Casein. α -Casein was prepared from commercial Hammarsten casein (Hopkin and Williams Ltd.) by fractionation with urea according to the method of Hipp, Groves & McMeekin (1955). β -Casein was a gift from Dr R. Aschaffenburg. The purity of the preparations was checked by electrophoresis at pH 8.6 in 0.04M-sodium borate buffer, in a starch gel. Both caseins migrated as single and distinct components. The α -casein contained 0.92% of phosphorus (range 0.6–1.1%, six samples). The β -casein contained 0.36% of phosphorus.

Phosvitin. This was obtained from Nutritional Biochemicals Corp., Cleveland, Ohio, U.S.A., having been prepared by the method of Mecham & Olcott (1949). The dry product contained 7% of phosphorus, all of which was released as inorganic phosphorus on incubation in N-NaOH for 16 hr. at 37°. Before use it was dissolved in 10 mM-sodium ethylenediaminetetra-acetate (EDTA), pH 7.4, dialysed first against two changes of 1.0 mM-EDTA (100 vol.) for 24 hr. and finally for at least 48 hr. against four changes of water (100 vol.). Radioactive phosvitin was prepared by incubating 10 mg. of phosvitin (after dialysis) with radioactive phosphate in the presence of rat-liver mitochondria prepared and used as described by Burnett & Kennedy (1954). After incubation, usually for 30 min., the mitochondria were removed by centrifuging and the radioactive phosvitin was isolated by precipitation of the manganese salt as described by Rabinowitz & Lipmann (1960). The preparation was freed from manganese by dialysis against EDTA and water as described by these authors.

Ovalbumin. This was a twice-recrystallized sample from Nutritional Biochemicals Corp., and was salt-free.

Pepsin and hexokinase. These were obtained from Sigma Chemical Co., St Louis, Mo., U.S.A. The pepsin was a threefold-recrystallized specimen. The hexokinase was crude type II prepared from yeast.

Casein phosphopeptones. These were prepared by the digestion of α -casein with crystalline pepsin by the method of Damodaran & Ramachandran (1941). The yield from 1 g. of α -casein was 25 mg. of peptone containing 1% of phosphorus.

Polyseryl phosphates. These were obtained from a partial acid hydrolysis of phosvitin by electrophoresis on paper and elution of the materials separated (Heald, 1961b).

Phosphoproteins from brain. These were prepared as described by Heald (1961a).

Sodium phosphoamidate. This was a gift from Albright and Wilson Ltd., Birmingham. It was purified by solution in water below 10° and addition of acetic acid dropwise to pH 4.0, when the monosodium salt was precipitated. The precipitate was washed with water and ether and air-dried. It was reconverted into the disodium salt by the addition of the stoichiometric amount of NaOH and the solution was

adjusted to pH 6.0 before use. This method of purification (cf. Stokespre, 1893, 1894) was communicated by Albright and Wilson Ltd.

Calgon PT, 'sodium triphosphosphate' and 'phosphate glass 696'. These polymetaphosphates were gifts from Albright and Wilson Ltd. (Alkaline Phosphates Division, Knightsbridge Green, London).

Radioactive phosphate. This was obtained as carrier-free inorganic orthophosphate from The Radiochemical Centre, Amersham, Bucks.

Analytical methods

Inorganic phosphate. This was normally determined by the method of Martin & Doty (1949), modified for use with smaller samples. Thus the sample containing 1–15 μ g. of phosphorus was transferred to a 15 ml. stoppered tube and brought to 3 ml. with water. Silicotungstate reagent (2 ml.) was added, followed by ammonium molybdate reagent (2 ml.) and isobutanol–benzene (1:1, v/v) (7 ml.). The mixture was shaken vigorously and centrifuged, and 5 ml. of the clear upper layer was mixed with stannous chloride reagent (1 ml.). The volume was brought to 10 ml. with ethanol–sulphuric acid (50:1, v/v). The extinction was read at 700 m μ against an ethanol–sulphuric acid blank. In the presence of labile phosphate esters inorganic phosphate was determined by the method of Lowry & Lopez (1946) as modified by Peel, Fox & Elsdon (1955).

Total phosphate. This was determined as inorganic phosphate after digestion of the sample with perchloric acid (King, 1932).

Determination of radioactive phosphate. This was determined by counting the final isobutanol–benzene solutions used for the determination of inorganic phosphate in an M-6.H liquid counter (20th Century Electronics Ltd.).

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

Phenol. This was determined by the method of Folin & Ciocalteu as described by Snell & Snell (1953).

Enzyme assay. Appropriate quantities of the enzyme solution were incubated with α -casein (1.0 ml.; 4%, w/v) and sodium succinate buffer (0.1M, pH 5.9). The final volume was 3.0 ml. After 15 min. at 37.5° the reaction was stopped by the addition of 2 ml. of 50% (w/v) trichloroacetic acid. After being chilled in ice for 20 min. the suspension was centrifuged and appropriate quantities were removed for the determination of inorganic phosphate. The large amount of trichloroacetic acid was necessary to ensure a complete precipitation of the α -casein. For the measurement of activity against various substrates (Tables 3 and 4; Figs. 5–7), the reaction mixture contained the enzyme (100–150 units), magnesium chloride (final concentration 0.3 mM), the substrate and sodium succinate, pH 5.9 (0.1M), to a total volume of 3.0 ml.

Units. One unit of activity is defined as the amount of enzyme liberating 1 μ g. of phosphorus/hr. from α -casein under the above-described conditions. Specific activity is defined as the number of units per mg. of nitrogen.

Purification procedure

Stage I: drying with acetone. Ox brain was obtained from the Metropolitan Cattle Market as soon as possible after death of the animal and was packed in a polythene bag surrounded by ice during transport to the laboratory.

After removal of membranes and adhering blood clots, the brain was dispersed for 3 min. in 10 vol. of acetone at -10° in a top-drive macerator. Suspensions were rapidly filtered with suction, washed with a further 3 vol. of acetone and finally with 3 vol. of diethyl ether. The powder was air-dried by suction and stored at -18° . Under these conditions no loss of enzymic activity was apparent for up to 10 weeks. Longer storage led to an appreciable loss of activity.

Stage II: extraction of the enzyme. The enzyme was extracted by grinding the powder, at room temperature, in a glass tube with a fitting pestle, with ten times its weight of water. The suspension was centrifuged for 15 min. at 78 400g and the supernatant retained. The residue was again extracted with a similar volume of water and after centrifuging for 30 min. at 78 400g the supernatant was removed. The combined supernatants constituted the 'water extract', in which the enzyme remained stable at 2° for up to 3 weeks.

Stage III: acetone fractionation. The water extract was chilled to 0° and stirred. Acetone (Hopkin and Williams Ltd., A.R.) at -10° was slowly added to a final concentration of 50% (v/v) the temperature of the mixture being lowered to -4° during the addition. For a typical preparation involving 200 ml. of the water extract the acetone was added over 40 min. The suspension was immediately centrifuged, at -5° , for 20 min. at 1500–1700g and the precipitate was discarded. The acetone concentration of the supernatant at -5° was raised to 55% (v/v) and the mixture centrifuged for 20 min. at 1500–1700g. The small inactive precipitate was discarded. The concentration of acetone in the supernatant was then rapidly raised to 90% (v/v) and the mixture kept for 1 hr. at -10° before being centrifuged for 20 min. at 1500–1700g. The precipitate was dissolved in one-fifth of the initial volume of water and stored at 2° . At this stage the enzyme retained full activity for up to 14 days.

Stage IV: absorption on calcium phosphate gel. The concentration of orthophosphate in the enzyme solution was adjusted to 700 $\mu\text{g./ml.}$ by the addition of 0.1 M KH_2PO_4 . At this stage the pH of the solution was 6.4–6.6. Calcium chloride (0.1 M), equivalent to the total inorganic phosphate present, was then added with stirring. Stirring was continued for a further 18 hr. at 4° and the suspension was centrifuged. The supernatant was discarded.

Stage V: elution from the gel. The precipitate was shaken for 30 min. at 4° , first with a volume of tris (0.5 M, pH 7.4)

equal to that of the solution at the beginning of stage IV. After centrifuging, the supernatant was discarded. The precipitate was shaken with a similar volume of sodium acetate (0.1 M, pH 6.4) for 30 min. at 4° and centrifuged. The precipitate was then shaken for 30 min. with a similar volume of sodium succinate buffer (0.1 M, pH 5.9) and centrifuged. This supernatant contained the enzyme purified some 90–100-fold over the original brain dispersion, in a yield of 20–25%. The overall purification and yields are summarized in Table 1.

RESULTS

Stability to fractionation. Fractionation of the water extracts by means of ammonium sulphate, added either stepwise or rapidly to near saturation, led to losses of 60–100% of the enzymic activity. Similar losses also occurred during fractionation with acetone though not if the enzyme was precipitated rapidly at a final concentration of acetone of 90% (v/v). A product of high specific activity was obtained first by adding acetone slowly to a concentration of 55% (v/v), discarding the precipitate and rapidly adding more acetone to a final concentration of 90% (v/v). Unless the first addition was slow and the second rapid a considerable loss in activity occurred. This procedure increased the purity about 10–12-fold but the product completely lost its activity within 5 days at 2° . A less pure product which retained activity for at least 2 weeks was obtained by the procedure detailed in the Materials and Methods section and in Table 1. In all experiments with both ammonium sulphate and acetone, activity once lost was not restored by recombination of any or all of the fractions nor by the addition of cysteine or thio-glycollate.

Stability to dialysis. The enzymic activity of the water extract was almost completely lost on dialysis against water at $0-4^{\circ}$; 50% of the loss occurred within the first 30 min. of the dialysis (Fig. 1). Recombination of the dialysis residue and the diffusate did not restore activity. The loss was

Table 1. *Purification of phosphoprotein phosphatase from ox brain*

Units are defined in the text. Percentages of acetone are by volume.

Stage	Description	Volume (ml.)	Total units	Total N (mg.)	Specific activity	Recovery (%)	Purification factor
I	Aqueous acetone-dried powder	210	35 280	656	54	100	1
II	Water extract	210	31 920	314	101	91	1.8
III	0–50% Acetone	95	5 700	—	—	17	—
	50–55% Acetone	50	0	—	—	—	—
	55–90% Acetone	55	23 100	5.8	397	66	7.4
V	Calcium phosphate gel eluates:						
	pH 7.4 eluate	50	0	—	—	—	—
	pH 6.4 eluate	50	2 500	—	—	7.0	—
	pH 5.9 eluate	35	6 650	1.3	5080	21.0	94
	Gel residue	25	2 000	—	—	6.0	—

not caused by contact with the dialysis tubing and was not prevented by dialysis against sodium chloride (0.1M), magnesium chloride (0.1M) or by the addition of bovine serum albumin (0.5%) before dialysis. However, dialysis against 100 vol. of 20 mM-cysteine at pH 6.4–6.6 for 3 days did not reduce the activity of the enzyme. Cysteine did not prevent a total loss of activity during the dialysis of the more purified fractions from stages III and V.

pH optimum and stability. The effect of pH upon the activity and stability of the enzyme is shown in Fig. 2. The pH optimum of 5.5 lies below the pH of maximum stability and is in the region where α -casein becomes increasingly insoluble. For this reason the assay was performed at pH 5.9.

Activators and inhibitors. The effect of various ions upon the activity of the purified brain phosphoprotein phosphatase is presented in Table 2. Of the metals examined both magnesium and manganese markedly activated the brain enzyme. The optimum activation was obtained with 0.3 mM- $MgCl_2$. Higher concentrations were inhibitory (Fig. 3). Other metal ions were either without effect or inhibitory. As might be expected for an enzyme activated by magnesium, fluoride and EDTA were markedly inhibitory. The enzyme was not activated by thiols but was strongly inhibited by iodoacetate.

Velocity and substrate concentration. The enzyme from stage III was used to determine the velocity of phosphate release from different concentrations of α -casein in the absence of Mg^{2+} ions (Fig. 4). A Lineweaver-Burk plot of the results gave K_m 3.74 mg.atoms of α -casein phosphorus. Because of its low solubility the concentration of α -casein was limited to 10.0 mg.atoms of α -casein phosphorus/l.

Activity towards various substrates. At pH 5.9 the partially purified enzyme did not release phosphate from phenyl phosphate, β -glycerophosphate, glucose 6-phosphate, fructose 1:6-diphosphate, *O*-phosphorylserine, adenosine 5'-phosphate, sodium phosphoamidate, phosphocreatine, polyseryl peptides, Calgon PT, tripolyphosphate and phosphate glass '696' or a mixture of brain phospholipids. It did not act on diphenyl phosphate to yield phenol or phosphate. Some preparations of the enzyme occasionally released phosphate from adenosine 5'-triphosphate and sodium pyrophosphate. This was considered to be due to the presence of adenosine triphosphatase, which could be demonstrated by assaying under the conditions optimum for these two enzymes (Gore, 1951; Gordon, 1953) (Table 3).

Activity towards other phosphoproteins. The activity of the partially purified enzyme towards several phosphoproteins is shown in Table 4. The enzyme liberated phosphate from α - and β -casein

and a derived peptone, phosvitin, ovalbumin and three preparations of brain phosphoprotein. No phosphate was released from yeast hexokinase or from pepsin. Failure to release phosphate from pepsin was not due to inactivation of the phosphatase since phosphate was released when α -casein was incubated in the system together with pepsin.

Time-course of the release of phosphate. Fig. 5 shows that the release of phosphate from α -casein

Fig. 1. Loss of phosphoprotein-phosphatase activity on dialysis against water. The water extract from stage II (see Materials and Methods section) at pH 6.4 was dialysed against water at 4°. Samples were removed periodically and assayed for activity in the system described in the Materials and Methods section. ○, Enzymic activity; ●, orthophosphate.

Fig. 2. pH stability and pH optimum of brain phosphoprotein phosphatase. ●, pH stability; enzyme from stage III was incubated in buffer at different pH values for 18 hr. at 2°, the pH adjusted to 5.9 and the enzyme assayed as described in the Materials and Methods section ○, pH optimum; the enzyme was assayed in buffers of varying pH. Buffer: pH 4.5–6.5, sodium succinate (0.2M); pH 7.4–9.2, tris-HCl (0.1M).

Table 2. Effects of added substances on the activity of brain phosphoprotein phosphatase

Reagents listed were tested in the system described in the Materials and Methods section, the enzyme from stage III or stage V being used. Superscript figures refer to the references listed below.

Substance	Concn. (mm)	Activity (% of control)	
		Brain enzyme	Enzymes from liver or spleen
MgCl ₂	0.5	200	100 ⁽¹⁾
MgCl ₂	1.0	180	200 ^(1, 3) , 100 ⁽⁶⁾
MnCl ₂	0.1	182	100 ⁽²⁾ , 150 ⁽¹⁾
CaCl ₂	1.0	100	150 ⁽¹⁾ , 100 ^(4, 5)
CuSO ₄	1.0	90	40 ⁽⁵⁾ , 200 ⁽⁴⁾ , 50 ⁽⁶⁾
ZnCl ₂	1.0	72	100 ⁽⁴⁾ , 50 ⁽⁶⁾
CoCl ₂	1.0	27	100 ⁽¹⁾ , 50 ⁽⁶⁾
FeCl ₂	0.1	0	60 ⁽³⁾ , 400 ⁽⁴⁾
MoO ₄	10.0	106	—
MoO ₄	0.1	95	0 ⁽⁴⁾ , 0 ⁽⁶⁾
NaF	1.0	40	100 ⁽⁴⁾ , 82 ⁽⁶⁾
Iodoacetate	10.0	12	70 ⁽⁶⁾
Iodoacetate	1.0	38	—
Iodoacetate	0.1	47	100 ⁽³⁾
NaCN	1.0	0	60 ⁽⁵⁾ , 150 ⁽¹⁾
NaCN	0.1	17	—
EDTA	1.0	25	100 ⁽⁶⁾
EDTA	0.1	60	—
Cysteine	10.0	100	200–400 ⁽⁵⁾ , 275 ⁽³⁾ , 250 ⁽⁴⁾
Thioglycollate	10.0	100	200–700 ⁽⁵⁾ , 200 ⁽³⁾ , 300 ⁽⁴⁾
Glutathione	10.0	100	—
Chloral	0.3	100	—
Phenobarbitone	3.0	100	—

(1) Feinstein & Volk (1949); (2) Hofman (1958); (3) Singer & Fruton (1957); (4) Paigen & Griffiths (1959); (5) Sundararajan & Sarma (1954); (6) Revel & Racker (1960).

Fig. 3. Effect of increasing concentrations of Mg²⁺ ions on the activity of brain phosphoprotein phosphatase. Enzyme from stage III. Experimental details were as described in the text.

was virtually complete within 20 min. in the absence of added Mg²⁺ ions and was complete within this time in the presence of the ions. Cessation of activity was not due to loss of enzymic activity since the addition of fresh α -casein, after incubation for 60 min., resulted in an immediate release of more inorganic phosphate. Addition of fresh enzyme was without effect once the action had ceased showing that the phosphatase was inert towards the product of dephosphorylation. In the

Fig. 4. Release of inorganic phosphate from α -casein with increasing concentrations of enzyme. The α -casein solution contained 10 mg.atoms of P/l. Enzyme from stage III. Incubation was for 15 min. at pH 5.9 and 37.5°.

presence of Mg²⁺ ions 85 % of the available phosphate was liberated within 10 min. A higher rate of phosphate release was obtained with phosvitin; the release was complete in 4 min. and 75–80 % of the available phosphate was liberated in 2 min.

This rate may not be maximal for phosvitin since release was measured under conditions optimum for α -casein; the optimum concentrations of Mg^{2+} ions may not be the same for both proteins. With a concentration of magnesium of 0.3 mM the optimum concentration of phosvitin was that yielding a concentration of phosphorus of 4.0μ -moles/ml. Higher concentrations of substrate inhibited the enzyme (Fig. 6), possibly owing to the binding of magnesium by excess of substrate. As with α -casein, once the reaction had ceased, the addition of further enzyme was without effect upon the dephosphorylated phosvitin.

Phosphate release from radioactive phosvitin. Cerebral tissues, yeast and liver are known to contain protein phosphokinases capable of phosphorylating added or intrinsic phosphoproteins (Burnett & Kennedy, 1954; Rabinowitz & Lipmann, 1960; Heald, 1961a). With a highly purified protein phosphokinase from yeast it was shown (Rabinowitz & Lipmann, 1960) that only a small fraction of the phosphorus from phosvitin was

capable of metabolic exchange, though the actual amount could not be assessed. Phosvitin was made radioactive by phosphorylation with the protein phosphokinase in a rat-liver mitochondrial preparation (Burnett & Kennedy, 1954), and the product subsequently dephosphorylated by the partially purified phosphoprotein phosphatase. The time-course of the dephosphorylation is presented

Fig. 5. Time-course of the release of inorganic phosphate from α -casein by the purified brain phosphoprotein phosphatase. System was as described in the Materials and Methods section. Enzyme from stage V. O, Phosphate release in the absence of Mg^{2+} ions; ●, phosphate release in the presence of 0.3 mM- Mg^{2+} ions. Fresh α -casein was added at A to one set of experimental flasks; fresh enzyme was added at B to the other set of experimental flasks.

Table 3. *Adenosine triphosphatase and inorganic pyrophosphatase in partially purified brain phosphoprotein phosphatase*

For the three substrates the assay systems were similar to those described in the Materials and Methods section and by Gore (1951) and Gordon (1953) respectively. Final concentrations: adenosine triphosphate and inorganic pyrophosphate, 7 mM; Mg^{2+} ion, 20 mM. For pH 7.4 the buffer was 0.1 M-tris-HCl.

pH	Phosphate released (μ g. of P/15 min.)		
	α -Casein	ATP	Inorganic pyrophosphate
5.9	27.0	5.0	1.0
7.4	7.0	35.0	5.0

Table 4. *Activity of phosphoprotein phosphatase from brain upon phosphoproteins*

Activity was assayed as described in the Materials and Methods section with the enzyme purified to stage V. All substrates except brain phosphoprotein were used in concentrations to yield 1–4.0 μ moles of P/ml. of assay system. Brain phosphoprotein was used at a concentration of 0.3 μ mole of alkali-labile P/ml. of assay system.

Phosphoprotein	Total P (%)	Alkali-labile P (%)	Phosphate released	
			Percentage of total P	Percentage of alkali-labile P
α -Casein	0.6	0.35	11	18
β -Casein	0.36	0.27	20	27
Phosvitin	7.0	7.0	5	5
Ovalbumin	0.09	0.09	6	6
Hexokinase	0.27	—	0	—
Pepsin	0.075	0.07	0	0
α -Casein phosphopeptide	0.8	0.50	17	26
Brain phosphoprotein				
I	—	0.05	—	7.0
II	—	0.05	—	8.5
III	—	0.035	—	7.0

Fig. 6. Inhibition of enzymic activity by increasing concentrations of phosvitin when added to the enzyme in the presence of 0.3 mM-Mg²⁺ ions. Enzyme from stage V.

Fig. 7. Time-course of the release of radioactive phosphate from phosvitin phosphorylated in the system of Burnett & Kennedy (1954) and incubated with phosphoprotein phosphatase, as described in the Materials and Methods section. Enzyme from stage III. O, Total phosphorus released; ●, radioactive phosphorus released.

in Fig. 7. The release of radioactivity paralleled that of inorganic phosphate and when the reaction has ceased 85% of the total radioactivity had been released.

DISCUSSION

The enzyme liberating inorganic phosphate from α -casein which has been prepared from ox brain can reasonably be described as a phosphoprotein phosphatase. In the absence of added Mg²⁺ ions the rate of liberation of phosphorus from α -casein by aqueous dispersions of ox brain was 25–30 μ moles/g. wet wt. of tissue/hr. and, for similar dispersions of rat and guinea-pig brain, 50–60 μ moles/g. wet wt. of tissue/hr.

The pH optimum of 5.5–6.0 and the Michaelis constant of 3.7 mM- α -casein phosphorus are similar

to the values found by Sundararajan & Sarma (1954) and Hofman (1958) for phosphoprotein phosphatase from spleen. In other respects, the two enzymes differ. The spleen enzyme, though purified to a state of homogeneity as determined by electrophoresis, ultracentrifuging and chromatography (Glomset, 1959; Glomset & Porath, 1960), was highly active towards phenyl phosphate, phosphocreatine, sodium phosphoamidate, inorganic pyrophosphate and adenosine triphosphate at pH 5.9. The brain-enzyme preparation did not act on the first four of these substrates and its slight activity towards the last-named two was probably due to the contaminating adenosine triphosphatase and inorganic pyrophosphatase (Table 3).

There are also sharp distinctions in the effects of activators and inhibitors on the two enzymes (Table 3). Thus the brain enzyme is activated by Mg²⁺ and Mn²⁺ ions but not by thiol compounds, whereas the spleen enzyme, reported variously to be activated or to be unaffected by Mg²⁺ and Mn²⁺ ions, has in all cases been stated to be activated by thiol compounds or ascorbate and in some cases to be inactive in the absence of thiols (Sundararajan & Sarma, 1957). In this respect the phosphoprotein phosphatase of rat or mouse liver (Feinstein & Volk, 1949; Paigen, 1958) appears to resemble more closely the spleen enzyme, being activated by thiols and reducing agents. Ferrous salts in concentrations up to 0.5 mM activate the phosphoprotein phosphatase of mouse liver (Paigen, 1958; Paigen & Griffiths, 1959) and of a stable spleen preparation (Revel & Racker, 1960), and 0.05 mM-molybdate strongly inhibits. The brain enzyme was inhibited by 0.1 mM-ferrous salts and not by molybdate at concentrations up to 10 mM. Unlike the stable spleen enzyme, that from brain was inhibited by sodium fluoride.

Brain phosphoprotein phosphatase is also less stable than this spleen enzyme, and in this respect the brain enzyme more closely resembles other phosphoprotein phosphatases obtained from ox spleen by Roche, Van Thoi & Pin (1953) and from chick embryo by Foote & Kind (1953). Both these enzymes were unstable in aqueous solution whereas the spleen enzyme was also inactivated when precipitated by ammonium sulphate. These last-named enzymes are distinct from the spleen preparations studied by Sundararajan & Sarma (1954), Hofman (1958), Singer & Fruton (1957) and Revel & Racker (1960), which are stable to dialysis and precipitation by ammonium sulphate and can be obtained by autolysis of spleen at pH 3.0 for 16 hr. In its instability, the brain enzyme resembles cerebral adenosine triphosphatase and inorganic pyrophosphatase (Gore, 1951; Gordon, 1953). These enzymes were also largely inactivated by dialysis or during precipitation with ammonium

sulphate, but their activities could be partially restored by incubation with cysteine.

Where comparable data exist the brain enzyme resembles in specificity at least the phosphoprotein phosphatase isolated from chick embryo by Foote & Kind (1953), or that studied in frog eggs by Harris (1946). Little additional information about the last-named two enzymes exists.

A further difference between the cerebral enzyme and that from spleen lies in its action upon the phosphoproteins α -casein and phosvitin. Thus the spleen enzyme liberates phosphate from casein or phosvitin over a period of several hours until the total phosphate has been removed (Sundararajan & Sarma, 1954; Hofman, 1958; Rabinowitz & Lipmann, 1960), whereas the brain enzyme rapidly liberates a definite amount of phosphate and then ceases to act. This phosphate includes most of the radioactive phosphorus incorporated by the action of a protein-phosphokinase preparation. This suggests that the brain phosphatase and the liver protein phosphokinase may be acting upon similar sites in the phosphoprotein molecule, but further work with more purified enzymes is needed. Phosvitin, as prepared by the method of Mecham & Olcott (1949), contains 25–30 phosphorus atoms/molecule. The release of 5–6 % of the total phosphorus by the phosphatase corresponds to the release of approximately a single phosphate group/molecule.

The phosphoprotein phosphatase did not act on pepsin, which has a single seryl phosphate in the sequence glutamic acid–serine–phosphorus–threonine (Flavin, 1954). On the other hand, both casein and phosvitin contain sequences of adjacent seryl phosphate residues (Williams & Sanger, 1959; Heald, 1961*b*) and it has been suggested (Williams & Sanger, 1959; Rabinowitz & Lipmann, 1960) that sequences of this type are responsible for the ability of these phosphoproteins to act as acceptors for protein phosphokinases. It seems significant that the substrates attacked by the phosphatase are those capable of acting as phosphate acceptors for the protein phosphokinases.

The release of phosphate from ovalbumin is not understood. Ovalbumin has been found to be attacked by spleen phosphoprotein phosphatase (Singer & Fruton, 1957) and to act, to a slight degree, as a phosphate acceptor for the liver protein phosphokinase (Burnett & Kennedy, 1954). Ovalbumin contains two phosphorus groups/molecule and studies upon phosphopeptides derived from partial acid hydrolysates (Flavin, 1954) have established the existence of two different sequences containing seryl phosphorus. Possibly the effects noted above are due to impurities in the preparation. The phosphoprotein phosphatase from spleen has been used previously in attempts to investigate

the nature of phosphate bonds in casein (Hofman, 1958) and has been studied as a possible means of detecting phosphorylated intermediates formed during oxidative phosphorylation (Revel & Racker, 1960). Such uses cannot yet be envisaged for the phosphoprotein phosphatase from brain, for though this enzyme is more specific than that from spleen, in the absence of further studies no firm conclusions can be drawn about the structures on which it acts.

SUMMARY

1. A phosphoprotein phosphatase acting on α -casein has been purified from ox brain 100-fold and in a yield of 25 %.

2. The pH optimum of the enzyme is 5.5. It is activated by Mg^{2+} or Mn^{2+} ions but not by thiol compounds. In the absence of Mg^{2+} ions $K_m = 3.7$ mg.atoms of α -casein phosphorus/l.

3. The enzyme lost activity when dialysed against water or salt solutions. With crude preparations the loss was prevented by dialysis against cysteine (20 mM). With more purified preparations cysteine did not prevent the loss of activity on dialysis. Activity was also lost during fractionation with ammonium sulphate and with graded concentrations of acetone.

4. At pH 5.9 the enzyme was inactive towards aliphatic and aromatic phosphonoesters, phosphoamides, a phosphodiester, inorganic polymetaphosphates, pyrophosphates, polyseryl phosphates and mixed phospholipids. It was active towards α - and β -casein, phosvitin, ovalbumin and preparations of brain phosphoprotein but not towards hexokinase or pepsin. Phosphopeptides prepared from α -casein were attacked by the enzyme.

5. The enzyme liberated only a small proportion of the total phosphate from α -casein and phosvitin before action ceased. The phosphorus released from phosvitin included 85 % of the radioactive phosphorus incorporated by incubation with a protein phosphokinase.

6. The brain enzyme differs from phosphoprotein phosphatases derived from spleen or liver.

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Enzymic Synthesis of 3:6-Anhydro-L-Galactose within Porphyrin from L-Galactose 6-Sulphate Units

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3:6-Anhydrogalactose is of general occurrence as a constituent of the mucilaginous intercellular polysaccharides of the red seaweeds (Rhodophyceae), sometimes occurring as the D- and sometimes as the L-enantiomorph (for a review, see Araki, 1958). It was first discovered in commercial agar (Hands & Peat, 1938), and has been found in an increasing number of these polysaccharides (Yaphe, 1959). Jones & Peat (1942) pointed out that it could arise from the L-galactose 6-sulphate units which they postulated to be present in agar, perhaps by chemical action during the processing of the polysaccharide, since it had been shown that methyl galactopyranoside 6-sulphate when heated with alkali gave rise to methyl 3:6-anhydrogalactopyranoside (Duff & Percival, 1941). This view was contested (Barry & Dillon, 1944; Percival, 1944; Araki, 1953), because agar had never been isolated containing sufficient ester sulphate or L-galactose to account on this basis for the high anhydrogalactose content, and also because agar was not oxidized by the periodate ion. Percival (1944) pointed out that, if the precursor of the anhydro sugar is a derivative of a sugar sulphate, it need not be that of a 6-sulphate, since a sugar 3-sulphate could equally well be imagined to give 3:6-anhydro-

galactose on suitable treatment. A further mechanism was proposed by Araki (1953), who suggested that oxidation at C-6 and reduction at C-1 would convert D-galactofuranose into 3:6-anhydro-L-galactose.

Hirst (1958) has again drawn attention to the possible role of galactose sulphate derivatives as precursors, but leaving open the nature of the derivatives involved. The interconversion and modification of monosaccharides usually occurs at the sugar nucleotide or sugar phosphate level (Hough & Jones, 1956; Hassid, Neufeld & Feingold, 1959). However, the possibility that this transformation from L-galactose 6-sulphate into 3:6-anhydro-L-galactose, if it occurs, takes place at the polysaccharide level, cannot be excluded, because the reaction might take place before the polysaccharide is deposited in the intercellular regions of the organism, and the fact that it was once highly sulphated would not then be evident (Percival, 1949).

Porphyrin, the major polysaccharide of the red seaweed *Porphyra umbilicalis*, is related to agar in that it contains residues of 3:6-anhydro-L-galactose (10.3%; Peat, Turvey & Rees, 1961). It also contains residues of L-galactose 6-sulphate (Turvey & Rees,