Turnover of Protein-Bound Phosphorylserine in Membrane Preparations from Ox Brain Catalysed by Intrinsic Kinase and Phosphatase Activity

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1. The turnover of protein-bound phosphorylserine in preparations of membrane fragments from ox brain cortex was studied. 2. Turnover was considered to arise from the action of intrinsic protein kinases and phosphatases on a membrane protein or proteins. 3. Properties of the kinase system were studied by measuring the rate of incorporation of ^{32}P from γ -labelled ATP into protein-bound phosphorylserine isolated from partial acid hydrolysates of membrane proteins. 4. Properties of the phosphatase system were studied by observing the rate of loss of ^{32}P from membrane preparations pre-labelled with $[\text{}^{32}\text{P}]\text{ATP}$. 5. Net phosphorylation and dephosphorylation of membrane protein was observed during incubation of membrane preparations with and without ATP. 6. The rate of turnover was about 4 nmol of P/h per mg of protein at 20°C; dephosphorylation was considered to be the rate-limiting step.

Turnover of the phosphorus of phosphorylserine contained in the cellular phosphoproteins was first observed in intact tissues some years ago (for reviews of the early work see Ågren, 1958; Heald, 1960). Subcellular studies have since shown that phosphoproteins from all parts of the cell are involved in this turnover. It presumably results, in part at least, from the action of protein kinases and phosphatases on specific protein substrates, although it is only in the case of cytoplasmic systems from muscle (Fischer & Krebs, 1966; Holzer, 1969; Wieland & Siess, 1970) and nuclei from liver and lymphocytes (Langan, 1966; Ord & Stocken, 1967, 1968; Kleinsmith & Allfrey, 1969*a,b*) that there is any firm evidence as to the nature of the processes concerned.

The present studies of the turnover of protein phosphorus in the cell membrane stem from an early observation that a fraction of the protein-bound phosphorylserine in respiring slices of cerebral cortex, apparently located in the neuronal membrane, increases in turnover rate on depolarization of the tissue with electrical pulses (Heald, 1962; Trevor & Rodnight, 1965; Jones & Rodnight, 1971). The physiological significance of this finding is not known: proposals (Heald, 1962; Judah & Ahmed, 1964) that this particular membrane phosphoprotein is directly involved as a carrier in transport of Na^+ have not been supported by detailed study of the enzyme system concerned in

ion movements across membranes (Albers, 1967; Kahlenberg, Galsworthy & Hokin, 1967; Chignell & Titus, 1968; Alexander & Rodnight, 1970; Alexander, 1971) although some other role for protein-bound phosphorylserine in cation transport remains possible. Recent work suggests that the increased turnover on stimulation may be related to the increased tissue concentration of cyclic AMP (adenosine 3':5'-cyclic monophosphate) which occurs on application of electrical pulses to cerebral tissues (Kakiuchi, Rall & McIlwain, 1969), but the significance of this event is also obscure. In any case, interpretation of these and other findings on phosphoprotein turnover in intact tissues is limited by the lack of any systematic knowledge of the nature, properties and complexity of the membrane-bound phosphoproteins and their associated enzymes.

If the turnover of phosphorylserine P in the membrane results from the action of membrane-bound kinases and phosphatases acting on membrane proteins, it should be possible, by using isotopically labelled ATP as a precursor, to demonstrate turnover in cell-free preparations of membrane fragments. It is already known that such preparations from brain and other tissues contain a system that catalyses the transfer of the γ -phosphate group of $[\text{}^{32}\text{P}]\text{ATP}$ to serine hydroxyl groups located in the membrane proteins (Rodnight & Lavin, 1966; Weller, 1969; Blat & Harel, 1970). This intrinsic

protein kinase activity appears to be distinct from a membrane-bound kinase that phosphorylates added phosphovitin and which may be termed the extrinsic protein kinase activity of the membrane (Rodnight & Lavin, 1964). It is notable that in several tissues the intrinsic, but not the extrinsic, reaction is stimulated by low concentrations of cyclic AMP (Weller & Rodnight, 1970; Rodnight & Weller, 1971). In previous work from this laboratory (Rodnight & Lavin, 1966) on the intrinsic system turnover was not observed and it was uncertain to what extent the observed transfer of phosphorus to the membrane consisted of a phosphorylation of vacant serine hydroxyl groups or of an exchange of labelled phosphorus with phosphorylserine P. Further, membrane-bound phosphatases dephosphorylating intrinsic membrane proteins have not been described and the protein phosphatase in brain which dephosphorylates casein (Rose & Heald, 1961) is mainly located in the mitochondria and cell cytoplasm (Rose, 1962). The present work demonstrates a turnover of protein-bound phosphorylserine in membrane fragments and supports the conclusion that the transfer of phosphorus to the membrane involves the phosphorylation of serine hydroxyl groups.

EXPERIMENTAL METHODS

Materials. Phosvitin was obtained from Nutritional Biochemicals Corp., Cleveland, Ohio, U.S.A., and purified by the procedure of Rose & Heald (1961). All chemicals were of the purest quality available.

Preparation of phosvitin kinase. The partially purified enzyme was prepared from whole guinea-pig brain by the procedure used by Rodnight & Lavin (1964), except that a potassium phosphate buffer containing KCl (0.1 M- KH_2PO_4 -0.05 M-KCl adjusted to pH 7.2 with KOH) was used instead of the sodium phosphate buffer to extract the acetone powder. Without the KCl less material was extracted but the results were still better than those obtained by using sodium phosphate alone.

Membrane preparations. Ox brain microsomal fractions were prepared by the method of Rodnight (1970). Crude preparations of synaptosomal membranes were prepared from the same source by the method of Rodnight, Weller & Goldfarb (1969). To facilitate comparison between different preparations each preparation was numbered with the code M for microsomal fractions and S for synaptosomal membranes.

Radioactive ATP. [^{32}P]ATP was prepared as the tris salt by the procedure of Rodnight, Hems & Lavin (1966).

Determination of protein. The method of Miller (1959) was used with bovine plasma albumin as standard.

Assay of phosvitin kinase activity. The procedure of Rodnight & Lavin (1964) was used. ^{32}P radioactivity (c.p.m.) in the precipitated and washed protein was determined by measuring Cerenkov radiation at an efficiency of about 40% in a Packard Tri-Carb liquid-scintillation spectrometer.

Intrinsic protein kinase activity. The method used

differed in several respects from that described by Rodnight & Lavin (1966). The standard procedure was carried out in a volume of 2.5 ml with constant stirring and an ATP/protein ratio of 2.5 nmol/ μg . The basal medium contained 30 mM-tris-HCl buffer (pH 7.4), 1 mM-tris-ATP (γ - ^{32}P -labelled, 5×10^5 - 30×10^5 c.p.m./mol), 1 μM - MgSO_4 and 1 mg of membrane protein; other additions were made where indicated. The reaction was started by addition of protein and stopped with 2.5 ml of cold 10% (w/v) trichloroacetic acid. The precipitated protein was centrifuged down and washed three times at 4°C in the centrifuge with 10% trichloroacetic acid containing 1 M- H_3PO_4 , twice with 10% trichloroacetic acid and twice with ethanol-diethyl ether (1:1, v/v). In trial experiments we found very similar yields of labelled phosphorylserine in the protein residue after extraction with ethanol-ether or neutral chloroform-methanol (2:1, v/v). With chloroform-methanol-conc. HCl (2:1:0.01 by vol.) the yield of [^{32}P]phosphorylserine was about 15% lower, presumably owing to the extraction by this reagent of phosphorylserine bound to the phosphatidopeptide fraction (Ledig & Mandel, 1969). The protein residue was then hydrolysed in stoppered tubes with 0.6 ml of 2 M-HCl in the presence of 0.1 μmol of carrier phosphorylserine for 6 h at 100°C and the hydrolysate freeze-dried in silicone-treated conical tubes. The residue was dissolved in water and the whole of the sample subjected to electrophoresis at pH 1.9 in the buffer used by Jones & Rodnight (1971) on Whatman 3MM paper at 50 V/cm for 1.5 h. The separation obtained is shown in Fig. 1; the labelled material remaining at the origin consists of peptides containing phosphorylserine. The paper was dried, stained for phosphate (Wade & Morgan, 1953) and the areas corresponding to phosphorylserine and P_i cut into four or five pieces and placed directly in vials containing 10 ml of scintillation fluid [0.5% 2,5-diphenyloxazole and 0.03% 1,4-bis-(4-methyl-5-phenyloxazol-2-yl)benzene in toluene]. Standards were set up at the same time by spotting a known quantity of

Fig. 1. Electrophoresis of a partial acid hydrolysate of membrane proteins from a microsomal preparation after labelling with [^{32}P]ATP for 1 min at 20°C by the standard procedure. The labelled protein was isolated and hydrolysed in the presence of carrier phosphorylserine as described in the Experimental Methods section and the equivalent of 1 mg of membrane protein applied to the paper. After electrophoresis the paper was dried, stained to detect phosphate and scanned for radioactivity.

[32 P]ATP on paper, drying and cutting out as described above. 32 P radioactivity (c.p.m.) was determined at an efficiency of about 95% in the Packard Tri-Carb spectrometer. The results have not been corrected for loss of protein-bound phosphorylserine during hydrolysis, which amounted to about 50% of the total P in the preparation labile to treatment with 1M-NaOH for 18 h at 37°C (see below).

In a few experiments protein phosphorylation was measured by the procedure of Ahmed & Judah (1965). The reaction was carried out at 20°C in a final volume of 2 ml; the same conditions of stirring were used as in the standard procedure. The medium contained 5 mM-ATP (γ - 32 P-labelled, 1.3×10^7 c.p.m./ μ mol), 5 mM-MgSO₄, 30 mM-tris-HCl buffer (pH 7.4), either 115 mM-NaCl or 115 mM-choline chloride, and finally 5 mg of membrane protein. After preincubation of the medium containing the enzyme protein with stirring for 10 min at 20°C the reaction was started by the addition of labelled ATP. The reaction was stopped with trichloroacetic acid and the precipitated protein washed with acid and solvents exactly as described by Ahmed & Judah (1965). The washed protein was hydrolysed with 2 ml of 2M-HCl for 6 h at 100°C; the hydrolysates were made up to 3.5 ml and three portions of 1 ml taken and freeze-dried in silicone-treated tubes. Phosphorylserine was separated and its radioactivity determined as described above.

Dephosphorylation of labelled membrane protein. Ox brain microsomal fractions (25 mg of protein) were phosphorylated for 1 h by the standard procedure. The reaction mixture was then cooled on ice and a sample (1 mg of protein) denatured with trichloroacetic acid and set aside for [32 P]phosphorylserine analysis. The remainder of the incubation mixture was centrifuged in the GSA rotor of a Sorvall RC2B centrifuge at 12500g for 15 min. The supernatant was decanted and the pellet resuspended at a concentration of about 1 mg of protein/ml in 4 mM-imidazole-HCl buffer, pH 7.2, by a brief homogenization then centrifuged again. The pellet was washed once more with the buffer and finally suspended in the same buffer at a concentration of at least 4 mg of protein/ml. At this stage a sample was taken for determination of protein. Throughout the washing procedure the sample was maintained as near as possible to 0°C. No radioactivity could be detected in the supernatant from the final wash, but in some preparations, particularly those of microsomal origin, up to 25% of the incorporated radioactivity was lost during the procedure. The recovery of protein was 75–80%.

Dephosphorylation was measured in 30 mM-tris-HCl buffer, pH 7.4, in a volume of 2.5 ml at 37°C with rapid stirring. The reaction was started by adding 0.25 ml of labelled protein (1 mg) and stopped with an equal volume of 15% (w/v) trichloroacetic acid. Protein-bound [32 P]-phosphorylserine was measured at zero time and after various intervals by the procedure described above and the rate of loss of radioactivity (c.p.m.) obtained by difference. The results are expressed as 'c.p.m. in phosphorylserine/mg of protein' since the specific radioactivity of the protein-bound phosphorylserine was unknown.

Treatment of membrane preparations with sodium iodide. The procedure described by Nakao, Tashima, Nagano & Nakao (1965) was adopted with modifications. All

operations were carried out at 2°C. The NaI reagent was freshly prepared and contained 4 M-NaI, 5 mM-EDTA and 0.5 mM-L-cysteine. The membrane preparation was suspended at a concentration of 3–5 mg of protein/ml in 50 mM-tris-HCl buffer, pH 8.3, and sufficient 50 mM-ATP (disodium salt) and 100 mM-MgCl₂ added to adjust the Mg²⁺ and ATP concentrations to 5 mM. After 10 min an equal volume of NaI reagent was added and the mixture left for 30 min with occasional shaking. It was then diluted to 0.8 M-NaI with water and centrifuged for 5.9×10^5 g-min in the no. 30 rotor of the Spinco model L centrifuge. The precipitate was washed by centrifugation twice with 60 ml of 4 mM-imidazole-HCl containing 5 mM-EDTA (pH 7.2) and twice with 60 ml of the same buffer without EDTA.

In some experiments the first supernatant after the treatment with NaI was dialysed against 10 vol. of 2 mM-imidazole-HCl buffer, pH 7.2, for 20 h with six changes. During the dialysis a precipitate formed which was centrifuged down for 4.7×10^6 g-min, suspended in buffer and stored at –20°C. The supernatant was concentrated by ultrafiltration dialysis under negative pressure and also stored at –20°C.

RESULTS

Intrinsic protein kinase system

Time-course and other properties. Typical time-courses obtained at 20°C and 37°C are shown in Fig. 2. In the curve relating to the microsomal

Fig. 2. Time-course at 20°C and 37°C of incorporation of the γ -phosphoryl group of [32 P]ATP into protein-bound phosphorylserine in preparations of synaptosomal and microsomal membranes. Standard reaction conditions (see the Experimental Methods section) were used. O, Microsomal preparation (no. M3) 20°C; ●, microsomal preparation, 20°C with addition of 100 mM-NaCl; ▲, synaptosomal membranes (no. S4), 20°C; △, synaptosomal membranes, 37°C.

preparation incubated without Na^+ , over 80% of the ATP had been hydrolysed by Mg^{2+} -activated adenosine triphosphatases by the time the peak of phosphorylation was reached. In the synaptosomal preparation the amount of [^{32}P]phosphorylserine recovered at the height of phosphorylation was about 0.95 nmol/mg of protein, compared with about 2.0 nmol/mg of protein for the same preparation by determination of [^{32}P]P_i labile to alkali (Table 6). This 50% loss of phosphorylserine P on acid hydrolysis of the protein is considerably less than the 80% loss reported by Jones & Rodnight (1971) for an unfractionated cerebral-cortex tissue from the guinea pig hydrolysed in the absence of carrier phosphorylserine.

The reaction followed the same curvilinear course observed by Rodnight & Lavin (1966) where the total labelled phosphate bound to the membrane was used as a measure of transfer, rather than [^{32}P]phosphorylserine. The time-course was not linear even when the first observation was made at 5s; at longer time-intervals product inhibition and site saturation probably contribute to the curvilinear nature of the time-course: 0.5 mM-tris-ADP inhibited the initial phosphorylation rate by 30% at 1 min from zero time. Under the standard conditions of assay the protein kinase systems were apparently saturated with donor since the initial rate was the same when either 1 mM- or 2 mM-ATP was used; with 0.2 mM-ATP, however, the rate was decreased by 66%. Increasing the Mg^{2+} concentration until it was 5 times the ATP concentration also had no effect on the initial rate. The rate of transfer over 1 min in a synaptosomal membrane preparation did not vary greatly over the pH range 7.2–7.7, but was lower at pH 7.0 and 8.0. Replacement of chloride in the medium by acetate or sulphate, or of the tris buffer by imidazole, did not affect the phosphorylation from 0 to 1 min.

Inhibition by cations. The inhibitory effect of Na^+ and K^+ noted by Rodnight & Lavin (1966) was studied further in the present work. The inhibitory action of Na^+ was time-dependent: little inhibition was observed at 1 min, but at longer time-intervals the degree of inhibition progressively increased (Fig. 2, Table 1). Inhibition by K^+ , on the other hand, tended to start earlier (Table 1). Of the other univalent cations examined Li^+ gave the greatest inhibition, Cs^+ inhibited more at 1 min than at 10 min from zero time, whereas Rb^+ behaved like Na^+ . A small inhibition at 10 min was also given by NH_4^+ (Table 1). The protein kinase activity of a microsomal preparation from guinea-pig kidney was similarly inhibited by Na^+ and K^+ .

We were unable to repeat the results of Ahmed & Judah (1965), who found that Na^+ stimulated

Table 1. *Inhibition by univalent cations of labelling of phosphorylserine*

Intrinsic kinase activity in preparations of synaptosomal membranes was determined at 20°C by the standard procedure with and without the addition of univalent cations as the chlorides. Results are the means $\pm$ S.D. with the number of observations given in parentheses.

Cation (mM)	Intrinsic protein kinase activity (% of control)	
	1 min	10 min
Reaction time ...		
Na^+ (10)	103 $\pm$ 11 (5)	82 $\pm$ 5 (6)
Na^+ (100)	91 $\pm$ 5 (5)	77 $\pm$ 4 (6)
K^+ (10)	86 $\pm$ 6 (5)	87 $\pm$ 7 (5)
K^+ (100)	72 $\pm$ 7 (4)	70 $\pm$ 5 (5)
Li^+ (10)	91 $\pm$ 5 (4)	—
Li^+ (100)	54 $\pm$ 9 (4)	40 $\pm$ 5 (3)
Rb^+ (100)	97 $\pm$ 8 (5)	78 $\pm$ 5 (6)
Cs^+ (100)	80 $\pm$ 6 (6)	97 $\pm$ 8 (6)
NH_4^+ (100)	103 $\pm$ 6 (3)	88 $\pm$ 3 (4)

incorporation of ^{32}P into protein-bound phosphorylserine when measured at 5s with 5 mM-ATP as donor. Two experiments were carried out in triplicate and from each hydrolysate three samples were taken; no statistically significant stimulation of labelling by Na^+ was found ($P > 0.1$).

Calcium chloride and manganese chloride (both 0.1 mM) inhibited the initial rate of phosphorylation by 40–50% with 1 mM-MgATP as donor. Zinc chloride and cupric chloride (both 0.1 mM) inhibited phosphorylation by 80–90%.

Effects of drugs. These were incorporated in the reaction mixture and phosphorylation measured at 1 min and 10 min by the standard procedure at 20°C. In view of the stimulating effect of cyclic AMP on the reaction (Weller & Rodnight, 1970) observations were made with or without 10 μM -cyclic AMP. The following drugs (1 mM, unless stated otherwise) showed no consistent stimulation or inhibition within $\pm 20\%$: chlorpromazine, sodium pentobarbitone, sodium phenobarbitone, L-amphetamine, morphine, cocaine, atropine, procaine, ouabain, reserpine (0.1 mM) and ethanol (1.7 mM). Strychnine hydrochloride (1 mM), theophylline (10 mM), caffeine (20 mM) and adenosine (1 M) all inhibited phosphorylation by about 50%. Metrazol (pentylene tetrazol; 1 mM) increased the phosphorylation rate measured at 1 min by about 60% in three out of four experiments, but only in the absence of cyclic AMP; at 10 min no stimulation was observed. The effect of the methylxanthines is noteworthy, since these drugs also inhibit the phosphodiesterase that hydrolyses cyclic AMP to 5'-AMP; in fact, we found that the inhibitory power of theophylline on membrane-bound

diesterase activity towards cyclic AMP was very similar to that exerted on the kinase system, the K_i in both cases being about 10mM. The soluble phosphodiesterase in brain, however, is considerably more sensitive to inhibition by methylxanthines (Butcher & Sutherland, 1962). The effect of metrazol will merit further study when more is known of the nature of the membrane-bound kinase system.

Disruption of the system by various treatments. To gain information on the degree to which the kinase system constitutes an integral part of the membrane structure, membrane preparations were treated with various disrupting agents and kinase activity examined in 'soluble' and particulate fractions obtained by centrifugation. During these experiments attempts were made to dissociate enzyme from substrate, and the extent to which kinase activity could be restored by recombination of fractions was investigated, where appropriate.

The disrupting action of several detergents was first examined by homogenizing a preparation in an aqueous solution of the detergent at 0°C and at a protein concentration of 5–10mg/ml and centrifuging the homogenate for 6×10^6 g·min. Kinase activity in the supernatant fractions was determined without further treatment; the sediments were resuspended in a 4mM-imidazole-HCl buffer, pH 7.2, containing detergent at the same concentration as used in the original treatment before determination of enzyme activity. The addition of detergent to the sediments was necessary since some of the detergents modified kinase activity in the unfractionated homogenate.

Homogenization of membrane preparations in 0.4% Lubrol W or 0.5% Triton X-100 had no

significant effect on kinase activity before fractionation. On centrifugation of Lubrol-treated samples 20–25% of the protein remained in the supernatant, which contained, however, only 10–20% of the kinase activity. About 60% of the original activity remained in the insoluble sediment, resulting in a 20% loss of activity that was not recovered on recombination of the fractions. With Triton X-100 less than 10% of the kinase activity was recovered in the supernatant, and since the detergent released 33% of the protein, kinase activity/mg of protein in the insoluble fraction was increased; all the original activity was present in the two fractions after centrifugation.

Sodium deoxycholate (0.5%) caused a major loss of activity even before centrifugation, although about 30% of the recovered activity was present in the supernatant. Sodium dodecyl sulphate (0.1%) almost completely inhibited kinase activity in the homogenate and fractionation studies were not pursued.

Brief treatment of microsomal fractions at pH 10.5 was found by Decsi & Rodnight (1965) to 'solubilize' 10–20% of the membrane protein and 50–70% of the extrinsic protein kinase activity with phosvitin as acceptor. In the present work the same treatment was applied to two different synaptosomal preparations. In both cases the sum of the kinase activities recovered in the 'soluble' and insoluble fractions indicated that a substantial loss of activity (59 and 75%) had occurred; activity was not restored by recombination of the fractions. Of the recovered activity about 80% remained in the soluble fraction. Phosvitin kinase activity, on the other hand, was fully recovered and 54% appeared in the supernatant fraction.

Table 2. *Distribution of protein kinase activities after treatment of a membrane preparation with sodium iodide*

A preparation of synaptosomal membranes (no. S1) was treated with NaI by a modification of the procedure of Nakao *et al.* (1965) described in the Experimental Methods section. Intrinsic protein kinase activity was determined at 20°C by the standard procedure given in the text and phosvitin kinase activity by the procedure of Rodnight & Lavin (1964). The distribution of kinase activities is expressed as the percentage of the total activity present in the original sample before treatment.

Fraction	Protein (%)	Intrinsic protein kinase activity (pmol of 32 P in phosphorylserine/min)		Distribution (%)	Phosvitin kinase activity (nmol of P transferred/min)		Distribution (%)
		Activity/mg of protein	Mean total activity		Activity/mg of protein	Mean total activity	
Original sample before treatment	100	58, 62	10100	—	0.23, 0.27	42	—
Particulate	53	1.2, 1.5	120	1.2	0.02, 0.03	2.2	5
Supernatant							
Soluble on dialysis	22	0.5	18	0.2	0.16, 0.20	6.6	16
Precipitated on dialysis	9	4, 3.2	54	0.5	0.53, 0.56	8.2	20

Table 3. *Fractionation of a labelled membrane preparation with sodium iodide*

A preparation of synaptosomal membranes (no. S2, 43 mg of protein) was labelled with [32 P]ATP under standard conditions for 75 min at 20°C. The reaction mixture was cooled to 0°C, centrifuged for 3.5×10^6 g·min and the pellet washed twice in the centrifuge with 4 mM-imidazole-HCl buffer, pH 7.2. Samples were taken before and after washing for determination of 32 P radioactivity in phosphorylserine. The labelled material was treated with NaI as described in the Experimental Methods section.

Fraction	Protein (%)	Intrinsic protein kinase activity (pmol of 32 P in phosphorylserine/mg of protein)	Distribution of [32 P]phosphorylserine (%)
Original sample	100	850	100
After washing	70	570	47
After treatment, before centrifugation	100	505	100
Supernatant	58	313	36
Particulate	42	635	53

Treatment of membrane preparations with a high concentration of sodium iodide was found by Nakao *et al.* (1965) to yield a particulate fraction enriched several times in the Na^+ - and K^+ -stimulated adenosine triphosphatase and containing only minimal Mg^{2+} -dependent adenosine triphosphatase activity. As reported briefly by Weller & Rodnight (1968) this treatment almost completely abolished the intrinsic protein kinase activity not only in the particulate fraction but also in the other fractions resulting from the procedure (Table 2). Recombination of the fractions did not result in any restoration of activity. Treatment with sodium iodide also resulted in the loss of 60% of the phosphatase activity of the preparation, the recovered activity appearing almost entirely in the supernatant fraction (Table 2).

Evidence for dissociation of the enzyme-substrate complex by sodium iodide was sought by treating a labelled membrane preparation with sodium iodide and determining the protein-bound [32 P]phosphorylserine in the two fractions (Table 3). Just over half of the [32 P]phosphorylserine was recovered in the particulate fraction and the remainder was in the supernatant, making it difficult to make firm conclusions about the distribution of substrates.

Intrinsic protein phosphatase activity

General features. On incubation at 37°C membrane preparations that had been labelled for 1 h and washed by the procedure described in the Experimental Methods section lost [32 P]phosphorylserine in a time-dependent fashion. When metal ions were not added the rate of loss was approximately linear over the first 0.5 h and was accelerated by Mg^{2+} and inhibited by EDTA (Fig. 3). The pH optimum for dephosphorylation was 7.4 both in the presence and absence of Mg^{2+} (Fig. 4). No loss of

[32 P]phosphorylserine was observed on incubation of labelled protein that had been denatured with trichloroacetic acid and washed with water until neutral. It appears therefore that dephosphorylation under these conditions is enzymically catalysed and that at least some of the serine hydroxyl sites accepting phosphate groups through kinase action are also associated with phosphatases that transfer the phosphate group to water. Less than 50% of the incorporated radioactivity was lost; no further loss occurred after incubation for about 1 h or less, depending on the initial rate (Fig. 3).

Effect of cations and other substances. The intrinsic phosphatase activity was stimulated by manganese chloride (which appears to substitute for Mg^{2+}) and strongly inhibited by zinc and cupric salts. To a lesser extent the molybdate ion also inhibited (Table 4). Some inhibition was given by thiol reagents but not by sodium fluoride.

Quite low concentrations of sodium chloride and potassium chloride inhibited dephosphorylation. At 10 mM K^+ was more inhibitory than Na^+ (Fig. 5a) but at 10 times this concentration Na^+ gave the greater inhibition at all times. Fig. 5(b), which summarizes results obtained from three different preparations, shows that the ions changed their relative affinities for the system at around 40–50 mM.

The intrinsic phosphatase activity was also inhibited by adenine nucleotides (Table 5); ITP and GTP at comparable concentrations were ineffective. The inhibition in this case appeared to be mainly due to the complexing of Mg^{2+} by the adenine compounds, since to some extent it was reversed by increasing the Mg^{2+} concentration in the medium. However, 9 mM-ATP still inhibited by 60% and 9 mM-ADP by 30%, even when the Mg^{2+} concentration was 15 mM.

Net phosphorylation and dephosphorylation of membrane protein. The demonstration in the

Fig. 3. Time-course at 37°C of the loss of ^{32}P from phosphorylserine in microsomal preparation (no. M4) previously labelled with [^{32}P]ATP by the standard procedure. The labelled membrane fragments were first washed with buffer as described in the Experimental Methods section. ●, Time-course in 30 mM-tris-HCl buffer, pH 7.4; ▲, with addition of 1 mM-MgCl₂; ■, a single value obtained with addition of 10 mM-EDTA.

membrane preparations of intrinsic protein kinase and phosphatase activity apparently acting on the same serine hydroxyl site shows that a turnover of protein-bound phosphorylserine can occur in a sub-cellular preparation. However, these experiments do not clearly prove that the phosphorylation initially involves a vacant hydroxyl group; if this were the case it should be possible to demonstrate a net phosphorylation of membrane protein, provided that some dephosphorylation had occurred *post mortem* during the preparation of the membrane fragments. The problem was investigated by taking the amount of bound phosphate labile to treatment with 1M-sodium hydroxide as a measure of protein-bound phosphorylserine (Table 6). Phosphorylation of synaptosomal membranes for 1 h at both 20°C and 37°C gave small but consistent increases in the concentration of alkali-labile P in the membrane. However, in the same experiments incorporation of ^{32}P from [^{32}P]ATP into the alkali-labile fraction was about twice the corresponding increase in total phosphoprotein-P in the membrane: the maximum incorporation of ^{32}P (corrected for zero-time radioactivity) ranged from 1.24 to 2.1 $\mu\text{mol}/\text{mg}$ of protein, as compared with an increase during the same period in total alkali-labile P of 0.4–1.3 $\mu\text{mol}/\text{mg}$ of protein. This discrepancy suggested that during incubation under phosphorylating conditions dephosphorylation as well as phosphorylation of membrane

Fig. 4. Rate of dephosphorylation at 37°C of a labelled microsomal preparation (no. M4) as a function of pH. The time-course over a 30 min period was determined as indicated in Fig. 3 for each pH and the initial rate estimated by drawing the tangent to the curve from zero time. ○, Δ, Mg²⁺ absent (left-hand ordinate); ●, with 1 mM-MgCl₂ (right-hand ordinate). ○, Tris-phosphate buffer; Δ, tris-HCl buffer.

protein was occurring. The same preparations were therefore incubated under identical conditions in the absence of ATP, and in some instances, of Mg²⁺. A loss of alkali-labile P was found at both 20°C and 37°C; the loss was greater in the presence of Mg²⁺ and in the preparation (no. S5) containing the highest initial concentration of alkali-labile P (Table 6). At both temperatures the losses occurring during incubation without ATP, when added to the net phosphorylation found in the presence of ATP, were adequate to account for the observed incorporation of ^{32}P .

The above experiments and inspection of the time-course shown in Fig. 2 show that dephosphorylation proceeds at a markedly slower rate than phosphorylation and is a rate-limiting step in the turnover of protein-bound phosphorylserine in the membrane. The initial rate of incorporation of ^{32}P should therefore depend on the degree to which the membrane proteins are phosphorylated at the start of the experiment. We tested this hypothesis (a) by preincubating a membrane preparation with non-radioactive ATP for various times and then labelling with [^{32}P]ATP and (b) by measuring the phosphorylation rate in preparations preincubated in the absence of ATP.

After preincubation with unlabelled ATP (1 mM) for 10 min the initial rate (at 1 min) of transfer of ^{32}P from labelled ATP was halved and the subsequent time-course was linear for at least 10 min (Fig. 6a). After preincubation for shorter times ^{32}P transfer was progressively increased until at 1 min it was nearly 80% of the control value (Fig.

Table 4. *Effect of various compounds on intrinsic protein phosphatase activity*

An ox brain microsomal fraction (no. M1) was labelled with [32 P]ATP and washed as described in the Experimental Methods section. Samples of the labelled protein (1 mg) were incubated for 30 min at 37°C in 30 mM-tris-HCl buffer, pH 7.4, with the additions indicated. The control sample contained 30 mM-tris-HCl and 1 mM-MgCl₂. The reaction was stopped with trichloroacetic acid and the residual protein-bound [32 P]phosphorylserine determined as described in the text. Phosphatase activity was calculated by subtracting this value from that obtained from a sample analysed at zero time.

Additions		Intrinsic protein phosphatase activity (% of control)
MgCl ₂ (mM)	Other compounds	
4	—	98
—	—	60
—	1 mM-MnCl ₂	202
1	10 mM-CaCl ₂	50, 70
1	10 mM-BaCl ₂	88, 100
1	0.1 mM-ZnCl ₂	18, 21
1	0.1 mM-CuCl ₂	13
1	0.05 mM-CuCl ₂	50, 70
1	1 mM-NaF	88, 96
1	1 mM-Ammonium molybdate	31, 45
1	1 mM- <i>p</i> -Chloromercuribenzoate	45, 68
1	1 mM- <i>N</i> -Ethylmaleimide	68, 96
1	1.75M-Ethanol	90, 100

Fig. 5. Inhibitory effect of NaCl and KCl on dephosphorylation of labelled membrane protein. (a) Time-course of inhibition in a microsomal preparation (no. M4). All media contained 30 mM-tris-HCl buffer, pH 7.4, and 1 mM-MgSO₄. ○, Control; □, with 10 mM-KCl; △, with 10 mM-NaCl. The closed symbols related to different membrane preparations, which gave comparable control time-courses (not shown): ▲, with 100 mM-NaCl; ■, with 100 mM-KCl; ▼, with 100 mM-LiCl. (b) Inhibition by different concentrations of Na⁺ and K⁺ in three preparations. Dephosphorylation was measured at 30 min as indicated in Table 4. Open symbols represent Na⁺ and closed symbols K⁺; each point is the mean of two observations. The regression line for each cation was calculated by assuming a linear relationship between concentration and inhibition in the range 10–100 mM; the difference in slope between the two lines in this range was highly significant ($P < 0.001$). △, ▲, Preparation no. S4; ○, ●, preparation no. S6; □, ■, preparation no. M1.

6b); the time-course of phosphorylation on addition of the radioactive ATP also became increasingly curvilinear as the preincubation period was decreased.

We were less successful in demonstrating a clear-cut effect on phosphorylation after preincubation under dephosphorylating conditions (Table 7). At 37°C preincubation for 30 or 60 min

Table 5. *Effect of nucleotides on intrinsic protein phosphatase activity.*

The intrinsic activity was measured in a microsomal preparation (no. M2) and the results are expressed as described in Table 4.

Additions		Intrinsic protein phosphatase activity (% of control)
Nucleotide (mM)	MgCl ₂ (mM)	
None	1.0	100
ATP (3)	3.0	78, 82
ATP (9)	9.0	30, 20
ATP (9)	15.0	45, 30
ADP (9)	9.0	60, 65
ADP (9)	15.0	65, 70
ITP (8.5)	8.5	90, 110
GTP (4.0)	4.0	96, 100
Cyclic AMP (0.6)	1.0	70, 50
Cyclic AMP (0.6)	3.0	90, 95

in tris buffer, with or without Mg²⁺, markedly inactivated the kinase system. At 20°C preincubation for 60 min had no apparent effect on the initial rate of phosphorylation, but it was found that after 10–15 min rather more ³²P had been incorporated than in the control sample, suggesting that the rate-limiting effect of the phosphatase system was acting later. The enhancement was not very marked, however, and it is possible that at 20°C as well as 37°C some inactivation occurs during preincubation.

DISCUSSION

Turnover of protein-bound phosphorylserine in membrane fragments. The above results clearly demonstrate a slow turnover of protein-bound phosphorylserine in membrane fragments incubated with ATP. This appears to arise from the coupled action of kinase and phosphatase enzymes acting on a tightly bound membrane protein or proteins. The initial step probably consists of the transfer of the γ -phosphoryl group of ATP to a free serine hydroxyl group, followed by transfer to water to give P_i. If exchange with phosphorylated serine hydroxyl groups were involved, or if the kinase reaction were reversible, ATP and ADP might have been expected to accelerate rather than retard the loss of ³²P radioactivity from the labelled protein. Phosphorylation by the kinase system therefore appears to be more analogous to the phosphorylation of a serine residue in phosphorylase *b* by phosphorylase kinase, a reaction that Krebs & Fischer (1962) considered to be essentially irreversible, than to the phosphorylation of phosphovitin by yeast protein kinase, which Rabinowitz &

Lipmann (1960) found under certain conditions to be reversible.

The absence of evidence for turnover of protein-bound phosphorylserine in previous studies (Rodnight & Lavin, 1966; Rodnight *et al.* 1966) led to the use of the term 'stable' to characterize this type of labelling of membrane proteins by [³²P]ATP (Skou & Hilberg, 1969). This description can now be seen to be inaccurate, although the rate of turnover of phosphorylserine is still many times slower than that of the acyl-bound phosphate associated with the Na⁺- and K⁺-stimulated adenosine triphosphatase system. In membrane preparations of the type used in the present study the latter system hydrolyses ATP at 20°C at a rate of about 40 μ mol/h per mg of protein; the rate of ATP hydrolysis at this temperature through phosphorylserine turnover, estimated from the linear phosphorylation rate in Fig. 6(a), amounts at the most to 4 nmol/h per mg of protein (after correction for a 50% loss of phosphorylserine on hydrolysis). However, the maximum capacity of the kinase step is considerably greater: taking ³²P transfer over the first minute as a minimum measure of this, rates at 20°C of about 12 and 24 nmol of phosphorus transferred/h per mg of protein in the absence and presence of cyclic AMP are obtained; at 37°C the corresponding rates would be approximately double.

Properties of the kinase system. It is difficult to draw any firm conclusions about these because, except over the first minute of incubation when it appears that vacant serine hydroxyl groups are mainly being phosphorylated, the rate of transfer is limited by the slower action of the associated phosphatase. However, there is enough evidence to conclude that the system studied here possesses several properties that appear to distinguish it from the other protein kinases that have been described in brain. Thus, in contrast with the intrinsic enzyme, the soluble extrinsic enzyme which phosphorylates phosphovitin (Rabinowitz & Lipmann, 1960; Rodnight & Lavin, 1964; Decsi & Rodnight, 1965) is stimulated by Na⁺ and K⁺, is not inhibited by thiol reagents and is not stimulated by cyclic AMP. Also the fraction of this latter enzyme associated with membrane preparations is relatively easily solubilized (Decsi & Rodnight, 1965), whereas the present study indicates that the intrinsic system is tightly bound to the membrane structure. Both these enzyme systems, however, are inhibited to various degrees by Ca²⁺, Zn²⁺, Mn²⁺ and Cu²⁺ and these properties distinguish them from the protein kinase studied by Miyamoto, Kuo & Greengard (1969; Ca²⁺-stimulated activity measured in the absence of cyclic AMP), and, for Ca²⁺, from the cyclic AMP-dependent kinase present in neurotubular subunits (Goodman,

Table 6. *Net changes in alkali-labile P on incubation of membrane preparations with and without ATP*

Preparations of synaptosomal membranes were incubated in 30 mM-tris-HCl buffer, pH 7.4, with the addition of 1 mM-MgSO₄ and/or 1 mM-[³²P]ATP as indicated. Samples containing 7 mg of protein were withdrawn at zero time and after incubation, 10 M-HClO₄ was added to give a final concentration of 0.5 M and the precipitated protein spun down. After washing with acid and lipid solvents and extraction of nucleic acids with hot trichloroacetic acid, alkali-labile P was determined by the procedure of Mollwain & Rodnight (1962). ³²P in the alkali-labile fraction was determined by measuring Čerenkov radiation in samples of the isobutanol-benzene layer. Results are means $\pm$ s.d. with the number of observations given in parentheses.

Membrane preparation (code no.)	Incubation conditions		Addition to medium		³² P transferred to alkali-labile P (nmol/mg of protein)	Total alkali-labile P (nmol/mg of protein)	Change in alkali-labile P from unincubated control (nmol/mg of protein)
	Time (min)	Temperature (°C)	MgSO ₄	[³² P]ATP			
S3	0	—	+	+	0.06 $\pm$ 0.01 (4)	7.3 $\pm$ 0.3 (4)	—
S3	60	20	+	+	0.30 $\pm$ 0.1 (4)	7.7 $\pm$ 0.5 (4)	+0.4
S4	0	—	+	+	0.05 $\pm$ 0.02 (8)	6.8 $\pm$ 0.26 (16)	—
S4	60	20	+	+	2.11 $\pm$ 0.1 (5)	7.7 $\pm$ 0.24 (5)	+0.9
S4	30	37	+	+	2.15 $\pm$ 0.1 (3)	7.7 $\pm$ 0.4 (3)	+0.9
S4	60	37	+	+	2.05 $\pm$ 0.2 (3)	8.1 $\pm$ 0.2 (3)	+1.3
S4	0	—	—	—	—	6.8 $\pm$ 0.26 (16)	—
S4	60	20	—	—	—	6.2 $\pm$ 0.2 (3)	-0.6
S4	60	20	+	—	—	5.9 $\pm$ 0.4 (3)	-0.9
S4	60	37	+	—	—	5.9 $\pm$ 0.3 (5)	-0.9
S5	0	—	—	—	—	7.9 $\pm$ 0.3 (3)	—
S5	60	37	—	—	—	5.8 $\pm$ 0.2 (3)	-2.1
S5	60	37	+	—	—	5.5 $\pm$ 0.3 (3)	-2.4

Fig. 6. Effect of preincubation with unlabelled ATP on the labelling of membrane protein with [^{32}P]ATP. Samples of synaptosomal membranes (no. S4, 1 mg of protein) were preincubated for various times in 30 mM-tris-HCl buffer, pH 7.4, containing 1 mM-ATP in a volume of 2.25 ml at 20°C. Labelling was carried out in the same tubes by the addition of 0.25 ml of 10 mM- ^{32}P]ATP, giving a concentration of ATP in the preincubated tubes of between 1.8 and 1.95 mM (depending on the time of preincubation) and a Mg^{2+} concentration of 0.9 mM. The exact initial concentration and specific radioactivity of the [^{32}P]ATP was calculated in each case in parallel experiments by determining the P_i released from the unlabelled ATP during preincubation. In other experiments it was shown that the phosphorylation rate was constant with 1–2 mM-ATP when the Mg^{2+} was maintained at 0.9 mM. (a) Time-course of labelling after preincubation for 2 and 10 min: $\circ$, control; $\triangle$, preincubated 2 min; $\square$, preincubated 10 min. (b) Effect of preincubation for various times on the initial rate of labelling over 1 min.

Table 7. *Effect of preincubation under dephosphorylating conditions on kinase activity*

Synaptosomal membranes were preincubated at a concentration of 0.5 mg of protein/ml in 37 mM-tris-HCl buffer, pH 7.4. Intrinsic protein kinase activity was determined at 20°C on 1 mg samples by the standard procedure, Mg^{2+} not being added where it was already present.

Preparation (code no.)	Preincubation conditions			Reaction time ...	Intrinsic protein kinase activity (pmol of ^{32}P in phosphorylserine/min)			
	Temperature (°C)	Time (min)	MgSO_4 (1.25 mM)		1 min	5 min	10 min	15 min
S5	—	0	—		46, 37	—	230, 242	—
S5	37	30	Absent		29, 26	—	136, 138	—
S5	37	30	Present		31, 32	—	150, 155	—
S5	37	60	Present		29, 23	—	105, 90	—
S4	—	0	—		90, 80	285, —	360, 375	475, 500
S4	20	60	Present		70, 100	250, —	400, 450	550, 580

Rasmussen, DiBella & Guthrow, 1970); the four enzyme activities are compared with respect to their sensitivities to bivalent cations in Table 8.

Interpretation of the inhibition of ^{32}P transfer by Na^+ and K^+ is complicated by the new observation that these ions also inhibit the phosphatase system. Since Na^+ gave virtually no inhibition over the first minute of incubation it is probable that its effect on kinase activity is minimal and that the apparent inhibition at longer time-intervals is due to a direct action on the phosphatase. For K^+ the results in Table 1 suggest that to a small extent the kinase is directly involved.

Stimulation of the kinase activity by cyclic AMP was found by Weller & Rodnight (1970) to be greatest at 1 min and to become progressively less as incubation continued so that after 10 min hardly any increase in activity was observed. The present experiments suggest that the decline in percentage stimulation is probably due to the rate-limiting effect of the phosphatase system increasing as the membrane protein becomes more fully phosphorylated. This is supported by the observation (M. Weller & R. Rodnight, unpublished work) that dephosphorylation of a synaptosomal preparation for relatively short periods at 37°C increased the

Table 8. *Effect of bivalent cations on different protein kinases from brain*

Membrane-bound intrinsic kinase activities were measured at 1 min with 1 mM-ATP (magnesium salt), 30 mM-tris-HCl buffer, pH 7.4, at 20°C as described in the text. Results for histone kinase are taken from Miyamoto *et al.* (1969) and for neurotubular intrinsic kinase from Goodman *et al.* (1970). References: ¹Rodnight & Lavin (1964) and Decsi & Rodnight (1965); ²Rabinowitz & Lipmann (1960).

Cation	Membrane-bound intrinsic kinase (% of control)	Extrinsic (phosvitin) kinase (% of control)	Histone kinase (% of control)	Neurotubular intrinsic kinase
Ca ²⁺	50-60 (0.1 mM)	0 (5 mM) ¹	At 2.5 mM substitutes for Mg ²⁺ , giving 70% activity	No effect at 2 mM
Zn ²⁺	10-20 (0.1 mM)	Inhibits ² at 1-2 mM	Substitutes for Mg ²⁺	—
Mn ²⁺	40-60 (0.1 mM)	Inhibits ² at 1-2 mM	Substitutes for Mg ²⁺ giving about 200% activity	—
Cu ²⁺	30 (0.1 mM)	Inhibits ² at 1-2 mM	Substitutes for Mg ²⁺ giving about 30% activity	—

percentage stimulation of kinase activity by cyclic AMP measured at 10 min; for example, after dephosphorylation for 10 min (when the increased effect was maximal), stimulation by 100 μ M-cyclic AMP at 10 min was 35% compared with 6% in a control untreated sample.

The reason why preincubation of the membrane preparations in the absence of ATP, especially at 37°C, results in an apparent loss of kinase activity is unclear. Rabinowitz & Lipmann (1960) found that extensive dephosphorylation of phosvitin destroyed its acceptor function for a protein kinase from yeast, but the results in Table 6 show that the preincubation conditions used in the present experiments only resulted in the dephosphorylation of about 15% of the total alkali-labile phosphorous in the membrane. Possibly, as suggested below, the protein-bound phosphorylserine remaining after preincubation occurs in a distinct protein or group of proteins which are not associated in the membrane structure with kinase or phosphatase enzymes.

Properties of the phosphatase system. The intrinsic phosphatase activity differs in several ways from the soluble casein phosphatase of brain studied by Rose & Heald (1961). The latter has a pH optimum of 5.9, is not significantly inhibited by Cu²⁺ or molybdate ions and is much less sensitive to zinc salts than is the intrinsic system. Conversely the soluble enzyme is more sensitive to inhibition by F⁻. Both enzymes, however, are stimulated by Mn²⁺.

The concentration-dependence of the inhibition of dephosphorylation by Na⁺ and K⁺ is particularly notable. Although there is considerable variation in the results illustrated in Fig. 5(b) they do indicate clearly that, between 10 and 100 mM, the inhibition by Na⁺ increases much more sharply than that by K⁺, which in this range shows relatively little concentration-dependence. If these relative sensitivities to the two cations apply to the phosphatase system in the intact cell it is possible that changes in Na⁺ concentration on the inside of the membrane may be a factor controlling the rate at which the membrane proteins are phosphorylated and dephosphorylated.

Complexity of protein-bound phosphorylserine in the membrane. We now consider whether the present work gives any further information about the possible heterogeneity of phosphorylated proteins in the cell membrane, and whether the observed intrinsic protein and phosphatase activities in our preparation are the result of one or more coupled systems. In discussing this point we assume that the membrane fragments studied are derived predominantly from neuronal sources and that contamination from other cell types can be neglected; the exact extent to which this is true is difficult to

judge with present techniques, but particularly for the synaptosomal membrane preparation it is probable that any phosphoprotein heterogeneity resulting from the presence of non-neuronal elements is minimal.

Considering first the heterogeneity of phosphoprotein, it appears that only about one-quarter of the total phosphorus in the membrane protein labile to mild alkali turns over under the experimental conditions used in the present work. This conclusion is supported by two observations not mentioned in the Results section. (a) Prolonged incubation under the dephosphorylating conditions used in Table 6 was not found to result in any further appreciable loss of alkali-labile phosphorus than was noted at 30–60 min (e.g. in one experiment on a microsomal preparation the value after incubation at 37°C for 5 h was 6.6 nmol of P/mg of protein compared with 7.2 at zero time). (b) Even after phosphorylation of a membrane preparation for 1 h the specific radioactivity of the alkali-labile phosphorus was still only 26% of that of the γ -P of ATP, roughly confirming the value of 10% found by Rodnight & Lavin (1966) after phosphorylation under slightly different conditions for 10 min. There is reason to believe that a similar situation exists in the intact tissue; thus after incubation of brain slices with ^{32}P for 3.5 h the specific radioactivity of the total protein-bound phosphorylserine in the tissue was 40% of that of the γ -P of ATP; similarly, in experiments *in vivo* with immature rats, equilibrium with the alkali-labile phosphorus was only approached 48 h after injection of radioisotope (Rodnight, 1971). It therefore seems possible that most of the membrane proteins containing phosphorylserine are metabolically relatively inactive and are phosphorylated either during the stage of protein synthesis or alternatively during membrane formation through the agency of a protein kinase which does not become incorporated into the membrane structure. Indeed, phosphorylation of this class of membrane protein might be a function of the extrinsic protein kinase studied by Rabinowitz & Lipmann (1960) and Rodnight & Lavin (1964); certainly the ease with which the small amount of this enzyme associated with membrane preparations can be brought into a soluble form (Decsi & Rodnight, 1965) is consistent with this hypothesis. However, the discovery by Mäenpää & Banfeld (1970) of a hepatic tRNA specific for O-phosphorylserine indicates that the former alternative is also a serious possibility. Conceivably both mechanisms may be involved, resulting in different proteins. The extent to which the metabolically less-active fraction is heterogeneous is unknown, but in an earlier attempt to fractionate a proportion of the membrane protein on DEAE-cellulose (Kothary,

1964; Rodnight, 1966) alkali-labile phosphorus was found to have a wide distribution in many different protein fractions.

It is more difficult to form a definite opinion at present on the possible complexity of the actively turning-over fraction and its associated enzymes. On the whole the evidence is consistent with the suggestion that there is mainly one coupled system in which the kinase component is truly cyclic AMP-dependent. The curvilinear nature of the time-course, which might be taken as evidence for the existence of several concurrent phosphorylation reactions, can reasonably be explained in terms of the partially dephosphorylated state of the membrane fragments as prepared and the rate-limiting action of the phosphatase component increasing as the vacant sites become phosphorylated; in addition it is possible that owing to the insoluble nature of the preparation some variation in accessibility of the sites to ATP may be a factor, as well as product inhibition through ADP. With regard to the phosphorylation that apparently occurs in the absence of added cyclic AMP, which apparently suggests a distinct system, it is notable that membrane preparations from brain are rich in adenylyl cyclase, which requires only ATP and Mg^{2+} to form cyclic AMP. Therefore, even when cyclic AMP is not added the membrane fragments may be producing sufficient endogenous cyclic AMP to maintain phosphorylation. To test this hypothesis we measured formation of cyclic AMP under standard phosphorylating conditions at 20°C and preliminary results suggest that the rate is of the order of 20 pmol of cyclic AMP/min per mg of protein, which is just sufficient to give a concentration of cyclic AMP in the medium within the range that stimulates kinase activity (Weller & Rodnight, 1970). [This is considerably lower than the rate of 500 pmol/min per mg of protein for rat brain synaptic membranes quoted by De Robertis, Rodriguez DeLores Arnaiz, Alberici, Butcher & Sutherland (1967); apart from the possibilities of a species difference, the fact that our preparations were frozen before analysis and were assayed at 20°C instead of 30°C may have contributed to the discrepancy.] Confirming the observations of Goldberg, Just, O'Dea, Weir & O'Toole (1970) we also find that the membrane fragments as prepared contain about 15–25 pmol of bound cyclic AMP/mg of protein and it is possible that this may be involved in the initiation of kinase activity before adenylyl cyclase has provided an adequate concentration.

A clear physiological role for the actively turning-over fraction of the membrane phosphoproteins does not emerge from this work. Probably the most reasonable hypothesis at present is that it plays a part in the regulation of the passive permeability

of the membrane to cations. For instance the conformation of the membrane proteins involved in this process could be related to their phosphorylated state. However, the slow rate of turnover appears to exclude a direct participation in the very rapid changes in permeability to Na^+ that occur on depolarization; it appears more likely that protein phosphorylation modifies the permeability response in some less direct way by influencing the ability of the membrane to respond on receipt of the appropriate stimulus. A theory of this type has been proposed in relation to the action of cyclic AMP on membrane phosphorylation (Rodnight & Weller, 1971).

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