Phosphoprotein Phosphatases from Rat Cerebral Cortex

SUBCELLULAR DISTRIBUTION AND CHARACTERIZATION*

HIROO MAENO AND PAUL GREENGARD

From the Department of Pharmacology, Yale University School of Medicine, New Haven, Connecticut 06510

SUMMARY

The subcellular distribution of phosphoprotein phosphatases which release orthophosphate from phosphoprotein was studied in rat cerebral cortex. In contrast to several other tissues examined, more than 50% of the total protein phosphatase activity in rat cerebral cortex was found in the particulate fractions; the activity was especially high in the crude mitochondrial fraction. Further subfractionation of the crude mitochondrial fraction by sucrose density gradient centrifugation showed that, among the membrane fractions, the specific activity of protein phosphatase was highest in the fractions rich in synaptic membranes and lowest in the mitochondria. A considerable amount of the enzyme activity in synaptic and microsomal membranes existed in a latent form which could be partially unmasked by treatment with Triton X-100. The specific activity of the protein phosphatase of the cell sap and of the synaptoplasma was considerably higher than that of the membrane fractions.

Column chromatography on DEAE-cellulose resolved the protein phosphatase activity of the cell sap into three distinct protein phosphatases, which were clearly distinguished from membrane enzyme by differences in substrate specificity and metal ion requirements. The soluble protein phosphatases, but not the enzyme released from the synaptic plasma membrane fraction by Triton X-100 treatment, were specifically activated by manganese chloride. Endogenous membrane protein was found to be the best among several phosphorylated proteins examined as substrates for membrane-bound protein phosphatase. Both membrane-bound and soluble protein phosphatase exhibited pH optima in the neutral range. Protein phosphatase catalyzed the stoichiometric release of orthophosphate from a phosphoserine residue of protamine. This was the only amino acid residue in protamine and histones which appeared to be phosphorylated by an adenosine 3',5'-monophosphate-dependent protein kinase purified from bovine brain.

Studies of the subcellular distribution in cerebral cortex of the enzymes related to the metabolism and function of cyclic adenosine 3',5'-monophosphate, namely, adenyl cyclase (1), cyclic AMP phosphodiesterase (1-3) and cyclic AMP-dependent protein kinase (4), have shown them to be considerably enriched in the synaptic membrane fractions. Furthermore, the subcellular distribution of endogenous proteins able to act as substrates for a partially purified cyclic AMP-dependent protein kinase from brain was found to parallel the distribution of endogenous cyclic AMP-dependent protein kinase activity (5). The localization in synaptic membrane fractions of these various enzymes and protein substrates of the cyclic AMP system, along with several other lines of evidence (e.g. 6-8), suggests an intimate involvement of the cyclic AMP system in the process of transmission at certain neural synapses.

Following the discovery of cyclic AMP-dependent protein kinases, initially in muscle (9), and then in liver (10) and brain (11), the hypothesis was suggested (6, 12-14) that the diverse biochemical and physiological effects of cyclic AMP may be mediated through regulation of cyclic AMP-dependent protein kinase activity, the specificity of the action of the cyclic nucleotide residing in the specificity of the enzyme and of its substrates in the various tissues. The dephosphorylation of the phosphoprotein products of protein kinase activity can, within this framework, be expected to be of importance in regulating the magnitude and duration of the effects of cyclic AMP. It, therefore, seemed of considerable importance, in evaluating the role of cyclic AMP in neural function, to study the subcellular location and properties of phosphoprotein phosphatase of neural tissue.

Phosphoprotein phosphatase capable of releasing phosphate from phosphoprotein has been reported in several tissues (15-21), including mammalian brain (18, 19, 21). In one study (20), a liver phosphoprotein phosphatase was found which is apparently specific for histone and other basic proteins. The present report deals with the subcellular distribution, heterogeneity, and some enzymological properties of protein phosphatases from rat cerebral cortex.

EXPERIMENTAL PROCEDURE

Histone mixture and arginine-rich histone from calf thymus were obtained from Mann. Salmon protamine sulfate, calf thymus lysine-rich histone (histone type III), and bovine serum albumin were from Sigma. ATP and cyclic AMP were purchased as the sodium salts from Schwarz BioResearch.
Other reagents were analytical grade. \([\gamma-^{32}P]ATP\) was prepared according to the method of Post and Sen (22).

Subcellular fractions of cerebral cortex were prepared in 0.32 M sucrose from 10 male Sprague-Dawley rats weighing 150 to 200 g each, and the identity of the fractions was verified by electron microscopy as described previously (4). In the subcellular fractionation experiments, including those of Table VIII, all particulate fractions were incubated with 0.1% Triton X-100 in 0.01 M Tris buffer, pH 7.2, prior to use as sources of protein phosphatase, to unmask latent enzymic activity. The subcellular fractionation experiments were performed several times, with consistent results. Therefore, data from representative experiments are shown.

The protein phosphatase of cell sap and synaptoplasm (M-3) was further purified by chromatography on a DEAE-cellulose column (2.2 x 11 cm), which had previously been equilibrated with 0.01 M Tris-HCl, pH 7.2. Protein phosphatase was eluted with a stepwise gradient of NaCl (0 to 0.7 M) containing 0.01 M Tris-HCl, pH 7.2, and 1 mM dithiothreitol.

Cyclic AMP-dependent protein kinase and succinic dehydrogenase were assayed as described previously (4). Protein was determined by the method of Lowry et al. (23) with bovine serum albumin as standard.

To prepare substrates for protein phosphatase, protamine and various histones were phosphorylated by incubating with \([\gamma-^{32}P]ATP\) in the presence of cyclic AMP-dependent protein kinase, the kinase having been partially purified from bovine brain by column chromatography on DEAE-cellulose according to the method of Miyamoto et al. (11). To prepare phosphorylated substrates, 1 ml of incubation mixture contained 46 \(\mu\)g of protein kinase; 50 \(\mu\)moles of sodium acetate buffer, pH 6.4; 1 mg of protamine or histone; 50 \(\mu\)moles of \([\gamma-^{32}P]ATP\) (5 to 10 \(\times\) 10\(^5\) cpm); 10 \(\mu\)moles of magnesium acetate, 10 \(\mu\)moles of sodium fluoride; 2.0 \(\mu\)moles of theophylline; 0.3 \(\mu\)mol of ethylene glycol bis(\(\beta\)-aminoethyl ether)-N\(^\text{N'}\)-tetraacetic acid; and 5.0 \(\mu\)moles of cyclic AMP. The incubation was carried out at 30\(^\circ\)C for 45 min. The phosphorylation reaction was terminated by the addition of 0.25 ml of 100% trichloroacetic acid. The resulting precipitate was centrifuged, washed twice by dissolving in water and reprecipitating with 20% trichloroacetic acid, and then dialyzed against distilled water for 24 hours.

Protein phosphatase activity, with protamine or histone as substrate, was assayed by measuring the release of radioactive orthophosphate from \(^{32}P\)-labeled protein. For routine assays, the reaction mixture contained, in a total volume of 0.1 ml, 10 \(\mu\)moles of Tris-HCl buffer, pH 7.2, 0.1 \(\mu\)mol of dithiothreitol, 100 \(\mu\)g of \(^{32}P\)-labeled protamine (containing 5 to 10 \(\times\) 10\(^5\) cpm), and 1 to 20 \(\mu\)g of enzyme protein. The incubation was performed at 30\(^\circ\)C for 10 min and terminated by the addition of 0.4 ml of 25% trichloroacetic acid. After the addition of 0.1 ml of 0.1 625% bovine serum albumin as a carrier for the precipitate, protein was removed by centrifugation. Orthophosphate was extracted from the deproteinized supernatant by a modification of the method of Plaut (24). To 0.4 ml of deproteinized supernatant were added 0.05 ml of 10\(^{-2}\) M KH\(_2\)PO\(_4\) and 0.15 ml of 5% ammonium molybdate. The resulting phosphomolybdate complex was extracted with 1.0 ml of isobutyl alcohol and the radioactivity of the isobutyl alcohol extract was measured. The amount of protein labeled with \(^{32}P\) and the amount of phosphate released from this substrate protein have been calculated from the specific activity of the radioactive ATP used as precursor in the protein phosphorylation reaction and neglecting the phosphate present in the protein substrate prior to its phosphorylation by radioactive ATP. One unit of protein phosphatase activity was defined as 1 pmole of \(^{32}P\) released under the above assay conditions.

In order to identify the site of phosphate incorporation, \(^{32}P\)-labeled proteins were hydrolyzed in 6 \(\times\) HCl at 108\(^\circ\)C in sealed ampoules for 6 hours. The hydrolysates were dried in vacuo over KOH and then subjected to high voltage electrophoresis on Whatman No. 3MM paper at pH 1.8 in formic-acetic acid buffer for 80 min (5, 25). After electrophoresis, the paper was dried and stained with ninhydrin to locate the amino acids. The paper was cut into 1-cm strips and the radioactivity of the strips was measured by liquid scintillation spectrometry. Authentic phosphoserine and phosphothreonine were hydrolyzed under the same conditions in order to correct for their decomposition during acid hydrolysis; the amount of orthophosphate formed during hydrolysis was determined colorimetrically according to Rockstein and Herron (26).

**RESULTS**

In view of the possible existence of multiple protein phosphatases with different substrate specificities, two different proteins, protamine and arginine-rich histone, were used as substrates in studying the distribution of activity in cerebral cortical subfractions. Table I depicts the distribution of protein phosphatase activity among the four primary subfractions. The crude mitochondrial fraction marked by the highest activity of succinic dehydrogenase and the high speed supernatant fraction (cell sap) each accounted for about 40% of the total activity. The specific activity of the phosphatase in the cell sap was much higher than that from any of the particulate fractions. Cyclic AMP-dependent protein kinase activity was also measured in these experiments; no significant difference in subcellular distribution of protein kinase was observed between the present studies, in which cerebral cortex was used, and the earlier studies (4) in which whole cerebral was used. Differences in the ratio of protein phosphatase activity to arginine-rich histone phosphatase activity among the different fractions suggested the existence of more than one type of protein phosphatase in rat cerebral cortex.

After osmotic shock of the crude mitochondrial fraction, about 40% of the protamine phosphatase activity remained in the particulate fraction, M-1, which contains synaptic membranes and mitochondria. The soluble fraction (synaptoplasm), M-3, contained a somewhat higher portion (56%) of the total activity. Arginine-rich histone phosphatase activity, on the other hand, was found slightly but reproducibly higher in the M-1 fraction than in the M-3 fraction (Table II). The fraction enriched in synaptic vesicles, M-2, exhibited very little of the total activity seen with either substrate. The specific activity of the phosphatase in the synaptoplasm was much higher than that in the M-1 or M-2 particulate mitochondrial subfractions. It is of interest that the specific activity of protamine phosphatase was slightly higher and that of arginine-rich histone phosphatase was slightly lower in the synaptoplasm than in the cell sap. The ratio of the two activities, accordingly, differed markedly in these two soluble fractions, synaptoplasm being relatively richer in protamine phosphatase activity. These differences between the enzymes from synaptoplasm and cell sap were not large, but they were consistent.
### Table I

**Distribution of protein phosphatase activity in primary subcellular fractions of rat cerebral cortex**

| Fraction          | Protein | Protein Phosphatase | Arginine-rich histone | Ratio of activity: protamine/argin-rich histone | RSA$^a$ of succinic dehydrogenase |
|-------------------|---------|---------------------|-----------------------|-----------------------------------------------|----------------------------------|
|                   | mg/g original tissue | units x 10$^{-3}$/g original tissue | units/mg protein | % | units x 10$^{-3}$/g original tissue | units/mg protein | % |                                |                                    |
| Nuclei            | 13.2    | 25.7                | 1,950                | 5.7 | 9.9                | 750                | 8.0 | 2.6                           | 0.6                                |
| Mitochondria      | 55.0    | 179.0               | 3,250                | 40.1 | 43.5               | 790                | 35.2 | 4.1                           | 2.0                                |
| Microsomes        | 25.3    | 66.3                | 2,520                | 14.8 | 17.9               | 710                | 14.5 | 3.7                           | 0                                  |
| Cytosol           | 23.4    | 1/2.0               | 1,920                | 39.4 | 52.2               | 2,230               | 42.3 | 3.4                           |                                    |
| Total             | 116.9   | 187.0               | 3,720                | 100.0 | 123.5              | 100.0               |     | 3.8                           |                                    |
| Starting material (homogenate) | 117.0   | 435.0               | 3,720                | 113.0 | 970                | 3.8                           | 3.8                           |
| Recovery (%)      | 99.9    | 102.6               | 109.0                |  |  |                                    |                                |                                    |

$^a$RSA (relative specific activity) = % recovered activity/% recovered protein.

### Table II

**Distribution of protein phosphatase activity in mitochondrial subfractions after osmotic shock**

| Fraction          | Protein | Protein Phosphatase | Arginine-rich histone | Ratio of activity: protamine/argin-rich histone | RSA of succinic dehydrogenase |
|-------------------|---------|---------------------|-----------------------|-----------------------------------------------|----------------------------------|
|                   | mg/g original tissue | units x 10$^{-3}$/g original tissue | units/mg protein | % | units x 10$^{-3}$/g original tissue | units/mg protein | % |                                |                                    |
| M-1 Myelin, free mitochondria and nerve endings without synaptic vesicles | 38.0    | 76.0                | 2,000                | 38.7 | 23.3               | 610                | 50.1 | 3.3                           | 1.5                                |
| M-2 Membranes and synaptic vesicles | 5.1     | 10.2                | 2,000                | 5.2 | 3.2                | 630                | 6.9 | 3.2                           | 0                                  |
| M-3 Soluble fraction | 12.2    | 110.0               | 9,000                | 56.1 | 20.0               | 1640               | 43.0 | 5.5                           | 0                                  |
| Total             | 55.3    | 126.2               | 100.0               | 46.5 | 100.0              |                                    |                                |                                    |
| Starting material (mitochondria) | 55.0    | 179.0               | 3,250                | 43.5 | 790                | 4.1                           | 4.1                           |
| Recovery (%)      | 100.0   | 109.0               | 107.1                |  |  |                                    |                                |                                    |
Further fractionation of the crude mitochondrial subfraction, M-1, by discontinuous sucrose density gradient centrifugation and identification of the subfractions were carried out as described previously (4). The data indicate (a) that the specific activity of protein phosphatase for both of the substrates was considerably lower in the mitochondrial subfraction than in the fractions rich in myelin and synaptic membranes, (b) that the M-1 (1.0) subfraction, which is enriched with synaptic membrane fragments, had the highest specific activity of protein phosphatase for both substrates, and (c) that the ratio of protamine phosphatase to arginine-rich histone phosphatase activity varied considerably among the subfractions of M-1. However, the recovery of enzyme activity from the sucrose gradient was low, ranging from 40 to 45% with protamine as substrate and from 55 to 60% with arginine-rich histone as substrate in three experiments, in contrast to a recovery from the gradient of about 75% (4) for cyclic AMP-dependent protein kinase activity, so that the pattern of distribution of protein phosphatase might have been altered by a selective loss of activity in some fractions. Therefore, data on the distribution of protein phosphatase for both substrates was varied considerably among the subfractions of M-1. The indicated amounts of particulate fractions were incubated with 0.1% Triton X-100 for 30 min in ice and then centrifuged at 150,000 × g for 60 min in a Beckman ultracentrifuge. The precipitates were suspended mechanically in 0.01 M Tris-HCl buffer, pH 7.2, by means of a glass homogenizer.

### Table III

| Fraction          | Protein | Specific activity (units/mg protein) |
|-------------------|---------|-------------------------------------|
|                   |         |                                    |
|                   |         | Protein phosphatase                 |
|                   |         | Activity | Specific |
|                   |         |          | activity |
|                   |         | (units x 10^-5) | (units x 10^-5) |
| Microsomes        |         |          |          |
| Starting material | 3.13    | 6.40     | 2.04     |
| Triton-insoluble  | 1.73    | 5.15     | 2.97     |
| Triton-soluble    | 1.45    | 6.75     | 5.90     |
| Total             | 2.88    | 11.91    |          |
| Recovery (%)      | 92      | 186      |          |
| M-2               |         |          |          |
| Starting material | 1.60    | 3.00     | 1.88     |
| Triton-insoluble  | 1.00    | 0.86     | 0.86     |
| Triton-soluble    | 0.59    | 2.35     | 3.98     |
| Total             | 1.59    | 3.21     |          |
| Recovery (%)      | 99      | 107      |          |
| M-1 (1.0)         |         |          |          |
| Starting material | 1.68    | 0.62     | 0.07     |
| Triton-insoluble  | 1.10    | 1.08     | 0.98     |
| Triton-soluble    | 0.60    | 1.00     | 1.67     |
| Total             | 1.70    | 2.08     |          |
| Recovery (%)      | 101     | 335      |          |

In order to determine whether particulate-bound protein phosphatase could be solubilized, the microsomal fraction, the synaptic vesicle (M-2) fraction, and a synaptic membrane (M-1 (1.0)) fraction were treated with 0.1% Triton X-100. Triton X-100, at this concentration, exhibited no inhibitory effect on cell sap protein phosphatases. It is evident from a comparison of protamine phosphatase activity in untreated fractions with activity in Triton X-100-treated fractions that large amounts of protamine phosphatase in the microsomal and the synaptic membrane fraction exist in a latent form (Table III). The M-2 fraction showed no latent activity, although most of the activity was found in the soluble fraction after treatment with the detergent. It is of interest that after Triton X-100 treatment of the microsomal or the synaptic membrane fraction, the precipitate showed higher specific activity than the corresponding fraction did prior to the treatment.
suggesting that the enzyme still remaining in the membranes after treatment with the detergent may have become more accessible to substrate.

In order to determine the cell sap protein phosphatase activity, the cell sap was subjected to column chromatography on DEAE-cellulose (Fig. 1). Over-all recoveries of protamine and arginine-rich histone phosphatase activity from this step were 72 and 95%, respectively. When protamine was used as substrate, three distinct loci of activity were invariably observed; in the experiments illustrated, peaks appeared at tube 51 (Fraction I) and at tube 58 (Fraction II), with a shoulder around tube 64 (Fraction III). Arginine-rich histone phosphatase activity showed two clear components; in the experiment shown in Fig. 1, these were manifested by a shoulder at tube 58 and a peak at tube 64. As shown in Table IV, fractions representing the three activity peaks were clearly distinguished from one another by their relative \( V_{\text{max}} \) values for dephosphorylation of protamine and arginine-rich histone, although apparent \( K_m \) values for either protamine or arginine-rich histone did not differ significantly among these three fractions. Chromatography of synaptoplasm (M-3) on DEAE-cellulose gave an elution pattern in which the position of the peaks was similar to that of cell sap for both types of phosphatase activity (data not shown). Moreover, the \( K_m \) for protamine, the \( K_m \) for arginine-rich histone, and the \( V_{\text{max}} \) (prolamine)/\( V_{\text{max}} \) (arginine) ratio of the three enzyme fractions from synaptoplasm were similar to those of the corresponding fractions from cell sap. Interestingly, the synaptoplasm differed from the cell sap in the relative amounts of the three enzyme fractions.

The enzymes which had been solubilized from the various particulate fractions resembled one another with respect to the values for each of these three kinetic parameters (Table IV). It is of considerable interest that the enzymes solubilized from the particulate fractions are distinguishable from the enzymes of the cell sap by having a much higher affinity for protamine. The combination of enzymes solubilized from any of the particulate fractions with any of the three cell sap enzymes exhibited only additive effects over a wide range of protamine concentrations. The latter results make it very unlikely that any loosely binding activators or inhibitors might be responsible for the difference in affinity of the various enzymes for protamine.

The ability of protein phosphatase in the synaptic membrane fractions to dephosphorylate protamine and histones was compared with the ability to dephosphorylate phosphoproteins intrinsic to the synaptic membrane fractions. For the measurement of intrinsic substrate activity, the synaptic membrane fractions were first phosphorylated with \([\gamma-^32P]ATP\) by the intrinsic protein kinase in the presence of cyclic AMP. As shown in Fig. 2 for the M-1 (1.0) fraction, phosphorylation reached a steady state level within a few minutes. At this point, EDTA, plus a large amount of nonradioactive ATP, was added to stop the incorporation of \(^32P\) into the membrane protein. (EDTA inhibits the protein kinase by about 60 to 70\% under the conditions used. Therefore, nonradioactive ATP was added to dilute the \([\gamma-^32P]ATP\) and minimize further incorporation of \(^32P\) into the protein.) Immediately after the addition of these reagents, rapid dephosphorylation due to intrinsic protein phosphatase activity could be observed. The time course of the removal of phosphate was biphasic; about half of the phosphate was removed in the first, more rapid phase, and the remainder more slowly. In order to evaluate the possibility that the release of phosphate was due to reversal of the kinase-catalyzed reaction, i.e. transfer of phosphoprotein...
phosphate to ADP, experiments were carried out in which the ability of ATP and ADP to reverse the level of membrane phosphorylation was compared. Whereas the addition of ATP alone gave results similar to those shown in Fig. 2 for ATP + EDTA, the addition of ADP alone did not result in the removal of phosphate from the membrane protein. These results indicate that protein phosphatase action, rather than reversal of the protein kinase reaction, was responsible for the removal of the membrane-bound phosphate. From the time course of dephosphorylation, the initial rate of dephosphorylation was calculated. This rate was compared with the rate of dephosphorylation of various exogenous proteins. Table V indicates that the fractions rich in synaptic membranes, M-1 (0.9) and M-1 (1.0), were dephosphorylated by the intrinsic protein phosphatase much more rapidly than were any of the exogenous substrates tested. The microsomal fraction, as another example of a membranous material, was also compared with various other proteins as potential substrate for its intrinsic protein phosphatase (Table V). The microsomal protein was the best among the proteins tested as substrate for the microsomal protein phosphatase, although its substrate activity relative to the other proteins tested as substrate, was not as striking as in the analogous case of the endogenous substrate activity of the synaptic membrane fractions. The data of Fig. 2 and Table V indicate a high turnover of phosphate in membrane proteins, particularly in the subcellular fractions rich in synaptic membranes. It is important to emphasize that the direct, quantitative comparison of endogenous with exogenous protein substrates suffers the uncertainty of meaning of the term "concentration" with respect to membrane proteins in the semisolid membrane phase. The differences found may be attributable to a greater accessibility of intrinsic membrane phosphatases to endogenous substrates than to exogenous substrates to the membrane-bound phosphatase. Nevertheless, the results do indicate unequivocally that these neuronal membranes contain intrinsic phosphatase activity.

![Table V](image-url)

**Comparison of various proteins as substrates for protein phosphatase of rat cerebral membranes**

Protein phosphatase activity was assayed in the same incubation medium as described for the assay of dephosphorylation shown in Fig. 2, except that [γ-32P]ATP was omitted when protamine or histone was used as substrate. When the membrane fractions were used simultaneously as enzyme and substrate, to measure intrinsic protein phosphatase activity, a total of 12.5 μg of radioactive membrane protein, prepared as in Fig. 2, was used. When the membrane fractions were used as enzyme only, 12.5 μg of nonlabeled membrane protein were used. Treatment of the membrane fractions with 0.1% Triton X-100 prior to the assay greatly increased the phosphatase activity towards protamine and histone; the data presented for these substrates are those obtained in the presence of the detergent. Intrinsic protein phosphatase activity was determined by measuring the amount of radioactive phosphate remaining in the membrane fraction with the use of the analytical conditions described in Fig. 2. When protamine or histone was used as substrate, enzyme activity was determined by measuring the amount of radioactive orthophosphate released, as described under "Experimental Procedure."

![Table VI](image-url)

**Stoichiometry of reaction catalyzed by phosphoprotein phosphatase**

The incubation conditions, with [32P]protamine as substrate, were the same as described for protein phosphatase assay under "Experimental Procedure" except that an incubation time of 60 min was used. After incubation, the reaction was stopped by the addition of 0.4 ml of 26% trichloroacetic acid; 0.2 ml of 0.625% bovine serum albumin was added as carrier and the concentration of trichloroacetic acid was readjusted to 20% by the addition of 100% trichloroacetic acid. After centrifugation at 20,000 x g for 30 min, radioactive orthophosphate was measured in the supernatant. The protein precipitate was washed twice with 20% trichloroacetic acid and then divided into two portions. One part was directly used for the measurement of radioactivity and the other portion was subjected to acid hydrolysis, followed by high voltage electrophoresis, to determine the amount of phosphoserine in the protein, as described under "Experimental Procedure." Phosphoserine values have been corrected for recovery of authentic phosphoserine.
Effect of metal ions on activity of brain protein phosphatase

Protein phosphatase activity was determined with 100 µg of [32P]protamine as substrate under the standard assay conditions in the presence of 2.5 mM metal ion. For the assay of protein phosphatase activity, 1.7 µg of Fraction I, 1.9 µg of Fraction II, 2.1 µg of Fraction III, and 6.5 µg of solubilized M-1 (1.0) were present in the incubation mixture. The M-1 (1.0) enzyme had been solubilized with 0.1% Triton X-100 as described in Table III. Activity is expressed as the percentage of that observed in the absence of added metal ions.

| Metal   | M-1 (1.0) | Fraction I | Fraction II | Fraction III |
|---------|-----------|------------|-------------|--------------|
| MgCl₂   | 110       | 100        | 101         | 91           |
| CaCl₂   | 101       | 103        | 100         | 91           |
| MnCl₂   | 100       | 233        | 210         | 155          |
| CoCl₂   | 22        | 38         | 72          | 105          |
| ZnSO₄   | 2         | 6          | 7           | 4            |
| CuCl₂   | 0         | 3          | 2           | 3            |
| FeCl₂   | 0         | 0          | 0           | 0            |

Table VII

Fig. 3. pH dependence of protein phosphatase activity of synaptic membrane fraction. The incubation mixture contained 100 µg of protamine (with 420 pmoles of [32P]) and 0.6 µg of enzyme, solubilized from the M-1 (1.0) fraction by treatment with 0.1% Triton X-100, as described in Table III. Incubation conditions were as described under "Experimental Procedure," except for the variation of pH of the reaction mixture, in which 0.1 M acetate buffer was used for pH 5 to 7 and 0.1 M Tris-HCl buffer was used for pH 7 to 9.

During incubation. After 60-min incubation of solubilized membrane enzyme or of cell sap enzyme with [32P]protamine as substrate, the sum of trichloroacetic acid-insoluble phosphate and inorganic phosphate released from the substrate accounted for all radioactivity in the substrate (Table VI). No significant radioactivity was detected in the trichloroacetic acid-soluble fraction after removal of inorganic phosphate (by extraction of the phosphomolybdate complex into isobutyl alcohol). Moreover, neither phosphoserine nor phosphothreonine, at 10⁻⁴ M, inhibited protein phosphatase activity of any enzyme fraction. These results indicate that the protein phosphatase activity is not to be attributed to the degradation by a proteolytic enzyme of substrate phosphoprotein into low molecular weight peptides or amino acids, followed by the action of a phosphoserine or phosphothreonine phosphatase.

Since it is important in the characterization of the protein phosphatases to know the site(s) of phosphorylation in the substrate, the [32P]protamine used as substrate was hydrolyzed in 6 N HCl at 108°C and subjected to electrophoresis on paper at high voltage. After correction for hydrolysis of phosphoserine and phosphothreonine, 91% of the total activity of the acid digest was recovered as phosphoserine and no significant radioactivity was detected in the phosphothreonine spot. These results indicate that the cyclic AMP-dependent protein kinase from bovine brain had phosphorylated serine residues of protamine primarily or exclusively, and that the various protamine phosphatases were capable of splitting phosphate from these residues. In fact, the protein phosphatases hydrolyzed the phosphoserine residues of protamine with nearly quantitative release of inorganic phosphate, as depicted in Table VI. In other experiments, in which cyclic AMP-dependent protein kinase from bovine brain had been used to phosphorylate arginine-rich histone, lysine-rich histone, and histone mixture, the amino acid residues which had been phosphorylated were identified as serine too.

The three cell sap enzymes and the solubilized fractions from microsomes, synaptic vesicles (M-2), and synaptic membranes (M-1 (1.0)) exhibited very similar pH dependence with optimal reaction rate at neutral pH. As an example, Fig. 3 illustrates the enzyme activity as a function of pH for the solubilized fraction from synaptic membranes. The liver histone phosphatase studied by Meisler and Langan (20) and the brain membrane-bound protein phosphatase studied by Weller and Rodnight (21) showed a similar pH dependence.

All of the protein phosphatases from cerebral cortex were inhibited approximately 50% by 10⁻² M NaF, as well as by 10⁻³ M orthophosphate, but were unaffected by any of the
organic phosphate compounds of low molecular weight which were tested (ATP, ADP, 5′-AMP, glucose 6-phosphate, β-glycerophosphate, phosphothreonine, phosphoserine at 10⁻³ M; cyclic AMP, cyclic guanosine 3′,5′-monophosphate, cyclic cytosine 3′,5′-monophosphate, cyclic uridine 3′,5′-monophosphate at 5 × 10⁻⁴ M). These results make it seem unlikely that these protein phosphatases from cerebral cortex are identical with any of the known 5′-nucleotidases, “nonspecific” phosphatases, ATPases, glucose 6-phosphatase, or cyclic nucleotide phosphodiesterases.

The effect of various ions on the activities of membrane and cell sap protein phosphatases is shown in Table VII. At the concentration tested (2.5 mM), MgCl₂ and CaCl₂ exhibited no specific activity on any of the four enzymes. CoCl₂ strongly inhibited the activity of the Triton-solubilized enzyme from the M₁ (1.0) fraction and the activity of cell sap Fraction I and slightly inhibited that from Fraction II, but did not inhibit that from Fraction III. ZnSO₄, CuCl₂, and FeCl₃ inhibited almost completely the activity of all fractions. Interestingly, MnCl₂ markedly stimulated Fractions I and II but had a lesser effect on Fraction III and no significant effect on the solubilized membrane enzyme. The effect of varying concentrations of MnCl₂ on each of these four enzymes is presented in Fig. 4.

Protein phosphatase activity, measured with histone as substrate, showed a pattern of subcellular distribution in non-neural tissue quite different from that observed with neural tissue (Table VIII). Thus, the protein phosphatase activity of particulate fractions, relative to the total activity of the particulate fraction plus cell sap, was much higher in brain (46 to 54% for cerebral cortex, cerebellum, caudate nucleus, and medulla) than in nonneurogenic tissues (18 to 29% for liver, lung, heart, kidney, and spleen). Recovery of phosphatase activity in the particulate fraction plus cell sap was approximately 100% for all tissues studied.

**DISCUSSION**

The subcellular distribution of protein phosphatase in rat cerebral cortex, found in the present study with phosphorylated protamine and histone as substrates, appears rather similar to the distribution of cyclic AMP phosphodiesterase reported by DeRobertis et al. (1). The results of studies of protein phosphatase distribution can be expected to vary, however, depending on the nature of the substrate used for the assay. For example, since membrane protein from the M₁ (1.0) fraction was a far better substrate for the protein phosphatase of M₁ (1.0) than any other protein tested, as indicated in Table V, it can be expected that the observed subcellular distribution of the enzyme activity might be greatly changed if this membrane protein could be isolated and used as substrate in the distribution studies.

As in the case of cyclic AMP-dependent protein kinase (4), protein phosphatase of membrane fractions is masked to a great extent and, therefore, it seemed desirable to determine the enzyme activity in the presence of the nonionic detergent, Triton X-100, which uncovered much latent activity. The low protein phosphatase activity in the particulate fractions, relative to that of the soluble, reported by Rose in the guinea pig brain (19), may be explained by the absence of unmasking detergent in his assay system. The subcellular distribution of protein phosphatase in guinea pig brain (data not shown), under our assay conditions, was found to be very similar to that reported here for rat brain.

It was shown previously that the synaptic membrane fractions are enriched with respect to cyclic AMP-dependent protein kinase activity (4). The association of protein phosphatase along with cyclic AMP-dependent protein kinase in these synaptic membrane fractions suggests an active turnover of phosphopeptide in the membrane protein. Indeed, a rapid turnover of phosphopeptide in vitro, particularly in the fractions rich in synaptic plasma membranes, as is illustrated in Fig. 2. Since the completion of these studies, Weller and Rodnight (21) have presented evidence for the turnover of protein-bound phosphorylserine phosphate in membrane preparations from ox brain cortex.

Viewed within the conceptual framework (6, 12-14) that the major biochemical action of cyclic AMP is to stimulate cyclic AMP-dependent protein kinase activity and thereby produce an increase of certain key phosphoproteins, the protein phosphatase can be regarded as a means of terminating the action of cyclic AMP by catalyzing removal of the phosphate group. It is important to emphasize that the presence in the plasma membrane fractions of high concentrations of adenyl cyclase (1), cyclic nucleotide phosphodiesterase (1-3), cyclic AMP-dependent protein kinase (4), substrate for the protein kinase (5), and protein phosphatase appears to be rather unique to brain tissue, supporting the hypothesis (3-6, 8) that the alterations in excitability of the plasma cell membrane, caused by cyclic AMP, result from altering the state of phosphorylation of the membrane protein.

Cell sap protein phosphatase activity was resolved into three fractions by column chromatography on DEAE-cellulose. These fractions could be clearly distinguished by the difference in relative rates of dephosphorylation of protamine and arginine-rich histone. Moreover, the protein phosphatase of the particulate fractions appears to be different from any of these soluble enzymes, as indicated by the K_m values for protamine and arginine-rich histone, as well as by the effect of MnCl₂. Thus, rat cerebral cortex would appear to contain at least four distinct protein phosphatases.

The demonstration in the present study of multiple forms

**TABLE VIII**

**Distribution of protein phosphatase activity in rat tissues**

Tissues from four rats were combined and the protein phosphatase activities of the whole homogenates, as well as of the supernatant and resuspended precipitate obtained following centrifugation at 150,000 × g for 60 min, were determined under the standard assay conditions with 100 μg of [³²P]histone as substrate.

| Tissue          | Activity of homogenate | Specific activity of homogenate | Activity in particulate fractions |
|-----------------|------------------------|--------------------------------|----------------------------------|
|                 | units × 10⁻⁴/g tissue  | units × 10⁻⁴/mg protein         | %                               |
| Brain           |                        |                                |                                  |
| Cerebral cortex | 141.0                  | 1.19                           | 48.8                             |
| Cerebellum      | 56.3                   | 0.44                           | 46.4                             |
| Caudate nucleus | 22.7                   | 0.10                           | 51.3                             |
| Medulla         | 41.9                   | 0.32                           | 54.2                             |
| Liver           | 48.5                   | 0.22                           | 19.8                             |
| Lung            | 47.5                   | 0.40                           | 24.3                             |
| Heart           | 33.8                   | 0.30                           | 18.2                             |
| Kidney          | 52.3                   | 0.28                           | 25.8                             |
| Spleen          | 63.7                   | 0.47                           | 18.1                             |
of protein phosphatase raises the interesting question as to whether multiple forms of this enzyme are present in a single cell type or whether the multiple forms merely reflect the heterogeneity of cell types in the cerebral cortex, with individual cells possessing only a single type of protein phosphatase. To answer this question, we have examined neuroblastoma cells and neuroglial cells, grown in tissue culture, for their protein phosphatase content. We have found that protein phosphatase in these cells grown in tissue culture exists in multiple forms, as in the case of the present studies with cerebral cortex. In addition, these single cell lines also were found to possess multiple forms of cyclic AMP-dependent protein kinase. The occurrence of several distinct protein kinase-substrate-protein phosphatase systems within a single cell makes good biological sense when we consider the multiple roles which cyclic AMP may play in any given cell. In the nervous system, for instance, the increase in cyclic AMP found to be associated with physiological activity (8) might be responsible for changes in the state of phosphorylation of proteins in the plasma membrane (controlling permeability), changes in the state of phosphorylation of nuclear histones (controlling new protein synthesis), and changes in the state of phosphorylation of various enzymes in the cell sap (controlling energy metabolism). The occurrence of several distinct protein kinase-substrate-protein phosphatase systems within a single cell could achieve these diverse physiological goals in an efficient way.

Acknowledgments—We thank Dr. E. G. Walton for valuable discussions, as well as for occasional experimental aid, and Mrs. C. Chu for her technical assistance.

REFERENCES

1. DelRoberto, E., Rodriguez deLorenzo, G., Alberici, M., Butcher, R. W., and Sutherland, E. W. (1967) J. Biol. Chem. 242, 3487–3493
2. Cheung, W. Y., and Salgianicoff, L. (1967) Nature 214, 90–91
3. Florence, N., Barnett, R. J., and Greenard, P. (1971) Science 173, 745–748

H. Maeno and P. Greenard, manuscript in preparation.
Phosphoprotein Phosphatases from Rat Cerebral Cortex: SUBCELLULAR DISTRIBUTION AND CHARACTERIZATION
Hiroo Maeno and Paul Greengard

J. Biol. Chem. 1972, 247:3269-3277.

Access the most updated version of this article at http://www.jbc.org/content/247/10/3269

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 0 references, 0 of which can be accessed free at http://www.jbc.org/content/247/10/3269.full.html#ref-list-1