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Partial Characterization of Endogenous Phosphorylation Conditions for Hen Brain Cytosolic and Membrane Proteins*

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The optimal conditions for endogenous protein phosphorylation with 5 μM [γ-^{32}P]ATP, 10 mM $MgCl_2$ in preparations containing synaptosomal cytosol or membranes (shocked crude mitochondrial fraction P_2) from adult hen brains were determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, autoradiography and microdensitometry. Phosphate incorporation increased linearly with protein concentration from 75–125 μg/200 μl in brain cytosol and was maximal at 75 μg/200 μl in brain membranes. Optimal incubation times were 60–90 s for brain cytosol and 10–15 s for brain membranes. With the exception of the 20 kilodalton myelin basic protein in the membrane fraction, pH 6.5 is generally optimal. While temperature optima varied considerably with different bands, most of them were found between 35 and 45 °C. When identical preparations from hen and rat brain were co-electrophoresed, one of the most striking differences was that the enhancement of phosphorylation of a 55 kilodalton doublet, which may be tubulin, by addition of 50 μM Ca^{2+} was at least 3 times greater in rat than in hen brain cytosol. Another species difference was apparent in the membrane fractions in which the 20 kilodalton hen brain presumptive myelin basic protein (MBP) was phosphorylated to approximately the same extent as that of the 16 and 20 kilodalton rat brain MBPs combined.

INTRODUCTION

Many proteins in the mammalian brain, some of which are neuron-specific, are subject to protein kinase-catalyzed phosphorylation, utilizing ATP as the phosphate donor. Endogenous protein phosphorylation is a dynamic regulatory mechanism in the nervous system. It mediates diverse physiological responses to neuronal second messengers such as the cyclic nucleotides, for which it is the primary, if not exclusive, type of response, and calcium, for which it is one of several effects, many of which require the intervention of the calcium-binding regulatory protein, calmodulin[15]. Neuronal substrates for calcium (Ca^{2+})- and calmodulin-activated protein phosphorylation include 3 of the 4 subunits of the acetylcholine receptor (δ and γ more than β)[36], the rate-limiting biogenic amine neurotransmitter synthetic enzymes tryptophan hydroxylase and tyrosine hydroxylase[18,44], α- and β-tubulin (DPH-L and DPH-M)[7–10], and the synaptic-vesicle associated protein Synapsin I (Protein I)[16]. Synapsin I and tyrosine hydroxylase are also substrates for the cAMP-dependent protein kinase[12,41], as is, ironically, the type II cAMP-dependent protein kinase's regulatory subunit[40].

Certain aspects of the normal physiological functioning of the nervous system and its perturbation by xenobiotics may be investigated by employing an in vitro assay for endogenous protein phosphorylation, using [γ-^{32}P]ATP. However, interpretation of such

* A preliminary account of this work has been presented, see refs. 2 and 28.

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experiments is not straightforward. The ^{32}P incorporation seen in brain proteins which have been separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) is the net result of an interplay of at least 3 types of enzymes: the protein kinases of various cofactor specificities, the phosphoprotein phosphatases and the ATPases. Incubation of acceptor proteins with [γ-^{32}P]ATP results in their labeling with ^{32}P, followed typically by net dephosphorylation after all excess ATP has been used up or hydrolyzed by membrane ATPase activity[32]. Furthermore, caution must be exercised in extrapolating from in vitro data to in vivo effects. For example, a net dephosphorylation of available substrate protein sites in vivo may, under certain conditions, lead to a net increase in the incorporation of radiolabeled phosphate in vitro[3]. However, despite potential quantitative differences in relative ^{32}P incorporation by different proteins, the substrate proteins for in vitro phosphorylation have, at least, been observed to comigrate with in vivo phosphoproteins[23].

This technique has been most commonly applied to the rat brain model system, although more recently it has been characterized for the brains of 6–8-day-old male chicks[5,37]. We have reported elsewhere increased in vitro protein phosphorylation in the brains of adult hens paralyzed as a result of administration of a single dose of the delayed neurotoxicant tri-*o*-cresyl phosphate (TOCP), 21 days previously[29]. Neither rats nor young chicks are susceptible to TOCP-induced delayed neurotoxicity[1,2]. Accordingly, we have undertaken to facilitate more extensive toxicological applications of the in vitro protein phosphorylation assay to adult hen brain cytosolic and membrane protein fractions by characterizing the conditions appropriate to the assay of these new model systems and by comparative studies of the phosphorylation of these neuronal protein fractions in hens and in rats, under the same conditions.

MATERIALS AND METHODS

Materials

[γ-^{32}P]ATP (4500 Ci/mmol) was obtained from ICN (Irvine, CA). Tris, ultrapure, was from Schwarz-Mann, Inc. (Spring Valley, NY) and glycerol was from Mallinkrodt (Paris, KY). All other materials used in the preparation of the polyacrylamide gels and associated buffers were of electrophoresis grade and were purchased from Bio-Rad Laboratories (Richmond, CA) as were Coomassie brilliant blue G-250 (protein assay dye reagent), bovine plasma gamma globulin, and Chelex 100. Ethyleneglycol-bis-(β-aminoethyl ether) N,N′-tetraacetic acid (EGTA), piperazine-N,N′-bis(2-ethane-sulfonic acid) (PIPES), N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid (HEPES), and phenylmethylsulfonyl fluoride (PMSF) were obtained from Sigma Chemical Co. (St. Louis, MO). Bovine brain calmodulin was prepared[42] and generously provided by Dr. Thomas C. Vanaman, Duke University Medical Center (Durham, NC). Trifluoperazine was the gift of Dr. Leon C. Greene of Smith, Kline and French Laboratories (Philadelphia, PA).

Care and treatment of animals

Adult leghorn laying hens (*Gallus gallus domesticus*) each 14 months old and weighing approximately 1.5 kg, were obtained from Featherdown Farm (Raleigh, NC). The hens were anesthetized with CO_2 and sacrificed by decapitation. In some experiments, young adult male rats, weighing approximately 200 g, were obtained from Harlan Sprague–Dawley (Indianapolis, IN) and were stunned and then also sacrificed by decapitation.

Preparation of subcellular fractions

Immediately following sacrifice, brains were removed and each was immersed in ice-cold 0.32 M sucrose, 0.3 mM PMSF, 50 mM Tris-HCl, pH 7.4. Motor driven Teflon-glass homogenization (6 strokes) in fresh aliquots of the same buffer at 10% w/v, followed by differential centrifugation of the homogenate at 1000 *g* and then the S_1 supernatant at 10,000 *g* yielded the crude mitochondrial fraction (P_2). Each P_2 fraction was subjected to hypoosmotic shock in 250 μl of 5.0 mM Tris, pH 8.0, 0.3 mM PMSF, per gram original tissue for 60 min with occasional vortex mixing, as described previously[29], to preferentially lyze the synaptosomes. The resulting lysate was centrifuged at 140,000 *g* for 30 min, and the pellet and supernatant obtained were used as sources of membranes and synaptosomal cytosol[25], respectively. The membranes were stored frozen at −70 °C for 1 week prior to assay[24]. In some experiments, the protease inhibitor PMSF (0.3 mM) was added to preparation

solutions based on its ability to preserve the Ca^{2+}-calmodulin tubulin kinase in rat brain[7].

Phosphorylation assays

The standard phosphorylation reaction was initiated by the addition of a 25 μl protein aliquot containing 100 μg (range = 25–150, when varied) of hen or rat brain synaptosomal cytosol or crude P_2 membranes, in 5.0 mM Tris, pH 8.0, to the reaction mixture. The standard reaction mixture also contained, in a final volume of 200 μl: 50 mM PIPES, pH 6.5 (range = 6.0–7.0, when varied; with 50 mM HEPES for pH 7.5, 8.0), 10 mM $MgCl_2$, 0.3 mM PMSF (only where indicated) and 5.0 μM [γ-^{32}P]ATP (4500 Ci/mmol) at 30 °C (range = 25–50, when varied). Additionally, cytosolic proteins were incubated in the absence or presence of 5 or 50 μM $CaCl_2$, 100 μM EGTA, or 50 μM $CaCl_2$ and 50 μM trifluoperazine, whereas membrane proteins were incubated in the absence or presence of 300 μM $CaCl_2$ alone or in combination with 1 μg calmodulin. The reaction was terminated and the samples prepared for electrophoresis after 60 s (range = 30–120, when varied) for cytosol or 20 s (range = 5–60, when varied) for membranes by the addition of 100 μl of 'stop solution', containing 0.125 M Tris-HCl, pH 6.8, 4.5% SDS, 20% glycerol and 10% β-mercaptoethanol, followed by heating for 3 min in a 90 °C water bath. Protein concentrations were determined by the method of Bradford[4] using Coomassie brilliant blue G-250 with bovine plasma gamma globulin as a standard. The deionized water used in the phosphorylation assays as well as in the homogenization, lysis and electrophoresis gel buffers was passed through a Chelex 100 column to ensure thorough removal of Ca^{2+}. The assays were performed in plastic (polyethylene) test tubes, and all solutions in contact with the tissue samples before the addition of stop solution were stored in plastic containers.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

The synaptosomal cytosolic and membrane proteins were resolved on 20 well 0.1% SDS-polyacrylamide vertical slab gels (12 × 16 × 0.15 cm) (Bio-Rad Laboratories, Richmond, CA). Electrophoresis was performed using the stacking, resolving, and running gel buffers described by Laemmli[19] with 6 and 10% acrylamide in the stacking and resolving gels, respectively (36.5:1 acrylamide:bisacrylamide). Aliquots (50 μl) of the samples were subjected to electrophoresis under conditions of constant current (30 mA/gel through the stacking gel and 40 mA/gel through the resolving gel) at 13–15 °C until the tracking dye reached the bottom of the gels (3.5–4.0 h). The apparent molecular weights of the resolved proteins were determined form M_r (electrophoretic mobility) standards (Bio-Rad Laboratories, Richmond, CA) which had been subjected to electrophoresis under conditions identical to those used for the tissue samples and are presented in kilodaltons. Following electrophoresis, the gels were fixed and stained for protein overnight with 0.1% Coomassie brilliant blue R-250 in 50% methanol and 10% acetic acid. The gels were destained with 50% methanol and 10% acetic acid (2–3 h) in a diffusion destainer employing activated charcoal (Bio-Rad Laboratories, Richmond CA) and then soaked (1 h) in 10% acetic acid and 1% glycerol (to prevent cracking) before drying for 2 h between two sheets of dialysis membrane (Bio-Rad Laboratories, Richmond, CA) at 80 °C using a water aspirator to withdraw the vapor.

Autoradiography

Autoradiographs of the ^{32}P-labeled proteins were obtained by placing the dried gels in close contact with Kodak X-OMAT RP X-ray film (0.5–3.0 days) at —70 °C using intensify screens (Dupont Cronex Lighting Plus, CGR Corp., Raleigh, NC)[20].

Microdensitometry

The amount of phosphoprotein in each autoradiographic band was quantified by integration of the area under the corresponding peak in the densitometric scan obtained by using a Quick-Scan (Helena Laboratories, Beaumont, TX) Transmission Microdensitometer or a Zeineh Soft Laser Densitometer (BioMed Laboratories, Inc., Fullerton, CA) interfaced with a PDP-11 Computer (Digital Equipment Corp., Maynard, MA) programmed for integration or an LKB Ultroscan Laser Densitometer interfaced with a Recording Integrator (LKB Instruments, Gaithersburg, MD). All data which were to be directly compared were scanned on the same instrument, taking the background darkness of the autoradiographs as the baseline. Since the darkness of

the autoradiographic bands, and therefore the peak areas in the densitometric scan, depend not only on the level of protein phosphorylation and specific radioactivity of the [γ-^{32}P]ATP but also on the duration and temperature of film exposure, during which the radioactivity of the incorporated ^{32}P may decrease significantly, all phosphorylation results are reported in arbitrary units[17].

Statistics

Statistical comparisons of protein phosphorylation between hens and rats or their tissues, were based on the Student's unpaired two-tailed *t*-test. Statistical comparisons of effects of cofactor concentration within the same animal were based on the Student's paired two-tailed *t*-test.

RESULTS

In order to determine the optimal in vitro protein phosphorylation assay conditions for hen brain synaptosomal cytosol and membranes (shocked P_2), the protein concentration, incubation time, temperature, and pH commonly employed for rat brain protein phosphorylation (100 μg/200 μl, 60 s for cytosol or 15 s for membranes, 30 °C, pH 6.5)[27] were each varied in turn while the other 3 were held constant. The ^{32}P-labeled proteins from each combination of assay conditions were electrophoresed on 10% polyacrylamide gels in the presence of SDS. Protein phosphorylation was measured in arbitrary units of area under peaks in the densitometric scans of the autoradiographs of the dried gels. Expressed in this way, it was plotted versus each varying parameter for the major peaks, identified by their electrophoretic mobilities (M_r) in the 4 preparations (Figs. 1–4).

Protein concentration

[^{32}P] phosphate incorporation increased linearly with protein concentration, at least over a range of 75–125 μg/200 μl assay volume, for all 3 major brain cytosolic bands of M_r 20, 55 and 70 kilodaltons (Figs. 1 and 3). In brain membranes, protein phosphorylation was maximal at 75 μg/200 μl, with no clear linear range, for all 3 major bands of M_r 20, 50 and 60 kilodaltons (Figs. 2 and 3).

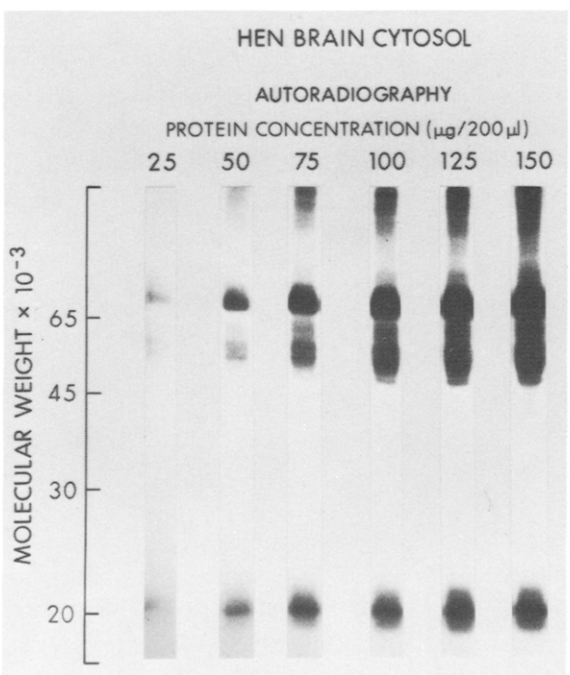

Fig. 1. Dependence of phosphorylation on protein concentration of hen brain cytosol. Representative SDS-PAGE autoradiographs of the in vitro^{32}P incorporation, from [γ-^{32}P]ATP in the presence of cofactor concentrations indicated under Methods.

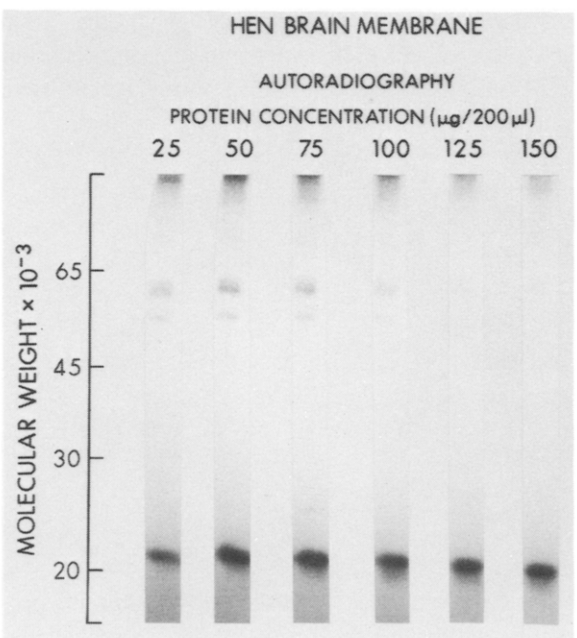

Fig. 2. Dependence of phosphorylation of protein concentrations of hen brain membranes. Representative SDS-PAGE autoradiographs of the in vitro ^{32}P incorporation, from [γ-^{32}P] in the presence of cofactor concentrations indicated under Methods.

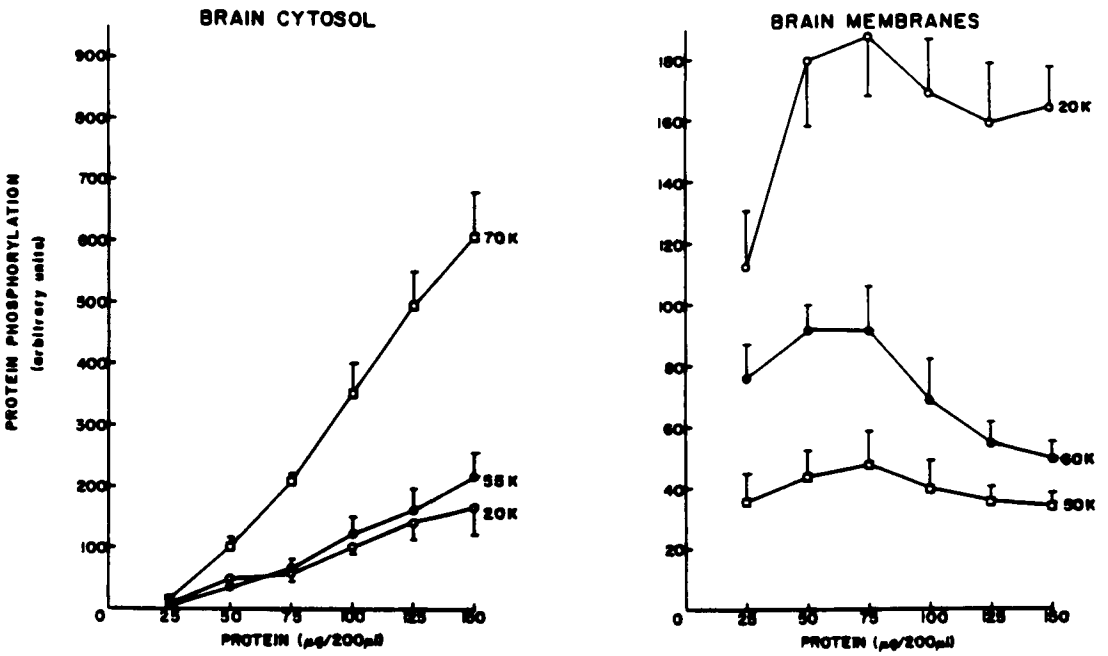

Fig. 3. Protein concentration curves for phosphorylation in hen brain cytosol and membranes. ^{32}P incorporation in vitro from [γ-^{32}P]ATP, under the conditions described in the Methods section, into various concentrations of neuronal proteins is plotted as the corresponding density ± S.E.M. (in arbitrary units) of the autoradiographs of the dried SDS-PAGE band with indicated electrophoretic mobilities (n=5).

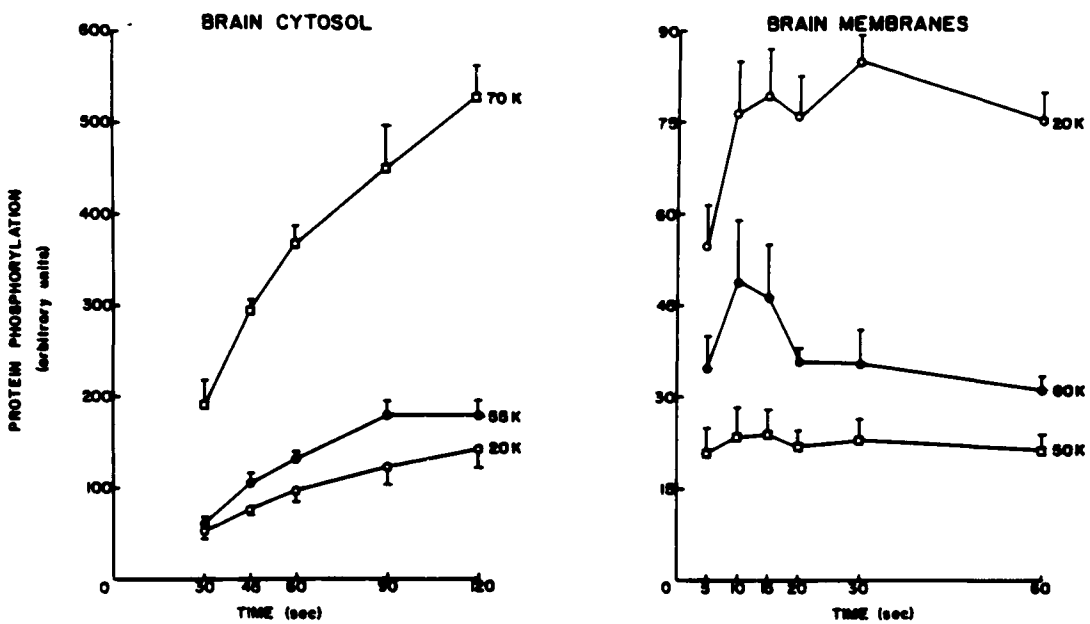

Fig. 4. Incubation time curves for phosphorylation in hen brain cytosol and membranes. Protein ^{32}P incorporation, over various times of incubation in vitro with [γ-^{32}P]ATP under the conditions described in the Methods section, is plotted as the corresponding density ± S.E.M. (in arbitrary units) of the autoradiographs of the dried SDS-PAGE bands with indicated electrophoretic mobilities (n=5).

Incubation time

Protein phosphorylation increased linearly with incubation time from 60–120 s for the 20 and 70 kilodalton and 45–60 s for the 55 kilodalton brain cytosolic bands (Fig. 4). The optimal time was 10–15 s for the 50 and 60 kilodalton brain membrane proteins (Fig. 4).

Incubation temperature

Different proteins responded differently to incubation temperature, although in brain cytosol, 40 °C seemed to be either the maximum temperature (70 kilodalton band), the temperature above which there is a leveling off (55 kilodalton band), or the temperature above which there is a decline in ^{32}P incorporation (70 and 20 kilodalton bands) (Fig. 5). In the brain membranes (Fig. 5), 35 °C appeared to be optimal for the 50 and 60 kilodalton proteins. In contrast, phosphate incorporation increased fairly linearly, at least from 35–45 °C, and then leveled off or declined in the 20 kilodalton protein band of brain membranes (Fig. 4).

Incubation pH

The protein phosphorylation response to pH varied considerably with the protein (Fig. 6). pH values of 6.5, 6.0 or less, and 7.5 were optimal for the 20, 55 and 70 kilodalton brain cytosolic proteins, respectively. The 20 kilodalton brain membrane protein was most phosphorylated at pH 7.0, 8.0, or greater, while the phosphorylation pH optimum was 6.5 for the 50 and 60 kilodalton bands.

Effect of phenylmethylsulfonyl fluoride (PMSF)

The effect of addition of the protease inhibitor phenylmethylsulfonyl fluoride to all preparative (homogenization, lysis and resuspension) buffers as well as to the actual reaction medium was investigated, since Burke and DeLorenzo[11] found it to be helpful protection for rat brain synaptic vesticle Ca^{2+}/calmodulin tubulin kinase activity. In the present study, such addition was not found to have a significant protective effect.

Comparisons between hen and rat brain protein phosphorylation

Comparisons of staining and autoradiographic patterns of phosphorylated brain proteins from the hen and the rat, which were co-electrophoresed on the same gel slab, disclose several striking differences.

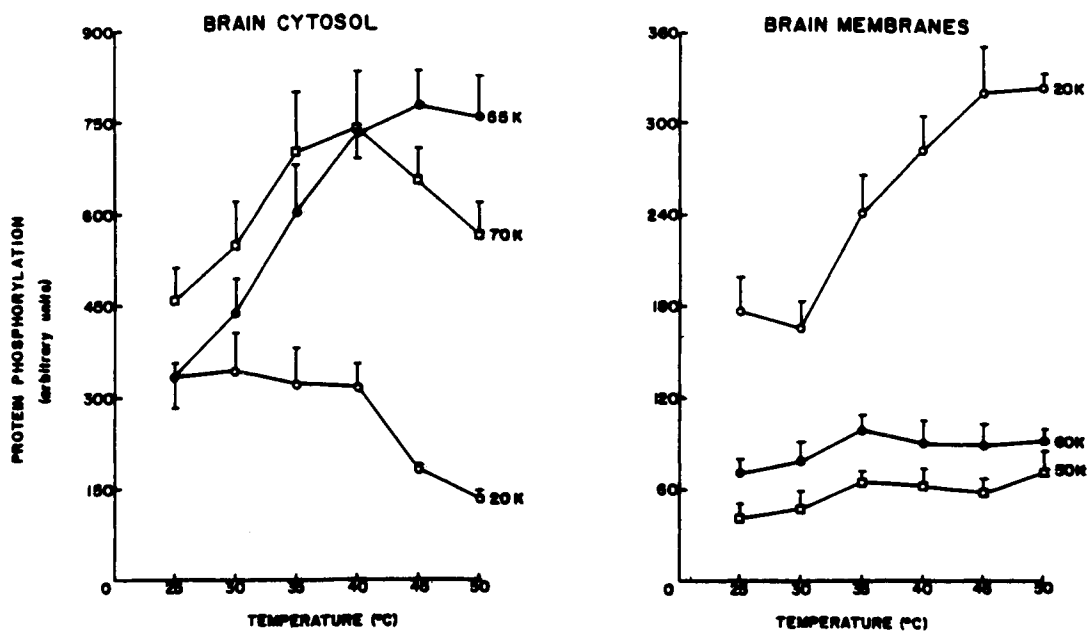

Fig. 5. Incubation temperature curves for phosphorylation in hen brain cytosol and membranes. Protein ^{32}P incorporation at various incubation temperatures in the presence of [γ-^{32}P]ATP under the conditions described in the Methods section, is plotted as the corresponding density ± S.E.M. (in arbitrary units) of the autoradiographs of the dried SDS-PAGE bands with indicated electrophoretic mobilities (n=5).

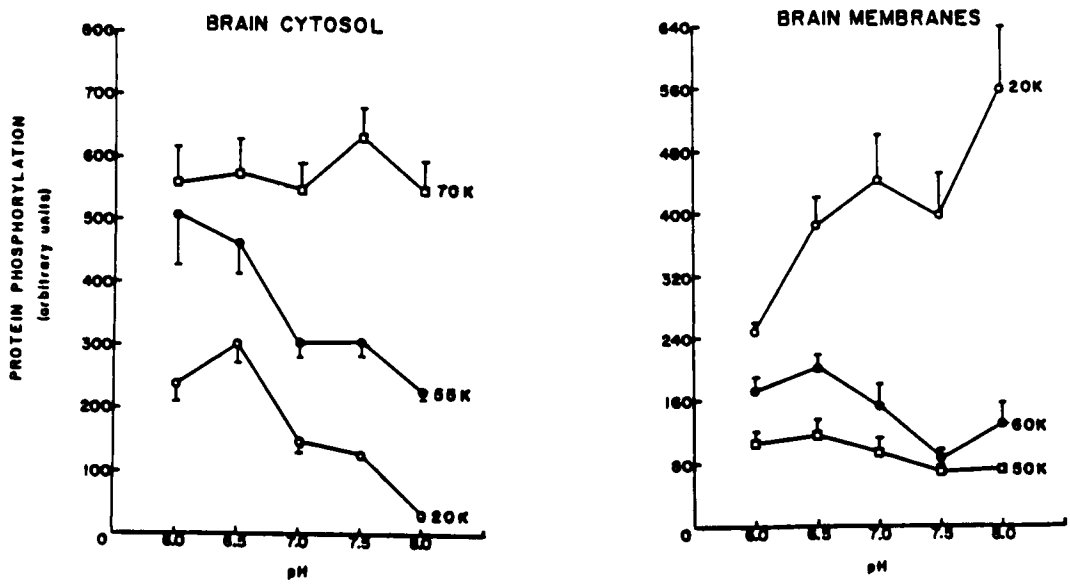

Fig. 6. Incubation pH curves for phosphorylation in hen brain cytosol and membranes. Protein ^{32}P incorporation in vitro in the presence of [γ-^{32}P]ATP under the conditions described in the Methods section, except at various pHs, is plotted as the corresponding density ± S.E.M. (in arbitrary units) of the autoradiographs of the dried SDS-PAGE bands with indicated electrophoretic mobilities (n=5).

For instance, there is an 80 kilodalton band in rat, but not hen, brain cytosol whose phosphorylation was nearly tripled by the addition of 50 μM Ca^{2+}, while there is an approximately 70 kilodalton band unique to hen brain cytosol whose phosphorylation was not significantly dependent on calcium, although it was inhibited in the presence of the calmodulin inhibitor trifluoperazine (Fig. 7, Tables I–III). The difference in the Ca^{2+}-dependence of protein phosphorylation between the two species is even more exaggerated for the 55 kilodalton doublet. The insignificant increase in its phosphorylation in hens upon addition of 50 μM Ca^{2+}, despite susceptibility to trifluoperazine (Table III), with the lower M_r member of the doublet incorporating much more phosphate than the higher M_r member, was overshadowed by an approximately 5-fold enhancement of the fairly symmetrically phosphorylated rat brain doublet. The phosphorylation of a minor brain cytosolic band of M_r 30 kilodalton was also more enhanced by calcium in the rat than it is in the hen. The calcium-dependence of the phosphorylation of the yet lower molecular weight proteins in the brain cytosol is principally evident from the effective inhibition of their phosphorylation by EGTA. The marginally significant effects of exogenous calcium on their phosphorylation in rats (insignificant in hens) may reflect calcium requirements of the kinase(s) phosphorylating these substrates that are low enough to be completely satisfied by endogenous calcium levels in hens and almost satisfied in rats.

The notably greater Ca^{2+}-independent phosphorylation of a 20 kilodalton band which may be a doublet in hens, in contrast to rats (Fig. 8, Tables IV and V), most likely results from the greater 20 kilodalton protein concentration evident in the hen brain membrane Coomassie blue staining pattern. Rat brain membranes, in contrast, have a 16 kilodalton protein band which is phosphorylated slightly more than their 20 kilodalton band.

The calmodulin-dependent phosphorylation of the 50 kilodalton band is roughly 4 times greater in rat brain membranes than in hen brain membranes, although the actual enhancement due to calmodulin is greater for hens. The Ca^{2+}/calmodulin-dependent phosphorylation of the 60 kilodalton doublet is much greater in the rat than in the hen brain membranes. Closer examination of the 60 kilodalton doublet in brain membrane autoradiographs (Fig. 8) reveals the further subtlety that phosphorylation of the lower M_r band predominates in hens, while phosphorylation of the higher M_r band predominates in rats. The relatively greater phosphorylation of an approximately

TABLE I

Comparison of hen and rat brain cytosolic protein phosphorylation

The net in vitro incorporation of ^{32}P from [γ-^{32}P]ATP, in the presence of various concentrations of calcium, into specific brain cytosolic proteins, separated by SDS-PAGE, was quantified from the densitometric scans of the autoradiographs of the dried gels and is expressed as the mean ± S.E.M. of independent observations from 5 hens and 4 rats. Values are in arbitrary units. Significance is that of the difference between hens and rats in ^{32}P incorporation, at the same calcium concentration, into a band of similar electrophoretic mobility in both species, as determined by the Student's unpaired two-tailed *t*-test. NP, band not present or undetectable in one species; S, a priori significant difference from a band with no counterpart in other species; n.s., not signficantly different.

Cofactors	*M_r (kilodaltons)*	*Hen*	*Rat*	*Hen/Rat*	*Significance*
None added	80	NP	53.0 ± 2.7	–	S
	70	38.0 ± 2.9	NP	–	S
	55	48.0 ± 4.1	50.6 ± 4.1	0.95	n.s.
	30	3.5 ± 0.2	3.4 ± 0.4	1.04	n.s.
	23	48.8 ± 0.7	40.4 ± 2.3	1.21	n.s.
	18	25.4 ± 0.7	22.7 ± 1.4	1.12	n.s.
	15	59.0 ± 0.7	59.2 ± 2.0	1.00	n.s.
100 μM EGTA	80	NP	21.5 ± 4.1	–	S
	70	22.4 ± 0.8	NP	–	S
	55	23.2 ± 0.9	25.9 ± 3.5	0.89	n.s.
	30	0.3 ± 0.1	NP	–	S
	23	4.8 ± 0.3	1.6 ± 0.7	2.97	n.s.
	18	3.6 ± 0.2	1.8 ± 0.5	2.04	n.s.
	15	1.8 ± 0.3	2.8 ± 0.9	0.64	n.s.
5 μM Ca^{2+}	80	NP	80.2 ± 4.9	–	S
	70	39.9 ± 1.0	NP	–	S
	55	56.3 ± 1.7	127.8 ± 3.8	0.44	n.s.
	30	4.5 ± 0.1	3.8 ± 0.5	1.20	n.s.
	23	50.6 ± 0.8	52.7 ± 0.9	0.96	n.s.
	18	31.2 ± 1.4	28.8 ± 1.3	1.08	n.s.
	15	62.0 ± 0.6	64.4 ± 2.7	0.96	n.s.
50 μM Ca^{2+}	80	NP	133.5 ± 15.1	–	S
	70	40.5 ± 2.6	NP	–	S
	55	75.5 ± 7.1	249.3 ± 15.8	0.30	$P < 0.05$
	30	5.8 ± 0.6	8.8 ± 1.3	0.66	n.s.
	23	43.7 ± 1.9	54.3 ± 3.9	0.81	n.s.
	18	34.1 ± 4.4	28.5 ± 3.0	1.20	n.s.
	15	61.6 ± 6.0	76.9 ± 5.5	0.80	n.s.

80 kilodalton band in rat than in hen brain membranes and of a weaker 70 kilodalton band in hen than in rat brain membranes, apparent from the autoradiographs (Fig. 8), is reminiscent of the brain cytosolic phosphorylation pattern for the two species.

DISCUSSION

We have prepared crude synaptosome (P_2) fractions [29] from the brains of rats and hens and subjected them to hypoosmotic lysis. The resulting synaptosomal cytosol and membrane fractions were phosphorylated in vitro with [γ-^{32}P]ATP under the same conditions in order to compare the protein phosphorylation in hen brain with established rat brain data. The extent of subcellular fractionation chosen was designed to minimize the preparation time delay-related tendency for decreased incorporation of ^{32}P into certain proteins as a result of kinase lability or proteolysis[10,32] while achieving approximately the same pattern and behavior of phosphate acceptor proteins, except for the addition of myelin basic protein, as would be attained upon further purification of the synaptosomes away from contaminating myelin and mitochondria by density gradient centrifugation[17]. We have also characterized the response of both fractions examined in the hen (brain synaptosomal cytosol and membranes) with respect

TABLE II

Stimulation of hen and rat brain cytosolic protein phosphorylation by calcium (mean % of control)

The net in vitro incorporation of ^{32}P from [γ-^{32}P]ATP into specific brain cytosolic proteins, separated by SDS-PAGE, was quantified from the densitometric scans of the autoradiographs of the dried gels and is expressed as the mean ± S.E.M. for 5 hens or 4 rats of the percentage the phosphorylation in the presence of the calcium concentrations indicated is of the phosphorylation for the sample from the same animal in the absence of exogenous calcium. NP, band not present or undetectable in one species.

Calcium concentration	*M_r (kilodaltons)*	*Hen*	*Rat*
5 μM Ca^{2+}	80	NP	149.2±5.9**
	70	111.2±11.9	NP
	55	123.7±19.3	249.9±14.7***
	30	131.9±14.6	105.2±19.7
	23	104.9±9.9	129.2±9.9*
	18	121.4±17.0	126.1±7.3*
	15	105.5±13.2	105.8±6.4
50 μM Ca^{2+}	80	NP	263.1±28.8**
	70	113.0±12.0	NP
	55	153.9±21.2	489.4±21.6***
	30	158.9±24.6	295.4±83.1
	23	90.8±10.9	134.9±2.3***
	18	127.1±33.9	126.7±8.4
	15	104.2±20.6	131.9±11.0

* Significantly stimulated by indicated calcium concentration, based on paired t-test, $P < 0.05$.

** Significantly stimulated by indicated calcium concentration, based on paired t-test, $P < 0.02$.

*** Significantly stimulated by indicated calcium concentration, based on paired t-test, $P < 0.005$.

to optimal protein concentration and incubation time, temperature, and pH in the in vitro protein phosphorylation assay. The observation that phosphorylation of the major bands in hen brain cytosol increases linearly with protein concentration, at least over the range of 75–125 μg/200 μl, is in good agreement with the standard use of 100 μg/200 μl for rat brain cytosol. However, the decline in phosphate incorporation above the apparent maximum of 75 μg/200 μl for most bands in hen brain membranes contrasts sharply with the choice of 100 μg/200 μl for rat brain membranes and cytosol. Burke and DeLorenzo[8] reported data indicating linearity in phosphorylation of the combined α- and β-tubulin components of rat brain 'nerve terminal cytoplasm' (NTC) up to 100 μg NTC/100 μl, while the time to reach maximal phosphorylation of tubulin (less than 50 s at 37 °C) was independent of protein concentration in the reaction mixture. O'Callaghan et al.[25] observed maximal phosphorylation of a 60 kilodalton rat brain synaptosomal cytosolic (100 μg/200 μl) band at 1 min and two bands of M_r 50 and 55 kilodalton at 2 min, all of which showed linearity of phosphorylation only up to 1 min at 30 °C. These observations are in reasonable agreement with our findings concerning the time dependence of hen brain cytosolic protein phosphorylation at 30 °C, 100 μg/200 μl, up to 2 min, although the only leveling off of protein phosphorylation in this fraction occurred at 90 s for the 55 kilodalton band. New increases in ^{32}P incorporation were observed only for the first 10–15 s in hen brain membranes. Perhaps this is because there are fewer acceptor proteins in brain membranes competing for the limited supply of ATP which is rapidly being hydrolyzed by the high level of membrane-bound ATPases in synaptic plasma membranes[38,43]. The overall optimal phosphorylation temperature observed for

TABLE III

Effect of the calmodulin inhibitor trifluoperazine on the in vitro phosphorylation of hen brain synaptosomal cytosolic proteins

The net in vitro incorporation of ^{32}P from [γ-^{32}P]ATP in the presence of 50 μM Ca^{2+}, under standard assay conditions, into hen brain synaptosomal cytosolic proteins, separated by SDS-PAGE, was quantified from the densitometric scans of the autoradiographs of the dried gels, and is expressed as the mean ± S.E.M. of independent observations from 6 hens in the absence of 50 μM trifluoperazine and 4 hens in its presence (phosphorylation was below the threshold for detection in the other two hens under these conditions). Values are in arbitrary units. The percentage that the mean protein phosphorylation in the presence of trifluoperazine is of that in its absence. Significance is that of the difference in ^{32}P incorporation between the presence and absence of trifluoperazine, based on the Student's unpaired two-tailed t-test.

M_r(kilodaltons)	*Control*	*Trifluoperazine*	*% of control*	*Significance*
70	425.5 ± 43.3	15.3 ± 6.0	3.6	$P < 0.001$
55	552.8 ± 118.0	19.2 ± 7.3	3.5	$P < 0.01$
20	242.1 ± 43.5	15.0 ± 4.0	6.2	$P < 0.005$

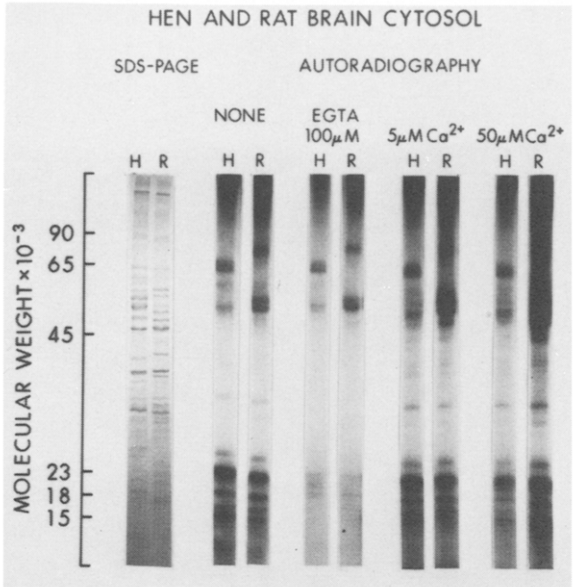

Fig. 7. Comparison of hen and rat brain cytosolic protein phosphorylation: dependence on calcium. Representative SDS-PAGE Coomassie blue R-250 staining profiles and autoradiographs of the in vitro ^{32}P incorporation, from [γ-^{32}P]ATP in the presence of indicated cofactor concentrations into polypeptides from hens (H) or rats (R), both at a protein concentration of 100 μg/200 μl for 60 s at 30 °C, pH 6.5.

the major hen phosphate acceptor proteins examined was 35 °C for two, 40 °C for one, and 45 °C for two, with the general trend favoring the high end of the temperature range. This is not surprising in view of the higher body temperature of chickens (41 °C) in comparison to rats (37 °C). With this in mind, we propose that in vitro phosphorylation assays be conducted at 35 °C on neuronal proteins from chickens, rather than the 30 °C, customary for rats[11,14,25,26,43]. A pH of 6.5 seems to be the best compromise for all of the phosphorylatable hen brain proteins and is indeed optimal, or nearly so, for all but two. This is in keeping with the general choice of pH 6.5 or 7.0 for rat brain protein phosphorylation[11,25,27]. The lack of effect of PMSF on the Ca^{2+}/calmodulin-dependent phosphorylation of hen brain P_2 lysate soluble proteins might be attributable to the relative brevity of the preparative procedure, although the contribution of species differences has not been excluded. Burke and DeLorenzo[8] have noted that prolonged handling, multiple washes, or increases in temperature decrease the Ca^{2+}-dependent phosphorylation of their vesicle fraction proteins.

A comparison of the characteristics of various phosphate acceptor proteins between the avian hen and the mammalian rat, members of two different phylogenetic classes, not surprisingly reveals several interesting differences as well as some similarities. Both the hen and the rat brain cytosol have a major Coomassie blue staining band with M_r approximately 43–45 kilodaltons which comigrates with purified chicken muscle actin (Sigma; data not shown) but, like actin, fails to incorporate ^{32}P. Both also have a major staining doublet at approximately 55 kilodaltons which comigrates with purified bovine brain α- and β-tubulin[34], (data not shown) and undergoes Ca^{2+}-activated phosphorylation, though to a much greater extent in the rat than in the hen. Subsequent experiments have also shown this phosphorylation to be calmodulin-dependent on the basis of its susceptibility to inhibition by the calmodulin inhibitor trifluoperazine[21] (data not shown), in agreement with other workers' observations on the phosphorylation of unidentified rat brain proteins with a 1-D M_r of 55 kilo-

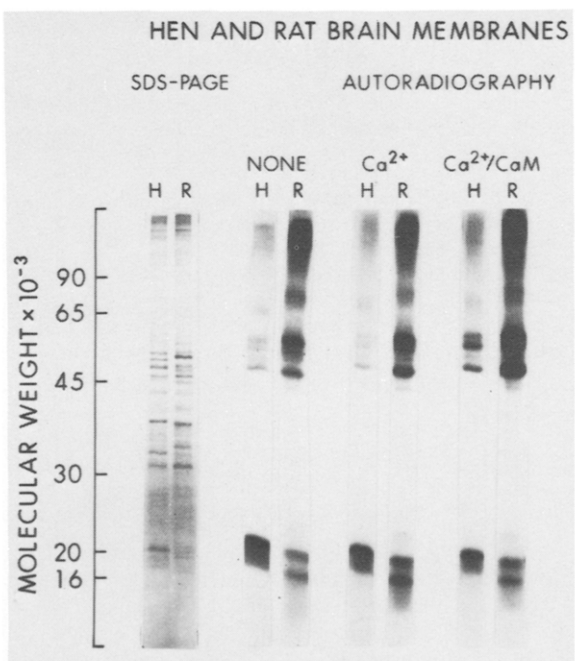

Fig. 8. Comparison of hen and rat brain membrane protein phosphorylation; dependence on calcium and calmodulin. Representative SDS-PAGE Coomassie blue R-250 staining profiles and autoradiographs of the in vitro ^{32}P incorporation from [γ-^{32}P]ATP, in the presence of indicated cofactor concentrations and into polypeptides from hens (H) or rats (R), both at a protein concentration of 100 μg/200 μl for 20 s at 30 °C, pH 6.5.

TABLE IV

Comparison of hen and rat brain membrane protein phosphorylation

The net in vitro incorporation of ^{32}P from [γ-^{32}P]ATP, in the absence or presence of cofactors at indicated concentrations, into specific brain membrane proteins, separated by SDS-PAGE, was quantified from the densitometric scans of the autoradiographs of the dried gels and is expressed as the mean ± S.E.M. of independent observations from 5 hens and 5 rats. Values are in arbitrary units. Significance is that of the difference between hens and rats in ^{32}P incorporation, under the same cofactor conditions, into a band of similar electrophoretic mobility in both species, as determined by the Student's unpaired two-tailed *t*-test. NP, band not present or undetectable in one species; S, a priori significant difference from a band with no counterpart in the other species; n.s., not significantly different.

Cofactors	*Mr (kilo daltons)*	*Hen*	*Rat*	*Hen/Rat*	*Significance*
None added	60	5.4 ± 1.1	16.9 ± 2.9	0.32	$P < 0.1$
	50	10.4 ± 2.0	52.5 ± 8.0	0.20	$P < 0.001$
	20	58.3 ± 7.3	20.7 ± 6.9	2.81	$P < 0.01$
	16	NP	26.6 ± 11.2	–	S
300 μM calcium	60	3.4 ± 0.4	9.1 ± 2.0	0.38	$P < 0.05$
	50	6.9 ± 1.0	58.8 ± 10.4	0.19	$P < 0.002$
	20	57.9 ± 9.9	24.7 ± 5.6	2.34	$P < 0.02$
	16	NP	38.5 ± 10.9	–	S
300 μM calcium, 5 μg/ml calmodulin	60	8.9 ± 0.9	25.5 ± 6.0	0.35	$P < 0.05$
	50	34.3 ± 3.5	116.2 ± 8.6	0.30	$P < 0.001$
	20	37.4 ± 4.7	20.9 ± 8.8	2.29	n.s.
	16	NP	24.5 ± 5.5	–	S

TABLE V

Stimulation of hen and rat brain membrane protein phosphorylation by calcium alone or with calmodulin (mean % of control)

The net in vitro incorporation of ^{32}P from [γ-^{32}P]ATP into specific brain membrane proteins, separated by SDS-PAGE, was quantified from the densitometric scans of the autoradiographs of the dried gels and is expressed as the mean ± S.E.M. for 5 hens or 5 rats of the percentage the phosphorylation in the presence of calcium ± calmodulin is of the phosphorylation for the sample from the same animal in the absence of exogenous cofactors. NP, band not present or undetectable in one species.

Cofactors	*M_r (kilo-daltons)*	*Hen*	*Rat*
300 μM calcium	60	70.8±12.8	53.4±5.8**
	50	72.8±11.3	110.3±12.8
	20	96.8±5.5	169.6±44.6
	16	NP	147.5±11.8
300 μM calcium, 5 γ/ml calmodulin	60	214.0±72.7	177.0±53.6
	50	378.6±77.6***	245.0±41.2***
	20	79.6±9.8	161.6±51.7
	16	NP	133.6±33.5

* Significantly affected by indicated cofactor(s), based on paired *t*-test, $P < 0.05$.

** Significantly affected by indicated cofactor(s), based on paired *t*-test, $P < 0.02$.

*** Significantly affected by indicated cofactor(s), based on paired *t*-test, $P < 0.005$.

daltons[14,25], and with DeLorenzo's observations on the Ca^{2+}/calmodulin-dependent phosphorylation of two rat brain proteins of M_r 60–64 and 52–54 kilodaltons, originally named DPH-L and DPH-M respectively, because of the ability of diphenylhydantoin to block their phosphorylation[10,11]. The major portion of these latter proteins has subsequently been shown to be composed of α- and β-tubulin by two-dimensional electrophoresis[7–9]. Furthermore, Breithaupt and Babitch[5] have found a major soluble polypeptide of M_r 43 kilodaltons in 6–8-day-old chicks, which is apparently not phosphorylated, as well as several synaptoplasmic polypeptides in the 53,000–56,000 dalton range, including α- and β-tubulin as shown by 2-D electrophoresis, which do incorporate ^{32}P, although the calcium and calmodulin dependence of that phosphorylation could not be easily ascertained in their whole synaptosomal system[6].

Hen and rat brain membranes show very minor phosphorylation at M_r 55 kilodaltons but major Ca^{2+}/calmodulin-dependent phosphorylatable substrates at M_r 50 and 60 kilodaltons. This accords well with the observations of O'Callaghan et al.[26] for rat

brain synaptic plasma membranes purified on discontinuous sucrose gradients. These authors have also noted that the polypeptides with apparent electrophoretic mobilities of 50 and 60 kilodaltons had matching counterparts in synaptosomal cytosol. Similar phosphoproteins can be seen on our brain cytosolic autoradiographs for both hens and rats, although they were not consistently resolved from the 55 kilodalton cytosolic band in microdensitometry. Consequently, their peak areas were incorporated with that of the 55 kilodalton band for quantitative purposes. Actually, a closer examination of the autoradiographs presented by O'Callaghan et al.[25] reveals that the 60 kilodalton band is really a doublet with the major band having the higher apparent molecular weight. This contrasts with our autoradiographs for hen brain membranes, in which the lower M_r member of the doublet predominated, but agrees perfectly with the autoradiographs of rat brain membranes presented here as well as with other reported findings of a 50 and two 60 kilodalton proteins which are prominent Ca^{2+}/calmodulin-dependent phosphorylation substrates in the crude particulate fraction from rat brain[33] and in partially purified preparations of one of the Ca^{2+}/calmodulin 'Synapsin I kinases' present in both the soluble and particulate fractions of brain homogenates[15], though apparently not in purified bovine brain synaptic vesicles[22]. Furthermore, Kennedy[15] has found that a monoclonal antibody raised against purified Synapsin I kinase, whose activity consists of phosphorylating the collagenase-sensitive tail of Synapsin I, co-precipitates this activity and the 50 kilodalton phosphoprotein. The latter thus appears to be an autophosphorylatable subunit of the enzyme or at least to exist in a complex with it.

Synapsin Ia, b, until recently called Protein Ia, b, is a 1:2 doublet of M_r 86 and 80 kilodaltons, respectively, associated principally with synaptic vesicles and probably only artifactually with postsynaptic densities[15]. In the electrophoretic mobility range of 80–86 kilodaltons, we observed a band phosphorylated in a Ca^{2+}/calmodulin (at least in the membranes)-dependent manner, which may be a poorly resolved doublet in rat brain cytosolic and membrane fractions, but which is undetectable in either of the hen neuronal protein fractions examined. A comparison of the stringent centrifugation conditions (186,000 *g* for 1 h) employed by Burke and DeLorenzo[8] when they isolated purified synaptic vesicles that had 'Ca^{2+}/calmodulin tubulin kinase' activity with the milder conditions (140,000 *g* for 30 min) to which we subjected our P_2 lysates raises the possibility that synaptic vesicles containing this Synapsin Ia, b in rats were present in both our synaptosomal cytosolic and P_2 membrane fractions. Sorensen and Babitch[37] have reported that baby chicks have Synapsin Ia but lack Synapsin Ib. Low concentrations of Synapsin I-like proteins with divergent properties have been detected in radioimmunoassay in non-mammalian species, including the chicken, suggesting that Synapsin I has been less conserved during evolution than has the cAMP-dependent protein kinase[15]. This could, conceivably, account for the lack of an observable phosphorylated band with that M_r in the hen and in *Torpedo californica*[31].

The hen does, however, have a prominent band with an M_r in the 65–70 kilodalton range (designated 70 kilodalton here, but 65 kilodalton previously[29]) in brain cytosolic autoradiographs, which is apparently minor or absent from rat brain cytosol. One identity worth considering for this autoradiographic band is that of the 68 kilodalton neurofilament subunit which is known to be phosphorylated as a single, identical site both in vivo and in vitro, though to a much greater extent in vitro, by a kinase which copurifies with it from rat spinal cord and brainstem[13], although its far greater phosphorylation in hens remains an anomaly. Also relevant to a consideration of phosphorylated proteins of M_r 70–80 kilodaltons are the chick synaptic plasma membrane polypeptide of M_r 75 kilodalton, pI 5.5, which has been found to label with ^{32}P as densely as the tubulins[6] and the rat whole brain cytosolic 77 kilodalton protein whose phosphorylation is stimulated by calcium in the presence of either the detergent SDS (sodium dodecyl sulfate), the hydrophobic probe ANS (8-anilino-1-naphthalene sulfonate), or phospholipids[14]. Juskevich et al.[14] also noted calcium and phospholipid, SDS, or ANS-mediated stimulation of the phosphorylation of a 47 kilodalton whole rat brain cytosolic band which has a calcium-dependent counterpart in our rat synaptosomal cytosol and a calcium, but not calmodulin,-dependent couterpart in our P_2 membranes from rat, but not hen, brain.

In agreement with the pattern of Ca^{2+}-dependence

described for the phosphorylation of the large (P_1, 19–20 kilodalton) and small (P_2, 16 kilodalton) rat myelin basic proteins[30], slight stimulation by calcium of ^{32}P incorporation into both the 16 and 20 kilodalton rat brain membrane proteins was observed (Table V, Fig. 8). This strongly suggests that they should be identified as myelin basic proteins. However, the tendency of the Ca^{2+}-dependent phosphorylation of the 16 kilodalton rat brain membrane protein to be inhibited by calmodulin (Tables IV and V), as has been reported for an approximately 15 kilodalton crude rat brain membrane protein[39] is somewhat unorthodox. In contrast with the 16 and 20 kilodalton rat brain membrane proteins, the slightly larger (22 kilodalton) counterpart in hens lacks any calcium effect on its relatively greater phosphorylation. We have tentatively identified this 22 kilodalton hen polypeptide as hen myelin basic protein on the basis of its specificity to the myelin fraction when hen brain P_2 membranes are subfractionated on discontinuous sucrose gradients[27]. Its phosphorylation also shows a tendency to be inhibited by calmodulin (Tables IV and V), a surprising finding, though not entirely without precedent[39]. Breithaupt and Babitch[5] have also observed a 22 kilodalton polypeptide which incorporated ^{32}P in whole synaptosomes or in synaptic plasma membranes of 6–8-day-old chicks. It may be the same as the one found in hens. It has been suggested that the variation in degree of membrane protein phosphorylation could be an important determinant of spatial relationships between membrane proteins and lipids[35]. Conformational changes resulting from dynamic protein phosphorylation in response to second messenger signals, for example, may mediate a variety of physiological effects[15].

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