

Phosphorylation of Proteins of Synaptosome-Enriched Fractions of Brain during Short-Term Training Experience: Biochemical Characterization^{1,2}

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Incorporation of radioactive phosphate into hot HClO₄ residue derived from synaptosomal particulate and soluble fractions was studied using a double-isotope technique. Increased phosphorylation was observed only in the particulate fractions of foot-shock avoidance-conditioned mice compared with quiet controls. Similar results were obtained irrespective of whether discontinuous sucrose or Ficoll-sucrose gradients were used to isolate the synaptosome-enriched fractions. Enzymatic and chemical characterization showed the radioactive label in the hot HClO₄ residue to be due to phosphorus covalently bound to proteins.

Neurochemical correlates of learning and memory have been the subject of numerous reviews and articles (Agranoff, 1971; Dunn *et al.*, 1974; Glassman, 1969; Kimble, 1965; Entingh *et al.*, 1975). Some of these neurochemical changes may be related to learning, while others could be related to some aspects of the training situation other than memory formation per se, such as sensory input, motor activity, and stress-induced hormonal action. However, it is becoming increasingly evident that phosphorylation of synaptic proteins may play a key role in nervous tissue function (Heald, 1962; Weller and Rodnight, 1970, 1973; Johnson *et al.*,

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1971, 1972; Perumal *et al.*, 1975; Routtenberg *et al.*, 1975). Such a phosphorylation could alter the conformation of proteins (Adler *et al.*, 1971), thus affecting interneuronal connectivity, a change that may be essential for the formation of new pathways mediating memory storage.

We have reported (Perumal *et al.*, 1975) that when naive mice are subjected to short-term avoidance conditioning after administration of radioactive orthophosphate, proteins derived from synaptosome-enriched fractions of brain contain more radioactivity than the corresponding proteins from the brains of untrained, yoked, or previously trained mice. We now report initial steps in the biochemical characterization of the material exhibiting increased phosphorylation.

MATERIALS AND METHODS

Animals, Injection, and Determination of Radioactivity

Male C57BL/6J mice, 6–8 weeks old (18–24 g), were obtained from Jackson Laboratories, Bar Harbor, Maine. In each experiment pairs of animals were matched for age and weight. Injections were made intracranially into the frontal cerebral cortex under mild ether anesthesia, by a method similar to that of Adair *et al.* (1968), except that no incision of skin was made. One mouse of each pair was bilaterally injected with 10 μ l of [32 P]orthophosphate (approx. 1 μ Ci/ μ l; total 20 μ Ci) and the other with 10 μ l of [33 P]orthophosphate (2 μ Ci/ μ l; total 40 μ Ci) in aqueous solution (carrier-free, from ICN, Irvine, Calif.). In view of the higher energy of 32 P-labeled β -particles, 32 P and 33 P radioactivity can be monitored simultaneously. All radioactive samples were counted using a Packard Tri-Carb liquid scintillation spectrometer. For each batch of isotope, a set of standards quenched to different degrees with different amounts of CHCl_3 was prepared. Counting efficiencies of 33 P and 32 P in their respective channels were about 40–50%.

Training

Twenty-nine minutes after injection of the isotope, a randomly chosen mouse was trained, while the other of the pair remained in its home cage, serving as a quiet control. The training apparatus was similar to that described previously (Adair *et al.*, 1968; Coleman *et al.*, 1971; Zemp *et al.*, 1966). The two-compartmented jump-box had a continuous grid floor. The training compartment had an escape shelf which was absent in the yoke compartment. The mouse to be trained was given 30 sec to explore the escape shelf and another 30 sec to explore the grid floor. Then a timer actuated the light and buzzer (conditioned stimuli) for 3 sec, after which a shock generator delivered 0.3 mA (unconditioned stimulus) to the grid floor until the animal jumped onto the shelf. The latency was recorded. On each successful avoidance or escape, the animal was allowed to remain on

the shelf for 15 sec and then lifted by its tail and put on the grid floor, where it remained for 10 sec before the conditioned stimuli were presented again, giving an intertrial interval of 25 sec. A trial session lasted 15 min, in which 30 trials were completed.

Synaptosome Isolation

All steps in the isolation procedure were carried out at 0–4°C. The animals were sacrificed under ether anesthesia immediately after training, and the brains of both trained and quiet animals (minus olfactory bulbs and cerebellum) were homogenized together in 10 ml of 0.32 *M* sucrose in 1 *mM* sodium phosphate buffer, pH 7.6, using a glass Potter–Elvehjem homogenizer with Teflon pestle (clearance 0.1–0.15 mm). One milliliter of the homogenate was saved for isolation of nucleoside monophosphates. The synaptosomes were isolated using hyperosmotic sucrose gradients as described by Whittaker (1969), or using isoosmotic Ficoll–sucrose gradients as described by Hershman *et al.* (1972). Electron micrographs of the various fractions from the gradient were used as morphological controls for the biochemical studies. From the micrographs of both types of gradients it was evident that the studies were indeed performed with fractions rich in synaptosomes and relatively free of contamination from myelin fragments and somatic mitochondria (Perumal, 1973). The synaptosome-enriched fraction sedimenting between the myelin and mitochondrial fractions was collected, diluted with 0.32 *M* sucrose in 1 *mM* sodium phosphate buffer, pH 7.6, and the final pellet was obtained by centrifugation at 63,000 g_{av} . This pellet was suspended in 5 ml of the 1 *mM* phosphate buffer, pH 7.6, and stored overnight at –20°C.

In one study, following hypoosmotic shock, freezing overnight, and thawing, the synaptosomal suspension was centrifuged in a Spinco 40 rotor at 105,000 g_{av} for 1 hr. The sediment thus obtained was the particulate fraction, and the supernatant solution was the soluble fraction.

Chemical Fractionating of the Synaptosomal Fractions

The frozen synaptosomal fraction was thawed at room temperature and an equal volume of cold 0.5 *N* HClO₄ was added. The pellet obtained by low-speed centrifugation was washed with cold 0.25 *N* HClO₄ and dispersed thoroughly in 3 ml of 0.25 *N* HClO₄. The lipids were extracted from this suspension by a modification of the Bligh and Dyer (1959) method, using 2% acetic acid in methanol and chloroform (1:1, v/v). The protein interphase was removed, washed with 0.25 *N* HClO₄, and then suspended in 0.25 *N* HClO₄. The suspension was then incubated in a water bath at 90°C for 15 min. The resulting protein pellet was obtained by sedimentation and dissolved in Soluene by incubation at 50–60°C for 2 hr.

Determination of Radioactivity in Nucleoside Monophosphates from the Brain

The nucleoside monophosphates from 1 ml of the brain homogenate were isolated as described by Zemp *et al.* (1966). The radioactivity in the adenylic acid spot was used to monitor the effectiveness of the injected isotope as an intracellular precursor for phosphorylation of proteins in the brain, and to correct for variations in delivery.

Characteristics of the Bound Phosphate in the Synaptosome-Enriched Fraction

(a) *Recovery of radioactive phosphate added to the synaptosome-enriched fraction.* To show that radioactive phosphate was not simply adsorbed to synaptosomal proteins, [^{32}P] and [^{33}P]orthophosphate were added to the synaptosomal fraction isolated from uninjected brains. The recovery of radioactivity through the various steps in chemical fractionation was monitored.

(b) *Enzymatic characterization of the hot HClO_4 residue from enriched synaptosomes.* (i) *Pronase and alkaline phosphatase digestion:* An aliquot of the synaptosomal fraction in 3 ml of 1 mM sodium phosphate buffer, pH 7.6, was boiled to denature endogenous enzymes and treated with 13 mg of pronase (B Grade, Calbiochem.), which had been preincubated for 2 hr at 37°C in 1 ml of the phosphate buffer. After the addition of 20 μg of actinomycin D, the mixture was incubated at 37°C for 22 hr with continuous agitation. In the control reaction mixture 1 ml of water was substituted for the enzyme solution. The reaction was terminated with an equal volume of 0.5 N HClO_4 . For alkaline phosphatase digestion, the hot HClO_4 residue from the synaptosome fraction of two mouse brains was suspended thoroughly in 0.5 ml of 0.1 M Tris-HCl buffer, pH 8.0. To this was added 2 mg of alkaline phosphatase (Sigma, *E. Coli* Type III; EC 3.1.3.1) in 0.1 ml of 0.1 M Tris, pH 8.0. The final incubation mixture contained 0.05 M MgCl_2 . This mixture was incubated with continuous agitation for 2 hr at 37°C. The reaction was terminated with 0.2 ml of 2 M HClO_4 , and the residue and supernatant solution were assayed for radioactivity.

(ii) *Tryptic digestion and paper electrophoresis of the tryptic peptides:* The digestion was performed by the method described by Gershey *et al.* (1968), with slight modifications. The protein residue from the synaptosome fraction of two brains was suspended in 0.1 M NH_4HCO_3 , pH 8.0. Initially, 0.4 mg of trypsin (Sigma, bovine pancreatic trypsin, Type I; EC 3.4.4.4) in 40 μl of 0.0001 N HCl was added to the sample and incubated at 37°C for 2 hr, at which time an additional 1 mg of the enzyme was added and the incubation was extended for an additional 18 hr. At the end of the incubation, the suspension was separated by centrifugation for 20 min at

30,000 g_{av} . The resulting supernatant solution was concentrated to about 100 μ l in an air stream and the tryptic peptides were separated electrophoretically as described by Langan (1969). The electrophoresis buffer used was 0.06 M NH_4HCO_3 , pH 7.9. Twenty-microliter aliquots were applied to paper strips (Grade 589, Green Ribbon, Schleicher and Schuell, 22 \times 3.6 cm) and electrophoresed at a constant 600 V for periods of 0.5 or 2 hr. The paper strips were then dried and scanned on a Packard radiochromatogram scanner, following which they were dipped in 0.2% ninhydrin in acetone. Color was allowed to develop at room temperature. The areas of the paper corresponding to radioactive peaks on the scan were cut into small pieces and then eluted in vials with 2 ml of 0.1 N HCl. Radioactivity in each eluate was then assayed by liquid scintillation. Radioautography was performed on some of the paper strips to visualize the zones of radioactivity.

Electrophoretic Separation of Phosphoserine, Phosphothreonine, and Phosphate from Hydrolysates of the Hot $HClO_4$ Residue from Synaptosome-Enriched Fractions

Two methods of hydrolyzing the hot $HClO_4$ were used.

(1) *Pronase digestion*. This was performed as described previously, and the supernatant solution resulting from centrifugation of the digest was air-dried.

(2) *HCl digestion*. Samples of the hot $HClO_4$ residue were hydrolyzed in 2 N HCl in vacuum-sealed tubes at 100°C for 10 hr. The resulting digests were evaporated *in vacuo* and dissolved in 0.5 ml of water. This was applied to a Dowex 50 \times 8 H^+ column (50 \times 4 mm). The phosphoamino acids were eluted with water and dried *in vacuo*.

The dried residues were dissolved in 30 μ l of a mixture of nonradioactive *l*-phosphoserine and *l*-phosphothreonine (5 mg of each/ml), and 10 μ l aliquots were used for electrophoresis as described by Langan (1969). The electrophoresis buffer contained 25 ml of 88% formic acid and 87 ml of glacial acetic acid per liter, pH 1.9. The samples were applied to Whatman No. 3 MM paper and electrophoresis was carried out for 2 hr at 500 V. A small strip of the paper was stained with phosphate reagent as described by Burrows *et al.* (1952). The rest of the strip was stained with 0.2% ninhydrin in acetone. The phosphate-positive bands were cut out, eluted in 0.1 N HCl, and the eluates were assayed for radioactivity.

Statistical Methods

Where ratios were involved, the median value with its interquartile range is presented. In order to test whether the ratios differed from 1.0, a two-tailed sign test was used and differences were assigned to be significant for $2P \leq 5\%$.

RESULTS

First, some methodological aspects are presented, second, the effect of training on the incorporation of phosphate into synaptosomal acid-insoluble material is reported, followed by a biochemical characterization of the labeled product.

Comparison of Synaptosome-Enriched Fractions Prepared on the Discontinuous Sucrose and Ficoll–Sucrose Gradients

Table 1A gives the distribution of radioactive label in the various fractions obtained from the discontinuous sucrose and Ficoll–sucrose gradients. The distribution profiles of the two types of gradients appear to be similar except in the myelin and synaptosomal fractions, where differences were evident. In the Ficoll–sucrose gradients, significantly more radioactivity could be recovered in the myelin fraction and significantly less radioactivity in the synaptosomal fraction than in the corresponding fractions from the sucrose gradient.

Table 1B shows the distribution of disintegrations per minute (dpm) in various components during the sequential extraction procedure. The distribution of the label in these components is independent of whether sucrose or Ficoll–sucrose gradients were used to isolate the synaptosomal fraction. It is to be noted that only about 3% of the total radioactivity in the synaptosome-enriched fraction is recovered in the hot HClO_4 residue. Most of the radioactivity of this fraction (74%) is acid soluble, and a

TABLE 1A
Distribution of Radioactivity in Discontinuous Sucrose
and Ficoll–Sucrose Density Gradients^a

Gradient fraction	Mean (dpm in fraction/total dpm in gradient) × 100 ± SD	
	Sucrose gradients (N = 8)	Ficoll–sucrose gradients (N = 10)
(S)	26.3 ± 7.8	28.8 ± 3.6
(My)	27.0 ± 2.6	31.8 ± 4.2
(IMySy)	5.6 ± 2.6	9.2 ± 1.1
(Sy)	27.4 ± 8.2	15.6 ± 2.2
(ISyMi)	5.7 ± 1.9	4.2 ± 1.0
(Mi)	7.9 ± 1.9	10.2 ± 3.6

^a The crude mitochondrial suspension was layered on a two-step discontinuous sucrose or Ficoll–sucrose gradient and centrifuged at $53,000g_{av}$ for 120 min in a Spinco SW 25.1 rotor (0–4°C). Aliquots from each of the fractions obtained were then assayed for radioactivity. N = Number of mice; SD = standard deviation from the mean. S = Sap or clear upper layer; My = myelin layer; IMySy = intermyelin–synaptosomal layer; Sy = synaptosome-enriched layer; ISyMi = intersynaptosomal–mitochondrial layer; Mi = mitochondrial pellet.

TABLE 1B
Distribution of Radioactivity in Various Chemical Components of the
Synaptosomal Fraction Derived from Sucrose and
Ficoll–Sucrose Gradients^a

Chemical component	Mean (dpm in chemical fraction/ total dpm in synaptosomal fraction) $\times 100 \pm$ SD	
	Sucrose gradients (<i>N</i> = 8)	Ficoll–sucrose gradients (<i>N</i> = 4)
HClO ₄ -soluble fraction	75.3 \pm 2.5	72.9 \pm 1.3
Methanol layer	1.31 \pm 0.43	1.07 \pm 0.44
Chloroform layer (lipid)	18.0 \pm 3.3	18.6 \pm 1.1
Hot HClO ₄ supernatant	2.61 \pm 1.0	4.37 \pm 0.63
Phosphoprotein residue	2.76 \pm 0.67	2.97 \pm 0.27

^a The synaptosomal fraction was subjected to 0.25 *M* cold HClO₄ extraction and then to lipid extraction using acidified chloroform:methanol (1:1, v/v). The interphase was then incubated in 0.25 *M* HClO₄ for 15 min at 90°C. The final residue obtained was dissolved in Soluene by heating for 2 hr at 50–60°C. Radioactivity was determined at each step of the procedure. *N* = Number of mice; SD = standard deviation from the mean.

considerable amount (18%) is recovered in the chloroform-extractable lipids.

Nucleoside Monophosphates

In order to correct for the relative intracellular availability of the injected radioactive precursor, the radioactivity in nucleoside monophosphates was monitored. The ratio of isotopes recovered in adenylic acid (AMP) was used, since this nucleotide contained more label than any of the others and, in the solvent system used, its separation from the other nucleoside monophosphates was complete. Also the phosphorus isotope ratios in the various nucleoside monophosphates recovered from any given pair of mice were all similar and, in fact, did not differ significantly from the ratio in the homogenate (Table 2).

TABLE 2
Radioactivity in Nucleoside Monophosphates and Homogenates
from the Brains of Trained and Quiet Mice

	<i>n</i>	dpm <i>T/Q</i>
Homogenate	12	0.69 (1.05) ^a
GMP	12	0.72 (1.10)
UMP	12	0.69 (1.65)
AMP	12	0.71 (1.06)
CMP	12	0.67 (1.19)

^a Median ratio with interquartile range in parentheses.

Effect of Avoidance Training on Phosphate Incorporation into the Hot HClO_4 Residue from the Synaptosome-Enriched Fraction

Pairs of mice were intracranially injected with $\text{H}_3^{32}\text{PO}_4$ and $\text{H}_3^{33}\text{PO}_4$, and one mouse of each pair was subjected to training in the jump-box while the other served as the quiet control. After sacrifice of the mice, their brains were processed as described previously.

Table 3 shows that, in 14 of 15 mouse pairs, the washed acid-insoluble residue, containing mainly proteins from synaptosomes, contained more radioactive phosphate from the trained mouse than from the quiet one. Although the difference was variable, it occurred very consistently throughout our experiments when training of naive animals was involved. Differences in other fractions obtained during the extraction of the synaptosome-enriched pellet were more variable, and the ratios did not differ significantly from 1.0.

Effect of Avoidance Training on Phosphorylation of the Hot HClO_4 Residue Obtained from Soluble and Particulate Fractions of Synaptosomes

Table 4 shows that the particulate fraction contains nearly seven times as much label in the hot HClO_4 residue as the residue from the soluble synaptosomal fractions contains. Moreover, it was found that the hot HClO_4 residue from the particulate fraction was the only one in which the ratio of the recovered dpm (T/Q), corrected for that in AMP, did increase as a result of training. In this series of experiments, both sucrose ($n = 6$) and Ficoll-sucrose ($n = 5$) gradients were used, giving about the same result in either case.

Analysis of the Hot HClO_4 Residue Obtained from Synaptosome-Enriched Fractions

(a) *Recovery of added orthophosphate.* When the recovery of radioactive phosphate added to the synaptosome-enriched fraction from uninjected brains was followed in the various extraction steps, it was found that almost all the radioactivity was accounted for in the cold HClO_4 extract. Less than 0.1% was recovered in all the other fractions combined. This is suggestive evidence that the radioactivity recovered in the hot HClO_4 residue is not due to adsorption of radioactive free phosphate.

(b) *Enzymatic characterization.* (i) *Pronase and alkaline phosphatase digestion:* Table 5 shows that pronase digestion of the hot HClO_4 residue obtained from quiet mice solubilizes about 80% of its radioactive label, while alkaline phosphatase solubilizes about 90% of the label. These data support the suggestion that the radioactive phosphate in hot HClO_4 residue is indeed covalently bound to protein.

(ii) *Tryptic digestion:* In this experiment the hot HClO_4 residue was obtained from synaptosomes from pooled brain homogenates of two pairs

TABLE 3
Incorporation of Phosphate into the Washed Acid-Insoluble Residue Obtained from Synaptosome-Enriched Fractions of Trained and Quiet Mice^a

Pair number	Isotope in trained	dpm in AMP		dpm ratio in AMP Trained/ quiet (A)	dpm in residue		dpm ratio in residue Trained/ quiet (R)	Percentage increase in dpm in residue from trained mouse [(R/A - 1) × 100]
		Trained	Quiet		Trained	Quiet		
113-114 ^b	³² P	25	116	0.22	49	166	0.30	36
117-118 ^b	³³ P	58	48	1.21	127	54	2.35	94
121-122	³² P	216	119	1.82	1470	660	2.23	23
123-124	³³ P	191	309	0.62	2003	1495	1.34	116
125-126	³³ P	829	328	2.53	3209	1094	2.93	16
127-128	³² P	131	428	0.31	491	1331	0.37	19
129-130	³³ P	1540	260	5.92	1815	142	12.78	116
131-132	³³ P	209	1499	0.14	677	4166	0.16	14
133-134	³² P	1249	476	2.62	1805	643	2.81	7
135-136	³² P	4273	838	5.10	3372	337	10.01	96
137-138	³² P	994	783	1.27	181	139	1.30	2
139-140	³³ P	1256	699	1.80	672	269	2.50	39
141-142	³² P	867	652	1.33	582	475	1.23	-8
143-144	³³ P	379	962	0.39	56	140	0.40	3
145-146	³² P	1532	1314	1.17	824	579	1.42	21
								21 (66) ^{c,*}

^a The methodology for this dual-isotope experiment is described in the text.
^b The initial crude mitochondrial (P₂) fractions for these pairs were obtained by centrifugation at 13,000g_{av} for 20 min rather than 55 min.
^c Median with interquartile range in parentheses.
* P < 0.001 (two-tailed sign test).

TABLE 4
The Incorporation of Radioactive Phosphate into Phosphoproteins
from Synaptosomal Soluble and Particulate
Fractions in Trained and Quiet Mice

	Soluble fraction	<i>n</i>	Particulate fraction	Particulate/ soluble
dpm Protein in <i>Q</i>				
³² P ^a	251 ± 96 ^b	6	1743 ± 720	7.1
³³ P	108 ± 44	5	771 ± 280	6.9
dpm Protein T/Q dpm AMP T/Q	1.00 (0.34) ^c	11	1.14 (0.30)*	

^a In five pairs the *T* received ³²P, in six pairs ³³P.

^b Mean ± SEM.

^c Median with interquartile range in parentheses.

* $2P < 0.001$.

of trained and quiet mice, with [³³P]orthophosphate in the trained animals. The residue was digested with trypsin and the concentrated digest was then subjected to paper electrophoresis. The electropherogram was scanned, autoradiographed, and then stained in ninhydrin to visualize the peptide material. Figure 1 represents such a scan, with the ninhydrin-positive areas indicated on the x-axis. Under these electrophoretic conditions, free phosphate was found to leave the paper in 20 min and there was no clear resolution of the peptides toward the anode. Most of the radioactivity was associated with the slower, ninhydrin-positive peak, while some label was associated with the faster, ninhydrin-negative peak, which has not been identified. Table 6 shows the results of a typical experiment.

TABLE 5
Effect of Enzymatic Treatment of the Hot HClO₄ Residue
Derived from the Synaptosome-Enriched Fraction^a

Treatment	Total dpm in hot HClO ₄ supernatant (S) after digestion		Total dpm in hot HClO ₄ resi- due (R) after digestion		[S/(S + R)] × 100	
	³³ P	³² P	³³ P	³² P	³³ P	³² P
Pronase	180	684	30	173	86	78.0
Control	8	72	240	773	3.1	8.5
Alkaline phosphatase	1571	886	176	109	89.9	89.0
Control	224	122	1445	1053	13.4	10.4

^a The final residue obtained *after* chemical fractionation of the synaptosomal pellet was digested with pronase or alkaline phosphatase, as described in the text. Each isotope was injected into one mouse, and 45 min later the two animals were sacrificed. The two brains were processed together. Neither mouse was trained.

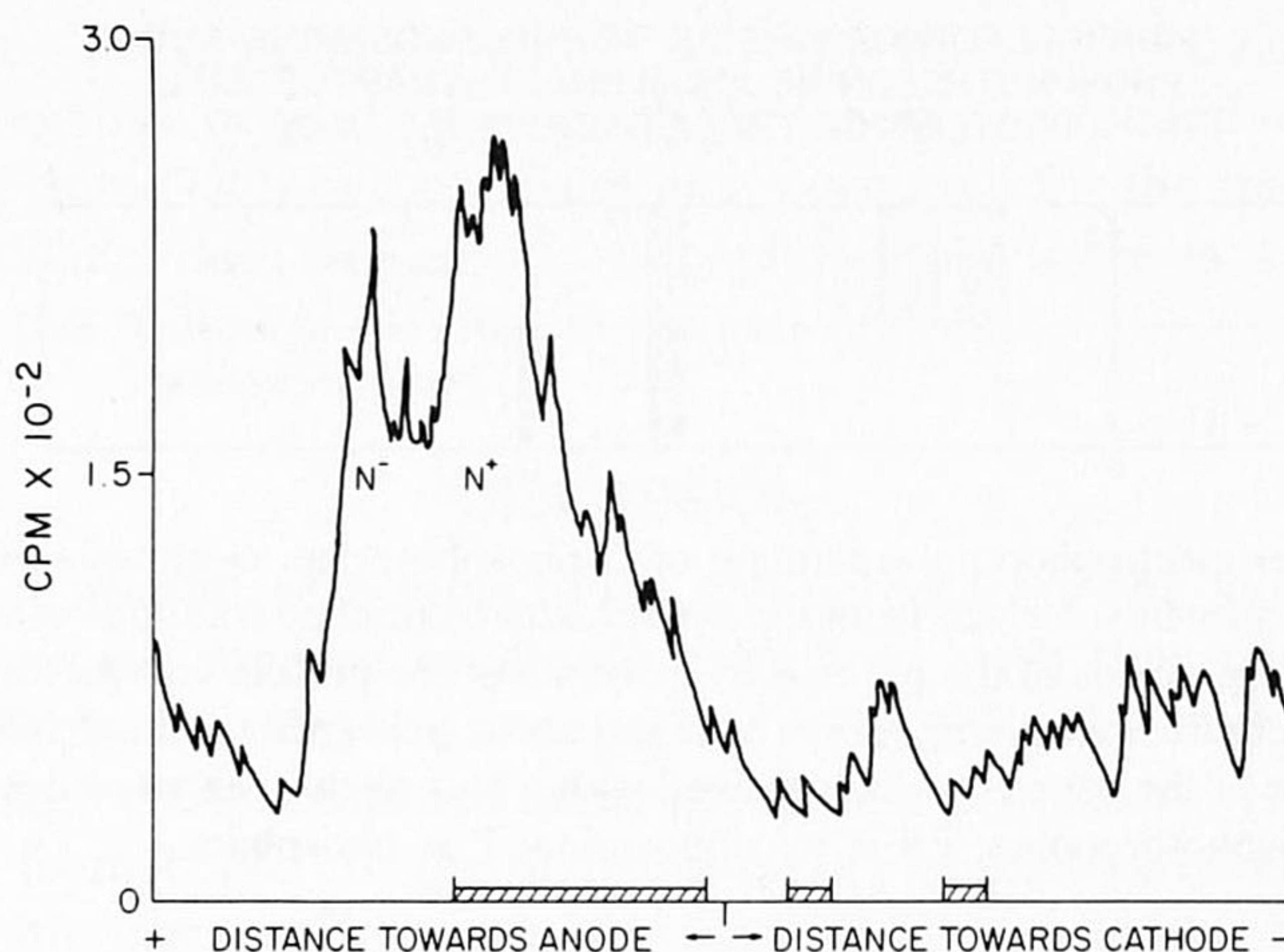


FIG. 1. Radioactivity scan of the paper electropherogram used to separate tryptic peptides (interelectrode distance, 20 cm). The electrophoresis was performed for 0.5 hr. The shaded areas on the X axis represent areas that were stained by ninhydrin. The two distinct peaks of radioactivity (ninhydrin positive, N^+ ; and ninhydrin negative, N^-) were then eluted with 0.1 N HCl. The radioactivity of each eluate was then determined (Table 6).

TABLE 6
The Incorporation of Radioactive Phosphate into Tryptic Peptides
of Synaptosomal Proteins from Trained and Quiet Mice^a

Duration of electrophoresis	0.5 hr		1 hr		2 hr	
	N^+	N^-	N^+	N^-	N^+	N^-
dpm T/Q	0.312	0.176	0.748	0.151	0.296	0.172
Percentage increase ^b	+71	-3	+311	-17	+63	-6
Mean percentage of total radioactiv- ity in the peak ^c	66	34	74	26	62	38

^a The peptides obtained after tryptic digestion of the final hot $HClO_4$ residue (pooled from two pairs) were subjected to paper electrophoresis, as described in the text. Then the ninhydrin-positive (N^+) and ninhydrin-negative (N^-) radioactive areas (Fig. 2) were eluted with 0.1 N HCl, and the radioactivity of each eluate was determined.

^b The percentage increase was corrected for variations in the relative amounts of injected radioactive phosphorus as follows:

$$\left(\frac{\text{Peptide } T/Q}{\text{AMP } T/Q} - 1 \right) \times 100, \text{ where } T/Q = \frac{\text{dpm of isotope from trained}}{\text{dpm of isotope from quiet}}$$

and $\text{AMP } T/Q = 0.182$. The isotope in the trained was [^{33}P]orthophosphate.

^c The percentage of total radioactive label in the (N^+) and (N^-) peaks was computed as follows: $[\text{dpm in peak } (N^+ \text{ or } N^-) / \text{dpm } (N^-) + \text{dpm } (N^+)] \times 100$, where dpm represents the sum of the radioactivity in the two isotopes [^{32}P] plus [^{33}P].

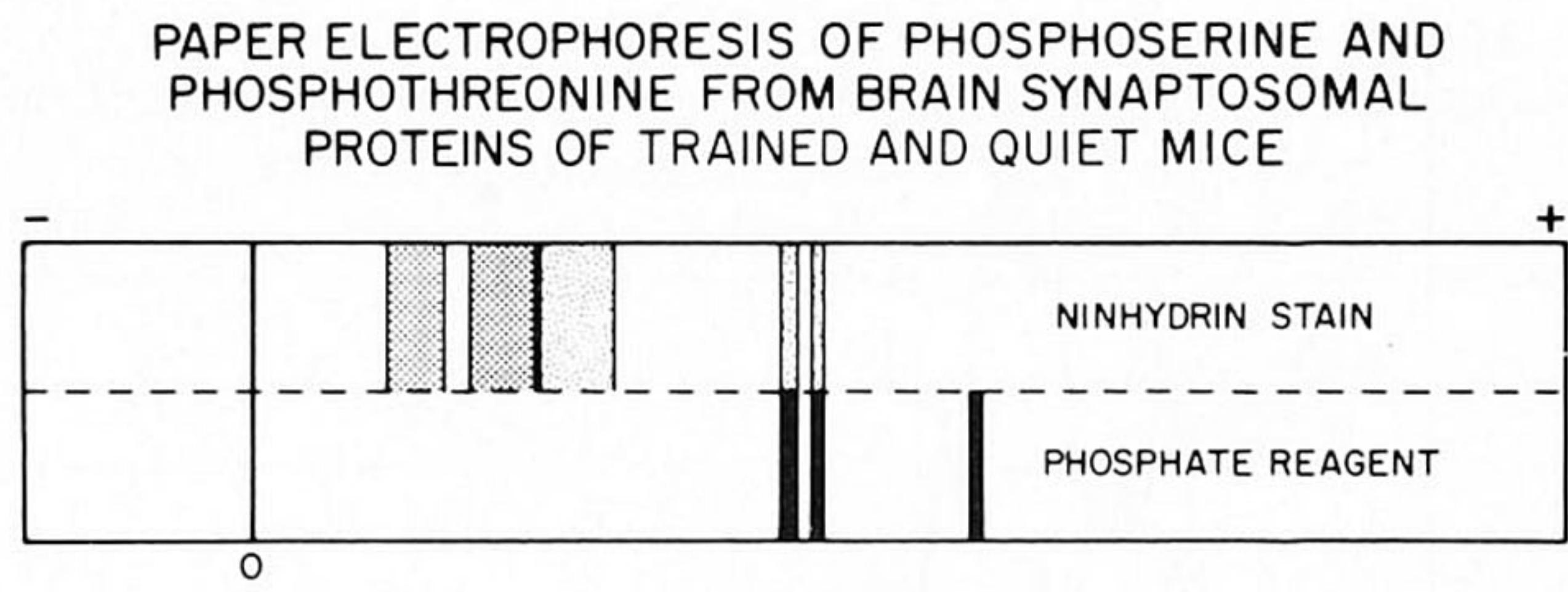


FIG. 2. Paper electrophoretic separation of *O*-phosphoserine, *O*-phosphothreonine, and phosphate from proteins derived from the synaptosome-enriched fractions of mouse brain. The paper electrophoresis of the pronase hydrolysate of the protein was performed for 2 hr at 500 V using a buffer containing formic acid and acetic acid (pH 1.9), and Whatman 3MM paper. A portion of the paper was later stained with a phosphate reagent as described in the text. PT = Phosphothreonine; PS = phosphoserine; P = phosphate.

The increase in the isotope ratio in the tryptic peptides (*T/Q*) corrected for that in AMP is consistently found in the ninhydrin-positive peak.

Electrophoretic Separation of Phosphoserine, Phosphothreonine, and Orthophosphate from the Hot HClO₄ Residue

In this experiment four pairs of mice were injected with [³²P]orthophosphate or [³³P]orthophosphate and 29 min later one mouse of each pair was trained while the other served as the quiet control. In two cases, the hot HClO₄ residue was hydrolyzed by pronase digestion, and in the other two by HCl digestion. The paper electropherogram (Fig. 2) showed a clear separation of phosphoserine and phosphothreonine. In addition, staining showed a phosphate-containing, ninhydrin-negative material migrating toward the anode. It was found that inorganic orthophosphate, under these conditions, had about the same mobility as this phosphate-containing ninhydrin-negative band. Table 7 shows the effect of training

TABLE 7
The Incorporation of Radioactive Phosphate into *O*-Phosphoserine, *O*-Phosphothreonine, and Phosphate Separated Electrophoretically from Synaptosomal Phosphoproteins of Trained and Quiet Mice

Isotope in trained mice	dpm AMP <i>T/Q</i>	$\frac{\text{dpm PS}^a \text{ } T/Q}{\text{dpm AMP } T/Q}$	$\frac{\text{dpm PT}^b \text{ } T/Q}{\text{dpm AMP } T/Q}$	$\frac{\text{dpm P}^c \text{ } T/Q}{\text{dpm AMP } T/Q}$
³² P	0.36	1.51	1.34	0.79
³³ P	0.16	1.38	1.64	0.94
³² P	3.34	1.61	1.54	0.90
³³ P	0.69	1.28	0.49	1.19

^a PS = *O*-Phosphoserine.
^b PT = *O*-Phosphothreonine.
^c P = Phosphate.

on the two phosphoamino acids and on the phosphate-containing ninhydrin-negative band. Even though the levels of radioactivity are low, the ratios of dpm (T/Q) in these fractions, corrected for the ratio in AMP, seem to indicate that, at least in phosphoserine, there is a significant increase in the radioactivity in the trained animals.

DISCUSSION

The lesser amount of radioactivity recovered from the synaptosomal fraction of Ficoll–sucrose gradients compared with that from sucrose gradients (Table 1A) may be accounted for by the greater purity of the synaptosomal fraction obtained using the former procedure. However, as can be seen from Table 1B, the distribution of radioactive label in various chemical components of the synaptosomal fractions derived from either the sucrose or the Ficoll–sucrose gradients do not differ significantly. We have also performed experiments in which the time of centrifugation used for preparation of the crude mitochondrial pellet was reduced to 20 min, in order to reduce microsomal contamination to a minimum. The perchlorate-insoluble residues from such synaptosome-enriched preparations were also used in the time-course studies reported in our previous paper (Perumal *et al.*, 1975), and the effects of the training experience on radioactive phosphate labeling were essentially the same as those we report here.

When radioactive precursors are injected into the brain in the manner described, autoradiographic studies indicate that there is a steep gradient of incorporation extending from the needle track and from all subdural and ventricular surfaces into the brain substance (D. Entingh, unpublished observations). This distribution of incorporation may be a fortuitous circumstance that enhances the differences seen in isotope incorporation during behavioral manipulations, since it may reduce incorporation in areas of the brain that are not changed by the experience. The intracranial injection itself has a minor effect on the mouse behavior (Adair *et al.*, 1968; Coleman *et al.*, 1971; Gispen *et al.*, 1977), but both trained and quiet mice underwent this experience.

In these studies AMP has been used as the “correction factor” for intracellular distribution of the injected isotopes. No attempt has been made to isolate the direct phosphate donor for protein kinase since: (a) there was uncertainty as to the exact nature of the donor; (b) even if ATP were the donor, its routine isolation would require strict experimental conditions that differ from ones that will allow isolation of synaptosomes and AMP from one homogenate; (c) corrections based on the “direct donor pools” are valid only if one accounts for intracellular compartmentation as well as specific activities of these pools; and (d) the dual isotope technique, by enabling one to subject the brains of trained and quiet

animals to identical conditions of homogenization and subsequent fractionation, eliminates experimental bias and is capable of detecting relatively small changes in incorporation. The fact that the median isotope ratios (T/Q) for homogenate, GMP, UMP, AMP, and CMP are rather close (Table 2) indicates that the increased phosphorylation response cannot be attributed to fluctuations in the intracellular pools of AMP.

The fact that only the particulate fraction of the synaptosomes exhibits the increased phosphorylation (Table 4), and that the increase is of the same order of magnitude as that detected with whole synaptosomes (Table 3), indicates that the observed response may be associated with the membrane or related structures. The finding that the increased phosphorylation of synaptosomal proteins in the trained mice compared with quiet controls is observed whether sucrose or Ficoll–sucrose gradients are used for isolation of the synaptosome-enriched fraction lends further evidence as to the authenticity of the phenomenon.

Characterization of the HClO_4 residue, both chemically and enzymatically, seems to indicate that the phosphate is covalently bound to protein. The phosphorus label is definitely not adsorbed to the proteins, since all the orthophosphate radioactivity added to the isolated synaptosomal fraction remains acid soluble. The finding that RNase does not release radioactivity from the synaptosome-enriched fraction indicates that the preparation is not contaminated with free polyribosomes. It may, however, contain membrane-bound polyribosomes, since it has been observed (Blobel and Potter, 1967; Murthy, 1970) that m-RNA associated with membrane-bound ribosomes is not degraded by RNase. Electron micrography indicates that any such contamination by microsomes derived from rough endoplasmic reticulum is minimal (Perumal, 1973). Pronase appears to render the radioactivity from both the whole synaptosome-enriched fraction (data not shown) and the final hot HClO_4 residue (Table 5) acid soluble, indicating that the label is initially attached to amino acids. Solubilization by alkaline phosphatase digestion proves that the phosphate is indeed covalently bound. Tryptic digestion of the final hot HClO_4 residue and detection of the increased phosphorylation in the ninhydrin-staining band of peptides derived from synaptosomes of trained mice are further evidence of this.

Additional proof of the incorporation of radioactive phosphate into proteins was obtained by hydrolyzing the hot HClO_4 residue with pronase or HCl, followed by separation of the phosphoserine, phosphothreonine, and inorganic phosphate (Table 7). HCl digestion appeared to be a more effective method of hydrolysis, since more radioactivity was recovered and less extraneous ninhydrin-staining material was obtained during electrophoretic separation. The combination of Dowex column chromatography with electrophoretic separation was the best way to separate phosphoserine, phosphothreonine, and orthophosphate from digests, as

suggested by Jones and Rodnight (1971). Most of the radioactivity was recovered in the phosphoserine and phosphothreonine, while the small amounts in orthophosphate may be attributed to hydrolysis of phosphate ester or anhydride bonds. The data show that the increase in phosphorylation may be attributed to a greater incorporation of phosphate into phosphoserine, although a phosphothreonine effect is not ruled out.

Some preliminary study was made of the nature of the phosphoproteins. Polyacrylamide gel electrophoresis revealed that two or more proteins were phosphorylated, but some difficulty was experienced in recovering sufficient radioactivity per gel slice. However, in gels with enough activity, it was possible to observe increases in the phosphorylation of proteins in the trained mouse compared with that in the quiet control (Perumal, 1973).

The report by Routtenberg *et al.* (1975) that 10 min of passive avoidance training in the rat affects the *in vitro* phosphorylation patterns of brain P_2 membrane proteins is in harmony with the results reported here, but direct comparisons between the two studies would be of doubtful significance because of the many differences in methodology.

From the above discussion it is apparent that the accumulation of radioactive phosphate in synaptosomal proteins increases in response to avoidance training. A study of the relationship of this increased accumulation to a specific aspect of the behavior is reported in the accompanying paper. The biochemical significance of the effect, however, is obscure at present; nor is it clear whether the increase represents an increased rate of phosphate incorporation or a decreased rate of phosphate hydrolysis. In view of the role of phosphorylation in other systems, e.g., enzyme activation and conformational changes, one might venture to predict that the change might in some way be concerned with altering interneuronal connectivity, which in turn might be of primary importance in the mechanisms underlying the formation of the memory trace or engram. This suggestion is supported by the report of Livet *et al.* (1975) that dibutyryl cyclic AMP mimics the effect of dopamine in producing a prolonged postsynaptic "memory trace" in rabbit superior cervical ganglion.

Numerous problems for future investigation are raised by the present report. One would like to know whether the phosphoproteins that respond to the training experience are located in the presynaptic or in the postsynaptic membrane and whether the duration of the change correlates well with the duration of short-term memory. There is also the question of the correspondence between the synaptic membrane phosphoproteins that are affected by behavior *in vivo* and those that are seen in various *in vitro* studies on these membranes. The ultimate goals would be to understand the roles played by these phosphoproteins in membrane function and how these molecular events are related to behavior and neural processing of information.

REFERENCES

- Adair, L. B., Wilson, J. E., and Glassman, E. (1968). Brain function and macromolecules. III. Uridine incorporation into polysomes of mouse brain during short-term avoidance conditioning *Proc. Nat. Acad. Sci. USA* **60**, 606–613.
- Adler, A. J., Schaffhausen, B., Langan, T. A., and Fasman, G. D. (1971). Altered conformational effects of phosphorylated lysine-rich histone (f_1) and f_1 -deoxyribonucleic acid complexes: Circular dichroism and immunological studies. *Biochemistry* **10**, 909–913.
- Agranoff, B. W. (1976). Learning and memory: Approaches to correlating behavioral and biochemical events. In R. W. Albers, G. J. Siegel, R. Katzman, and B. W. Agranoff (Eds.), "Basic Neurochemistry," pp. 765–782. Boston: Little, Brown.
- Bligh, E. G., and Dyer, W. J. (1959). A rapid method for total lipid extraction and purification. *Canad. J. Biochem. Physiol.* **37**, 911–917.
- Blobel, G., and Potter, V. R. (1967). Studies on free and membrane-bound ribosomes in rat liver. I. Distribution as related to total-cellular RNA. *J. Mol. Biol.* **26**, 279–292.
- Burrows, S., Gryllis, F. S. M., and Harrison, J. S. (1952). Paper chromatography of phosphoric esters. *Nature (London)* **170**, 800–801.
- Coleman, M. S., Pfingst, B., Wilson, J. E., and Glassman, E. (1971). Brain function and macromolecules. VIII. Uridine incorporation into brain polysomes of hypophysectomized rats and ovariectomized mice during avoidance conditioning. *Brain Res.* **26**, 349–360.
- Dunn, A., Brogan, L., Entingh, D., Entingh, T., Gispen, W. H., Machlus, B., Perumal, R., and Rees, H. D. (1974). Biochemical correlates of brief behavioral experiences. In F. O. Schmitt and F. G. Worden (Eds.), "The Neurosciences: Third Study Program," pp. 679–684. Cambridge: MIT Press.
- Entingh, D., Dunn, A., Glassman, E., Wilson, J. E., Hogan, E., and Damstra, T. (1975). Biochemical approaches to the biological basis of memory. In M. S. Gazzaniga and C. Blakemore (Eds.), "Handbook of Psychobiology," pp. 201–238. New York: Academic Press.
- Gershoy, E. L., Vidali, G., and Allfrey, V. G. (1968). Chemical studies on histone acetylation. The occurrence of ϵ -N-acetyllysine in f_{2a1} histone. *J. Biol. Chem.* **243**, 5018–5022.
- Gispen, W. H., Perumal, R., Wilson, J. E., and Glassman, E. (1977). Phosphorylation of proteins of synaptosome-enriched fractions of brain during short-term training experience: The effects of various behavioral treatments. *Behav. Biol.* **21**, 358–363.
- Glassman, E. (1969). The biochemistry of learning: An evaluation of the role of RNA and protein. *Annu. Rev. Biochem.* **38**, 605–646.
- Heald, P. J. (1962). Phosphoprotein metabolism and ion transport in nervous tissue: A suggested connexion. *Nature (London)* **193**, 451–454.
- Hershman, H. R., Cotman, C., and Mathews, D. A. (1972). Serological specificities of brain subcellular organelles. I. Antisera to synaptosomal fractions. *J. Immunol.* **108**, 1362–1369.
- Johnson, E. M., Maeno, H., and Greengard, P. (1971). Phosphorylation of endogenous protein of rat brain by cyclic adenosine 3',5'-monophosphate dependent protein kinase. *J. Biol. Chem.* **246**, 7731–7739.
- Johnson, E. M., Ueda, T., Maeno, H., and Greengard, P. (1972). Adenosine 3',5'-monophosphate-dependent phosphorylation of a specific protein in synaptic membrane fraction from rat cerebrum. *J. Biol. Chem.* **247**, 5650–5652.
- Jones, D. A., and Rodnight, R. (1971). Protein-bound phosphorylserine in acid hydrolysates of brain tissue: The determination of (32 P) phosphorylserine by ion-exchange chromatography and electrophoresis. *Biochem. J.* **121**, 597–600.
- Kimble, D. P. (Ed.) (1965). "The Anatomy of Memory." Palo Alto: Science and Behaviour Books.

- Langan, T. A. (1969). Phosphorylation of liver histone following the administration of glucagon and insulin. *Proc. Nat. Acad. Sci. USA* **64**, 1276-1283.
- Libet, B., Kobayashi, H., and Tanaka, T. (1975). Synaptic coupling into the production and storage of a memory trace. *Nature (London)* **258**, 155-157.
- Murthy, M. R. V. (1970). Membrane-bound and free ribosomes in the developing rat brain. In A. Lajtha (Ed.), "Protein Metabolism of the Nervous System," pp. 109-127. New York: Plenum Press.
- Perumal, R. (1973). Doctoral dissertation. Chapel Hill: University of North Carolina.
- Perumal, R., Gispen, W. H., Wilson, J. E., and Glassman, E. (1975). Phosphorylation of proteins from the brains of mice subjected to short-term behavioral experiences. *Progr. Brain Res.* **42**, 201-207.
- Routtenberg, A., Ehrlich, Y. H., and Rabjohns, R. R. (1975). Effect of a training experience on phosphorylation of a specific protein in neocortical and subcortical membrane preparations. *Fed. Proc.* **34**, 293.
- Weller, M., and Rodnight, R. (1970). Stimulation by cyclic AMP of intrinsic protein kinase activity in ox brain membrane preparations. *Nature (London)* **225**, 187-188.
- Weller, M., and Rodnight, R. (1973). Protein kinase in membrane preparations from ox brain. *Biochem. J.* **132**, 483-492.
- Whittaker, V. P. (1969). The synaptosome. In A. Lajtha (Ed.), "Handbook of Neurochemistry, Volume II," pp. 327-362. New York: Plenum Press.
- Zemp, J. W., Wilson, J. E., Schlesinger, K., Boggan, W. O., and Glassman, E. (1966). Brain function and macromolecules. I. Incorporation of uridine into RNA of mouse brain during short-term training experience. *Proc. Nat. Acad. Sci. USA* **55**, 1423-1431.

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