Incorporation of ^{32}P *in vivo* into phosphatidopeptides of rat brain

Some of the inositol phosphatides of brain occur bound to protein as phosphatidopeptides[1], and in work recently reviewed[2], these substances have been shown to incorporate ^{32}P at a relatively high rate. Subsequent reports have described the incorporation of labeled inositol into phosphatidopeptides and other lipids[3] and the incorporation of ^{32}P into phosphatidopeptide fractions in tissues other than brain[4,5]. In kidney, inositol monophosphate was demonstrated chromatographically to be a constituent of phosphatidopeptides[4]. In liver, the ^{32}P taken up into the phosphatidopeptide preparation was present in a substance which yielded glycerylphosphorylinositol and inositol monophosphate on mild alkaline hydrolysis, and was presumably similar to phosphatidylinositol[5]. In view of these findings and the fact that a major part of the phosphoinositide of brain contains inositol diphosphate[6], with phosphatidylinositol accounting for only a small proportion of the combined inositol, we have investigated the nature of the labeled phosphate compound which is obtained from rat brain phosphatidopeptides after P incorporation *in vivo*. We have found that this compound is an inositol phosphate, the chromatographic behavior of which differs from that of inositol monophosphate but is similar to that of the compound obtained from pure brain diphosphoinositide.

Adult, white, Wistar rats, weighing 300–500 g were injected with about 1 μC ^{32}P/g body weight, as unbuffered neutral sodium phosphate solution in a total volume of about 0.2 ml. After 3 h, the rats were sacrificed by decapitation, the brains removed immediately, and the phosphatidopeptides prepared by the method previously reported[1], except that the protein residue was dried by lyophilization rather than with acetone just prior to the final extraction with acidified solvent. In three separate experiments, brains of 3 to 6 rats were pooled for each extraction. It should be emphasized that this procedure does not involve a preliminary extraction of the tissue with cold 10% trichloroacetic acid, a step included in most other work on phosphatidopeptides which has thus far been reported. Because of the acidity of trichloroacetic acid, its use before extraction of lipids by neutral solvents could alter the nature of lipid–protein complexes subsequently extracted. We are currently investigating this possibility.

The phosphatidopeptide extracts were dried and the resulting solids hydrolyzed in 1 *N* alkali at 37° for 16 h[8]. The resulting hydrolysate, after acidification and treatment with trichloroacetic acid, was exhaustively extracted with ether to remove trichloroacetic acid and fatty acids, and the residual ether was removed from the water phase by warming. Soluble phosphates were prepared from this solution by precipitation with lead and decomposition of the lead precipitate with H_2S (see ref. 6), and were chromatographed on paper using the solvent of SCHORMÜLLER AND WÜRDIG[9]. The papers were sprayed with the molybdate reagent of HANES AND ISHERWOOD[10] and exposed briefly to u.v. light to make the phosphate spots visible.

After development and identification, the spots were cut out, eluted with distilled water, and transferred to aluminum planchets for counting in a Robinson gas flow counter[11]. After counting, the residue on the planchet was washed off with distilled water and total P determined[1].

Only one radioactive spot was found on the chromatograms prepared from phosphatidopeptides as described above. This spot was blue and moved to a different

position than did inositol monophosphate*, sodium glycerophosphate and inorganic phosphate standards alone or mixed with the unknown. Chromatography (solvent: 90% aq. acetone; color development with $AgNO_3$ (see ref. 12) of a deionized hydrolysate (6 *N* HCl, 109°, 40 h) of the radioactive material yielded only one spot corresponding to inositol.

To obtain further information about the nature of the radioactive phosphate compounds obtained in this manner from rat brain, similar but non-radioactive preparations were made on a larger scale from beef-brain white matter. For comparison, a soluble phosphate preparation was made from beef-brain potassium diphosphoinositide[6] by identical alkaline hydrolysis and lead-precipitation procedures. The phosphate solutions thus prepared were run on the same chromatogram, both separately and in mixture with standard solutions of sodium glycerophosphate and inositol monophosphate. The inositol phosphates prepared from potassium diphosphoinositide and from phosphatidopeptides behaved identically in the system used and were clearly distinct from inositol monophosphate or glycerophosphate. The diphosphoinositide also showed a small amount of inorganic phosphate which in this system is not well separated from glycerophosphate but appears yellow rather than blue. The molar ratio of P to inositol in such preparations varied from somewhat over 2 to somewhat over 3.

Thus it seems that the incorporation of ^{32}P *in vivo* into rat-brain phosphatidopeptides occurs in an inositol phosphate compound which is more highly phosphorylated than monophosphate. The presence of either the di- or triphosphate, or of a mixture of the two, would be consistent with the data, provided these two substances are indistinguishable in the chromatographic system used. We feel that the single spot found cannot be a glycerylphosphoryl diester of inositol since no glycerol could be demonstrated in the chromatograms of the acid hydrolysis products. While it is true that some loss of glycerol could have occurred by evaporation during preparation of the sample for chromatography, every attempt was made to minimize such losses. Had glycerol been present in an equimolar ratio to inositol, it would have been demonstrable on the chromatogram.

Preliminary experiments, similar to those described above, had shown that the specific activity of the P in the phosphatidopeptide fraction was about ten times as great as that of the P in the washed total lipid extract, *i.e.* the extract obtained in the same experiment by homogenizing the tissue directly with $CHCl_3$–CH_3OH (2:1). This finding was confirmed in subsequent experiments in which the lipid extracts were washed[13] and dialyzed until they were essentially free of contaminating radioactivity, and aliquots were taken for the determination of total P and for counting. The data in Table I show that the incorporation of ^{32}P into the P of phosphoinositide bound to proteins in phosphatidopeptides is about 15 times as high as the incorporation into total phospholipid-P. ANSELL *et al.*[14,15] have reported that after a 3-h equilibration period with ^{32}P *in vivo*, brain lipid inositide-P also had a specific activity about 15 times as high as total phospholipid-P. Although these workers used young rats, their data suggest the possibility that the P metabolism of phosphatidopeptides may be related to that of brain-lipid phosphoinositide. Assuming that any changes in the composition of the lipid extract occasioned by the differences between our

* Purchased from California Corporation for Biochemical Research, Los Angeles, Calif. (U.S.A.)

TABLE I

SPECIFIC ACTIVITIES OF BRAIN PHOSPHATIDOPEPTIDES AND TOTAL PHOSPHOLIPIDS 3 H AFTER INJECTION OF ^{32}P INTO ADULT RATS

Expt.	*Specific activity**		*Ratio* $\frac{B}{A}$
	Total phospholipid-P (A)	*Inositol phosphate-P from phosphatidopeptides (B)*	
1	—	60.7	—
2	6.8	89.3	13.1
3	3.0	53.4	17.8

* Specific activities are expressed as $\frac{\text{counts/min}/\mu\text{g P}}{10^6 \text{ counts/min injected/g rat}}$

preparation methods and those used by ANSELL *et al.* did not substantially affect the radioactivity data, it would appear that the P of the phosphatidopeptide has approximately the same specific activity as the P of the lipid inositide and may originate from a common pool.

This work was supported in part by research grant B-572 from the National Institute of Neurological Diseases and Blindness, National Institutes of Health, and by a research grant from the Ford Foundation for the Study of the Dynamic Biochemistry of Brain Structural Components.

Addendum (received June 26th, 1960): Since this paper was submitted, DITTMER AND DAWSON[16] have reported isolation of inositol triphosphate from a fraction obtained by steps similar to those used in our studies to prepare phosphatidopeptides.

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Received June 14th, 1960