AMIDATED PHOSPHOPEPTIDES IN NERVOUS TISSUE

P.MANDEL and M.LEDIG

Centre de Neurochimie du C.N.R.S. and Institut de Chimie Biologique, Faculté de Médecine, Strasbourg, France

Received June 9, 1966

After lipid extraction and removal of acid-soluble compounds by diluted TCA, treatment by CH_3OH:$CHCl_3$:HCl 12 N (1:2:0.01 by vol.) of nervous tissue yields an extract which contains some diphosphoinositides and amino acids included in polypeptidic chains (Folch, 1952). We studied the peptidic fraction of this extract and observed that it contains phosphopeptides (PP) of which the phosphorus shows a very high turnover in vivo and in vitro (Ledig and Mandel, 1964). Here we describe a method which allows the isolation of 28 different PP from the phosphatidopeptidic extract of ox brain and also demonstrate that these are amidated PP mostly localized in membranous structures.

Methods.

1) Isolation of the phosphopeptidic fraction as a whole. Nervous tissue is treated with 20 vol. of CH_3OH:$CHCl_3$ (1:2) ; the residue is washed successively with acetone, water, HCl 0.03 N and acetone, and then extracted with CH_3OH:$CHCl_3$:HCl 12 N (1:2:0.01). One fifth volume water is added to this extract. After separation of the alcoholic aqueous upper phase, the lower chloroformic phase is washed twice with a methanol-water-chloroform mixture (48:47:3). The pooled alcoholic aqueous phases represent fraction A and the chloroformic phase, fraction B.

2) Isolation of various phosphopeptides. After lyophilisa-

tion, the compounds of fraction A are dissolved and introduced on an activated charcoal column ; the efluent is separated ; the adsorbed fraction is eluted with an ethanol-ammonia-water mixture (50:2:48). The efluent and the eluate are lyophilised, then submitted to paper electrophoresis (15 h, 9 V/cm, pyridin-acetic acid-water buffer 6:20:974, pH 3.5). The samples are applied as a narrow strip 20 cm long. After electrophoresis, two lateral strips are cut out : one of them is treated with Hanes and Isherwood (1949) reagent, the second one with ninhydrin. On the sheets of the efluent and eluate electrophoresis, a total of 8 areas (Aa, Ab, Ac, Ad, Ae, Af, Ag, Ah) containing PP can be detected they are eluted with water, lyophilised, dissolved in water and submitted to descending paper chromatography (solvent : n-propanol-acetic acid-water 3:1:1). The samples are applied as a narrow strip on the whole length of the sheet (15 cm) as for the electrophoresis and the spots are located in the same way. Two areas containing PP are obtained from each Aa, Ab, Ac, Ae, Af, Ag fraction (Aa_1 + Aa_2, Ab_1 + Ab_2, etc.) and a single one from Ad and Ah fraction (Ad_1 and Ah_1). After elution, the chromatography on Dowex 1X8 of the 14 PP shows a single peak for each one.

Fraction B is hydrolysed with alkali (KOH N, 15 h at 37°C), $HClO_4$ is added until the final concentration is 1 N ; the acid-soluble fraction is separated and the $HClO_4$ is removed as $KClO_4$. The supernatant is submitted to the same treatment as fraction A : separation on charcoal, electrophoresis, paper and column chromatography. Fourteen other PP (Ba_1 + Ba_2, Bb_1 + Bb_2, etc.) are thus obtained. In addition, we find some glycerophosphate, phosphoinositol and phosphoserine.

The amino acid distribution of each PP was determined by the

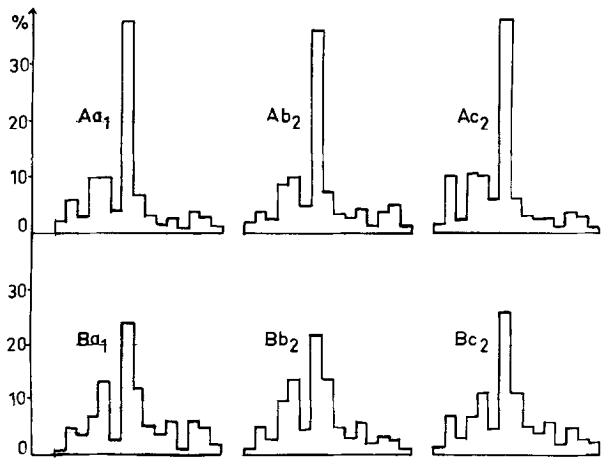
Technicon Amino Acid Analyser. The phosphorus was measured according to Briggs (1922). The nitrogen content was determined by Kjeldahl procedure and titration of $SO_4(NH_4)_2$ was done by Mann's method (1963). The ammonia which could possibly contaminate the PP was removed by steam after treatment with $CO_3(Li)_2$ in the Yovanovitch apparatus (1925).

Results and Discussion.

The various treatments lead to 28 compounds with N/P ratios between 4.0 and 28.0. The homogeneity of each compound was tested by paper chromatography in different solvents (butanol-acetic acid-water 4:1:5, phenol-water 4:1, pyridine-alcohol-ammonia-water 3:3:3:1, propanol-ammonia-water 6:3:1, isobutyric acid-ammonia 0.5 N 5:3). In each case, only one spot is obtained.

After hydrolysis (HCl 2 N, 8 h at 100°C) each one of the isolated compounds liberates phosphoserine as the only phosphorylated amino acid. Complete acid (HCl 6 N, 20 h at 110°C) or alkaline hydrolysis (KOH N, 3 h at 110°C) of each compound yields the following amino acids : cys, asp, thr, ser, glu, pro, gly, ala, ileu, leu, tyr, phe, lys, his, arg. No tryptophane nor methionine could be detected ; alanine is always the terminal amino acid. If the least abundant amino acid is taken as an unit, the total number of amino acids of the peptidic chain varies from 50 to 105, and differs from one another. The distribution of the amino acids in 6 different PP is shown in the figure.

In all the PP, glycine content is very high : from 19 to 39 p.100. The level of the other amino acids is in the following range : glutamic acid 8 to 15 p.100, serine 7 to 27 p.100, for the remaining amino acids, the amount varies from 1 to 13 p.100.



The columns indicate the amino acid distribution as percentage of total ; the amino acids are in the following order : cys, asp, thr, ser, glu, pro, gly, ala, val, ileu, leu, phe, lys, his, arg.

The high quantity of ammonia appearing after acid hydrolysis of the PP suggested the presence of amide bonds in them. Indeed, mild acid hydrolysis (HCl N, 5 min at 100°C) releases 40 to 60 p.100 of the nitrogen as ammonia depending on the PP. On the other hand, alkaline hydrolysis (NaOH 0.5 N, 3 h at 100°C) liberates only 3 p.100 of the amount of ammonia obtained after mild acid hydrolysis as is the case in phosphoamides (Rathlev and Rosenberg, 1956). The parallel titration of the native and deamidated PP indicates that the second acidity of the phosphate is amidated.

The following table shows the distribution of the PP in the subcellular fractions of the brain, indicating that these compounds are present in high concentration in myelin sheaths, in the membranous structures and in the nuclei.

Our results demonstrate that in membranous structures and

Table. - Amidated phosphopeptides in subcellular fractions of rat brain.

myelin sheaths	3.96 ±1.14
nuclei	1.48 ±0.20
endoplasmic reticulum membranes	0.93 ±0.11
endoplasmic reticulum	0.49 ±0.11
mitochondrial membranes	0.67 ±0.10
mitochondria	0.29 ±0.05
cytoplasm	0.17 ±0.07

Values are expressed in µg P of PP/mg protein.

especially in myelin sheaths, there are PP displaying the properties of amidated polyanions similar to cations exchangers. Their presence has also been observed in other organs (Ledig, unpublished results) and has been shown in bacterial membranes (Rebel and Ledig, unpublished results). The phosphorus of these PP has an extremely fast turnover. Our data suggest that these amidated PP are liable to participate in transport phenomena through cellular membranes.

This investigation was supported by grants from the Délégation Générale à la Recherche Scientifique et Technique and the Commissariat à l'Energie Atomique.

References

Briggs, A.P., J. Biol. Chem., 53, 13 (1922).
Folch, J., in "Phosphorus Metabolism", Ed. McElroy, W.D. and Glass, B., Johns Hopkins Press, Baltimore, vol. II, 186 (1952).
Hanes, F.C., and Isherwood, F.A., Nature, 164, 1107 (1949).
Ledig, M., and Mandel, P., Biochim. Biophys. Acta, 90, 408 (1964).
Mann, L.T., Analyt. Chem., 35, 2179 (1963).
Rathlev, T., and Rosenberg, T., Biochim. Biophys. Acta, 65, 319 (1956).
Yovanovitch, A., Bull. Soc. Chim. Biol., 7, 665 (1925).