PHOSPHOLIPIDES CONTAINING AMINO ACIDS OTHER THAN SERINE

I. DETECTION*

By JOHN WESTLEY,† J. J. WREN, and HERSCHEL K. MITCHELL

(From the Kerckhoff Laboratories of Biology, California Institute of Technology, Pasadena, California)

(Received for publication, May 21, 1957)

Several years ago experiments on the biochemistry of *Drosophila melanogaster* (1) directed our attention to a class of substances that are soluble in dry ether but yield a variety of amino acids upon hydrolysis. Moreover, it was observed that in very young larvae essentially all of the non-protein amino acids are found in a lipide-soluble fraction. During subsequent rapid growth and development of the organism, this kind of material diminishes strikingly and free amino acids and peptides appear in increasing concentrations. Protein synthesis is also rapid during this period as indicated by a greater than 100-fold increase in tissue weight.

We have now found similar amino acid-containing lipides in phospholipide preparations from several different sources. In recent years the presence in a wide variety of tissues of unidentified phospholipides which contain amino acids other than serine has been reported. Some confusion has resulted from the power of phospholipides to render polar molecules soluble in non-polar solvents. Thus when an amino acid was detected in a lipide hydrolysate, ambiguity remained as to whether or not it had been chemically bound. Nevertheless a number of workers have adequately demonstrated the removal of contaminants, yet have found various amino acids after hydrolysis of their phospholipide preparations. Barbier and Lederer (2) concluded that hydroxylysine in the phospholipides of *Mycobacterium phlei* is bound analogously to serine in phosphatidylserine. However, nearly all of the amino acids so far reported as lipide constituents have no alcoholic hydroxyl groups and it is generally believed, so far with little direct evidence, that they must be joined to ethanolamine or serine phospholipides as peptides. The complex bacterial phospholipides contain both sugars and amino acids (3), and there is abundant evidence that these are chemically bound (4–10). Masamune and his collaborators (11–14) have found similar constituents in blood group lipides, which they believe

* This investigation was supported in part by grants from the Research Corporation (Williams-Waterman Fund) and the National Science Foundation.

† National Science Foundation Postdoctoral Fellow. Present address, Department of Biochemistry, University of Chicago, Chicago 37, Illinois.

to be peptidyl-phosphatidylethanolamine derivatives. Phosphoinositides from brain appear to contain bound polypeptides (15).

There have been several investigations of the nitrogenous constituents of blood lipides since Wynn and Williams (16) first reported that lipides obtained from plasma and purified by dialysis yielded several amino acids upon hydrolysis. From paper chromatography, German workers (17, 18) deduced the existence of "lipopeptides" in dialyzed blood lipide preparations. Their deduction was later attacked on the grounds that chromatography in a second dimension resolved the "lipopeptide" spots into separate lipide and peptide spots (19). Since the work of others (see below) leaves little doubt of the existence of "lipopeptides" in blood, this observation might be explained by cleavage of peptide chains on the chromatograms. Cheftel *et al.* (20) have reported a subsequent study of serum lipides in which a preparation purified by chromatography on cellulose gave three ninhydrin-positive spots of low R_F (presumed peptides) on a paper chromatogram in addition to the fast running phospholipide spot. When this fast running spot was separately eluted and again chromatographed, the same three spots of low R_F reappeared. Such a phenomenon has been observed in this laboratory in paper chromatography of non-lipide peptides.[1] Douste-Blazy and Polonovski (21, 22) have also isolated amino acid-containing phospholipides from human, dog, and rat sera by countercurrent distribution, and Blass *et al.* (23) have subjected the lipides of human and equine plasma and erythrocytes to prolonged dialysis or to electrodialysis and identified the amino acids liberated by subsequent hydrolysis.

In addition to the instances already mentioned, there is evidence that similar phospholipides occur widely, *i.e.* in rabbit liver (24, 25), rat liver (25–27), ox heart and liver (28, 29), beef brain (30, 31), limpets (32), cytochrome oxidase (33), bakers' yeast (25), rye and wheat germ (34), soya (35, 36), and groundnut (36). Hen's egg, rat tissues (37–39), cockerel serum (40), and sheep erythrocytes (41) have recently afforded unidentified phospholipides which may well also be similar.

In view of the widespread occurrence of the amino acid-containing lipides and the unique course of their metabolism during development of *Drosophila*, we have carried out extensive investigations concerned with their isolation and chemical nature. The present report is concerned primarily with techniques and procedures developed to insure removal of non-lipide contaminants from preparations of these lipides.

EXPERIMENTAL

Extraction and Precipitation of Phospholipides—Whole *Drosophila*, rat tissues, hen's egg yolk, and mycelial pads and spray-dried cultures of

[1] Mitchell, H. K., unpublished.

Eremothecium ashbyii were macerated with sand and extracted with cold ether. The materials were usually carefully dried by lyophilization but, if not, they were washed with acetone before extraction and the extracts were cooled to −20° and decanted from ice crystals. Addition of alcohol to the ether used for extraction, which increased the total yield of phospholipide, appeared to decrease the proportion of amino acid-containing phospholipide in the extract. The phospholipides were purified by four to six precipitations by acetone (5 to 10 volumes) from concentrated solutions in ether or chloroform. During further precipitations by ethanol, the amino acid-containing lipides (except phosphatidylserine) accumulated in the ethanol-soluble (lecithin) fraction;[2] however, they were not found in the lecithin-$CdCl_2$ precipitate (42).

Color Tests Used for Paper Chromatography and Paper Electrophoresis—The ninhydrin reaction was performed by the dipping technique (43). Choline and its derivatives were detected by means of phosphomolybdic acid (44), phosphates by the method of Bandurski and Axelrod (45), and all compounds containing an —NH— group by the chlorination method (46, 47).

Chromatography on Paper and on Cellulose Columns—Of a variety of solvent systems employed for ascending chromatography on Whatman No. 1 paper, the following four were most suitable for resolution of free amino acids and peptides from phospholipides and for partial resolution of the phospholipides themselves. (*a*), *n*-Butanol saturated with 0.05 M phthalate buffer, pH 4.1; the paper was first sprayed with buffer solution and dried. Free amino acids have $R_F \leq 0.3$. Fly phospholipides give ninhydrin-positive bands at R_F 0.4 and 0.7, and larval phospholipides at 0.8, but their amino acid content is similar. (*b*), *n*-Propanol-0.25 N aqueous NH_4OH-acetone (4:1:1, v/v). Free amino acids have $R_F \leq 0.5$. Fly phospholipides give ninhydrin-positive bands at R_F 0.65 and 0.85, but the two fractions contain the same amino acids. (*c*), Ether-ethanol-concentrated NH_4OH (15:5:3). Fly phospholipides give ninhydrin-positive bands at R_F 0.8 and 0.9. (*d*), Methanol-benzene-*n*-butanol-water (2:1:1:1) (48). Free amino acids have $R_F \leq 0.65$, phospholipides $R_F \leq 1$.

These solvent systems were also employed, in preparative experiments, on columns of cellulose and on strips of heavy filter paper. Phospholipide bands on the heavy filter paper were excised and eluted with methanol or chloroform. Amino acids contained in the filter paper itself were removed by preliminary chromatographic washing with the developing solvent to be used. However, it was still possible that during development the phospholipides could liberate and entrain amino acid-containing materials or-

[2] In countercurrent distribution of serum lipides Polonovski and Douste-Blazy (22) found that bound amino acids were most abundant in the lecithin fraction.

dinarily unavailable to the solvent. This possibility was eliminated by showing that egg lecithin (N:P ratio 1.02), purified by Pangborn's method (42) and containing no amino acids, could be chromatographed on washed paper without gaining any amino acids. Moreover, rechromatography showed the absence of free amino acids, and phospholipides from the several sources purified on Hyflo Super-Cel contained the same amino acids as when purified on paper.

Chromatography on Hyflo Super-Cel—1 gm. of adsorbent was used per micromole of phosphorus in the specimen to be chromatographed. In one procedure, a phospholipide solution in ligroin (b.p. 60–70°) was run on to a column of Hyflo Super-Cel, which was washed successively with ligroin and ethyl acetate. Methanol then eluted the phospholipides in a single band containing no free amino acids. In another procedure, a solution in ligroin (b.p. 30–60°) was used and the column was developed successively with ligroin, ethyl acetate, and 90 per cent methanol. The phospholipides were eluted in fractions by a linear gradient from 90 per cent methanol to 90 per cent methanol, 0.01 N in NH_4OH. Phosphorus anlaysis (49) of the fractions obtained from fly phospholipides indicated that three overlapping bands were eluted, but attempts to resolve these were unsuccessful. None contained free amino acids, and the lipide-bound amino acids were all in the last band.

Paper Electrophoresis—Electrophoresis was carried out at 15 to 20 volts per cm. during 2 to 4 hours on Whatman No. 3MM paper, with use of 0.025 M phthalate buffer, pH 4.15, in a closed chamber. Specimens were applied to the paper in ethereal solution or, better, in aqueous emulsion. Phospholipides remained at the origin while amino acids, the lactams of γ-aminobutyric and δ-aminovaleric acids, the mixed cyclic dipeptides of leucine and valine (prepared by Sannié's method (50)), and a mixture of pyrrolidonecarboxylic acid and acidic cyclic peptides (prepared from glutamic acid by the same method) all migrated.

Hydrolysis and Identification of Nitrogenous Products—Phospholipides were hydrolyzed by being heated in a sealed tube at 110° with 6 N HCl for 18 hours. The tube was then opened, HCl removed under reduced pressure, the residue extracted with water, and the extract washed twice with ligroin. Its nitrogenous constituents were then identified by two-dimensional paper chromatography, with (*a*) *n*-propanol-0.25 N aqueous NH_4OH (2:1) and (*b*) the Wiggins and Williams solvent (51)-ethanol-water (2:2:1). Amino acids were further identified by paper chromatography of their dinitrophenyl derivatives (52–54).

γ-Aminobutyric and δ-aminovaleric acids and their lactams were identified chromatographically and by paper electrophoresis in the system described above.

RESULTS AND DISCUSSION

The following are the amino acids consistently found in hydrolysates of chromatographically purified phospholipides from different sources. *E. ashbyii*, alanine, γ-aminobutyric acid, δ-aminovaleric acid, aspartic acid, cystine, glutamic acid, leucine, serine, threonine (?), valine; *Drosophila*, aspartic acid, cystine, glutamic acid, serine; *hen's egg*, aspartic acid (?), glutamic acid, serine; *rat brain*, aspartic acid, glutamic acid, serine; *rat kidney*, aspartic acid, cystine, glutamic acid, serine.

The presence of γ-aminobutyric and δ-aminovaleric acids in *E. ashbyii* phospholipides was surprising. The acids were also found free in the medium in which the mold was grown and in 50 per cent (v/v) aqueous alcoholic extracts of spray-dried cultures. They were isolated from the latter source by ion exchange chromatography on buffered (pH 4.2) Amberlite XE-97 and positively identified by comparison of the acids, their lactams, and their dinitrophenyl derivatives, with authentic specimens.[3] Since the relatively non-polar lactams were found in the culture medium, it seemed likely that these might not have been separated from the phospholipides by chromatography. This proved to be so. The lactams were added to purified hen's egg lecithin, which was then repurified by acetone precipitation and paper chromatography; both γ-aminobutyric and δ-aminovaleric acids appeared in hydrolysates of the product. When the *E. ashbyii* phospholipides were further purified by paper electrophoresis, these two acids no longer appeared in hydrolysates.

The experience with lactams suggested that the amino acids in purified phospholipide preparations might simply be present as relatively non-polar cyclic peptides not chemically bound to lipide. It was indeed found that added small cyclic peptides remained with phospholipides through the chromatographic purification. However, they were readily removed by paper electrophoresis, leaving the phospholipides with their usual amino acid content.

The evidence described in this paper confirms the existence of lipides containing various bound amino acids. Chromatography usually removes

[3] The decomposition points obtained in a Fischer type apparatus for the isolated δ-aminovaleric acid and γ-aminobutyric acid were 153° (literature, 157° (55)) and 197° (literature, 202° (56)), respectively, with some variation, depending on the rate of heating. The isolated amino acids were indistinguishable from authentic δ-aminovaleric acid and γ-aminobutyric acid (California Foundation for Biochemical Research) in the paper electrophoretic or two-dimensional chromatographic systems described above, although both of these systems separate them from all of the α-amino acids, β-alanine, ε-aminocaproic acid, and from each other. Moreover, both the lactams and the dinitrophenyl derivatives prepared from the isolated compounds and from the authentic specimens were identical, respectively, in chromatographic behavior.

unbound amino acids and peptides from phospholipide preparations, but paper electrophoresis is a necessary test to establish the absence of cyclic peptides and lactams. The way in which the amino acids are bound is still not clear. Dinitrophenylation (53, 57) of fly and *E. ashybii* phospholipides, followed by hydrolysis and paper chromatography, has so far failed to reveal any dinitrophenylamino acids; a similar observation was recently reported by Cheftel *et al.* (20). Hydrolysates of all preparations contain major amounts of ethanolamine and this might indicate a peptidyl-phosphatidylethanolamine structure (an alternative is the phosphatidyl-seryl-peptide structure considered by Baer (58)).

Work is now in progress on the isolation of the presumed peptidyl-phospholipides in quantity, and the larger scale work has revealed the presence of components which have a greater variety of amino acids than is found in the fractions described here. Studies on the biochemical function of these substances, particularly in relation to a possible role in protein synthesis, have been instituted.

SUMMARY

1. Evidence is presented which demonstrates the existence, in a variety of tissues, of amino acid derivatives of lipides. Amino acids found include several which do not contain hydroxyl groups. Among these are alanine, leucine, aspartic acid, glutamic acid, and cystine.

2. Methods are presented that permit a clear distinction between bound amino acids and those rendered soluble by phospholipides. Paper electrophoresis is particularly satisfactory for this purpose.

3. The substances under consideration appear to be peptide derivatives of phospholipides.

4. γ-Aminobutyric acid and δ-aminovaleric acid have been identified as normal constituents in the fungus *Eremothecium ashbyii*, and the corresponding lactams appear as contaminants in phospholipide preparations obtained from it.

BIBLIOGRAPHY

1. Hadorn, E., and Mitchell, H. K., *Proc. Nat. Acad. Sc.*, **37,** 650 (1951).
2. Barbier, M., and Lederer, E., *Biochim. et biophys. acta*, **8,** 590 (1952).
3. Asselineau, J., and Lederer, E., *Fortschr. Chem. org. Naturstoffe*, **10,** 170 (1953).
4. Gendre, T., and Lederer, E., *Ann. acad. sc. Fennicae, Series A, II, No. 60*, 313 (1955).
5. Vilkas, E., and Lederer, E., *Compt. rend. Acad.*, **240,** 1156 (1955).
6. Michel, G., and Lederer, E., *Compt. rend. Acad.*, **240,** 2454 (1955).
7. Asselineau, J., *Bull. Soc. chim. biol.*, **38,** 1397 (1956).
8. Blass, J., *Bull. Soc. chim. biol.*, **38,** 1305 (1956).
9. Westphal, O., and Lüderitz, O., *Angew. Chem.*, **66,** 407 (1954).

10. Cmelik, S., *Z. physiol. Chem.*, **293,** 222 (1953); **296,** 67 (1954); **299,** 227 (1955); **300,** 167 (1955); **302,** 20 (1955).
11. Masamune, H., Hakomori, S., Maehara, T., and Suzuki, Y., *Tôhoku J. Exp. Med.*, **59,** 231 (1954).
12. Hakomori, S., *Tôhoku J. Exp. Med.*, **60,** 331 (1954).
13. Masamune, H., Hakomori, S., Numabe, H., Akama, J., and Kamiyama, S., *Tôhoku J. Exp. Med.*, **61,** 283 (1955).
14. Akama, J., *Tôhoku J. Exp. Med.*, **63,** 335 (1956).
15. Folch, J., and LeBaron, F. N., *Canad. J. Biochem. and Physiol.*, **34,** 305 (1956).
16. Wynn, V., and Williams, T. N. W., *Nature*, **165,** 768 (1950).
17. Becker, G., Bode, F., and Schrade, W., *Klin. Wochschr.*, **31,** 593 (1953).
18. Schrade, W., Becker, G., and Böhle, E., *Klin. Wochschr.*, **32,** 27 (1954).
19. Bode, F., and Ludwig, U. M., *Klin. Wochschr.*, **32,** 1097 (1954).
20. Cheftel, R. I., Moretti, J., and Polonovski, J., *Bull. Soc. chim. biol.*, **39,** 291 (1957).
21. Douste-Blazy, L., *Compt. rend. Acad.*, **239,** 460 (1954).
22. Polonovski, J., and Douste-Blazy, L., Proceedings of the 2nd international conference on biochemical problems of lipids, London, 64 (1956).
23. Blass, J., Rouhi, A., Lecomte, O., and Macheboeuf, M., *Bull. Soc. chim. biol.*, **35,** 959 (1953).
24. Hawthorne, J. N., *Chem. and Ind.*, 1171 (1956).
25. Michelazzi, L., *Experientia*, **11,** 389 (1955).
26. Levine, C., and Chargaff, E., *Exp. Cell Res.*, **3,** 154 (1952).
27. Pilgeram, L. O., and Greenberg, D. M., *J. Biol. Chem.*, **216,** 465 (1955).
28. Hecht, E., and Ottens, H., *Acta Haematol.*, **8,** 265 (1952).
29. Hecht, E., and Mink, C., *Biochim. et. biophys. acta*, **8,** 641 (1952).
30. Chargaff, E., Levine, C., and Green, C., *J. Biol. Chem.*, **175,** 67 (1948).
31. Levine, C., and Chargaff, E., *J. Biol. Chem.*, **192,** 465 (1951).
32. Etienne, J., *Bull. Soc. chim. biol.*, **38,** 1475 (1956).
33. Marinetti, G. V., Scaramuzzino, D. J., and Stotz, E., *J. Biol. Chem.*, **224,** 819 (1957).
34. Schulte, K. E., and Krause, H., *Biochem. Z.*, **322,** 168 (1951).
35. Hawthorne, J. N., and Chargaff, E., *J. Biol. Chem.*, **206,** 27 (1954).
36. van Handel, E., quoted by Aylward, F., *Chem. and Ind.*, 1360 (1956).
37. Marinetti, G. V., and Stotz, E., *Biochim. et biophys. acta*, **21,** 168 (1956).
38. Marinetti, G. V., Witter, R. F., and Stotz, E., *Federation Proc.*, **15,** 308 (1956).
39. Rouser, G., Marinetti, G. V., Witter, R. F., Berry, J. F., and Stotz, E., *J. Biol. Chem.*, **223,** 485 (1956).
40. Hillyard, L. A., Entenman, C., and Chaikoff, I. L., *J. Biol. Chem.*, **223,** 359 (1956).
41. Turner, J. C., *J. Exp. Med.*, **105,** 189 (1957).
42. Pangborn, M. C., *J. Biol. Chem.*, **188,** 471 (1951).
43. Toennies, G., and Kolb, J. J., *Anal. Chem.*, **23,** 823 (1951).
44. Bevan, T. H., Gregory, G. I., Malkin, T., and Poole, A. G., *J. Chem. Soc.*, **841** (1951).
45. Bandurski, R. S., and Axelrod, B., *J. Biol. Chem.*, **193,** 405 (1951).
46. Rydon, H. N., and Smith, P. W. G., *Nature*, **169,** 922 (1952).
47. Reindel, F., and Hoppe, W., *Naturwissenschaften*, **40,** 221 (1953).
48. Mason, M., and Berg, C. P., *J. Biol. Chem.*, **188,** 783 (1951).
49. King, E. J., *Biochem. J.*, **26,** 292 (1932).
50. Sannié, C., *Bull. Soc. chim.*, **9,** 487 (1942).

51. Wiggins, L. F., and Williams, J. H., *Nature*, **170**, 279 (1952).
52. Sanger, F., *Biochem. J.*, **39**, 507 (1945).
53. Sanger, F., and Thompson, E. O. P., *Biochem. J.*, **53**, 353 (1953).
54. Blackburn, S., and Lowther, A. G., *Biochem. J.*, **48**, 126 (1951).
55. Schotten, C., *Ber. chem. Ges.*, **21**, 2235 (1888).
56. Tafel, J., and Stern, M., *Ber. chem. Ges.*, **33**, 2224 (1900).
57. Ellman, G. L., and Mitchell, H. K., *J. Am. Chem. Soc.*, **76**, 4028 (1954).
58. Baer, E., *Canad. J. Biochem. and Physiol.*, **34**, 288 (1956).