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The Reported Occurrence of Sphingosylphosphorylcholine in Animal Tissues

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Little is known about the intermediary metabolism of sphingomyelin. Rossi (1935) and Goebel & Seckfort (1948) reported an uncharacterized sphingomyelinase in extracts of spleen, brain and liver, and Thannhauser & Reichel (1940) and Fujino (1952) have produced some evidence that this acts by splitting the bond between lignoceryl-sphingosine and phosphorylcholine. Booth (1935) and Booth & Milroy (1935) described the isolation of a water-soluble choline precursor from various animal tissues, and identified this as the choline ester of sphingosinephosphoric acid. King & Small (1939) provisionally identified a similar substance from pig pancreas as the same compound. Aloisi (1947) also isolated the substance from pancreas and studied the rate at which it was oxidized by various tissues.

It was considered by the author that if sphingosylphosphorylcholine occurred in tissues some insight into the metabolism of sphingomyelin might be obtained by studying the relationship between the phosphodiester and the phospholipid after the injection of isotopes. Analogous studies have indicated the metabolic relationship between glycerylphosphorylcholine and lecithin and also between glycerylphosphorylethanolamine and phosphatidylethanolamine in the liver of the rat (Dawson, 1955*a*). However, from fractions obtained by methods equivalent to those described by Booth (1935) and King & Small (1939) no evidence could be obtained for the presence of

sphingosylphosphorylcholine, nor could the compound be detected chromatographically in various tissues of the rat or sheep. These negative results are reported because of the frequency with which the isolation of the phosphodiester is quoted in the literature, e.g. Celmer & Carter (1952), Artom (1952), Rouser, Berry, Marinetti & Stotz (1953).

Attempts were made to prepare sphingosylphosphorylcholine both by the acid and alkaline methanolysis of sphingomyelin. Whereas the results confirm the findings of Rennkamp (1949) that sphingosylphosphorylcholine is a minor component of the acid hydrolysate of sphingomyelin, none of the diester could be obtained from alkaline hydrolysates where its presence has been reported by Rouser *et al.* (1953).

EXPERIMENTAL

Materials. The sphingomyelin used was generously provided by Dr J. Olley and had been prepared from ox spinal cord (Found: N, 3.45; P, 3.8%; N:P molar ratio 2.01:1. Calc. for lignoceryl-sphingosylphosphorylcholine: N, 3.4; P, 3.7%; N:P molar ratio 2:1). No carbohydrate could be detected by the Molisch test. A small sample of sphingomyelin prepared by Dr D. N. Rhodes on a silicic acid column and having an N:P ratio of 2.08:1 gave the same hydrolysis products as the main sample. Phosphorylcholine and glycerylphosphorylcholine were prepared as described by Dawson (1955*b*, 1956). A solution containing sphingosylphosphorylcholine was prepared by hydrolysis of sphingomyelin (20 mg.) with 2 ml. of methanolic 1.4N-HCl for 2 hr. in a sealed ampoule at 100°. The

hydrolysate was evaporated to dryness *in vacuo* and the residue shaken with 4 ml. of water plus 10 ml. of ether. After centrifuging, the ethereal supernatant was withdrawn, and the aqueous layer was shaken with 5 ml. of CHCl_3 . The emulsion was centrifuged for 15 min. at 10 000 g, the aqueous layer was withdrawn, concentrated *in vacuo* and applied as a strip on a paper chromatogram which was developed in butan-1-ol-acetic acid-water (4:1:5, by vol.; upper phase). The sphingosylphosphorylcholine was located by spraying marker strips first with ninhydrin (0.25% in acetone) and then with a reagent for detecting phosphorus: it had an R_f of approx. 0.62. The strip containing the compound was cut out and chromatographically eluted with methanol (5 ml.). This solution was used to provide a reference marker.

Estimations. Phosphorus was determined by the procedure of Fiske & Subbarow (1925), after organic P had been converted into inorganic P by oxidation with perchloric acid (King, 1951). Nitrogen was estimated after digestion with the reagent of Hiller, Plazin & Van Slyke (1948) and distillation of NH_3 in a Pregl apparatus; occasionally digestion was carried out with the reagent of Borsook & Dubnoff (1939), followed by estimation of NH_3 in a Conway microdiffusion unit. Choline was estimated by the procedure of Appleton, La Du, Levy, Steele & Brodie (1953) and glycerylphosphorylcholine after acid hydrolysis of the labile choline in N-HCl at 100° for 20 min. Sphingosine was determined by the method of McKibbin & Taylor (1949).

Chromatography. Washing of the paper before chromatography, the method of chromatography, and the detection of phosphorus and ninhydrin-reacting spots have been described (Dawson, 1955a). Choline was detected on chromatograms, occasionally by the method of Chargaff, Levine & Green (1948) or more usually by the iodine-vapour procedure of Brante (1949). With the latter method it was found essential to dry thoroughly any acid solvent from the paper before applying the test, else low concentrations of choline (3–5 μg .) were not detected. Solvents used for the chromatography of free choline were those of Whittaker & Wijesundera (1952) and also butanol-acetic acid-water (4:1:5, by vol.; upper phase). For the separation of sphingosylphosphorylcholine from other methanol- and water-soluble phosphate esters which occur in tissues it was necessary to employ two-dimensional paper chromatography. Phenol saturated with water or 0.1% aq. NH_3 soln. was used in the first run; in this solvent sphingosylphosphorylcholine, glycerylphosphorylcholine and phosphorylcholine (R_f values approx. 0.9) separate from other phosphate esters which are present. The second solvent used was either butanol-acetic acid-water or ethanol-aq. NH_3 soln. (sp.gr. 0.88)–water (6:3:1, by vol.). In the acid solvent sphingosylphosphorylcholine had R_f approx. 0.6 whereas glycerylphosphorylcholine and phosphorylcholine both ran to R_f 0.11. In the alkaline solvent both phosphorylcholine and sphingosylphosphorylcholine had R_f 0.20 and glycerylphosphorylcholine R_f 0.45.

For the chromatographic detection of water- and methanol-soluble phosphate esters the tissue (0.5 g.) was homogenized in a blender with 25 ml. of methanol and centrifuged. The methanolic extract was evaporated to dryness *in vacuo* and the residue was shaken with 5 ml. of water and 10 ml. of ether. The mixture was centrifuged, the ethereal layer was withdrawn and the aqueous layer was

shaken with 5 ml. of CHCl_3 . After centrifuging, the aqueous layer was neutralized to pH 7, then concentrated to low volume and applied to the paper.

RESULTS

Isolation and examination of the fraction described by Booth (1935)

This fraction, described by Booth (1935) as the choline ester of sphingosine phosphoric acid, was isolated from sheep kidney and brain by essentially the same procedure. The products obtained were snow-white light solids which were readily soluble in both ethanol and water. The product from brain had an N:P molar ratio of 1.6:1 and that from kidney 1.3:1. On two-dimensional paper chromatography of the fractions in phenol-aq. NH_3 soln. and ethanol-aq. NH_3 soln., no ninhydrin-reacting and phosphorus-containing spot appeared in the sphingosylphosphorylcholine position. Phosphorus-containing spots appeared corresponding to glycerylphosphorylcholine and phosphorylcholine. There were also a number of ninhydrin-reacting spots, not containing phosphorus, corresponding with various amino acids. Again on two-dimensional paper chromatography of the fraction with phenol-aq. NH_3 soln. and butanol-acetic acid-water, no sphingosylphosphorylcholine spot was visible. Neither product contained sphingosine, when this base was estimated after the fractions had been hydrolysed for 16 hr. at 100° in 5N-HCl. Acid-labile choline determinations showed that 42% of the phosphorus in the product from kidney was present as glycerylphosphorylcholine, whereas the corresponding figure for the brain fraction was 28%. Both products contained traces of glycerylphosphorylethanolamine, revealed by paper chromatography.

Examination of the pig-pancreas fraction described by King & Small (1939)

This fraction was isolated from fresh pig pancreas as a syrupy liquid. On examination by two-dimensional paper chromatography, no sphingosylphosphorylcholine could be detected, but the major part of the phosphorus was present as a mixture of phosphorylcholine and glycerylphosphorylcholine; the concentration of the phosphodiester was 2–3 times higher than that of the monophosphoester. There were also numerous ninhydrin-reacting spots which did not contain phosphorus and which were probably amino acids.

Examination of tissues for the presence of sphingosylphosphorylcholine

The water- and methanol-soluble phosphate esters from rat kidney, brain, spleen and liver, sheep brain and pancreas, and pig pancreas were

examined for sphingosylphosphorylcholine by two-dimensional paper chromatography. In no instance was a ninhydrin-reacting phosphorus-containing spot located in the position of the diester, but this does not eliminate the possibility that very small amounts of diester may be present in these tissues.

Breakdown of sphingomyelin with methanolic acid and alkali

Rennkamp (1949) reported the occurrence of two phosphorus-containing acid-hydrolysis products of sphingomyelin, which when isolated as Reineckate salts gave analyses corresponding to phosphorylcholine and sphingosylphosphorylcholine, the diester being present as only a very minor component. The present findings agree with the results of Rennkamp on these points. On hydrolysis of sphingomyelin in methanolic 1.4N-HCl, either under reflux or in a sealed tube at 100°, two water-soluble phosphorus-containing hydrolysis products could be separated by paper chromatography with butanol-acetic acid-water. The predominant phosphorus-containing spot (R_f 0.11) was not ninhydrin-reacting and was identified as phosphorylcholine. The other phosphorus-containing spot (R_f 0.58–0.64) was strongly ninhydrin-reacting and reacted with the choline-detecting reagents of Chargaff *et al.* (1948), but as pointed out by Rouser *et al.* (1953) this spray reacts with sphingosine as well as choline. However, when the compound was eluted and hydrolysed for 18 hr. in 5N-HCl at 100°, choline was detected chromatographically among the hydrolysis products as well as inorganic phosphate and a ninhydrin-reacting substance which is presumably sphingosine. The choline and phosphorus were in equal molar ratios and the N:P ratio was approx. 2:1.

This ninhydrin-reacting phosphate ester occurred in acid hydrolysates in such low concentrations that it was possible to obtain it in solution only, but the present findings leave little doubt that it is sphingosylphosphorylcholine, thus confirming the finding of Rennkamp (1949).

Attempts were made to prepare sphingosylphosphorylcholine by hydrolysis of sphingomyelin with KOH in a sealed tube at 100° according to the directions of Rouser *et al.* (1953). The methanolic solution was freed from alkali either by passage through a column of Amberlite IRC 50 anion-exchange resin which was equilibrated with methanol, or by neutralizing the solution with methanolic HCl and removing the precipitated KCl after cooling. The methanolic solution was shown by paper chromatography to contain, as well as unchanged sphingomyelin, a ninhydrin-positive and phosphorus-containing spot similar to that of sphingosylphosphorylcholine, but in the ethanol-

aq. NH_3 soln. solvent it remained at the origin, whereas the diester isolated from the acid hydrolysate had an R_f of about 0.2. The compound was insoluble both in CHCl_3 and water and, when shaken with these two solvents and centrifuged, it formed a white pellicle between the two layers of solvent. On dissolving this in CHCl_3 -methanol (1:1, v/v) it could be reprecipitated with 3 vol. of acetone. On drying, the product became resinous and brown, and all attempts to crystallize it were unsuccessful. Two successive preparations had P:N molar ratios of 1:0.97 and 1:0.96, and on total hydrolysis in 5N-HCl at 100° such that all the phosphorus was converted into the inorganic form, no free choline could be detected in the hydrolysate. Although the identity of the compound was not ascertained it was clearly not sphingosylphosphorylcholine. The phosphorus content of the preparations (4.6, 5.0%) was too low for that of sphingosine phosphate. No evidence was obtained that the methanolic-KOH hydrolysate of sphingomyelin contained more than traces of water-soluble phosphorus-containing hydrolysis products, and sphingosylphosphorylcholine could not be detected.

DISCUSSION

The finding that glycerylphosphorylcholine is present as the main component in the fraction described by other workers as sphingosylphosphorylcholine is perhaps not surprising, as this former compound has comparatively recently been described as a constituent of animal tissues (King & Aloisi, 1945; Schmidt, Hershman & Thannhauser, 1945; Schmidt, Greenbaum, Fallot, Walker & Thannhauser, 1955). Moreover, free phosphorylcholine is known to be widely distributed in appreciable concentration in various tissues (Dawson, 1956). Both these phosphate esters are relatively soluble in water and ethanol and are not precipitated from aqueous solutions by common heavy-metal ions such as Ba^{2+} , Hg^{2+} or Pb^{2+} . Moreover, the present investigation has shown them to be precipitated from ethanolic solution with mercuric chloride, one of the purification steps used in the Booth (1935) procedure. King & Aloisi (1945) found that glycerylphosphorylcholine was isolated from dried ox pancreas by a method similar to that which yielded 'sphingosylphosphorylcholine' from fresh pig pancreas, and Schmidt *et al.* (1945) suggested that glycerylphosphorylcholine, together with a nitrogenous impurity, might account for the material in the fraction isolated by King & Small (1939).

Neither Booth (1935) nor King & Small (1939) positively identified sphingosine in their samples, but at the time no method was available for the estimation or detection of sphingosine, nor was

paper chromatography available. The suggestion that sphingosine was present was made by Booth (1935) because of the total N and amino-N analysis of the isolated samples, which gave an N:P molar ratio of 2:1; in the present work a puzzling factor has been that the N:P molar ratios of the equivalently isolated fractions were lower than 2:1. Chromatographic results of the present paper suggest that sphingosylphosphorylcholine is not present in any of the animal tissues examined in any appreciable concentration, but its presence as a very minor constituent cannot be excluded.

The failure to confirm the chromatographic findings of Rouser *et al.* (1953) of the presence of sphingosylphosphorylcholine in alkaline hydrolysates of sphingomyelin is somewhat puzzling. The main if not the sole hydrolysis product, although ninhydrin-reacting and phosphorus-containing, contains no choline and possesses equimolar proportions of nitrogen and phosphorus. No chromatographic spot equivalent to sphingosylphosphorylcholine was detectable in the hydrolysate. It is suggested that a complete chemical investigation of the alkaline hydrolysis of sphingomyelin is necessary before the confusion in this subject can be clarified.

SUMMARY

1. Fractions of animal tissues which were reported by other workers to contain the choline ester of sphingosine phosphoric acid were isolated by equivalent methods and analysed.

2. The fractions contained glycerylphosphorylcholine, phosphorylcholine and amino acids in varying proportions but no sphingosylphosphorylcholine.

3. Sphingosylphosphorylcholine was not detected chromatographically in the water- and methanol-soluble phosphate esters extracted from tissues.

4. Methanolic hydrochloric acid treatment of sphingomyelin yielded phosphorylcholine and a small amount of sphingosylphosphorylcholine. Methanolic-potassium hydroxide hydrolysis gave an unidentified water- and chloroform-insoluble compound with phosphorus and nitrogen in equi-

molar proportions: no sphingosylphosphorylcholine was detected in the alkaline hydrolysate.

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