THE MEASUREMENT OF ^{32}P LABELLING OF INDIVIDUAL KEPHALINS AND LECITHIN IN A SMALL SAMPLE OF TISSUE

by

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There is at present no method of lipid fractionation available which can be used for measuring the ^{32}P labelling of individual phospholipids present in a small sample of tissue. However, it has recently been found possible to measure the labelling of some of the phospholipids by isolating identifiable fragments of their molecules after they had been subjected to various hydrolytic procedures (NORMAN AND DAWSON[1], DAWSON[2]). Using this same principle, a method has now been developed whereby the ^{32}P labelling of phosphatidyl ethanolamine, phosphatidyl serine, phosphatidyl choline and also diphosphoinositide can be quickly measured in a single small sample of tissue (150–400 mg). The lipids isolated from the tissue are hydrolysed with methanolic KOH, and after the removal of alkali and fatty acids, the breakdown products of the phospholipids are completely resolved by two-dimensional filter paper chromatography.

The method has been used to measure the degree of labelling of the phospholipids present in a guinea-pig brain dispersion after this had been incubated under conditions which ensured a lively uptake of labelled phosphate into its lipid fraction. The results confirm that in such dispersions there is a rapid synthesis of diphosphoinositide and of a lipid which is probably phosphatidic acid, while the incorporation of ^{32}P into lecithin and the other kephalins is negligible (DAWSON[2]).

METHODS

The labelled sample of tissue (150–400 mg according to its phospholipid content) was precipitated with ice-cold 5% trichloroacetic acid solution, and the lipids extracted from the precipitate by a method described previously (DAWSON[3]). The extraction with chloroform/methanol was carried out for one hour by which time over 95% of the solvent-soluble lipids were in solution. Contaminating acid-soluble phosphorus was removed from the extract by shaking for 2 min with 2 volumes of ice-cold 0.1 *N* HCl. The $CHCl_3$ layer which separated was clarified from suspended aqueous phase by centrifuging and taken to dryness *in vacuo* at room temperature. The lipid residue was then taken up in 0.8 ml of carbon tetrachloride and to the solution was added 6.3 ml of methanol and 0.9 ml of water. The mixture was then treated with 2.0 ml *M* methanolic NaOH and immediately incubated at 37° for 15 min. After cooling in ice the hydrolysate was diluted with 16 ml of ice-cold water and the milky fluid quickly passed through a column of Amberlite IRC 50 resin (7 cm × 0.8 cm^2). After washing through with 10 ml of water the ice-cold hydrolysate which now gave a slightly acid reaction (pH 6) was shaken successively with 25 ml portions of carbon tetrachloride, ether and petroleum ether. The aqueous phase which still remained turbid was shaken with 2 vols of *iso*butyl alcohol,

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References p. 379.

which removed the turbidity, and also considerably reduced the volume of the hydrolysate. The aqueous solution was filtered, neutralized to pH 7 with ammonia and taken to dryness under reduced pressure below 50°. The residue was taken up in a little water and applied to a filter paper chromatogram which was developed in two dimensions with phenol/NH_3 (18 h) and *tert*-butanol/trichloroacetic acid (20 h) solvents. The spots were located by spraying with the acid-molybdate reagent of HANES AND ISHERWOOD[4] and irradiation with ultraviolet light (BANDURSKI AND AXELROD[5]).

Phosphorus and radioactivity determinations

The phosphorus contained in the spots was brought into solution by a technique described in a previous paper (ANSELL AND DAWSON[6]). Radioactivity of the solution was measured in a liquid counter tube as previously reported (DAWSON AND RICHTER[7]). Phosphorus was estimated by the method of FISKE AND SUBBAROW[8]. It was shown that quantitative recoveries of phosphorus were obtained when known amounts of inorganic phosphate were spotted on paper, sprayed with acid-molybdate and then irradiated with ultraviolet light before they were assayed for phosphorus.

Chromatographic solvents

Phenol/NH_3; phenol A.R. saturated with 0.1% w/v NH_3/solution.

Butanol/trichloroacetic acid; tert-butanol, water 62/38 v/v, trichloroacetic acid 10% w/v. After using this solvent, the chromatogram was dried in air and trichloroacetic acid removed by washing the chromatogram in ether.

Propanol/ammonia; (HANES AND ISHERWOOD[4]).

Propanol/formic acid; n-propanol: formic acid (88%) water (80/15/5; v/v).

Ethylene glycol/butanol; (LEVINE AND CHARGAFF[9]).

Descending chromatography was used for the phenol/NH_3 solvent and ascending for the other solvents. Whatman No. 1 filter paper sheets were well washed with 2 *N* acetic acid before use.

RESULTS

Mild alkaline hydrolysis of isolated or synthetic phospholipids

On subjecting phosphatidyl serine, phosphatidyl ethanolamine and phosphatidyl choline to mild alkaline hydrolysis, as described in the experimental section, the fatty acids were rapidly removed, leaving the glycerylphosphoryl "base" residue as the dominant phosphorus containing product. After a short period of hydrolysis only traces of free glycerophosphoric acid could be detected in the hydrolysate.

Phosphatidyl ethanolamine

Synthetic DL-α-dimyristyl kephalin and phosphatidyl ethanolamine isolated from ox brain gave on mild alkaline hydrolysis a water-soluble phosphorus-containing derivative of ethanolamine. This derivative was inseparable from synthetic L-α-glycerylphosphorylethanolamine on paper chromatography in propanol /NH_3 (R_F 0.42) propanol/formate (R_F 0.08) ethylene glycol/butanol (R_F 0.28) phenol/NH_3 (R_F 0.62). On hydrolysis in acid (18 min, *N* HCl, 100°) the derivative was completely decomposed and ethanolamine and glycerophosphoric acid were chromatographically detected in the hydrolysate. The yield of glycerylphosphorylethanolamine obtained from a phenol/NH_3 chromatogram after 15 minutes alkaline hydrolysis of the synthetic kephalin was 55% of theoretical. The yield did not change appreciably on prolonging the hydrolysis to 3 hours.

Phosphatidyl choline

Mild alkaline hydrolysis of synthetic DL-α-dipalmityl lecithin yielded a phosphorus derivative which was inseparable from α-glycerylphosphorylcholine (prepared from ox pancreas by the method of SCHMIDT, HERSHMAN AND THANNHAUSER[10]) on chromato-

References p. 379.

graphy in the following solvents: propanol /NH_3 (R_F 0.41) propanol/formate (R_F 0.06) ethylene glycol/butanol (R_F 0.26) phenol/NH_3 (R_F 0.88). The derivative was water-soluble and on hydrolysis for 15 min. at *N* HCl at 100° it was completely decomposed, giving glycerophosphoric acid which was identified chromatographically, and choline which was precipitated as its characteristic reineckate. After 15 min. alkaline hydrolysis 84% of the theoretical yield of glycerylphosphorylcholine was obtained, but this decreased somewhat on prolonging the hydrolysis.

Phosphatidyl serine

When phosphatidyl serine isolated from ox brain was mildly hydrolysed with alkali, there appeared in the hydrolysate a water-soluble phosphorus derivative of serine. When this was hydrolysed with *N* HCl (1 h, 100°) it was completely decomposed and serine and glycerophosphoric acid were chromatographically identified in the hydrolysate. From this evidence and by analogy with the behaviour of phosphatidyl choline and phosphatidyl ethanolamine on alkaline hydrolysis, there seems little doubt that the derivative is glycerylphosphorylserine. About 55% of the phosphorus of the original phosphatidyl serine was recovered as "glycerylphosphorylserine" after 15 min alkaline hydrolysis; the yield was smaller after longer periods of hydrolysis.

Phosphatidic acid

A sample of phosphatidic acid was prepared from cabbage leaves by the method of CHIBNALL AND CHANNON[11]. This method depends upon the activity of a recently discovered enzyme which splits off the bases from phospholipids present in the cabbage (HANAHAN AND CHAIKOFF[12]). On mild alkaline hydrolysis of phosphatidic acid for 15 minutes a water-soluble phosphorus derivative was split off with a minimum phosphorus yield of 50%. The derivative was stable when heated for 2 h in *N* HCl at 100°, and was inseparable from α-glycerophosphoric acid on paper chromatography in propanol /NH_3 (R_F 0.18); propanol/formate (R_F 0.09); ethylene glycol/butanol (R_F 0.29) and phenol/NH_3 (R_F 0.19).

Diphosphoinositide

The dominant water-soluble phosphorus-containing derivative obtained after the mild alkaline hydrolysis of pure K diphosphoinositide was found on chromatography to run a little slower than "glycerylphosphorylserine" in both phenol/NH_3 and butanol/trichloroacetic acid solvents. The structure of the derivative was not established, although in these solvents it had R_F-values which are similar to those of inositolmetadiphosphate. However, although the structure of diphosphoinositide has not yet been completely established it is known to contain glycerol and it seems likely, therefore, that the release of fatty acids on alkaline hydrolysis may initially produce a phosphorus-containing breakdown product containing a glycerol residue. After 15 min. mild alkaline hydrolysis, 50% of the phosphorus of diphosphoinositide was recovered from the spot formed by the derivative on a two-dimensional chromatogram developed with phenol/NH_3 and butanol/trichloroacetic acid.

Mild alkaline hydrolysis of lipid extracts from tissues

Mild alkaline hydrolysis has been carried out on samples of liver and brain lipids obtained from the guinea-pig and rat. Fig. 1 illustrates the type of chromatogram

References p. 379.

obtained after the acid-soluble phosphorus had been separated on the paper with phenol/NH_3 and butanol/trichloroacetic acid solvents. The parent phospholipid responsible for each spot was identified by carrying out hydrolysis experiments with single isolated phospholipids and known mixtures of these. Spraying with ninhydrin before the acid-molybdate reagent also helped in the location of "glycerylphosphorylserine" and glycerylphosphorylethanolamine. The glycerylphosphorylcholine and glycerylphosphorylethanolamine are seen to be well separated from the other phosphorus-containing hydrolysis products in the phenol/NH_3 run. The butanol/trichloroacetic acid solvent was the only one of a number tried which adequately separated "glycerylphosphorylserine", glycerophosphoric acid and the phosphorus-containing hydrolysis product of diphosphoinositide. It seems likely that owing to the slow running and strongly acid nature of this solvent a little decomposition of the acid-labile glycerylphosphoryl derivatives of serine, ethanolamine and choline may occur during the development of the chromatogram. This, however, will not affect the specific activities of the phosphorus contained

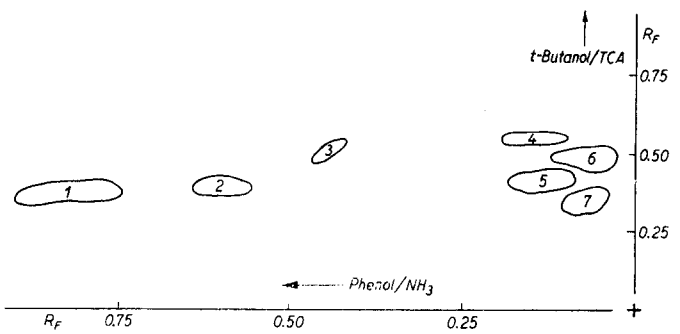

Fig. 1. Tracing of a paper chromatogram of the P-containing spots in an alkaline hydrolysate of the lipids extracted from a guinea-pig brain dispersion. *P-containing spots:* 1. Glycerylphosphorylcholine; 2. Glycerylphosphorylethanolamine; 3. Unidentified (also seen in hydrolysates of rat liver lipids); 4. Glycerophosphoric acid; 5. Glycerylphosphorylserine; 6. Unidentified (also seen in hydrolysates of rat liver lipids); 7. Hydrolysis product of diphosphoinositide.

in their respective spots. Free glycerophosphate was never found in aklaline hydrolysates of the lipids extracted from fresh tissue which would suggest that phosphatidic acid is either absent or only present as a trace constituent of the lipid fraction. Inorganic phosphorus which contaminates crude lipid extracts shows as a yellow spot on the chromatogram immediately after spraying with the acid-molybdate reagent. This was not however seen on chromatograms obtained after the alkaline hydrolysis of purified lipid extracts obtained from trichloroacetic acid precipitates of tissue.

Application of the method of alkaline hydrolysis to determine the specific activities of the phospholipids present in a labelled brain dispersion

A guinea-pig brain dispersion was prepared and labelled with ^{32}P by a method previously described (DAWSON[3]). The comparative specific activities of the phospholipids were measured after a purified lipid extract of the dispersion had been hydrolysed with alkali and the hydrolysis products separated by two-dimensional chromatography. It is seen (Table I) that in such dispersions which had actively incorporated labelled phosphate into their lipids there was little or no deposition of ^{32}P in phosphatidyl serine, phosphatidyl ethanolamine and phosphatidyl choline. On the other hand there was an appreciable synthesis of diphosphoinositide. The glycerophosphate spot on the chromatogram which is probably derived mainly from phosphatidic acid present in the dispersion also had a high specific radioactivity. The results of a previous investigation (DAWSON[1]) using similar tissue preparations but different methods of measuring specific radio-

References p. 379.

activities are included in Table I for comparison, and it is seen that a good measure of agreement was obtained.

TABLE I

SPECIFIC RADIOACTIVITIES OF PHOSPHOLIPIDS IN A LABELLED GUINEA-PIG BRAIN DISPERSION COMPARED WITH THE SPECIFIC ACTIVITY OF THE WHOLE LIPID EXTRACT

Phospholipid	*Specific radioactivities as % of specific radioactivity of whole lipid fraction*	
	Present investigation	DAWSON[2]
Phosphatidyl choline	1	< 10
Phosphatidyl ethanolamine	2	< 5
Phosphatidyl serine	3	< 5
Diphosphoinositide	960	950
"Glycerophosphate spot" phosphatidic acid?	4,340	—

DISCUSSION

It was concluded in a previous paper that a guinea-pig brain dispersion incubated under favourable conditions was able to synthesise both diphosphoinositide and another acidic glycerophosphate-containing lipid, although it had little capacity for labelling the phosphorus in the other kephalins or lecithin (DAWSON[2]). These results have been confirmed using the method of mild alkaline hydrolysis described in the present paper for measuring the specific radioactivities of the phospholipids.

It is interesting that the small quantity of glycerophosphate present in the alkaline hydrolysate prepared from the lipids of a labelled brain dispersion had a high specific radioactivity. This high specific radioactivity cannot be due to glycerophosphate originating from phosphatidyl serine, phosphatidyl ethanolamine, or phosphatidyl choline as their specific radioactivities are less than 1% of that of the glycerophosphate. Moreover the glycerophosphate is not likely to be derived from plasmalogens as these like sphingomyelin are reported to be stable on alkaline hydrolysis (FEULGEN AND BERSIN[13]). It would therefore appear probable that this high specific activity glycerophosphate had originated from phosphatidic acid present in the dispersion. This lipid has been shown in the present investigation to yield glycerophosphate on alkaline hydrolysis. KORNBERG AND PRICER[14] have recently isolated an enzyme from liver which is capable of condensing activated long chain fatty acids, with glycerophosphate to give phosphatidic acid. The present results would therefore suggest that a similar enzyme is present in brain tissue, and that when isolated brain tissue is incubated with labelled phosphate this enzyme is responsible for most of the incorporation of ^{32}P into the lipid fraction.

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References p. 379.

SUMMARY

1. A method has been devised which enables the specific radioactivities of phosphatidyl ethanolamine, phosphatidyl serine and phosphatidyl choline, and also diphosphoinositide, to be determined in a single small sample of tissue (150–400 mg) which has been labelled with ^{32}P.

2. A lipid extract of the tissue is hydrolysed under mild alkaline conditions, and identifiable phosphorus-containing hydrolysis products of the phospholipids are completely resolved by two-dimensional filter paper chromatography.

3. The method has been used to examine a lipid extract prepared from a guinea-pig brain dispersion after incubation with labelled phosphate.

4. In such dispersions there is little incorporation of ^{32}P into phosphatidyl ethanolamine, -serine or, -choline, while there is an appreciable synthesis of diphosphoinositide and a lipid-solvent soluble substance which is probably phosphatidic acid.

RÉSUMÉ

1. L'auteur décrit une méthode qui permet la détermination, sur un petit échantillon d'un tissu (150–400 mg) marqué par ^{32}P, des radioactivités spécifiques de la phosphatidyléthanolamine, de la phosphatidylsérine et de la phosphatidylcholine, ainsi que du diphosphoinositide.

2. Un extrait lipidique du tissu est hydrolysé dans des conditions alcalines douces et les produits d'hydrolyse identifiables contenant du phosphore, issus des phospholipides, sont complètement séparés par chromatographie bidimensionnelle sur papier.

3. La méthode a servi à l'examen d'un extrait lipidique préparé à partir d'une dispersion de cerveau de cobaye après incubation avec du phosphate marqué.

4. Dans ces dispersions, l'incorporation de ^{32}P dans les phosphatidyléthanolamine, -sérine ou -choline est faible. Au contraire il se produit une synthèse appréciable de diphosphoinositide et d'une substance soluble dans les solvants des lipides qui est probablement l'acide phosphatidique.

ZUSAMMENFASSUNG

1. Eine Methode wurde entwickelt, welche es ermöglicht in einer einzigen kleinen Gewebsprobe (150–400 mg), welche mit ^{32}P gezeichnet wurden, die spezifische Radioaktivität von Phosphatidyl-Ethanolamin, -Serin und -Cholin und auch von Diphosphoinosit zu bestimmen.

2. Ein Fettextrakt des Gewebes wird unter milden alkalischen Bedingungen hydrolysiert. Die identifizierbaren phosphorhaltigen Hydrolyseprodukte der Phospholipoide werden durch zweidimensionale Filterpapierchromatographie vollständig getrennt.

3. Die Methode wurde benutzt um einen Fettextrakt zu prüfen, der aus Meerschweinchen-Gehirn-Dispersion hergestellt war, die mit gezeichnetem Phosphat inkubiert war.

4. In solchen Dispersionen wird wenig ^{32}P eingebaut in Phosphatidyl-Ethanolamin, -Serin und -Cholin, während beträchtlich synthetisiert werden sowohl Diphosphoinosit als auch eine fettlösende Substanz, die in Fettlösungsmitteln löslich und wahrscheinlich Phosphatidsäure ist.

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