Studies on Phospholipids

4. DETERMINATION OF ETHANOLAMINE AND SERINE*

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(Received 22 October 1957)

A reliable and sensitive method for the determination of ethanolamine and serine, the bases present in kephalin, is necessary in the study of phospholipid metabolism. The procedure described by Artom (1945) is reliable but not sensitive (range 10–50 μ moles), whereas that described by Levine & Chargaff (1951) is sensitive (range 0.1–0.3 μ mole) but unreliable. The method devised by Axelrod, Reichenthal & Brodie (1953) involves the hydrolysis of the fat, the reaction of the free bases with 1-fluoro-2:4-dinitrobenzene (FDNB), the separation of the *N*-dinitrophenyl (DNP) bases and their quantitative estimation by their light absorption at a wavelength at which FDNB does not interfere. The present paper describes a method which involves the reaction of the lipids with FDNB, the complete removal of excess of reagents, the hydrolysis of the DNP-lipids and the determination of DNP-ethanolamine and DNP-serine by a differential colour reaction which does not involve their separation.

MATERIALS

All solvents used were reagent grade. The tetramethylammonium hydroxide was used as supplied as a 25% (w/v) solution in water. DNP-ethanolamine and DNP-serine were prepared as described by Wheeldon & Collins (1957).

N-(2:4-Dinitrophenyl)-ethanolamine-*O*-phosphoric acid (423 mg.) (Californian Foundation for Biochemical Research) with FDNB (700 mg.) and NaHCO₃ (1 g. in 20 ml. of water). The mixture was left at 20° for 16 hr. in the dark and extracted with diethyl ether. The aqueous layer was cooled and acidified with 10*N*-HCl until an oil separated. This solidified at -10° and crystallized from ethanol. The yield was 0.24 g., m.p. 163–165° (uncorr.) (Found: C, 31.7; H, 3.4; N, 13.0; P, 9.6. C₈H₁₀O₈N₂P requires C, 31.3; H, 3.3; N, 13.7; P, 10.1%). In 0.1*N*-HCl $\lambda_{\max}$ was at 350 m μ (ϵ =15 500). On paper chromatography a single spot was obtained.

N-(2:4-Dinitrophenyl)-serine-*O*-phosphoric acid. This was prepared by treating serine-*O*-phosphoric acid monohydrate (406 mg.) (Californian Foundation for Biochemical Research) with FDNB (1 g.) and NaHCO₃ (1.5 g. in 30 ml. of water). The mixture was left for 16 hr. at 20° in the dark.

The solution was then extracted with diethyl ether to remove excess of FDNB, acidified (pH 5) with 10*N*-HCl and concentrated to a syrup *in vacuo*. The free acid did not crystallize readily but on the addition of an equimolecular amount of triethylamine the salt was crystallized from propan-2-ol. The yield was 0.40 g., m.p. 147° (uncorr.) (Found: C, 39.1; H, 5.9; N, 12.0; P, 7.2. C₉H₁₀O₁₀N₂P, C₉H₁₂N requires C, 39.9; H, 5.5; N, 12.4; P, 6.9%). In 0.1*N*-HCl $\lambda_{\max}$ was at 350 m μ (ϵ =14 900). On paper chromatography a single spot was obtained.

METHODS

The extraction of the lipids and their dinitrophenylation was carried out as described by Wheeldon & Collins (1957).

Paper chromatography. Ascending chromatography of the DNP-bases was carried out on Whatman no. 1 paper either with *m*-NaH₂PO₄ and 0.5*M*-Na₂HPO₄ (Levy, 1954) or with *tert*-pentanol-pyridine-water (5:1:5, by vol.).

RESULTS

Hydrolysis of N-(2:4-dinitrophenyl)-lipids. The most satisfactory medium for hydrolysis of the DNP-lipids was ethanolic *N*-HCl; hydrolysis was complete in 3 hr. at 100°. Table 1 shows that the recoveries of total DNP-bases after hydrolysis of DNP-lipids were quantitative. The hydrolysis procedure was complicated by the fact that DNP-serine in ethanolic *N*-HCl formed the ethyl ester and that DNP-serine was light-sensitive. The

Table 1. *Recovery of N*-(2:4-dinitrophenyl)-bases after the hydrolysis of *N*-(2:4-dinitrophenyl)-lipids

DNP-lipids were hydrolysed by heating with *N*-HCl in ethanol for 3 hr. The amount of DNP-bases was determined from the light absorption; DNP-lipids were measured in light petroleum and the free DNP-bases were measured in water after removing the fatty acids.

Lipid	DNP-base (μ moles)		Recovery (%)
	Before hydrolysis	After hydrolysis	
Sheep brain	6.0	5.9	98
Sheep brain	32.6	32.3	99
Guinea-pig brain	60.5	61.3	101
Rat liver	3.7	3.4	92
Rat liver	8.3	8.7	105

* Part 3: Wheeldon & Collins (1958).

solutions were shielded from light. As the ethyl ester of DNP-serine could not be distinguished from DNP-ethanolamine in the colour reaction to be described the initial hydrolysis in ethanolic $N\text{-HCl}$ was followed by hydrolysis in aqueous $N\text{-HCl}$. The bases present, after hydrolysis, were identified by paper chromatography. In no case were there more than 2 % of DNP-compounds other than DNP-ethanolamine and DNP-serine, and these impurities were in the positions expected for the corresponding phosphoric acid derivatives. The amounts present were estimated by cutting out the spots, and eluting with water and measuring the absorption at $350\text{ m}\mu$. Table 2 gives the R_F values for the DNP-bases and the phosphoric acid derivatives.

Colour reaction for $N\text{-(2:4-dinitrophenyl)-bases}$. Porter (1955) described the reaction between tetramethylammonium hydroxide dissolved in dimethylformamide and various nitroderivatives of aromatic compounds. DNP-ethanolamine and DNP-serine in this system gave rise to coloured ions with λ_{max} at $430\text{ m}\mu$ having dissociation constants of 0.1 and 6 mM respectively and which on the addition of 20 % (v/v) ethanol became 3 mM and 0.2M respectively. Fig. 1 shows the effect of varying concentrations of tetramethylammonium hydroxide on the values of the molecular-extinction coefficient at $500\text{ m}\mu$. At a concentration of quaternary base of 27 mM the DNP-ethanolamine is completely ionized whereas the DNP-serine is only 10 % ionized, and this difference forms the basis of the present method. Fig. 2 shows the absorption spectra of the two DNP-bases under these conditions and demonstrates that $500\text{ m}\mu$ is a convenient wavelength to use. Measurements at $393\text{ m}\mu$, where the two compounds have the same molecular-extinction coefficient, enable the combined concentration of the DNP-bases to be calculated.

The reliability of the colorimetric procedure depends on the concentration of tetramethylammonium hydroxide being the same for unknown and for standards. In a hydrolysate of a DNP-lipid the

Fig. 1. Colour development and concentration of quaternary base. Molecular-extinction coefficients at $500\text{ m}\mu$ of DNP-ethanolamine and DNP-serine were measured in dimethylformamide-ethanol (4:1, v/v) and known amounts of tetramethylammonium hydroxide. Theoretical curves have been calculated assuming a dissociation constant of 3 mM for DNP-ethanolamine (upper curve) and of 0.2M for DNP-serine (lower curve); experimental values are indicated by the circles.

Fig. 2. Absorption spectra of DNP-ethanolamine and DNP-serine in 27 mM-tetramethylammonium hydroxide. DNP-ethanolamine (curve A) and DNP-serine (curve B) were dissolved in a mixture of dimethylformamide-ethanol-*n*-butylamine (4:1:0.04, by vol.).

Table 2. R_F values for some $N\text{-(2:4-dinitrophenyl)-bases}$ related to $N\text{-(2:4-dinitrophenyl)-lipids}$

The solvent system used was A, that described by Levy (1954) or B, *tert*-pentanol-pyridine-water (5:1:5, by vol.). Whatman no. 1 paper was used in an ascending system. Time of run, 18 hr.

	R_F in solvent system	
	A	B
DNP-ethanolamine	0.28	0.90
DNP-serine	0.46	0.38
DNP-ethanolamine- <i>O</i> -phosphoric acid	0.60	0.24
DNP-serine- <i>O</i> -phosphoric acid	0.80	0.10

water-soluble fraction will contain, in addition to the DNP-bases, phosphoric acid, glycerophosphoric acid, inositol phosphoric acid, choline phosphate or chloride and free hydrochloric acid. The last component is removed by evaporation *in vacuo*. If the concentration of tetramethylammonium hydroxide were to change by 10 %, i.e. from 27 to 24 mM, it can be calculated from the equations given below and from the data in Fig. 1 that the error introduced into the method will not exceed 2 %. This change in concentration could be caused by the presence of 30 $\mu\text{g.}$ equivalent of acid (or 15 $\mu\text{g.}$ atoms of P) in the hydrolysate, and the sample of hydrolysate taken for analysis should not contain more than 15 $\mu\text{g.}$ atoms of P.

The addition of 0.8 % (v/v) of *n*-butylamine to the solvent system was found to assist in buffering the solution.

Determination of ethanolamine and serine in lipids

On the basis of the foregoing results the following procedure is recommended. The lipids are obtained from the tissues and dinitrophenylated as described by Wheeldon & Collins (1957). A quantity of DNP-lipid which corresponds to between 0.1 and 1.5 μmoles of amino N is dissolved in 5 ml. of ethanolic *N*-HCl and hydrolysed by boiling under reflux for 3 hr. The hydrolysate is evaporated to dryness *in vacuo*, 2 ml. of *N*-HCl is added and the mixture is again refluxed for a further 3 hr. After adding about 0.1 g. of Hyflo Supercel the mixture is filtered, the filter is washed with 5 ml. of water and the filtrate is reduced to dryness by heating *in vacuo*. The residue is dissolved in 10 ml. of a mixture of dimethylformamide-ethanol-*n*-butylamine (4:1:0.04, by vol.), 0.1 ml. of 25% (w/v) tetramethylammonium hydroxide is added and, after mixing, the extinctions are measured at 393 and 500 $\text{m}\mu$. If *e* and *s* respectively represent the amounts of DNP-ethanolamine and DNP-serine in μmoles in the 10.1 ml. of solution and if E_1 and E_2 represent the extinctions respectively at 393 $\text{m}\mu$ and at 500 $\text{m}\mu$ then it follows that $E_1 = ae/1.01 + bs/1.01$ and $E_2 = ce/1.01 + ds/1.01$. The constants *a*, *b*, *c* and *d* are equal to 10^{-4} times the molecular-extinction coefficients; the mean values and standard errors (12 determinations) are: *a*, 1.13 ± 0.007 ; *b*, 1.13 ± 0.007 ; *c*, 1.23 ± 0.007 and *d*, 0.184 ± 0.006 . Hence

$$e = 0.965E_2 - 0.157E_1;$$

$$s = 1.05E_1 - 0.965E_2;$$

$$e + s = 0.894E_1$$

and

$$e/(e + s) = 1.18 - 1.08E_2/E_1.$$

The recoveries from mixtures of known amounts of DNP-ethanolamine and of DNP-serine are shown in Table 3. Known amounts of DNP-serine were added to hydrolysates of guinea-pig lipids and, as shown in Table 4, the average recovery was 104.5 %.

Reaction of other *N*-(2:4-dinitrophenyl)-compounds. The phosphoric acid derivatives of DNP-ethanolamine and DNP-serine, and dinitrophenylated lipids, gave $\lambda_{\text{max.}}$ at 365 $\text{m}\mu$ in the colour test

whereas the methyl ester of DNP-serine, and methylated and dinitrophenylated lipids (Collins & Wheeldon, 1957), gave $\lambda_{\text{max.}}$ at 430 $\text{m}\mu$.

Occurrence of ethanolamine and serine in some lipids. The proportions of ethanolamine and serine were determined on a number of lipids, with the results shown in Table 5.

DISCUSSION

The method described in this paper for the determination of serine and ethanolamine has certain features in common with the procedure described by Axelrod *et al.* (1953). Although it is as convenient to treat the lipid with FDNB before hydrolysis as afterwards (cf. Axelrod *et al.* 1953), the former procedure has the advantage that removal of the excess of reagents is readily

Table 3. Application of the colour reaction to mixtures of *N*-(2:4-dinitrophenyl)-ethanolamine and *N*-(2:4-dinitrophenyl)-serine

Known amounts of DNP-ethanolamine and DNP-serine were mixed and analysed. All figures are in μmole .

DNP-serine		DNP-ethanolamine	
Added	Found	Added	Found
0.84	0.85	0.24	0.23
0.56	0.60	0.48	0.47
0.28	0.32	0.72	0.72

Table 4. Recoveries of *N*-(2:4-dinitrophenyl)-serine added to hydrolysates of *N*-(2:4-dinitrophenyl)-lipids

DNP-lipids prepared from guinea-pig tissues were hydrolysed and the DNP-bases determined before and after adding known amounts of DNP-serine. All figures in μmole .

Lipid	DNP-ethanolamine	DNP-serine		
		In sample	Added	Recovered
Brain	0.47	0.06	0.22	0.24
Kidney	0.58	0.06	0.22	0.22
Liver	0.85	0.10	0.11	0.13
Heart	0.37	0.06	0.11	0.10

Table 5. Ethanolamine and serine contents of some lipids

All figures are $\mu\text{moles}/100 \text{ mg.}$ of lipid.

Source	Ethanolamine	Serine
Guinea-pig kidney	12	1.4
Guinea-pig liver	21	2.6
Guinea-pig heart	11	2.1
Guinea-pig brain	23	3.5
Rat brain	19	6.5
Sheep brain	24	8.9
Egg yolk	18	<0.2
A serine-rich fraction of ox-brain lipids	2.4	14

accomplished. Further, the use of a differential colour reaction is less time-consuming than alternative methods which involve separation of the two DNP-bases (cf. Axelrod *et al.* 1953).

In the rare instances where more than two DNP-bases are present, as, for example, in *Neurospora* lipids, which contain ethanolamine, serine and 1-amino-2-methylpropan-2-ol (Ellman & Mitchell, 1954), the procedure described by Levy (1954) for DNP-amino acids can be used. When this method was applied to a mixture of DNP-ethanolamine and DNP-serine the recovery was good (98 %) but the colorimetric method was quicker.

The experimental results on the application of the colour test to the DNP-bases suggest that the acidic group in the DNP-serine is responsible for the difference in the dissociation constants of the coloured forms. This is confirmed by the fact that the ethyl ester of DNP-serine reacts like DNP-ethanolamine and that DNP-ethanolamine-*O*-phosphoric acid reacts like DNP-serine. These observations also explain why the colour reaction with DNP-lipids simulates DNP-serine and methylated DNP-lipids simulate DNP-ethanolamine, as in the first case the phosphoric acid groups are free and in the second case they are in the form of methyl esters.

The procedure described in this paper was developed as an adjunct to the study of DNP-

lipids (Collins & Wheeldon, 1957) but, if used in conjunction with the method described by Wheeldon & Collins (1957) for total amino N, should be of wide applicability.

SUMMARY

1. A method depending on a differential colour reaction for the determination of ethanolamine and serine in phospholipids is described.

2. The advantages of the method, as compared with those of other methods, are discussed.

Our thanks are due to Mrs M. Labutis for her technical assistance.

REFERENCES

- Artom, C. (1945). *J. biol. Chem.* **157**, 585.
Axelrod, J., Reichenthal, J. & Brodie, B. B. (1953). *J. biol. Chem.* **204**, 903.
Collins, F. D. & Wheeldon, L. W. (1957). *Biochem. J.* **66**, 441.
Ellman, G. L. & Mitchell, H. K. (1954). *J. Amer. chem. Soc.* **76**, 4028.
Levine, C. & Chargaff, E. (1951). *J. biol. Chem.* **192**, 465.
Levy, A. L. (1954). *Nature, Lond.*, **174**, 126.
Porter, C. C. (1955). *Analyt. Chem.* **27**, 805.
Wheeldon, L. W. & Collins, F. D. (1957). *Biochem. J.* **66**, 435.
Wheeldon, L. W. & Collins, F. D. (1958). *Biochem. J.* **70**, 43.

Substrates for Rumen β -Glucosidase

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(Received 27 January 1958)

Substrates used for β -glucosidase include natural products such as cellobiose and salicin as well as synthetic compounds like the nitrophenyl glucosides (Aizawa, 1939; Conchie, 1954). Enzyme extracts from single micro-organisms have been shown to vary in their specificities. Jermyn (1952) separated eight components in an *Aspergillus oryzae* extract, with varying degrees of specificity towards different substrates, whereas a *Stachybotrys atra* preparation hydrolysed *p*-nitrophenyl β -glucoside but not *o*-nitrophenyl β -glucoside or cellobiose (Jermyn, 1955). Differences in relative cellobiase and salicinase activities in preparations from different sources were observed by Grassmann, Zechmeister, Tóth & Stadler (1933) and Reese & Levinson (1952). Barnett, Ingram & Swain (1956) studied the β -glucosidase activity of different yeasts and found that all split aesculin,

but many would not split any one of arbutin, salicin or cellobiose. In the present investigation *o*-nitrophenyl β -glucoside, cellobiose and salicin have been used in a comparative study of β -glucosidase activity from cell-free extracts of mixed micro-organisms from sheep rumen.

EXPERIMENTAL

Materials. *o*-Nitrophenyl β -D-glucoside was kindly provided by Dr J. Conchie. Cellobiose (shown to be chromatographically pure) and glucono-1:4-lactone were obtained from L. Light and Co. Ltd., salicin from Hopkins and Williams Ltd., glucose oxidase from Sigma Chemical Co., and peroxidase from C. F. Boehringer und Soehne.

β -Glucosidase preparations. These were produced by butanol extraction of sheep-rumen micro-organisms centrifuged at 1500 g, as described previously (Festenstien, 1958).