Enzymic Hydrolysis of Sphingomyelin by Rat Liver

By M. HELLER AND B. SHAPIRO

Department of Biochemistry, Hebrew University-Hadassah Medical School, Jerusalem, Israel

(Received 16 August 1965)

1. An enzyme that hydrolyses sphingomyelin to ceramide (*N*-acylsphingosine) and phosphorylcholine was isolated from rat liver. 2. The enzyme is particle-bound (mitochondria or lysosomes) and can be solubilized by ultrasonic treatment and freezing and thawing. 3. It has been partially purified by precipitation at pH 5.2, neutralization and ammonium sulphate fractionation. 4. The enzyme is activated by Triton X-100 (0.2%) or low concentrations of cetyltrimethylammonium bromide (0.02%), higher concentration being inhibitory. 5. The optimum pH is 5.5. 6. Of synthetic substrates tested, the *erythro* isomers of *DL-trans*-2-*N*-palmitoyl-1-*O*-phosphorylcholinesphingosine or dihydrosphingosine were hydrolysed at a rate similar to the natural compound. The *threo* isomer was hydrolysed much more slowly. The enzyme had little activity on lecithin. 7. The split products of the hydrolysis have little inhibitory effect.

The hydrolysis of sphingomyelin by enzymes derived from animal tissues has not been studied in detail. This is in contrast with the considerable information gained on the hydrolysis of lecithin and other glycerophosphatides (Dawson, 1962; Kates, 1960).

Enzymes that split sphingomyelin so far described belong to the group of phospholipases C (EC 3.1.4.3). These enzymes degrade sphingomyelin to phosphorylcholine and ceramide (*N*-acylsphingosine). They also split glycerophosphatides, producing diglyceride and a phosphoryl base. Phospholipases C were found mainly in bacteria and have been isolated from *Clostridium welchii* and *Bacillus cereus* (Kates, 1960). A specific sphingomyelin-hydrolysing enzyme has been identified in the strain $\alpha\beta$ of *Staphylococcus pyogenes* (Doery, Magnusson, Cheyne & Gulasekharan, 1963). The phospholipase C activity is calcium-dependent (Macfarlane & Knight, 1941). Ether accelerates considerably the rate of hydrolysis of the phosphatides (Hanahan & Vercamer, 1954). The purified enzyme from *C. welchii* is isoelectric at pH 5.5, and requires positively charged ions or positive amphipathic compounds for maximal activity (Bangham & Dawson, 1962). The existence of an enzyme in calf liver splitting sphingomyelin into ceramide, phosphoric acid and choline has been reported (Thannhauser & Reichel, 1940; Thannhauser & Boncoddio, 1946). A similar enzyme was described in pig spleen (Fujino, 1952). In both cases, activity was very low and prolonged incubation times were necessary to obtain significant hydrolysis. This paper deals with the isolation of an enzyme from

rat liver which hydrolyses sphingomyelin (sphingomyelinase), its partial purification, properties and specificity. A preliminary account has been published (Heller & Shapiro, 1963).

EXPERIMENTAL

Materials

Sphingomyelin was prepared from various tissues, after extraction with chloroform and methanol according to Folch, Lees & Sloane-Stanley (1957). The lipid material, obtained after evaporation of the organic layer, was subjected to mild alkaline hydrolysis with aq. *N*-NaOH at 37° for 16–24 hr. with continuous shaking, according to Thannhauser, Benotti & Boncoddio (1946), or in methanolic *N*-NaOH for 2 hr. at 37°. After the hydrolysis, which affects only the glycerolipids, the sphingomyelin was purified by silicic acid column chromatography (Hanahan, 1960). The sphingomyelin thus obtained gave rise to two spots (depending on the fatty acid residue) when chromatographed on thin layers of silicic acid according to Mangold (1961). Analysis of the sphingomyelin constituents according to Sweeley (1963), and to Bötter, Preis & Vant Gent (1961), gave the following ratios: choline/P, 1.0; sphingosine/P, 0.77; amide bond/P, 0.9.

The synthetic sphingomyelins, *N*-palmitoyl derivatives of *D-erythro*-sphingomyelin and *DL-erythro*-dihydrosphingomyelin, and *DL-threo*-dihydrosphingomyelin, were a gift from Professor D. Shapiro of the Weitzman Institute, Rehovoth, Israel.

Lecithin was prepared from egg yolk according to Pangborn (1951), or from rat livers by silicic acid column chromatography (Hanahan, 1960). Its purity was confirmed by thin-layer chromatography and by ester/P ratio. Aqueous suspensions of the phosphatides were prepared by ultrasonic treatment at 20 key/sec. (Branson Instruments

Inc.) for several minutes at 0–4°. The suspensions were stable when kept in glass-stoppered tubes in the refrigerator at concentrations below 0.01 M.

Phosphatidic acid was prepared from egg lecithin by the action of phospholipase D (EC 3.1.4.4) according to Kates (1956).

Phosphorylcholine and *p*-nitrophenyl phosphate were purchased from the Sigma Chemical Co. (St Louis, Mo., U.S.A.). Triton X-100 was obtained from City Chemical Corp. (New York, N.Y., U.S.A.). Cetavlon (cetyltrimethylammonium bromide) was from British Drug Houses Ltd. (Poole, Dorset).

Methods

Thin-layer chromatography. The phosphatides were separated on chromatoplates prepared according to Mangold (1962), with the solvent system chloroform-methanol-water (65:25:4, by vol.) (Wagner, Hörhammer & Wolff, 1961). Ceramides were chromatographed with chloroform-methanol-acetic acid (95:1:5, by vol.). Phosphorylcholine was identified on thin-layer chromatography by the solvent system described by Skidmore & Entenman (1962). The spots were made visible by iodine vapour or by sulphosalicylic acid reagent. Protein determinations were carried out according to Lowry, Rosebrough, Farr & Randall (1951), with bovine serum albumin as standard. Phosphorus was estimated by a slight modification to the methods of Fiske & Subbarow (1925) and Bartlett (1959) and nitrogen was determined by a slight modification to the method of Lang (1958).

Tissue fractionations. Rat livers were homogenized in 4 vol. of 0.25 M-sucrose in 1 mM-tris-HCl buffer, pH 7.4. These homogenates were subjected to differential centrifugation, according to the scheme suggested by de Duve, Pressman, Gianetto, Wattiaux & Appelmans (1955). The centrifugations were performed with a Servall centrifuge, rotor no. SS-34, at 0–4°. The nuclear fraction (N) was obtained by centrifugation of the homogenate at 600 *g* for 10 min. The heavy-mitochondrial fraction (M) was obtained by centrifuging the nuclear supernatant for 10 min. at 3500 *g*. The light-mitochondrial fraction (L) was a sediment of the heavy-mitochondrial supernatant subjected to a centrifugal force of 18000 *g* for 20 min. The rest, namely the microsomal fraction and cell sap (post-mitochondrial fraction), were not separated further and were designated (S). All sediments were washed once with the suspending medium (0.25 M-sucrose in 1 mM-tris-HCl buffer, pH 7.4), centrifuged and resuspended in the original volume.

Purification of enzyme. The rat-liver homogenates in 0.25 M-sucrose containing 1 mM-tris-HCl buffer, pH 7.4, were centrifuged at 600 *g* for 10 min. to remove the nuclei and cell debris, and the particles that sedimented between 600 *g* and 18000 *g* were used. All operations were carried out at 0–4° unless otherwise stated. The particle suspension was subjected to ultrasonic treatment for several minutes and subsequently to repeated freezing and thawing (about 10 times), between –70° (acetone-solid CO₂) and 50°. This was followed by centrifugation for 90 min. at 105 000 *g* in a Spinco model L preparative ultracentrifuge. The precipitate was discarded and the clear supernatant (supernatant 1) was acidified with dilute acetic acid to bring it to pH 5.2–5.3. After centrifugation at 10 000 *g* for 20 min. the supernatant was discarded, and the sediment (precipitate pH 5.3) was

resolubilized by adding 0.25 M-sucrose containing 0.1 M-tris-HCl buffer, pH 7.4, and 0.08% of sodium cholate. All the insoluble material was removed by centrifugation at 105 000 *g* for 90 min. (supernatant 2). The clear yellowish supernatant was then subjected to fractionation with (NH₄)₂SO₄ at pH 7.5–7.8. Ammonium sulphate was added as the solid; the fraction precipitating between 15 and 40% (w/v) contained most of the enzyme.

Assays of enzyme activity. Total acid-phosphatase (EC 3.1.3.2) activity was assayed by the method of Wattiaux & de Duve (1956), with some modification in the presence of 0.1% of Triton X-100 as releasing agent. The buffer was 0.05 M-pyridine-HCl, pH 5.5, and the substrate was 0.01 M-*p*-nitrophenyl phosphate. The reaction was stopped after 10 or 20 min. by the addition of 5% trichloroacetic acid, the mixture was centrifuged and a sample taken to measure the production of *p*-nitrophenol at 400 mμ after the addition of 0.5 N-NaOH. The activity is expressed as μmoles of *p*-nitrophenol formed/min.

Sphingomyelinase. In the earlier experiments this enzyme was assayed by determining the decrease in sphingomyelin (Heller & Shapiro, 1963). In later experiments the formation of water-soluble phosphorus compounds was followed. The reaction mixture of 0.5 ml. or 1 ml. final volume contained 1.5–3.2 μg. atoms of sphingomyelin phosphorus, 0.05–0.075 M-buffer (pyridine-HCl or acetate-acetic acid), pH 5.5, 2 mg. of Triton X-100 and the enzyme (the amount of protein used did not exceed 1 mg./ml.). Incubations were carried out at 37° in glass-stoppered tubes with shaking for 90–180 min. The reaction was terminated by adding 6 ml. of chloroform-methanol (2:1, v/v). Potassium chloride solution was added to form the two-phase system of Folch *et al.* (1957). The amount of phosphorus in the upper phase was measured. Units of activity were defined as the amount of phosphorylcholine (μmoles/min.) formed under the conditions specified. The specific activity of the enzyme was defined as units/mg. of protein.

All the estimations of enzymic activity were performed under optimum conditions, giving zero-order kinetics. This was always checked by carrying out the assays with two enzyme concentrations and frequently with two incubation periods.

RESULTS

Nature of the enzymic reaction. In preliminary observations (Heller & Shapiro, 1963) it was found that rat-liver homogenates can hydrolyse sphingomyelin, since the amount of phosphatide resistant to both mild alkali and acid treatments decreases. Examination of the water-soluble and lipid products of reaction showed clearly that the reaction is a hydrolytic cleavage of sphingomyelin:

Intracellular distribution of the enzyme. It was revealed that the sphingomyelinase activity is associated with particles sedimenting between 600 and 18000 *g*, which contain both heavy- and light-mitochondrial fractions. No activity could be detected in the microsomal fraction or the cell sap.

To correlate the sphingomyelinase with the subcellular fraction, acid phosphatase, which is known to be located in the lysosomes (de Duve, 1959), was chosen as a reference enzyme (Table 1). It seems that most of the sphingomyelinase activity accompanies the acid phosphatase. Further evidence for the localization of sphingomyelinase in the lysosomes was obtained by keeping fresh rat livers frozen for several days; on thawing and centrifuging homogenates prepared therefrom at 105 000 *g* for 90 min., no activity was sedimented, but all the enzyme was found in the soluble cell sap. In addition it is known that Triton X-100 releases the lysosomal hydrolases into solution and a 0.5% solution of this non-ionic detergent was found to liberate sphingomyelinase.

Purification. The particles obtained by centrifuging the homogenate between 600 and 18 000 *g* and which are known to contain mitochondria and lysosomes served as the source of enzyme for purification. Although Triton X-100 released the enzyme from these particles, it also solubilized the bulk of the other mitochondrial and lysosomal proteins. Another disadvantage in using this detergent was the difficulty of removing it for

further purification. de Duve (1959) has suggested several agents and treatments for disruption of the lysosomal membrane to release the hydrolases. Accordingly we tried to treat the particles ultrasonically for 3–20 min., but found that after this treatment and high-speed centrifugation the separation of the enzyme from the total protein in the soluble and insoluble fractions was inadequate for further purification. However, when ultrasonic treatment was followed by repeated freezing and thawing, the supernatant obtained after high-speed centrifugation contained most of the enzyme with only 30% of the total protein. The purification procedure is given in the Experimental section; Table 2 summarizes the purification pattern, indicating a 21-fold purification. The calculated enzyme activity of fresh liver is 0.31 unit/g.

pH-dependence. The optimum activity was between pH 5 and pH 5.5 with different buffer systems. Pyridine–hydrochloric acid, phthalate–sodium hydroxide and acetic acid buffers at 0.05–0.1 M gave the best results, whereas in citrate and maleate systems lower values were obtained. Fig. 1 shows the pH curves as measured with two different buffer systems.

Table 1. *Distribution pattern of sphingomyelinase and acid phosphatase*

The subcellular fractionation pattern is described in the Methods section. 'Relative specific activity' is the ratio between the percentage of total activity and the percentage of total protein.

Fraction	Volume (ml.)	Protein (%)	Acid phosphatase		Sphingomyelinase	
			Activity (%)	Rel. sp. activity	Activity (%)	Rel. sp. activity
Homogenate	24	100	100	—	100	—
Nuclear fraction	29	34	22	0.65	19	0.56
Heavy mitochondria	24.5	9	7.8	0.87	19	2.1
Light mitochondria	26.5	15	40.5	2.7	60	4.0
Post-mitochondrial fraction	120	41	30	0.73	0	0

Table 2. *Purification of sphingomyelinase*

Homogenate was obtained from 200 g. of rat liver in a final volume of 1 l. 'Particles': heavy and light mitochondria. 'Supernatant 1': obtained after high-speed centrifugation of 'particles' treated ultrasonically and by freezing and thawing. 'Precipitate (pH 5.3)': obtained from 'supernatant 1'. Supernatant 2: the supernatant obtained after neutralizing 'precipitate (pH 5.3)' to pH 7.5 and high-speed centrifugation. 'Ammonium sulphate': the precipitate obtained from 'supernatant 2' by ammonium sulphate at 15–40% (w/v) concn. Incubation time, 120 min.

Fraction	Protein (g.)	Activity (units)	10 ³ × Sp. activity	Yield (%)	Purification
Homogenate	51	68.0	1.33	100	1
Particles	10.2	48.5	4.75	71	3.55
Supernatant 1	3.6	28.2	7.82	41.5	5.9
Precipitate (pH 5.3)	1.8	21.5	11.9	31.5	8.9
Supernatant 2	1.31	20.8	15.9	30.5	12
Ammonium sulphate	0.36	10.3	28.5	15	21.6

Kinetics. The hydrolysis of sphingomyelin by the enzyme was linear with time for at least 4 hr. Proportionality was also found with enzyme concentration (Fig. 2). The dependence of the velocity on substrate concentration indicated an apparent K_m 0.5–0.9 mm (Fig. 3).

Activation and inhibition. Both the crude homogenate and the particles were capable of hydrolysing sphingomyelin without any other additions. The partially purified preparations, however, required some surface-active agent such as Triton X-100 or Cetavlon. Emulsification of

Fig. 1. pH-activity curve of sphingomyelinase. O, Acetate-acetic acid buffer, 0.05M; Δ , pyridine-HCl buffer, 0.05M.

Fig. 2. Kinetics and enzyme concentration-dependence of sphingomyelinase. This was performed with the 'ammonium sulphate fraction' (see Table 1). Enzymic protein: (1) 0.21 mg.; (2) 0.42 mg.; (3) 0.84 mg.

Fig. 3. Dependence of rate on substrate concentration. All components of the reaction mixture were constant, but sphingomyelin concentration was varied from 0.2 mm to 2 mm as shown. The experiment was performed with 'supernatant 1' fraction (0.45 mg. of protein). Incubation time, 90 min.

Fig. 4. Effect of Triton X-100 (O) and Cetavlon (Δ) on sphingomyelinase activity. Both detergents were added to the reaction mixture, as aqueous solutions in the concentrations (mg./ml.) indicated, at pH 5.5. The experiment was performed with 'supernatant 1' fraction; 100% for Triton X-100 effect was 0.35 μ g. atom of 'water-soluble phosphorus' formed; 100% for Cetavlon effect was 0.31 μ g. atom of 'water-soluble phosphorus' formed. Incubation time, 120 min.

sphingomyelin by ultrasonic treatment for 8 min. was insufficient to initiate enzymic hydrolysis. The activating effect of Triton X-100 reached a plateau at approx. 2 mg./ml. Cetavlon gave maximal results at a concentration of 0.2 mg./ml., higher concentrations causing inhibition (Fig. 4). When Triton X-100 was added to inhibitory amounts of Cetavlon, the inhibition was not counteracted. The surface-active agent may be required for both solubilization of the enzyme, which is precipitated at the pH of the reaction, and for proper dispersion of the substrate. The addition of diethyl ether or Ca^{2+} ions, either alone or in combination, or the addition of chelating agents such as EDTA, citrate or maleate, was essentially without effect. Oleic acid (1 mM) or sodium chloride (up to 0.5 M) did not influence the rate of hydrolysis in the presence of Triton X-100. In the absence of the detergent no activity could be detected under any of the above conditions.

Stability. The enzyme is heat-labile, being inactivated after 1 min. in boiling water or 10 min. at 65° in the absence of the substrate.

The enzyme preparations, at various stages of purification, were relatively stable when kept frozen at -18°. A 30–35% loss of activity during a period of about 3 months was observed when the enzyme solutions were repeatedly withdrawn from the freezer for assay, thawed and refrozen. This relative stability was greatly dependent on the

presence of some preserving agent. Sucrose (0.25 M), glycerol (20%) or ammonium sulphate (33% saturation) were satisfactory agents, sucrose being the one generally used. In solutions containing buffer only, more than 80% loss in activity was observed within a day of storage at -18°.

Specificity. The phospholipases C from microbial sources are relatively non-specific, hydrolysing most of the phosphatides (Kates, 1960; Matsumoto, 1961). The rat-liver enzyme has a much greater specificity. Marked differences in rates of hydrolysis were found with different sphingomyelin isomers. The naturally occurring sphingomyelin (which has been shown to be *D-erythro-trans-2-N-acyl-1-O-phosphorylcholinesphingosine*; Shapiro & Flowers, 1962), as well as synthetic *D-* or *DL-erythro* isomer, were hydrolysed at approximately the same rate. Synthetic sphingomyelins containing dihydro-sphingosine were hydrolysed by the enzyme to an extent almost equal to those containing sphingosine. Much lower activity, however, was found with the *threo* isomer of the dihydrosphingomyelin tested (Table 3). The following phospho compounds tested were hydrolysed at a much slower rate than sphingomyelin: o-oleoethin, synthetic lecithin, phosphorylcholine.

Phosphatidic acid, however, was split almost at the same rate as sphingomyelin by the crude particles. These particles contain a specific

Table 3. *Action of sphingomyelinase on sphingomyelins from various sources*

Expt. 1 was performed with 'particles' (0.96 mg. of protein in the reaction mixture). Expt. 2 was performed with 'supernatant 1' (0.75 mg. of protein in the reaction mixture) (see Table 1).

Expt.	Substrate	Source	Substrate added (μ moles)	Product formed (μ mole)
1	Sphingomyelin	Rat liver	1.79	0.665
		Red blood cells	1.58	0.560
		Sheep liver	1.64	0.495
		Cattle liver	1.56	0.485
		Cattle brain	1.40	0.705
		Synthetic	0.72	0.315
	<i>D-erythro-N</i> -Palmitoyl-sphingomyelin		1.44	0.515
			2.16	0.681
			2.88	0.762
	<i>DL-threo-N</i> -Palmitoyl-dihydrosphingomyelin	Synthetic	1.44	0.247
			2.16	0.167
			2.88	0.180
			3.60	0.145
			4.32	0.187
2	Sphingomyelin	Rat liver	1.00	0.332
	<i>D-erythro-N</i> -Palmitoyl-sphingomyelin	Synthetic	1.00	0.322
	<i>DL-erythro-N</i> -Palmitoyl-dihydrosphingomyelin	Synthetic	1.28	0.269
	<i>DL-threo-N</i> -Palmitoyl-dihydrosphingomyelin	Synthetic	1.00	0.02
			2.00	0.08

phosphatidic acid phosphatase, as shown by Wilgram & Kennedy (1963), but this activity was lost during purification. It could no longer be detected in the 'ammonium sulphate fraction', even when tested at pH 7.5, known to be at the optimum of this enzyme (Table 4). The acid-phosphomonoesterase activity toward *p*-nitrophenyl phosphate is, however, carried along during the purification steps.

Product inhibition. To test whether a product inhibition occurs, both ceramide and phosphorylcholine were added, one at a time or in combination, with the concentrations varied from 0.5 to 1.6 mM. Only 23% inhibition was obtained.

Stoichiometry of the reaction. A reaction mixture (containing purified enzyme) was incubated and the reaction terminated as described above; the upper methanol-water phase was analysed for total phosphorus and for inorganic phosphorus. Since no inorganic phosphorus was found, the total phosphorus represented phosphorylcholine. The lower phase was analysed for total phosphorus.

Another sample of the lower phase was evaporated to dryness under nitrogen and the residue was extracted with propan-2-ol-heptane-sulphuric acid according to Dole (1956). The heptane layer contained all the ceramide (Roitman & Gatt, 1963). However, some sphingomyelin was also carried into

Table 4. *Action of sphingomyelinase on phosphorus-containing substrates*

Expts. 3 and 4 were performed with 'particles' (0.9 mg. of protein and 0.31 mg. of protein respectively). Expt. 5 was performed with 'supernatant 1' (0.6 mg. of protein). Expts. 6 and 7 were performed with 'ammonium sulphate fraction' (0.34 mg. of protein and 0.15 mg. of protein respectively). All the reactions were carried out at the pH optimum of sphingomyelinase (5-5.5), except the experiments marked with asterisks, which were performed also at pH 7.5 for comparison. (Note: the hydrolysis of phosphorylcholine and phosphatidic acid was followed by measuring production of inorganic phosphorus.)

Expt.	Substrate	Source	Substrate added (μ moles)	Product formed (μ mole)
3	Sphingomyelin	Rat liver	1.56	0.4
	Lecithin	Egg	1.63	0.023
	Lecithin (dipalmitoyl)	Synthetic	1.52	0.032
4	Sphingomyelin	Rat liver	1.05	0.160
	Phosphatidic acid	Egg lecithin	1.15	0.143
	Phosphorylcholine	Synthetic	1.05	0.038
5	Sphingomyelin	Cattle brain	1.68	0.335
	Sphingomyelin	Cattle brain	1.68	0.118*
	Phosphatidic acid	Egg lecithin	1.4	0.091
	Phosphatidic acid	Egg lecithin	1.4	0.121*
6	Sphingomyelin	Cattle brain	1.56	0.36
	Lecithin	Egg	1.0	0
7			2.0	0.037
	Sphingomyelin	Cattle brain	1.68	0.183
	Sphingomyelin	Cattle brain	1.68	0.038*
	Phosphatidic acid	Egg lecithin	1.4	0
	Phosphatidic acid	Egg lecithin	1.4	0*

Table 5. *Stoichiometry of the reaction*

Experiments were performed with the purified enzyme at the stage of 'ammonium sulphate fraction'. Results are expressed in μ g. atoms or μ moles. Sphingomyelin was assayed as 'chloroform-soluble phosphorus', phosphorylcholine as 'water-soluble phosphorus' (Folch *et al.* 1957), ceramide as 'heptane-soluble nitrogen' (Dole, 1956).

Expt.	Time (min.)	Sphingomyelin	Ceramide	Phosphorylcholine	P _i
1	0	1.56	0	0	—
	150	0.72	1.12	1.01	—
2	0	5.32	0	0	—
	150	2.56	2.50	2.0	—
3	0	6.3	0	0	—
	150	3.76	3.46	2.54	—
4	0	6.73	0	0	0
	150	4.70	1.9	2.03	0

this phase, as shown by phosphorus analysis of this phase and by thin-layer chromatography. Therefore the heptane layer was washed with 'Dole's lower phase solvents' and the interfering sphingomyelin could be removed almost completely (phosphorus estimations revealed removal of more than 95% of the accompanying sphingomyelin). The amount of ceramide was estimated by determining total nitrogen in the washed heptane phase. Table 5 shows four analyses done by this procedure.

DISCUSSION

The enzymic breakdown of sphingomyelin by rat-liver preparations, demonstrated in this paper, is similar to the cleavage brought about by phospholipase C from various bacterial sources, yielding phosphorylcholine and ceramide. The animal phospholipase C differs, however, from the bacterial enzyme in its substrate specificity, showing very little activity towards lecithin. Further, the configuration of the sphingosine moiety influences the enzymic activity considerably.

No cleavage of the fatty acid from sphingomyelin was found, even with crude liver preparations. It seems likely therefore that the sequence of degradation of sphingomyelin is to form ceramide, which may then be hydrolysed to sphingosine and fatty acid by an enzyme described by Gatt (1963) in rat tissues.

Part of this work is included in a Ph.D. Thesis submitted by M. H. to the Senate of the Hebrew University, Jerusalem.

REFERENCES

- Bangham, A. D. & Dawson, R. M. C. (1962). *Biochim. biophys. Acta*, **59**, 103.
 Bartlett, G. R. (1959). *J. biol. Chem.* **234**, 466.
 Böttcher, C. Y. F., Preis, C. & Van Gent, C. M. (1961). *Rec. Trav. chim. Pays-Bas*, **80**, 1169.
 Dawson, R. M. C. (1962). In *Comparative Biochemistry*, vol. 3, p. 265. Ed. by Florkin, M. & Masson, H. S. New York: Academic Press Inc.

- de Duve, C. (1959). In *Subcellular Particles*, p. 128. Ed. by Hayashi, T. New York: The Ronald Press Co.
 de Duve, C., Pressmann, B. C., Gianetto, R., Wattiaux, R. & Appelmans, F. (1955). *Biochem. J.* **60**, 604.
 Doery, H. M., Magnusson, B. J., Cheyne, I. M. & Gulasekharan, J. (1963). *Nature, Lond.*, **198**, 1091.
 Dole, V. P. (1956). *J. clin. Invest.* **35**, 150.
 Fiske, C. H. & Subbarow, Y. (1925). *J. biol. Chem.* **66**, 375.
 Folch, J., Lees, M. & Sloane-Stanley, G. H. (1957). *J. biol. Chem.* **226**, 497.
 Fujino, Y. (1952). *J. Biochem., Tokyo*, **39**, 55.
 Gatt, S. (1963). *J. biol. Chem.* **238**, pc3131.
 Hanahan, D. J. (1960). *Lipide Chemistry*, p. 11. New York: J. Wiley and Sons Inc.
 Hanahan, D. J. & Vercamer, R. (1954). *J. Amer. chem. Soc.* **76**, 1804.
 Heller, M. & Shapiro, B. (1963). *Israel J. Chem.* **1**, 204.
 Kates, M. (1956). *Canad. J. Biochem. Physiol.* **34**, 967.
 Kates, M. (1960). In *Lipide Metabolism*, p. 165. Ed. by Bloch, K. New York: J. Wiley and Sons Inc.
 Lang, C. A. (1958). *Analyt. Chem.* **30**, 1692.
 Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951). *J. biol. Chem.* **193**, 265.
 Macfarlane, M. G. & Knight, B. C. J. G. (1941). *Biochem. J.* **35**, 884.
 Mangold, H. K. (1961). *J. Amer. Oil Chem. Soc.* **38**, 708.
 Mangold, H. K. (1962). In *Dünnschicht Chromatographie*, p. 141. Ed. by Stahl, E. Berlin: Springer-Verlag.
 Matsumoto, M. (1961). *J. Biochem., Tokyo*, **49**, 23.
 Pangborn, M. C. (1951). *J. biol. Chem.* **188**, 471.
 Roitman, A. & Gatt, S. (1963). *Israel J. Chem.* **1**, 1960.
 Shapiro, D. & Flowers, H. M. (1962). *J. Amer. chem. Soc.* **84**, 1047.
 Skidmore, W. P. & Entenman, C. (1962). *J. Lipid Res.* **3**, 471.
 Sweeley, C. C. (1963). *J. Lipid Res.* **4**, 402.
 Thannhauser, S. J., Benotti, J. & Boncoddio, N. F. (1946). *J. biol. Chem.* **166**, 677.
 Thannhauser, S. J. & Boncoddio, N. F. (1946). *J. biol. Chem.* **166**, 699.
 Thannhauser, S. J. & Reichel, M. (1940). *J. biol. Chem.* **135**, 1.
 Wagner, H., Hörhammer, L. & Wolff, P. (1961). *Biochem. Z.* **334**, 174.
 Wattiaux, R. & de Duve, C. (1956). *Biochem. J.* **63**, 606.
 Wilgram, F. G. & Kennedy, E. P. (1963). *J. biol. Chem.* **238**, 2615.