6. The new evidence presented is fully in keeping with our previous finding as to the composite nature of phrenosinic acid. Klenk & Clarenz's claim that it is pure, or nearly pure α -hydroxy-*n*-tetracosanoic acid is rejected.

We should like to thank Prof. E. Stenhagen (Uppsala) for a sample of synthetic ($\pm$)- α -hydroxy-*n*-docosanoic acid and Dr J. C. Smith (Oxford) for a sample of synthetic ($\pm$)- α -hydroxy-*n*-tetracosanoic acid. We are indebted to Dr T. Malkin for two X-ray analyses (B 504; B 505).

REFERENCES

- Ashton, R., Robinson, R. & Smith, J. C. (1936). *J. chem. Soc.* p. 283.
 Blount, B. K., Chibnall, A. C. & Mangouri, H. A. (1937). *Biochem. J.* **31**, 1375.
 Chibnall, A. C., Latner, A. L., Williams, E. F. & Ayre, C. A. (1934). *Biochem. J.* **28**, 313.
 Chibnall, A. C., Piper, S. H. & Williams, E. F. (1936). *Biochem. J.* **30**, 100.
 Chibnall, A. C., Piper, S. H. & Williams, E. F. (1953). *Biochem. J.* **55**, 711.
 Francis, F., King, A. M. & Willis, J. A. V. (1937). *J. chem. Soc.* p. 999.
 Francis, F., Piper, S. H. & Malkin, T. (1930). *Proc. Roy. Soc. A*, **128**, 214.
 Klenk, K. & Clarenz, L. C. (1939). *Hoppe-Seyl. Z.* **257**, 263.
 Klenk, K. & Diebold, W. (1933). *Hoppe-Seyl. Z.* **215**, 79.
 Piper, S. H., Chibnall, A. C., Hopkins, S. J., Pollard, A., Smith, J. A. B. & Williams, E. F. (1931). *Biochem. J.* **25**, 2072.
 Rosenheim, O. (1916). *Biochem. J.* **10**, 142.
 Thudichum, J. L. W. (1881). *Ann. chem. Med.* **2**, 1.
 Weitkamp, A. W. (1945). *J. Amer. chem. Soc.* **67**, 447.

The α -Hydroxy-*n*-fatty Acids of Yeast Cerebrin

By A. C. CHIBNALL, S. H. PIPER AND E. F. WILLIAMS

Department of Biochemistry, University of Cambridge and the Wills Physical Laboratory, University of Bristol

(Received 28 May 1953)

Cerebrin, the main cerebroside of yeast, was first prepared by Reindel (1930), who showed that it was of relatively simple composition, consisting of a base of unknown constitution coupled to an α -hydroxy acid which was considered to be α -hydroxy-*n*-hexacosanoic acid. The brain cerebroside phrenosin yields a similar acidic product (phrenosinic or cerebronic acid) of somewhat lower molecular weight, which was shown by Chibnall, Piper & Williams (1936) to be a mixture of α -hydroxy-*n*-docosanoic, α -hydroxy-*n*-tetracosanoic and α -hydroxy-*n*-hexacosanoic acids. It seemed probable that the yeast acid might also be of a composite nature, for naturally occurring long-chain normal aliphatic products have usually been shown, on searching investigation, to be mixtures of near homologues, cf. the *n*-fatty acids and primary alcohols of waxes, and the *n*-fatty acids of glycerides. This paper describes an investigation of the yeast acid on lines similar to those employed by Chibnall *et al.* (1936) for phrenosinic acid. The work was started in 1936 and was completed within two years, but publication has been withheld pending the completion of the investigation described in the preceding paper (Chibnall, Piper & Williams, 1953). Meanwhile, Reindel, Weickmann, Picard, Luber & Turula (1940) have published further observations on cerebrin, which deal in large part with the constitution of the basic component.

EXPERIMENTAL AND RESULTS

The melting points recorded in this paper were obtained by the methods described by Chibnall *et al.* (1953) and are corrected. Carbon and hydrogen analyses were by Schoeller (Berlin-Schmargendorf).

Investigation of the hydroxy acid prepared by the method of Reindel (1930)

The first sample of hydroxy acid prepared by Reindel's procedure to be examined was an amorphous powder, m.p. 103.5–103.7°, which showed no evidence of crystalline structure when viewed under the microscope. (Found: mol. wt. by titration, 433. $C_{26}H_{52}O_3$ requires 412.4.) It was recrystallized repeatedly from various solvents without appreciable change, the material separating in all cases in masses of microscopic irregular shaped rods which gave very poor and uninterpretable X-ray photographs.

Oxidation of the hydroxy acid. The procedure, which is an oxidative titration with a sharp end-point, is the one employed in earlier work dealing with the oxidation of the primary alcohols of waxes and of phrenosinic acid (Chibnall *et al.* 1936). The hydroxy acid (5 g.) in glacial acetic acid (600 ml.) was treated at 60° with CrO_3 (3.2 g.) in the same solvent (150 ml.). The reagent, added in 5 ml. portions at a time, turned the solution red, but as the oxidation proceeded the colour faded and the solution became green by reflected light. Not until this stage had been reached was the next portion of the reagent run in. The reaction was considered to be complete when the red colour persisted for 3 min. Excess water was then added, the precipitated fatty

Table 1. *Melting points and crystal spacings of fatty acid fractions obtained on oxidation of yeast α -hydroxy acid*

Substance	Weight (g.)	Melting point (°)	Series no.	Crystal spacings in Å		
				Pressed (P) or melted (M) layer	B	C
Fraction 1	0.21	80.5–81.0 (Misty)	—	—	—	—
Fraction 2	0.33	82.2–82.4	B453	P	57.45	—
				M	—	56.3
Fraction 3	0.50	82.4–82.7	B454	P	57.45	—
				M	—	56.8
Fraction 4	0.65	82.4–82.7	B455	P	57.45	—
				M	57.7	—
Fraction 5	0.32	81.9–82.1	B457	P	57.45	—
				M	—	55.0
C ₂₅ acid	—	83.2–83.4	B459	P	57.7	—
				M	57.7	—
90% C ₂₅ + 10% C ₂₃	—	81.8–82.0	B433	P	57.5	—
				M	—	55.6

acids filtered off, and thoroughly washed with water. The product was then dissolved in ether and treated with sodium ethoxide (Chibnall *et al.* 1936). The acid obtained through the purified sodium soap crystallized in large rhombs showing clean straight cleavages; m.p. 81.5–82.0°. (Found: mol. wt. 385.9. C₂₅H₅₀O₂, requires 382.4.) The material (3.65 g.) was then fractionated with ethanolic lithium acetate by the method of Klenk (1928) and the acid fractions crystallized once from acetone at 25°. The melting points and crystal spacings are given in Table 1.

Fraction 1 had an indefinite melting point and obviously contained a small amount of unoxidized hydroxy acid. The behaviour of the freshly prepared crystals of fractions 2, 3 and 5, was in good agreement with that of binary mixtures of odd-number *n*-fatty acids containing about 10% of the lower homologue (Chibnall *et al.* 1936); in this case 90% C₂₅ with 10% C₂₃. The pressed layers of the crystals gave a B spacing of the right magnitude, and on melting the C form crystals appeared. The melting point and pressed spacing of fraction 4 both suggested about 5% of C₂₃ in C₂₅ and the contamination of this acid is certainly not greater, or the C form would have appeared on melting. The X-ray analysis showed no indication of the presence of C₂₇, for 2% would have raised the B spacing to about 58.3 Å and 5% to 59 Å. Furthermore, a synthetic mixture of 5% C₂₃ + 90% C₂₅ + 5% C₂₇ gave a photograph (series no. B458) which did not resemble any of these fractions, for it had very diffuse lines and both B and C spacings for a pressed layer.

The oxidized acid was therefore C₂₅ with not more than 10% of C₂₃, so that the yeast hydroxy acid must have been a mixture of about 90% α -hydroxy-*n*-hexacosanoic acid with 10% α -hydroxy-*n*-tetracosanoic acid. Accordingly, the high molecular weight exhibited by the yeast acid must have been due to some non-homologous contaminant that had been removed during the oxidation treatment.

Purification of the hydroxy acid

The hydroxy acid (2.0 g.) was treated under reflux with acetyl chloride (20 ml.) for 3 hr., the acetylated product recovered in the usual way and crystallized from acetone-petroleum (b.p. 40–60°) (1.903 g.), m.p. 72.0–72.5°. The

acetate was then extensively fractionated by a judicious combination of the following treatments: (1) Crystallization at 0° from equal volumes of acetone and light petroleum (b.p. 40–60°). (2) Extraction at 35° with acetone, filtering the warm solution from the insoluble residue and treating the clear filtrate as under (1). There was thus obtained 0.87 g. of material; m.p. 84–84.5°. (Found: C, 73.9; H, 12.1, mol. wt. by titration 456.5. C₂₈H₅₄O₄ requires C, 73.9; H, 11.9%; mol. wt. 454.3.)

This acetyl derivative (0.3 g.) was saponified with 30 ml. of 5% ethanolic NaOH for 3 hr., the volume reduced to 20 ml. and the solution cooled slowly to room temperature. The sodium soap crystallized in large oblong plates (0.3 g.). The free acid was prepared by partitioning between aqueous HCl and ether, washing the ethereal solution and transferring to warm acetone. On cooling the product separated as a gel, but could be filtered free from solvent on a Büchner funnel; m.p. 104–104.5°. (Found: mol. wt. by titration 417.2; C₂₆H₅₂O₃ requires 412.4.) The acid was optically active, a property that had been masked by the impurities present in the original preparations; $[\alpha]_D^{20}$ in pyridine = +2.1 (c, 2.8).

Another batch of yeast acid melted at 101–101.5° and had a molecular weight by titration of 504.9 showing that the cerebrin had been incompletely hydrolysed. The acid (10.0 g.) was therefore saponified with 600 ml. of 5% ethanolic NaOH for 4 hr. Crystals of sodium salt separated fairly early during the initial stages of the saponification, and at the end of the operation were removed by filtration on a hot funnel. The weight of sodium soap, after washing with hot ethanol, hot acetone and ether was 5.62 g. (fraction A). On cooling the filtrate to 0° a yellowish amorphous powder separated, which was filtered off, washed three times with boiling acetone, then with ether and air dried. It weighed 3.4 g. (fraction B).

Fraction A. The crystalline soap (2.0 g.) was converted to the free acid, which was taken up in warm acetone. On cooling, the product separated as a gel. It was filtered and dried to constant weight *in vacuo* at 40°. (Found: mol. wt. by titration 415.8.) As this fraction of the sodium soap had separated so early in the saponification it was deemed advisable to resaponify the free acid and the remainder of soap for 3 hr. with 500 ml. of 5% benzene:ethanolic (1:1,

v/v) NaOH, which held the soap in solution. On cooling to 0° the sodium soap crystallized out and, after washing, it was converted to the free acid. The product separated as a gel from the following solvents: methyl, ethyl, propyl or amyl alcohol, ethylene dichloride, chloroform, carbon tetrachloride, benzene, benzene-ethanol, xylene, acetic acid and dioxan. The gel could, however, be readily filtered from cold acetone. For analysis the material was dried *in vacuo* at room temperature, m.p. 104.0–104.5; $[\alpha]_D^{20}$ in pyridine = +2.45 (c, 2.56). (Found: C, 75.6; H, 12.7%; mol. wt. by titration 413. $C_{26}H_{52}O_2$ requires C, 75.7; H, 12.7%; mol. wt. 412.5.) The acetyl derivative was prepared as before; the material shrank at 79.5° and melted sharply at 85.2–85.5°. The ethyl ester melted at 74.2–74.4°.

The hydroxy acid (0.5 g.) was cautiously oxidized at 65° with 0.32 g. of CrO_3 in 16 ml. of glacial acetic acid, the resulting acid being purified through the sodium soap. The product (0.33 g.) crystallized in large rhombs and melted at 81.6–81.8°. It was recrystallized from 35 ml. and then twice from 25 ml. of acetone at 37° (0.15 g.); m.p. 83.0–83.2°. (Found: mol. wt. by titration 384. $C_{25}H_{50}O_2$ requires 382.4.) The X-ray photograph (series no. B480) gave eleven and higher orders measuring 58.0 Å for a pressed layer and nine orders measuring 55.8 Å for a melted layer, showing that the substance was C_{25} with not more than 5% of C_{23} . The collected mother liquors gave a second fraction (0.13 g.), m.p. 81.6–81.8°. (Found: mol. wt. by titration 382.) The X-ray photograph (series no. B483) was diffuse but strong and the spacing 57.8 Å. Taken in conjunction with the melting point this suggested 15% C_{23} + 85% C_{25} . The melted layer had a spacing 55.2 Å, corresponding to about 20% of C_{23} + 80% C_{25} . This sample of the yeast hydroxy acid was therefore α -hydroxy-*n*-hexacosanoic acid with not more than 10% of α -hydroxy-*n*-tetracosanoic acid.

We were, nevertheless, not satisfied that full purification had been effected because we had been unable to obtain the product in sufficiently good crystalline form for the X-ray photograph to exhibit the intensity distribution characteristic of an α -hydroxy acid. In the previous work on phrenosinic acid (Chibnall *et al.* 1936) a series of synthetic inactive acids of the series from C_{12} to C_{18} had been examined, as also a few selected mixtures. All of them separated from appropriate solvents in crystalline form and all gave good X-ray photographs with measured spacings that were proportional to the chain length. We were loth to believe that this yeast acid (and also phrenosinic acid, cf. Chibnall *et al.* 1953) could not be obtained in good crystalline form, for it appeared to differ from the synthetic acids in only one property, that of being optically active. Much time and material were accordingly spent in attempts to attain this end, and it was not until we recognized that the gelling effect on cooling a hot solution was brought about by the separation of very minute imperfect crystals which may initially be in some liquid crystal form that we were able to make progress.

Under the following conditions the material separated in satisfactory crystalline form. The acid (0.3 g.) was dissolved in dry ethanol (50 ml.) at 50° and the solution run into a Dewar flask. Cooling was slow, and at the end of 20 hr., when granular material was beginning to separate, the temperature was 35°. The crop was still granular at 33°, when it was rapidly filtered off. The mother liquor gelled on further cooling. The operation was repeated giving 0.19 g. Under the microscope the material was a mass of small crystalline transparent flakes with straight cleavage edges

and, in appearance, was very similar to the synthetic higher ($\pm$)- α -hydroxy-*n*-fatty acids that we have handled in the course of this work. These crystals gave, as one would expect, a very good X-ray photograph (series no. B500) showing 16 orders with the intensity distribution characteristic of an α -hydroxy-*n*-fatty acid. The spacing was 67.9 Å. The A spacing of α -hydroxy-*n*-hexacosanoic acid (extrapolated) is 66.7 Å and the B spacing 59.7 Å. The pure acid should give the B form, and the fact that the observed value is slightly above that of the A form implies, according to our experience, contamination with a small amount of homologous material and is thus in keeping with the deduction from chemical analysis and melting-point data that the yeast product is about 90% α -hydroxy-*n*-hexacosanoic acid with 10% α -hydroxy-*n*-tetracosanoic acid.

Fraction B. The sodium soap gave an acid, m.p. 101.8–102.3°, which separated from hot solvents as a granular powder similar to that of the original preparation. Resaponification was without effect on the melting point and the molecular weight, and the resulting product showed no tendency to gel: $[\alpha]_D^{20}$ in pyridine = +2.04 (c, 2.6). (Found: C, 75.6; H, 12.7, mol. wt. 419. $C_{26}H_{52}O_2$ requires C, 75.7; H, 12.7%, mol. wt. 412.5.)

The acetyl derivative shrank at 73° and melted indistinctly at 78–84°. The hydroxy acid (0.5 g.) was oxidized as before and the product recrystallized thrice from acetone at 37°; m.p. 82.4–82.6°; mol. wt. 384.6. The X-ray photograph (series no. B481) gave 58.0 Å for a pressed layer and 55.5 Å for a melted layer, agreeing with a composition of approximately 90% C_{25} + 10% C_{23} .

Fraction B was clearly the same α -hydroxy acid mixture as fraction A, but the impurity present in the original preparation had not been completely removed. The persistence, in fact, with which this impurity clings to the hydroxy acid, even after fairly drastic saponification, recalls the observation, well known to those who have worked on the structure of phosphatides, that the fatty acids of the latter substances cannot be entirely freed from the last traces of phosphorus except by distillation.

DISCUSSION

The clear demonstration given above that the acid from the yeast cerebroside is a mixture of two even-numbered α -hydroxy-*n*-fatty acids provides yet one more instance for the general rule, so well illustrated by earlier work on waxes (Chibnall, Piper, Pollard, Williams & Sahai, 1934) and on phrenosinic acid (Chibnall *et al.* 1936, 1953) that when long-chain normal aliphatic products occur in nature it is as components of mixtures with one or more homologues of the same odd or even carbon series as the case may be. Of more practical interest, perhaps, is the observation that the yeast hydroxy acid can separate from hot solvents as a gel, in which condition it is very probably in one of the liquid-crystal forms of the type described by Friedel (1922), for this led to the explanation of the 'cauliflower-like' masses of phrenosinic acid that have been repeatedly obtained by earlier workers (cf. Chibnall *et al.* 1953). The same phenomenon was observed by Francis, King & Willis (1937) during the purification of synthetic ($\pm$)- α -hydroxy-*n*-tetratriacontanoic

acid, and it seems legitimate to conclude that this property is inherent in α -hydroxy-*n*-fatty acids of very long chain length.

SUMMARY

1. The α -hydroxy-*n*-fatty acid component of yeast cerebrin has been investigated in detail.

2. The pure product obtained after alkaline saponification is optically active.

3. Under the usual conditions of crystallization from hot solvents the product separates as a gel. On very slow cooling from hot ethanol under special conditions crystalline material was obtained.

4. The crystals gave a very good X-ray photograph showing the characteristic intensity distribution of an α -hydroxy-*n*-fatty acid. The observed spacing was that of the A form of α -hydroxy-*n*-hexacosanoic acid, indicating that a small amount of homologous contaminant was present.

5. Melting-point and X-ray data for the *n*-fatty acids given on oxidation show that the cerebrin hydroxy acid is α -hydroxy-*n*-hexacosanoic acid with not more than 10% of α -hydroxy-*n*-tetra-cosanoic acid.

It is a pleasure to thank Prof. F. Reindel for samples of the yeast hydroxy acid.

REFERENCES

- Chibnall, A. C., Piper, S. H. & Williams, E. F. (1936). *Biochem. J.* **30**, 100.
 Chibnall, A. C., Piper, S. H. & Williams, E. F. (1953). *Biochem. J.* **55**, 707.
 Chibnall, A. C., Piper, S. H., Pollard, A., Williams, E. F. & Sahai, P. N. (1934). *Biochem. J.* **28**, 2189.
 Francis, F., King, A. M. & Willis, J. A. V. (1937). *J. chem. Soc.* p. 999.
 Friedel, M. G. (1922). *Ann. Phys., Paris*, **18**, 273.
 Klenk, E. (1928). *Hoppe-Seyl. Z.* **174**, 221.
 Reindel, F. (1930). *Liebigs Ann.* **480**, 76.
 Reindel, F., Weickmann, A., Picard, S., Luber, K. & Turula, P. (1940). *Liebigs Ann.* **544**, 116.

The Estimation of Oxidized Glutathione

By L. W. MAPSON

Low Temperature Station for Research in Biochemistry and Biophysics, University of Cambridge, and Department of Scientific and Industrial Research

(Received 29 April 1953)

Several methods have been published for estimating reduced glutathione (GSH) (Guthrie & Wilcoxon, 1932; Grunert & Phillips, 1951; Miller & Rockland, 1952), but no satisfactory method for estimating the oxidized form (GSSG). Hence the oxidized form can be estimated if it can be reduced quantitatively. Chemical methods for achieving this were tried (Goddard & Michaelis, 1934; Clarke, 1932; Folin & Marenzi, 1929) but were unsatisfactory quantitatively, owing to interference from phenolic compounds present in plant extracts.

Reduction of GSSG by glutathione reductase appears to be a very suitable means of estimating GSSG because the reaction is relatively rapid, the enzyme appears highly specific for GSSG, cystine and other derivatives of cystine and glutathione are not reduced (Conn & Vennesland, 1951), and the equilibrium point of the reaction between reduced triphosphopyridine nucleotide and GSSG greatly favours the formation of GSH (Mapson & Goddard, 1951; Conn & Vennesland, 1951).

Reduction of GSSG is achieved enzymically by means of malic dehydrogenase, together with glutathione reductase and triphosphopyridine nucleotide (TPN) as previously described (Mapson & Goddard, 1951). The essential reaction catalysed by glutathione reductase is

where TPNH = reduced TPN and TPN^+ = oxidized TPN. Malic dehydrogenase enzyme with its substrate is used to regenerate reduced TPN. These enzymes are present in acetone powder preparations from pea seeds, dialysed to remove all soluble —SH and —S—S— compounds. On the addition of malate, manganese, TPN and GSSG to the dialysed extract under anaerobic conditions, hydrogen readily transfers from malate to GSSG.

Diphosphopyridine nucleotide (DPN), if present in the preparation of TPN, does not interfere in the estimation of GSSG, since the only substrate added is malate, and the malic dehydrogenase of peas is