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Further Observations on Phrenosinic Acid

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

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

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In an earlier paper dealing with the fatty acids of phrenosin and kersin we advanced the view that phrenosinic (cerebronic) acid was a mixture of 85% α -hydroxy-*n*-tetracosanoic acid and 15% α -hydroxy-*n*-hexacosanoic acid with perhaps a small amount of α -hydroxy-*n*-docosanoic acid, thereby disposing, as we thought, of the rival claims of Levene, that it was α -hydroxyisopentacosanoic acid and of Klenk, that it was α -hydroxy-*n*-tetracosanoic acid (Chibnall, Piper & Williams, 1936). This conclusion was based on evidence derived from three sets of observations. (1) Comparison of the melting point and X-ray crystallographic data with those of a series of synthetic ($\pm$)- α -hydroxy-*n*-fatty acids from C_{13} to C_{18} (higher members were not available at the time) and of various mixtures thereof. (2) Comparison of the melting point of the acetyl derivative with those of the synthetic series mentioned above. (3) Comparison of the melting point and X-ray data for the *n*-fatty acid given on oxidation with those of synthetic *n*-fatty acids from C_{21} to C_{27} and of mixtures thereof.

We were aware, however, that our finding left

unexplained three observations to which attention was drawn at the time, but which were not emphasized because they had no immediate bearing on the point then at issue. First, phrenosinic acid, as normally prepared, always separates in the 'cauliflower-like' masses first described by Thudichum (1881), and shown later by Rosenheim (1916) to be conglomerates of irregular spherocrystals. Such a product gave an X-ray photograph that was difficult to interpret. We were at a loss to explain this phenomenon, for according to our experience, a similar mixture of synthetic α -hydroxy-*n*-fatty acids would crystallize in rhombic plates with rather broken edges. Secondly, the molecular weight, as determined by titration, was too high for the postulated mixture, as also were the analytical figures for carbon and hydrogen, suggesting the presence of some, probably non-homologous, impurity which was destroyed on oxidation. Thirdly, the melting point of the acetyl derivative, 66.5° , seemed low, even for a mixture, because the extrapolated value for ($\pm$)- α -acetoxy-*n*-tetracosanoic acid was about 83° .

Investigations to throw further light on these apparent anomalies have been in progress since 1936, and we have benefited from our experience in the parallel investigation on the constitution of the α -hydroxy-*n*-fatty acids in yeast cerebrin (Chibnall, Piper & Williams, 1953). For various reasons the work on both products had to be suspended in 1939, although considerable progress had already been made. In the case of phrenosinic acid especially we felt the need to confirm our conclusions by reference to synthetic ($\pm$)- α -hydroxy-*n*-docosanoic and ($\pm$)- α -hydroxy-*n*-tetracosanoic acids, and it is only recently that samples of these have been placed at our disposal.

In the meantime, Klenk & Clarenz (1939) had synthesized ($\pm$)- α -hydroxy-*n*-tetracosanoic acid and had found that the acetyl derivative melted at 65.2–65.7°, the same temperature as that of their phrenosinic acid. On oxidation, moreover, this synthetic hydroxy acid gave an *n*-fatty acid whose melting point, molecular weight and X-ray analytical data appeared to be identical with that given by phrenosinic acid under similar treatment. The new evidence led them to challenge our conclusions as to the composite nature of phrenosinic acid, and to reiterate the former claim of Klenk & Diebold (1933) that it was α -hydroxy-*n*-tetracosanoic acid, with a negligible amount, if any, of a higher or lower homologue.

In the present paper we describe the preparation of phrenosinic acid in true crystalline form. A study of its properties and derivatives, interpreted in the light of similar observations on an appropriate series of synthetic ($\pm$)- α -hydroxy-*n*-fatty acids, leads us to re-affirm our view as to its composite nature.

EXPERIMENTAL

The melting points of the *n*-fatty acids and α -hydroxy-*n*-fatty acids recorded in this paper were obtained by the method of Piper *et al.* (1931). In the case of the acetoxy acids the crystals were packed in the melting-point tubes in the usual way and the bath temperature was raised at a rate of about 0.5°/min. All melting points are corrected. Carbon and hydrogen analyses were by Schoeller (Berlin-Schmargendorf) or by Weiler and Strauss (Oxford).

($\pm$)- α -Acetoxy-*n*-fatty acids. A sample of synthetic ($\pm$)- α -hydroxy-*n*-docosanoic acid was kindly made available by Prof. E. Stenhagen, and of ($\pm$)- α -hydroxy-*n*-tetracosanoic acid (Ashton, Robinson & Smith, 1936) by Dr J. C. Smith. Both acids crystallized in clusters of long needle-like plates showing sharp cleavage edges. The hydroxy acid (0.2 g.) was treated with acetyl chloride (2 ml.) at the boil for 3 hr., the acetylated product recovered in the usual way and crystallized twice from light petroleum (b.p. 40–60°). ($\pm$)- α -Acetoxy-*n*-docosanoic acid, m.p. 79.1–79.4°. (Found: C, 72.6; H, 11.5. $C_{24}H_{46}O_4$ requires C, 72.5; H, 11.4%.) ($\pm$)- α -Acetoxy-*n*-tetracosanoic acid, m.p. 81.9–82.3°. (Found: C, 72.9; H, 11.8. $C_{26}H_{50}O_4$ requires C, 73.2; H, 11.8%.)

Table 1. *Melting points and long crystal spacings of a series of ($\pm$)- α -hydroxy-*n*-fatty acids*

Acid (no. of carbon atoms)	Melting point (°)	Series no.	Crystal spacings in Å			Melting point of acetyl derivative (°)	Difference between melting point of acid and its acetyl derivative
			Pressed (P) or melted (M) layer	A	B	C	
13	78.0	B409	P	34.55	—	—	—
14	81.5	B408	P	—	33.7	31.8	56.0–56.5
			M	38.7*	33.75	—	—
15	84.5–84.7	B396	P	39.5	—	—	49.5–50.0
			M	39.1	—	—	—
16	86.9–87.2	B389	P	—	38.0	—	63.2–63.5
			M	42.3	38.05	—	—
17	89.3–89.6	B395	P	44.6	—	—	59.3–59.5
18	91.5–91.7	B394	P	—	42.25	39.0	70.1–70.3
			M	—	42.3	40.2	—
22	97.8–98.0	B505	P	—	51.0	—	79.1–79.4
24	100.3–100.5	B462	P	—	55.5	—	81.9–82.3
34†	109–110	—	P	—	77.0	—	—
Phrenosinic acid	101.8–102.1	B504	P	—	54.2	—	66.0–66.5
Yeast hydroxy acid‡	104.0–104.5	B500	P	—	67.9	—	85.2–85.5
70% C_{15} + 30% C_{17}	78.3–78.8	B399	P	42.6	—	—	—
70% C_{18} + 30% C_{16}	79.8–80.0	B400	P	—	39.7	37.6	54.5
40% C_{14} + 40% C_{18}	74.6	—	—	—	—	—	39.0
+ 20% C_{18}							35.6

* Only first order observed.

† Francis, King & Willis (1937).

‡ Chibnall *et al.* (1953).

Preparation of crystalline phrenosinic acid. It has been customary in the past to hydrolyse phrenosin with 10% methanolic H_2SO_4 in order to conserve the sphingosine and sugar component, both of which would suffer decomposition if alkaline saponification were employed. Recent work with the yeast hydroxy acid, however, convinced us that this treatment was insufficient to free the phrenosinic acid from all extraneous products, and we have accordingly followed the initial separation with an alkaline saponification.

The starting material was a sample of phrenosinic acid which had undergone some degree of purification and had separated from acetone at 25° in the usual 'cauliflower-like' masses. (Found: C, 75.5; H, 12.5; mol. wt. by titration 396. $\text{C}_{24}\text{H}_{48}\text{O}_8$ requires C, 74.9; H, 12.6%; mol. wt. 384.5.) The X-ray analyses (series B, no. 405) gave five orders measuring 54 Å, but the lines were diffuse. The acid (0.3124 g.) was saponified for 4 hr. in 24 ml. benzene:ethanol (1:1, v/v) containing 1.2 g. KOH. The volume was then reduced to 10 ml. and on cooling slowly to room temperature the sodium soap crystallized in plates. The free acid was prepared by partitioning between aqueous HCl and ether, washing the ethereal solution and transferring to warm acetone. On cooling to 0° the product separated as a granular powder (0.2786 g.). It was then crystallized from ethanol at 60° in a Dewar flask by the procedure employed for the yeast hydroxy acid (Chibnall *et al.* 1953), when it separated in masses of very small transparent flakes showing sharp cleavage edges, m.p. 101.8–102.1°. (Found: C, 74.6; H, 12.5%; mol. wt. by titration 388.6. $\text{C}_{24}\text{H}_{48}\text{O}_8$ requires C, 74.9; H, 12.6%; mol. wt. 384.5.) The X-ray photograph (series B, no. 504) showed the broad lines characteristic of a mixture, but the intensity distribution in the seven orders registered was the same as in the synthetic α -hydroxy-*n*-tetracosanoic acid. The measuring spacing, 54.2 Å, which may be in error by ± 0.5 Å as the lines are too broad for accurate computation, is lower than that of the synthetic acid (55.5 Å), suggesting the presence of a small amount of α -hydroxy-*n*-docosanoic acid. The observed molecular weight now agrees fairly closely with that deduced from our previous work on the constitution of the *n*-fatty acids given on oxidation (Chibnall *et al.* 1936), which was a mixture of 85% α -hydroxy-*n*-tetracosanoic acid and 15% of α -hydroxy-*n*-hexacosanoic acid with perhaps a small amount of α -hydroxy-*n*-docosanoic acid. The acetyl derivative, recrystallized from light petroleum (b.p. 40–60°) melted, as before, at 66.0–66.5°, emphasizing the probable presence of three components (Table 1).

DISCUSSION

The observations recorded above show that phrenosinic acid, as normally prepared, contains a small amount of possibly non-homologous material which can be removed after alkaline saponification, and that it is probably this impurity which favours the separation of the acid under the usual conditions of crystallization in the initial form of a liquid that solidifies on further cooling to give the 'cauliflower-like' masses of Thudichum, whose crystals are very minute and imperfect giving very few diffuse reflexions (cf. the corresponding yeast hydroxy acid, Chibnall *et al.* 1953).

The data collected in Table 1 show that the melting points of the synthetic ($\pm$)- α -hydroxy-*n*-fatty acids between C_{13} and C_{24} fall on a smooth curve. In all cases the X-ray photographs give very good reflexions. The orders are recorded up to the extreme angle of rock, and the intensity distribution differs characteristically from that of a *n*-acid. As usual, the binary mixtures give poorer reflexions and more diffuse lines, and it is only when one of the members constitutes about 80% or more that sufficient orders of reflexion are registered to establish the intensity distribution characteristic of the series. Phrenosinic acid is clearly a mixture of this latter type. Weitkamp (1945) showed that the optical isomer of α -hydroxy-*n*-tetracosanoic acid melted at 88.5° and that of α -hydroxy-*n*-hexadecanoic acid at 93.6°, i.e. 6.8° and 6.4° higher than the corresponding inactive acids respectively. Phrenosinic acid, therefore, which is optically active, may well be a mixture, even though it melts about 0.6° higher than ($\pm$)- α -hydroxy-*n*-tetracosanoic acid.

There is a pronounced odd-even alternation in the melting-points curve for the ($\pm$)- α -acetoxy-*n*-fatty acids, but the even members lie on a smooth curve. Our preparation of ($\pm$)- α -acetoxy-*n*-tetracosanoic acid melted at 81.5–82.0°, as one would have expected, and we are thus unable to confirm the much lower value, 65.2–65.7° which Klenk & Clarenz (1939) record, and incidentally use as a weighty argument in the present dispute.

The new evidence for the composition of phrenosinic acid is fully in keeping with that deduced by us from our former study of the *n*-fatty acids given on oxidation (Chibnall *et al.* 1936). The objection voiced by Klenk & Clarenz (1939) to our use of chromic acid as an oxidant, in that it might have given rise to lower homologues by multiple oxidation, is rejected. In our procedure (cf. Chibnall *et al.* 1953) the oxidant is never present in any appreciable excess and had oxidation beyond the α -carbon atom occurred we should have found evidence for the production of even-number *n*-fatty acids, the presence of which we were able definitely to exclude from a consideration of melting-point and X-ray data. We have, moreover, in our previous researches on waxes, always used this same procedure to oxidize the long-chain primary alcohols to the corresponding *n*-fatty acids, and these latter could never have been obtained pure by the simple after-treatment we employed if multiple oxidation had occurred.

Klenk & Clarenz (1939) include in their paper a report by Prof. Thiessen on four of their products that had been investigated by X-ray analysis. These were (I) their synthetic *n*-tricosanoic acid, m.p. 78.5–79°; (II) the oxidation product, m.p. 77.5–78°, of their synthetic α -hydroxy-*n*-tetra-

cosanoic acid; (III) the oxidation product, m.p. 77.7–78.1°, of their phrenosinic acid; and (IV) a purified fraction of the latter, m.p. 78.0–78.5°. The X-ray data were obtained by the Debye-Scherrer method. The long lattice-plane spacing d_1 for sample I (53.1 Å) was markedly lower than those of samples II (57.1 Å), III (56.7 Å) and IV (56.4 Å), which are not very different from each other. The d_2 and d_3 spacings, which do not depend on the chain length and have the same value for all members of a series, were similar in all cases, as one would expect. Prof. Thiessen concluded that sample I must be different from the products represented by samples II–IV. The latter appeared to be identical within the limits of the procedure employed, and he concluded that if they were impure the impurities must be the same in kind and quantity. He considered that the presence of *n*-pentacosanoic acid was a possibility, but if, from the method of preparation and purification, this were considered improbable, then he thought that serious attention should be given to a second possibility, namely, that sample I, and the products represented by samples II–IV, were two different allotropic modifications of the same substance.

We would point out that the X-ray photographs for *n*-tricosanoic acid obtained by Francois, Piper & Malkin (1930) gave only one spacing, that of the B form. There are four known crystal forms adopted by odd-numbered *n*-fatty acids, and for *n*-tricosanoic acid the spacing values for these are B, 53.8 Å, and the others, (extrapolated), A, 60.0 Å, C, 51.8 Å and D, 45.5 Å. These spacings are not in accord with any of those quoted in Prof. Thiessen's report. It is only in the lower members of the odd-number acid series that all four crystal modifications have been observed, and all modifications, incidentally, have the same melting point.

Klenk & Clarenz claim that phrenosinic acid must be pure, or nearly pure, α -hydroxy-*n*-tetracosanoic acid because their synthetic preparation of the latter acid gave derived chemical and X-ray data very similar indeed to that given by the former acid. We suggest that their data point to the opposite conclusion, namely, that their synthetic acid must have contained as much homologous contaminant as their phrenosinic acid. Finally we may mention their complaint that the discussion concerning the nature of phrenosinic acid, which has been proceeding for many years, has been pushed back step by step from its starting point, namely, the question whether it was an α -hydroxyisopentacosanoic acid, or an α -hydroxy-*n*-tetracosanoic acid, until it is now concerned solely with the purity of the substance. From the viewpoint of the biochemist this must surely be the logical outcome of any such inquiry. We know that in glycerides and waxes the *n*-fatty acids belong to the even-number series and that in

some cases one particular member may be present in predominating amount though never to the complete exclusion of others. Their synthesis in nature must therefore involve a stepwise increase or decrease in chain length. When a ketonic acid is one of the components of a wax, however, our present evidence suggests that its synthesis is unique (Chibnall, Latner, Williams & Ayre, 1934; Blount, Chibnall & Mangouri, 1937). It is therefore of biochemical importance to have shown that the α -hydroxy-*n*-fatty acids of cerebrosides are not of unique occurrence, but may represent α -oxidation products of the *n*-fatty acid series. Phrenosinic acid and the yeast α -hydroxy acid are similar mixtures that differ only in the proportions in which the two main components, C_{24} and C_{26} , are present. We may expect that investigation of cerebrosides from various sources will disclose, in due course, the presence of other even-number members of the series.

SUMMARY

1. Evidence was presented in a former paper (Chibnall *et al.* 1936) that phrenosinic acid was a mixture of about 85% α -hydroxy-*n*-tetracosanoic acid and 15% α -hydroxy-*n*-hexacosanoic acid with, probably, a small amount of α -hydroxy-*n*-docosanoic acid, and not pure α -hydroxy-*n*-tetracosanoic acid as had been claimed by Klenk & Diebold (1933).

2. This evidence was based on melting point and long crystal spacing data for phrenosinic acid itself, and of the *n*-fatty acid obtained from it on oxidation. Parallel data were also presented for a series of synthetic ($\pm$)- α -hydroxy-*n*-fatty acids from C_{13} to C_{18} . It was pointed out, in particular, that the melting point of acetylphrenosinic acid (66.5°) was much too low for the extrapolated value (83°) for α -acetoxy-*n*-tetracosanoic acid.

3. Klenk & Clarenz (1939) disputed these conclusions and having found that, among other things, their sample of synthetic α -acetoxy-*n*-tetracosanoic acid melted at 65.7° they reiterated the former claim of Klenk & Diebold.

4. ($\pm$)- α -Acetoxy-*n*-docosanoic and ($\pm$)- α -acetoxy-*n*-tetracosanoic acids have been prepared. Their melting points, 79.4° and 82.3° respectively, fall on a smooth curve with other even-number members of the ($\pm$)- α -acetoxy-*n*-fatty acid series.

5. Phrenosinic acid has been prepared for the first time in crystalline form, and it gives an X-ray photograph similar to that of mixtures of α -hydroxy-*n*-fatty acids. The 'cauliflower-like' masses, which have hitherto been regarded as characteristic of this acid, are shown to consist of very minute imperfect crystals which might be due to the separation from hot solution of liquid material.

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).

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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

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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