The Haemolytic Acid present in Horse Brain

1. PURIFICATION AND IDENTIFICATION AS *cis*-OCTADEC-11-ENOIC ACID

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The occurrence of haemolytic substances in the tissues, secretions and excretions of animals has occasionally been reported. McKee (1915) and Ponder (1921) found in human urine a lytic factor which was soluble in organic solvents and which it was suggested might be a bile acid or some related compound. Drury & Weil (1934) and Drury, Miles, Platt, Plaut, Weil & Hughes (1936) studied the lytic action of psychosin (lignoceryl sphingosine), and showed that it was lost on deamination; however, no evidence could be found that psychosin, or even sphingosine, existed in the free state in animals. Bergenhem & Fåhraeus (1936) reported the presence of a haemolytic substance in normal blood and, on rather slender evidence, suggested that it was a lysolecithin derivative.

Laser & Friedmann (1945) isolated from human blood a solid acid, m.p. 45°, which contained neither nitrogen nor phosphorus and which had a strong haemolytic action. They advanced for it a formula $C_{22}H_{42}O_5$ suggestive of a hydroxylated fatty acid. Linoleic, oleic and various other fatty acids are weakly haemolytic, and their action on erythrocytes and on the ciliated protozoon, *Glaucoma piriformis*, has been studied by Chaix & Baud (1947). Laser (1946) carried out physiological studies and showed that this haemolytic acid was of importance in the metabolism of the malarial parasite. He also recognized that the original preparation was not homogeneous, and considered that the active haemolytic component was a mono-unsaturated fatty acid with a probable chain length C_{18}. Later, the same author (Laser, 1948, 1949) showed that the acid was more widely distributed than had at first been thought, and he obtained it from various body tissues, brain tissue being a particularly rich source.

In the present investigation, the free fatty acids of brain were prepared by the normal ethanolic extraction procedure and, by lead salt precipitation, the unsaturated fatty acid fraction was separated from the relatively inactive saturated acid fraction. The unsaturated acid fraction was now subjected to molecular distillation, a series of empirical fractions being collected. As can be seen from Fig. 1, haemolytic activity (assayed by the method of Laser (1949) using horse erythrocytes) was largely confined to the earlier fractions. The individual active distillates were now subjected to low temperature crystallization from acetone-light petroleum (Hilditch, 1948). The fractions insoluble at −20° consisted of saturated acids (confirmed by iodine value determinations), while those soluble at −20° and insoluble at −70° comprised monoethylenic acids; polyethylenic acids were soluble at −70°. The haemolytic activity of various crystallizates from fraction II of the molecular distillate is shown in Fig. 2; activity is almost wholly confined to the monoethylenic fraction. One of the most highly active fractions obtained in this way (fraction III-2-2) was obtained from fraction III of the molecular distillate. The haemolytic activity of this fraction is very similar to that of synthetic *cis*-octadec-11-enoic acid (Fig. 3). The ultraviolet absorption spectrum of fraction III-2-2 and other similar fractions showed only general absorption in the region 2000–2200 A., whereas simple saturated acids and α, β-unsaturated acids show maxima near 2040 and 2100 A., respectively. The infrared spectrum was in its main features identical with that of oleic acid (*cis*-octadec-9-enoic acid) and no groupings of the types $-C(CH_3)=CH-$, $-CH=CH_2$ or *trans*-$-CH=CH-$ could be detected. The infrared data, coupled with the iodine value (83), strongly suggested that fraction III-2-2 consisted wholly of C_{18} monoethylenic fatty acids; indeed it seemed likely that these fractions were substantially the strongly haemolytic C_{18} acid, contaminated, in all probability, with oleic acid. The contamination with oleic acid would be expected from the method of preparation. The impracticability of complete removal of contaminants from the purified concentrate of haemolytic acid using the small amount of material available caused us to proceed to structural determination by chemical methods, using fraction III-2-2 and other fractions which were closely similar in potency, iodine value and other characteristics (e.g. fractions II-2-1, IV-2-1-2).

As a first step, hydroxylation of the double bond was carried out on a semi-micro scale. The method of Lapworth & Mottram (1925) could not be satisfactorily adapted to our needs, but oxidation with osmic acid in pyridine (Criegee, Marchand & Wannowius, 1942) gave an α-glycol fraction, m.p. 124·5–125°, in over 90% yield. Comparative oxidations

were carried out on oleic acid which yielded a glycol, m.p. 131° (cf. Bader, 1948) and petroselinic acid (*cis*-octadec-6-enoic acid) which gave a glycol, m.p. 122–122·5°. Osmic acid oxidation of synthetic *cis*-octadec-11-enoic acid gave a glycol, m.p. 125·5–126°, in agreement with the findings of Ahmad, Bumpus & Strong (1948), who used potassium permanganate as oxidizing agent. Mixtures of the glycol from fraction III-2-2 and other similar fractions with those from oleic acid or *cis*-octadec-11-enoic acid melted at 125–126°, but a mixture of the same glycol with that from petroselinic acid melted at 80–100°.

The α-glycol from fraction III-2-2 and similar fractions was now subjected to periodate oxidation, using the procedure of King (1938) modified for use on 70–100 mg. scale; for this purpose the apparatus described by Erdös & Laszlo (1938) was found suitable. Fission of dihydroxy fatty acid, prepared as above, should yield a mixture of an aldehyde and an aldehydo-acid thus:

$$CH_3(CH_2)_xCHOHCHOH(CH_2)_yCOOH \rightarrow CH_3(CH_2)_xCHO + OHC(CH_2)_yCOOH.$$

After completing the oxidation with periodate, the aldehyde was steam distilled from the product, and from it the semicarbazone and 2:4-dinitrophenylhydrazone were prepared. These derivatives were expected to be mixtures, since we suspected that the concentrate used contained some oleic acid in addition to the strongly haemolytic acid. The semicarbazone obtained had m.p. 92–93°; it gave intermediate values in mixed melting-point determinations with the semicarbazones of the straight chain C_7 and C_9 aliphatic aldehydes, and marked melting-point depression when mixed with those of the C_5, C_6 and C_8 aldehydes. The 2:4-dinitrophenylhydrazone prepared from the aldehyde fraction had m.p. 80–88°, and a synthetic mixture of the corresponding C_7 and C_9 aldehyde derivatives melted at 81–86°. It was found impossible to fractionate the material, m.p. 80–88°, or indeed to separate artificial mixtures of the C_7 and C_9 aldehydo-derivatives by chromatography, and attempts to purify the aldehyde itself by distillation failed.

The portion of the periodate oxidation product which did not steam distil was further oxidized with potassium permanganate. Fractional crystallization of the resulting acid mixture yielded azelaic acid (*n*-heptane-1:7-dicarboxylic acid) and *n*-nonane-1:9-dicarboxylic acid.

From these results, it is justifiable to conclude that the haemolytic acid is *cis*-octadec-11-enoic acid (*cis*-heptadec-10-ene-1-carboxylic acid, *cis*-vaccenic acid) and that the isolated natural product is contaminated with oleic acid. The results of surface film and X-ray studies (Goddard & Alexander, 1950) are in accord with this conclusion. Further confirmation was obtained upon synthesizing *cis*-octadec-11-enoic

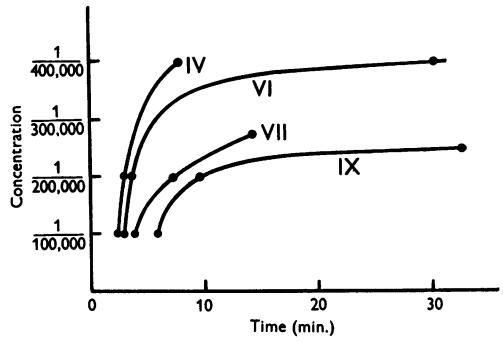

Fig. 1. Haemolytic activity of molecular distillates. Roman numerals refer to fraction numbers. 10 ml. phosphate buffer (0·15 M), pH 7·3, 0·1 ml. 5% erythrocyte suspension. The time in this and subsequent figures is the time taken to reach the end point of haemolysis as described by Laser (1949).

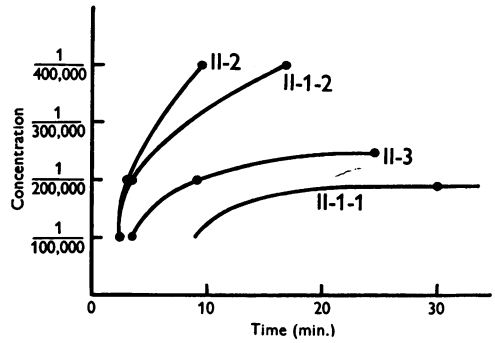

Fig. 2. Haemolytic activity of fractions crystallized at low temperatures. Numerals refer to fraction numbers. 10 ml. phosphate buffer (0·15 M), pH 7·3, 0·1 ml. 5% erythrocyte suspension.

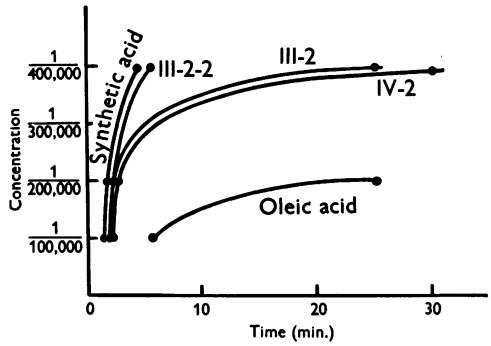

Fig. 3. Haemolytic activity of natural concentrates and pure acids. Numerals refer to fraction numbers. 10 ml. phosphate buffer (0·15 M), pH 7·3, 0·1 ml. 5% erythrocyte suspension.

acid according to the method of Ahmad *et al.* (1948); the synthetic acid had haemolytic properties similar to the natural material (Fig. 3). It is of interest to note that *cis*-octadec-11-enoic acid has not hitherto been found in nature, although its *trans* isomer, vaccenic acid (Bertram, 1928), has been known as a constituent of natural fats for many years. Although no precise figure can be given, consideration of the amount of purified material isolated, and its haemolytic activity relative to synthetic *cis*-vaccenic acid, suggests that the latter acid is present in brain in quantity at least 0·025 % of the undried tissue.

EXPERIMENTAL

Isolation of the free fatty acid fraction from horse brain

Eight horse brains, each weighing approx. 400–500 g., were minced and freeze-dried at −10° to +10° in a high vacuum for 72 hr. to yield a powder which was ground with sand and extracted in a large vapour-jacketed Soxhlet apparatus with ethanol (10 l.). After extraction (18 hr.), the cooled liquor was filtered to remove sterols (395 g.), concentrated to smaller bulk (1·5 l.) in an atmosphere of N_2 and additional sterols (28 g.) filtered off. The ethanolic filtrate was evaporated yielding a solid residue which was taken up in ether (1 l.). Extraction with NaOH (2 %, w/v) in 200 ml. batches yielded the Na salts of the free fatty acids in solution. The solution was acidified to pH 7·1–7·3 and extracted with ether. The ether extract was washed thoroughly with water and extracted with K_2CO_3 solution (2 %, w/v); the alkaline extract was acidified and the free acids again extracted with ether. A final extraction of the ethereal solution with NaOH solution (2 %) yielded a solution of Na salts which was then acidified with acetic acid to pH 7·1–7·3 and lead acetate (50 ml.; 5 %, w/v) added following the usual Pb salt precipitation procedure (Hilditch, 1947). The Pb salts of the monoethylenic fatty acid fraction, contaminated with acetic acid, were extracted with ether to yield the free fatty acids (10·6 g.).

Molecular distillation of the free fatty acid fraction

The free fatty acids (approx. 9 g.) were distilled slowly in a large 'cold finger' molecular still, and the distillate collected in ten (arbitrary) fractions, one per day. Fractions I–X each weighed approx. 700–900 mg. and distilled at approx. 60°/10^{-5} mm.; they were stored under N_2 and protected from light.

Fractionation of the fatty acid mixtures

The various fractions were dissolved in light petroleum (b.p. 40–60°) or acetone, moisture being excluded, and cooled to the desired temperature for at least 60 min. (Hilditch, 1948). The fractions were filtered through a Jena G4 sintered-glass funnel surrounded by a cooling bath, and the filtrate was collected in a receiver immersed in an ethanol/CO_2 bath. The filtration was carried out as quickly as possible and, as far as possible, under an N_2 atmosphere. All I_2 values were determined in duplicate or triplicate by the method of Trappe (1938) on 2–3 mg. samples.

Fraction II (1150 mg.; iodine number 71) in acetone (16 ml.) was cooled to −10° and filtered. The filtrate was further cooled to −70° and filtered at that temperature. The following fractions were thus obtained:

II-1; insol. −10°, iodine number 50, 507·4 mg.

II-2; sol. −10°, insol. −70°, iodine number 90, 349·1 mg.

II-3; sol. −70°, iodine number 112, 280 mg.

Fraction III (705 mg., iodine number 71) was dissolved in acetone (10·5 ml.) and filtered at −10°, and the filtrate cooled to −70° and filtered at that temperature, to yield fractions:

III-1; insol. −10°, iodine number 33, 144·4 mg.

III-2; sol. −10°, insol. −70°, iodine number 79, 335·1 mg.

III-3; sol. −70°, iodine number 74, 154·5 mg.

Other fractions from the molecular distillation were treated in a similar manner. As a second solvent, light petroleum (b.p. 40–60°) was used.

Fraction III-2 (330 mg.) was dissolved in light petroleum (10 ml.), cooled to −50° and filtered at that temperature to yield:

III-2-1; insol. −50°, iodine number 87, 142·6 mg.

III-2-2; sol. −50°, iodine number 83, 134 mg.

Fraction III-2-1 (130 mg.), dissolved in light petroleum (5 ml.), was cooled to −70° and filtered at that temperature to yield:

III-2-1-1; insol. −70°, 103·8 mg.

III-2-1-2; sol. −70°, 21·4 mg.

Other highly active fractions IV-2-1-2 and II-2-1 were obtained in a similar manner.

Oxidation experiments with potassium permanganate and osmic acid

(1) Oleic acid (426·2 mg.) was oxidized by the procedure of Lapworth & Mottram (1925). The acid was dissolved in a solution of KOH (0·5 g.) in water (50 ml.) and cooled to 3°. $KMnO_4$ solution (40 ml., 1 %) was added, the mixture briskly stirred for 5 min., SO_2 passed in to decolorize, conc. HCl (15 ml.) added and the mixture allowed to stand, then filtered and the filter residue washed with light petroleum (5 ml., b.p. 80–100°). The yield of crude 9:10-dihydroxyoctadecanoic acid was 235 mg. (48 %), m.p. 122°. Decreasing the time of oxidation decreased the yield of the product and increasing the amount of material oxidized gave an increase in percentage yield.

(2) Oleic acid (332 mg.) was oxidized with osmic acid in ether and pyridine at 0° (Criegee *et al.* 1942) to yield the pure α-glycol (336·9 mg., 91 %), m.p. 131°.

(3) Osmic acid oxidation of the haemolytic acid concentrate (fraction IV-2-1-2; 288 mg.) in the same way yielded a solid dihydroxy acid (290·5 mg.), m.p. 117–118°. Recrystallization from ethanol: ether raised the m.p. to 124·5°. (Found: C, 67·8; H, 11·2. Calc. for $C_{18}H_{36}O_4$: C, 68·3; H, 11·4 %.) Mixed with 9:10-dihydroxyoctadecanoic acid (m.p. 131°) and with 11:12-dihydroxyoctadecanoic acid (m.p. 126°), the melting point was 125–126°.

Oxidation of the dihydroxy acid with periodic acid

The procedure of King (1938) was followed closely and adapted to a semi-micro scale. Model experiments were first carried out with 9:10-dihydroxyoctadecanoic acid. This glycol (105·1 mg.) was dissolved in ethanol (6 ml.) at 40°, and a solution of KIO_4 (78·9 mg.) in N-H_2SO_4 (3·8 ml.) at

40° added. The mixture was stirred briskly for 120 sec., cooled with stirring for 60 sec., water (30 ml.) added and the whole extracted with ether (2 × 100 ml.). After removal of ether, the residual oil was steam distilled in the apparatus described by Erdös & Laszlo (1938); the aldehyde was extracted from the distillate with ether and converted to its semicarbazone. The non-volatile residue in the apparatus was transferred with ether to a small beaker, the ether removed, 0·1 N-$KMnO_4$ (12 ml.) and N-H_2SO_4 (3 ml.) were added and, after 3 hr. at room temperature, SO_2 was passed in to decolorize and the azelaic acid (43 mg., 98%; m.p. 105°) extracted with ether. In order to obtain satisfactory yields in these experiments, it was necessary to use peroxide-free ether.

The glycol from the haemolytic acid concentrate was now oxidized with periodic acid in the same manner. The α-glycol (79·2 mg.) oxidized for 1·5 min. using KIO_4 (64·6 mg.) yielded the aldehyde semicarbazone (18·6 mg.), m.p. 84°. Recrystallization of the semicarbazone raised the melting point to a constant 92–93°. Mixed melting points with authentic semicarbazones of the straight chain C_5–C_9 aliphatic aldehydes were: C_5, 75–80°, depression; C_6, 77–88°, depression; C_7, 92–103°, intermediate; C_8, 88–89°, depression; C_9, 93–96°, intermediate.

In further fission experiments, attempts were made to distil the oily aldehyde from the steam distillate in the apparatus of Craig (1936, 1937), but only polymers were obtained. Conversion of the aldehyde fragment to the 2:4-dinitrophenylhydrazone gave a derivative, m.p. 82–88°. Chromatography on talc, magnesium sulphate or alumina, using light petroleum (b.p. 40–60°) and benzene as developing solvents, failed to separate it into components. A synthetic mixture of the C_7 and C_9 aldehyde 2:4-dinitrophenylhydrazones (m.p. 81–86°) behaved similarly. The use of paper chromatography with butanol-acetic acid-water as developer was also tried without success, as was anhydrous paper saturated with methanol and light petroleum (b.p. 100–120°)-methanol as developer.

From the non-volatile fragment of the fission product, permanganate oxidation gave a dicarboxylic acid fraction, m.p. 91° (45·4 mg.). Fractional crystallization of this material from benzene yielded a less soluble fraction, m.p. 107° which, mixed with *n*-nonane-1:9-dicarboxylic acid (m.p. 110°), had m.p. 108°, and a more soluble fraction, m.p. 105°, undepressed in admixture with an authentic sample of azelaic acid (m.p. 105°).

cis-*Octadec*-11-*enoic acid*

The method of Ahmad *et al.* (1948) was followed and furnished a product, m.p. 10–11°. Oxidation with osmic acid gave an α-glycol, m.p. 125·5–126°. A mixed melting point with that from the haemolytic acid showed no depression.

SUMMARY

1. An unsaturated fatty acid present in horse brain and showing high haemolytic activity *in vitro* has been obtained in the form of a highly active concentrate containing oleic acid as a contaminant.

2. Chemical evidence is presented showing that the haemolytic acid is *cis*-octadec-11-enoic acid (*cis*-heptadec-10-ene-1-carboxylic acid), an acid not hitherto found in nature.

3. Synthetic *cis*-octadec-11-enoic acid appears to have all the properties of the natural haemolytic acid.

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