# 169. THE CONSTITUTION OF ARACHIDONIC ACID (PRELIMINARY COMMUNICATION)

By DORIS ELAINE DOLBY, LESLIE CHARLES ALFRED NUNN and IDA SMEDLEY-MACLEAN

*From the Lister Institute, Chelsea Bridge Road, S.W. 1*

(*Received 2 October 1940*)

Arachidonic acid is widely distributed in the animal body and its presence has been shown to be essential for the maintenance of the normal health of the rat. In rats which have suffered for many months from the fat deficiency disease first described by Burr & Burr [1930] the liver was found to be entirely free from this acid and only very small amounts were detected in other parts of the body. It was, however, retained if linoleic or arachidonic acid itself was included in the diet of the rats. The conclusion was drawn by Nunn & Smedley-MacLean [1938] that the rat can probably synthesize arachidonic acid using linoleic acid as its starting material. At this time all that was known as to the structure of arachidonic acid was that it contained a normal chain of 20 carbon atoms since it yielded arachidic acid on reduction [Bosworth & Sissons, 1934], that four double bonds were present and that, from the normal iodine value, these were unconjugated. We were therefore anxious to determine the position of the double bonds in the chain and to compare the structure of this acid with those of linoleic acid.

Shinowara & Brown [1940] have, however, recently published some preliminary results on the structure of this acid and suggested a formula though they emphasize that it is only tentative. The determination of the diene number of methyl arachidonate is interpreted by these authors as indicating the presence of about 5% of conjugated bonds derived either from the presence of a small proportion of an isomeric ester or from a rearrangement of the ester under the conditions of the diene determination. However, in the bulk of the acid examined, the bonds were not conjugated. The methods of oxidation used by these authors were ozonolysis and oxidation with permanganate in acetone solution. We, on the other hand, have used the action of an aqueous alkaline permanganate solution and have arrived at entirely different conclusions from those drawn by Shinowara & Brown. We should have preferred to repeat and extend our experiments before publishing our results but in view of the communication of these authors and of the postponement of our experiments necessitated by present conditions, we have decided to publish them.

Shinowara & Brown, with their methods of oxidation, did not detect any aldehyde higher than acetaldehyde, nor did they detect oxalic acid but they obtained indications of adipic and succinic acids and concluded that a double bond existed between the 18th and 19th carbon atoms and that the group $:C.CH_2.C:$ characteristic of linoleic and linolenic acids was not present in the molecule. We on the other hand deduce from our findings that the terminal chains of ten carbon atoms are similar in structure in both arachidonic and linoleic acids and that certainly three and almost certainly four of the groups $:CH.CH_2.CH:$ occur in the arachidonic molecule.

We used as our starting material the acid prepared by debromination of the ether-insoluble arachidonic octabromide obtained by brominating the fatty acids of ox suprarenal glands and extracting the product with ether in a Soxhlet apparatus following the directions given by Brown [1928]: I.V. = 334 (calc. 334·2). In order to avoid rearrangement of the position of the double bonds, the acid was not distilled. Farmer & Van den Heuveland [1938], in working with the cod-liver oil acids, had found evidence of increased molecular refraction appearing after the methyl esters had been distilled even at low pressure; but there was no increased refraction when the process of "molecular distillation" was used.

*Oxidation by alkaline permanganate*

3·18 g. arachidonic acid were dissolved in a solution of 30 g. KOH in 2 litres water and to this were added 50 g. $KMnO_4$ dissolved in 1·5 litres water. Both solutions were cooled to 0° and the $KMnO_4$ solution added to the well-stirred soap solution during rather less than 10 min. The reaction mixture which at the end of 2·5 hr. at room temperature was still alkaline and contained excess of permanganate was decolorized by passing in $SO_2$ and 60 ml. conc. $H_2SO_4$ were diluted and added. The mixture was then steam-distilled; the distillates containing $SO_2$ and volatile acids were neutralized, evaporated to dryness and the residual aqueous solution extracted 25 times by ether.

*Volatile acids.* The salts of the volatile acids were acidified and again steam-distilled, the distillates being now free from sulphite or sulphate. The distillate was collected in successive portions of 30 ml. and the equivalents of the acids present in these fractions determined by comparing the amount of 0·1 *N* NaOH required for neutralization with the dry weight of sodium salt obtained. The distillation was continued until 30 ml. of distillate required less than 1 ml. 0·1 *N* NaOH for neutralization. The first fraction was distinctly milky, the equivalent of its acid being 102·2; the acid equivalents of the next three fractions lay between 96·6 and 98·7. The final 635 ml. of distillate contained acid of average equivalent 60 but in the final 60 ml. of this distillate one-third of the acidity was due to formic acid. The acid of the first fractions (equiv. 102) smelt strongly of valeric acid. Since, however, lower acids were present in the mixture, the presence of a higher homologue was not excluded, the most probable interpretation being the presence of valeric with possibly some hexoic acid. A test tube experiment showed that under the conditions of our experiment if sodium valerate was left with alkaline permanganate some reduction takes place, so that some amount of lower acid will in any case probably be formed.

0·12 g. Na salt prepared from the first fraction was boiled under a reflux for 1·5 hr. with 0·3 g. *p*-bromophenacyl bromide in aqueous alcoholic solution and rapidly cooled; 0·18 g. crystals (M.P. 46–50°) was obtained, and the M.P. raised by recrystallization to 58–60°. *p*-Bromophenacyl butyrate, valerate and hexoate were prepared for comparison. M.P.: butyrate 61·2°; valerate 72–73·5°; hexoate 70–71·5°. Crystals prepared from the 2nd and 3rd fractions melted at 55–57°.

Mixtures of crystals (M.P. 58–60°) with butyrate (M.P. 61·2°): softened at 45°, melted at 49–50°; valerate (M.P. 72·3°): softened at 63°, melted at 71·2°; hexoate (M.P. 70·5–71·5°): softened at 65°, melted at 68–70°. These results seemed to exclude the presence of butyric acid and suggested the possible presence of a mixture of hexoic and valeric acids. A mixture of *p*-bromophenacyl-valerate and -hexoate softened at 63° and melted at 67°.

By further crystallization from aqueous methyl alcohol the melting-point of the crystals was raised to 55–60°. Two kinds of crystals were present, transparent plates and opaque clumps. When the transparent plates were separated as far as

possible, these melted from 65 to 70°; mixed with *p*-bromophenacyl valerate, M.P. 73°, they softened at 65° and melted completely at 71–72°; with the hexoate, M.P. 70–71°, they softened at 55° and melted at 60–65°. From a consideration of the equivalent, the behaviour of the *p*-bromophenacyl ester and the odour, the chief acid present is certainly valeric, and it is most probably accompanied by some hexoic acid.

*Identification of dibasic acids.* The residual aqueous solution was extracted with ether 25 times; this extract gave no precipitate with a solution of 2:4-dinitrophenylhydrazine; ketonic groups were therefore absent.

The ethereal solution of the first 14 extracts contained 2·59 g. solid of which 0·44 g. separated as sparingly soluble white crystals losing 28·24 % of their weight in the desiccator; M.P. 188–189° (anhydrous). $(COOH)_2\ H_2O$ loses 28·5 %; anhydrous oxalic acid, M.P. 189°. A small amount of the crystals washed with ether on a tile and dried gave an equivalent by titration of 49·2. The equivalent weight of anhydrous oxalic acid is 45. A solution of the sodium salt gave with $CaCl_2$ solution and ammonia the white flocculent ppt. characteristic of oxalic acid.

The total amount of $(COOH)_2$ present was therefore calculated as follows. From the ether-soluble fractions 1–14, 0·316 g. anhydrous oxalic acid was isolated. A portion of the remainder was titrated with $N/10$ $KMnO_4$ in acid solution and the amount of oxalic acid estimated. From similar titrations carried out on the subsequent ether extracts the total amount of oxalic acid was computed as 1·9 g., representing approximately 2 molecular proportions of oxalic acid for each molecule of arachidonic acid oxidized.

The residual aqueous liquid after repeated ether extraction was then treated with $N/10$ $KMnO_4$ in acid solution to remove any remaining oxalic acid. This liquid, however, reacted only slowly with the permanganate using in all 16·0 ml. corresponding with a possible maximal amount of 0·07 g. oxalic. The residual aqueous solution from the first 14 fractions which had been treated with acid permanganate to remove all oxalic acid present contained about 0·5 g. of some substance other than oxalic acid. It was therefore concentrated to a small bulk and ten times extracted with ether. 0·69 of somewhat sticky crystals was obtained. From this, by recrystallization from water, 0·1 g. crystals melting at 142–150° was obtained. This suggested the presence of adipic acid, M.P. 154°, but the mixed melting point of the crystals with a specimen of adipic acid (M.P. 149–150°) was 125–135°, with suberic acid (M.P. 140°), 122–130°. The crystals were therefore neither adipic nor suberic acid. By recrystallization from ether the M.P. was raised to 160–165° and a mixed melting point with succinic acid (M.P. 186–187°) was 165–180°, on remelting 170–178°. No evidence was obtained of the presence of any dibasic acid other than oxalic and succinic.

The aqueous residue from fractions 15–19 was added to the residual aqueous solution from fractions 1 to 14 and extracted with ether but no satisfactory product was obtained.

The final ether extracts were not treated with $KMnO_4$: the solid residue was extracted with benzene; after washing on a porous tile 14 mg. white needles were obtained (M.P. 85–89°), mixed M.P. with glutaric acid (M.P. 92–95°), 88–91°.

The benzene-insoluble residue melted at 95–105°: it dissolved in ether and from this solution two sets of crystals were separated; these were picked out by hand and consisted of

(*a*) 0·21 g. of well-defined cubes, M.P. 90–100°, equiv. by titration 65. These crystals were extracted with $CHCl_3$ and the extract filtered; the solution deposited crystals (M.P. 95–98°), mixed with glutaric acid (M.P. 93°), M.P. 71°. These were probably hydrated oxalic acid.

(*b*) A very small quantity of needles, M.P. 180°. These were probably succinic acid.

The final residual liquid which had been repeatedly extracted with ether and then treated with acid permanganate was precipitated with barium chloride with the object of separating an acid-soluble barium salt from the mass of $BaSO_4$ thrown down. This attempt was not successful.

From 3·14 g. arachidonic acid, there is therefore evidence of the formation of approximately 2 molecular proportions of oxalic acid, of succinic and valeric with probably some hexoic acid, of lower volatile acids and of a very small amount of glutaric acid.

Since from determinations of the iodine value and of the diene number arachidonic acid probably does not contain conjugated double bonds, a chain of 11 carbon atoms is the shortest that will include the four double bonds without conjugation, i.e. inserting one methylene group between each pair of double bonds. The presence of valeric acid with probably small amounts of hexoic acid, indicates a terminal *n*-amyl group attached to the ethylene group at one end of the chain of unsaturated linkages; the valeric acid would be formed if the ethylene group were oxidized to oxalic, the hexoic acid by fission of the double bond. If the terminal ethylene group nearest to the carboxyl were oxidized to oxalic acid, we should be left with succinic acid formed from carbon atoms 1–4. This structure seems confirmed by the isolation of the small amount of glutaric acid, which must have come from fission of the terminal group at the carboxyl end of the chain. The formula of arachidonic acid would therefore be regarded as

$$CH_3(CH_2)_4CH{:}CH.CH_2.CH{:}CH.CH_2.CH{:}CH.CH_2CH{:}CH.(CH_2)_3.COOH.$$

This formula would agree well with the formation of arachidonic from linoleic acid since the chain of ten terminal carbon atoms is identical in the two compounds, these two double bonds representing the 9:10 and 12:13 positions in the 18 C chain. If we suppose that the lengthening of the chain takes place by the condensation of the carboxyl group of the $C_{18}$ acid with some compound such as pyruvic acid or acetic acid, unsaturation must then occur in the 6:7 and 3:4 positions in the $C_{18}$ chain or in the 8:9 and 5:6 positions in the $C_{20}$ chain.

Since Shinowara and Brown used different methods of oxidation it is difficult to compare their results with ours. Using oxidation by permanganate in acetone solution they were unable to identify any oxalic acid. They agree with us in obtaining evidence of the presence of succinic acid which they confirmed by the analysis of the Ba salt. They obtained some evidence of the presence of an acid with M.P. 154°, equiv. 69·9 (adipic acid has M.P. 151–153°, equiv. 73) which they regarded as adipic acid. As described above, we found an acid with M.P. 142–150° which we thought might be adipic acid but this was disproved by a lowering of about 20° when the mixed melting point was taken. Shinowara and Brown identified acetaldehyde in an ozonolysis experiment; possibly this might originate from the oxidation of a $:CH.CH_2CH:$ group but the large proportion of volatile fatty acids with five and six carbon atoms obtained by oxidation with alkaline aqueous permanganate clearly indicates that the terminal ethylene linkage is not in the 17:18 position in the arachidonic chain. The formula suggested by Shinowara and Brown is not in accordance with the results of the oxidation with alkaline aqueous permanganate.

It may also be noted that in work on the oxidation of the hydroxy derivatives of linoleic and linolenic acids Green & Hilditch [1937] have shown that whereas in the oxidation of the dihydroxy derivatives of the monoethylenic acids the main fission is into oxalic acid and acids containing eight carbon atoms, with the tetra-

and hexa-hydroxy derivatives only 20 % of the acid is broken up in this manner. In the oxidation of arachidonic acid at least 50 % of the ethylene groups are separated as oxalic acid.

SUMMARY

Oxalic, succinic, acetic, valeric with probably some hexoic and small amounts of glutaric and formic acids have been identified among the oxidation products of arachidonic acid with alkaline permanganate.

A formula is suggested in which the ten terminal atoms are similar in structure to those of linoleic acid.

REFERENCES

Bosworth & Sissons (1934). *J. biol. Chem.* **107**, 489.
Brown (1928). *J. biol. Chem.* **80**, 435.
Burr & Burr (1930). *J. biol. Chem.* **86**, 587.
Farmer & Van den Heuveland (1938). *J. chem. Soc.* p. 427.
Green & Hilditch (1937). *J. chem. Soc.* p. 764.
Nunn & Smedley-MacLean (1938). *Biochem. J.* **32**, 2178.
Shinowara & Brown (1940). *J. biol. Chem.* **130**, 331.