AUTOXIDATION AS A CAUSE OF ANTIBACTERIAL ACTIVITY IN UNSATURATED FATTY ACIDS

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PLATE XVIII

It has long been recognised that certain long-chain fatty acids have antibacterial properties (Nieman, 1954; Kodicek, 1949, 1958; Galbraith *et al.*, 1971); but, although various hypotheses have been advanced, the mechanism of the inhibition has remained uncertain. Moreover, results obtained by different workers with different groups of micro-organisms have not been consistent. It is known that not only the primary interaction of unsaturated fats with molecular oxygen but also the further breakdown of the lipid peroxides can affect cell function (Schauenstein, 1967; Dormandy, 1969, 1971; Slater, 1972; Wills, 1961). The term "autoxidation" is used here to include this process of secondary fragmentation. We have investigated the antibacterial action of several lipids as a function of their autoxidation.

MATERIALS AND METHODS

The four fatty acids, chosen for their varying degrees of unsaturation, were oleic (18:1), linoleic (18:2), linolenic (18:3), and arachidonic (20:4). They were obtained 99% pure from Sigma Chemicals (Kingston-upon-Thames KT2 7BH) in sealed vials. Each fatty acid was dispensed in 100- μ l portions into a series of five 7 $\times$ 1-cm glass tubes. To the first tube in each of the four series, 10 μ l 0.2% butylated hydroxytoluene in methanol (BHT) was added immediately to prevent autoxidation. This tube was flushed with oxygen-free nitrogen, sealed, and stored for reference at -4°C . The remaining tubes were left exposed to atmospheric oxygen at room temperature. At intervals from 7 hours to 7 days, autoxidation in these tubes was stopped by the addition of BHT and sealing and storage as for the "reference" tube. The chemical changes in the materials were monitored by serial gas-liquid (figure) and thin-layer chromatograms.

Antibacterial activity was tested against four organisms: *Staphylococcus aureus*, no. NCTC6571 (Gram-positive aerobe), *Escherichia coli*, no. NCTC10418 (Gram-negative aerobe), *Clostridium welchii*, wild strain (Gram-positive anaerobe) and *Bacteroides* sp., wild strain (Gram-negative anaerobe). The *Clostridium* and *Bacteroides* were cultured on 10% layered blood-agar plates prepared from Digest Agar (Southern Group Laboratories). They were incubated in MacIntosh and Fildes jars for 18 hours at 37°C . The *Staphylococcus* and *Escherichia* were cultured on "Wellcotest" Sensitivity Test Agar (Wellcome Reagents) by aerobic incubation for 18 hours at 37°C . Growths from overnight blood-agar cultures were used to prepare saline suspensions of the organisms sufficient to give confluent growth when flooded on the appropriate test plates. Excess fluid was removed and the plates were dried at 37°C . Of each fatty acid in stages of progressive autoxidation, 20 μ l was pipetted on 10-mm sterilised paper disks and the impregnated disks were applied to the surface of the medium. After incubation at 37°C for 18 hours, the zone of inhibition around each disk was measured.

RESULTS

The table shows the degree of growth inhibition as indicated by the diameter of the inhibition zone around the disks. Except for the *Staphylococcus* which was inhibited by

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polyunsaturated fatty acids at zero time, i.e., when theoretically no autoxidation had yet occurred, the increasing inhibition with time and the corresponding chromatograms suggest that antibacterial activity was largely the function of progressive autoxidation. As would be expected, inhibition was greatest and earliest with arachidonic acid, the most unsaturated of the fatty acids tested. Limitations on the diffusion of water-soluble autoxidation products into the gel probably accounts for the plateaux reached after 2–7 days. Even in the case of the staphylococcus the relation of the degree of inhibition at zero time and the relative susceptibility of the four fatty acids to autoxidation raises the strong possibility that antibacterial activity was due to the presence in low concentrations of autoxidation products in the original material. The antioxidant BHT in 10 times the concentration in which it was used to arrest autoxidation had no inhibitory effect on its own.

TABLE

Inhibition of four organisms by 20 μ l of four unsaturated fatty acids that had been exposed to the atmosphere for various periods of time

Organism	Width (mm) of inhibition zone* around disk containing indicated fatty acid (degree of unsaturation)																			
	Oleic (18 : 1): duration of exposure (days)					Linoleic (18 : 2): duration of exposure (days)					Linolenic (18 : 3): duration of exposure (days)					Arachidonic (20 : 4): duration of exposure (days)				
	0	1	2	3	7	0	1	2	5	6	0	1	2	4	5	0	1	2	3	6
<i>Staphylococcus aureus</i>	0	0	0	1	1	1	7	9	11	15	6	8	8	8	17	14	16	19	20	20
<i>Clostridium welchii</i>	0	0	0	0	0	0	0	0	2	3	0	0	5	6	6	0	2	6	6	6
<i>Escherichia coli</i>	0	0	0	0	0	0	0	0	0	0	0	0	2	3	4	0	2	3	4	5
<i>Bacteroides</i> sp.	0	0	0	0	0	0	1	4	5	7	0	5	9	11	11	0	6	9	11	10

* Total diameter minus diameter of the disk (10 mm).

DISCUSSION

It has been recognised since the earliest days of autoxidation studies that the rapid breakdown of lipid peroxides generates many water-soluble, small-molecular fragmentation products (Gutteridge, Stocks and Dormandy, 1973). In recent years, Schauenstein (1967), who used mainly chromatographic techniques, showed that several of these substances had marked anti-tumour activity. The main implication of our own findings is that some antibacterial effects attributed in the past to long-chain fatty acids may also be due to their autoxidation products. The high antibacterial potency and water solubility of some of these compounds suggest their possible value as chemotherapeutic agents.

SUMMARY

Four unsaturated fatty acids were allowed to undergo varying degrees of autoxidation before being tested for antibacterial activity against *Staphylococcus aureus*, *Escherichia coli*, *Clostridium welchii*, and *Bacteroides* sp. The degree of inhibition was proportional to the degree of unsaturation of the fatty acid and to the duration of its autoxidation. Some of the

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FIGURE.—Gas-liquid chromatograms showing three stages in the autoxidation of linolenic acid. Glass columns (4 mm $\times$ 1.5 m) packed with 20% polyethylene glycol 20M coated on 100–120 mesh Diatomite C. Detection by flame ionisation. Separations shown are isothermal runs at 180°C; (A) immediately after exposure to air; (B) after 1 day's exposure; (C) after 5 days' exposure.

antibacterial activity of fatty acids described in the past could be accounted for by autoxidation products.

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