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Effects of non-ionic surfactants that modify experimental tuberculosis on lipase activity of macrophages

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Summary

1. Six series of non-ionic surface active polyethylene glycol ethers, whose effects on experimental tuberculosis have previously been correlated with their polyoxyethylene chain lengths, were examined for their influence on the activity of a lipase present in homogenates of normal mouse peritoneal macrophages. The surfactants are concentrated in the lysosomes of macrophages—a cell type in which the host-parasite confrontation takes place. A preparation of soy bean oil was used as triglyceride substrate; and hydrolysis at pH 4·5 was compared in the presence and absence of surfactant, the products of hydrolysis being assayed by photodensitometry of thin-layer chromatograms.

2. The compounds with short polyoxyethylene chains inhibited the release of fatty acid, compared with surfactant-free standard, more than did those with long chains; and some of the latter showed actual enhancement of release. Accumulation of monoglyceride was observed in the presence of six of the seven long-chained compounds, but with none of the seven short-chained compounds.

3. The similarity between this correlation of chain length of the surfactants with their effect on macrophage lipase activity, and the known correlation of their chain length with their effect on experimental tuberculosis, suggests a possible connection. How this connection might relate to the mechanism of the varying effects on tuberculosis is briefly discussed.

Introduction

The activities of lipoprotetin lipase and pancreatic lipase, and of some liver lysosomal phospholipases, are inhibited by the non-ionic surface active agent " Triton WR-1339 " (Brown, Boyle & Anfinsen, 1953; Schotz, Scanu & Page, 1957; Robinson, Jeffries & French, 1954; Janicki & Aron, 1962; Jorolan & Janicki, 1965; Fowler & de Duve, 1969; Stoffel & Trabert, 1969. This surfactant and other, structurally related compounds—poloxyethylene ethers of alkylphenol-formaldehyde polymers—show differing effects on experimental murine tuberculosis (Cornforth, Hart, Nicholls, Rees & Stock, 1955; Hart & Rees, 1955). If the phenolic nucleus is the same but the average number of ethylene oxide units per phenolic group is varied, the compounds with short polyoxyethylene chains tend to suppress and those with long chains to enhance the disease. Similar contrasting effects are shown on the multiplication of tubercle bacilli within cultured macrophages, which are a main site of attack in tuberculosis (Hart, 1968).

These surfactants probably produce their antituberculous and 'protuberculous' effects by acting on the host cell rather than directly on the bacterium, but the mechanism is still obscure. During administration of some of the compounds to mice and rabbits, neutral lipids accumulate in the blood plasma (Kellner, Correll & Ladd, 1951; Cornforth *et al.*, 1955) and within macrophage lysosomes (J. A. Armstrong & D'Arcy Hart, unpublished observation), suggesting that alterations in tissue lipase activity might be involved. We have therefore tested selected compounds, having specified polyoxyethylene chain lengths, for their possible varying influence on the degradation of a defined triglyceride (TG) substrate by the enzymes in homogenates of mouse peritoneal macrophages; the hydrolysis products have been assessed by thin-layer chromatography.

Methods

Non-ionic surfactants

Fifteen of the available members of six series of polyethylene glycol ethers (prepared by Professor J. W. Cornforth and his colleagues) were selected as representative. Each of five of the series had a different phenolic nucleus and—within that series—a range of average numbers of ethylene oxide units per phenolic group, so that a final product can be referred to as, for example, HOC-15. The nuclear structures were as follows: 'HOC'=a *p-tert*-octylphenol-formaldehyde cyclic tetramer; 'LOC'=a stereoisomer of HOC; 'HHC'=as HOC but *p-tert*-hexylphenol; 'D_2'=a *p-tert*-octylphenol-formaldehyde linear polymeric mixture; 'DoD_2'=as D_2 but *p-tert*-dodecylphenol. The nucleus of the sixth series was 2,2-di-*n*-octadecylpropane-1,3-diol ('OPD'). Triton WR-1339 (Rohm & Haas Co. Philadelphia) was also included.

Macrophage homogenate ('enzyme')

Macrophages were obtained from the (unstimulated) peritoneal cavities of normal (uninfected) mice of the albino P strain, and were maintained as monolayers on glass coverslips in Leighton tubes containing 1·0 ml of medium (without surfactant) suitable for long-term survival (Hart, 1968). After 1–3 weeks in culture at 37° C, coverslips were withdrawn from the tubes and, after washing in several changes of 0·9% NaCl, four were crushed manually and sequentially with a glass pestle in a single dry test-tube. The fragments were covered with 1·0–1·2 ml of 0·9% NaCl, cooled to 4° C, exposed for 30 s to ultrasonic vibration (400 kHz supplied by a 200 W generator), and cooled again; this procedure disrupted the cells. The homogenate was transferred with a Pasteur pipette to a second test-tube containing fragments from a further four coverslips, and a second exposure of 30 s to vibration was made. The final pooled homogenate (derived from eight coverslips or about 2×10^6 cells, and containing about 500 μg protein measured by the copper-Folin method) was made up to 1·2 ml with 0·9% NaCl—sufficient for six assay tubes—and was stored at 4° C for up to 1 hour.

Triglyceride substrate

The emulsion 'Intralipid 20%' (Vitrum, Stockholm, obtained from Paines & Byrne, Greenford, Middlesex) was used. Its composition is given as 20% (w/v)

fractionated soy bean oil (consisting of TG mainly of long-chain unsaturated fatty acids (FA)); 1·2% (w/v) fractionated egg-yolk lecithin; 2·5% (w/v) glycerol; in water.

Procedure

Surfactant, 0·1 ml of 1·25% (w/v) in 0·9% NaCl, was added to test-tubes (75 mm × 15·5 mm) containing 0·004 ml of a 10% aqueous dilution of Intralipid emulsion (80 μg TG). Two further tubes received 0·1 ml of 0·9% NaCl instead of surfactant. After incubation at 37° C for 1 h, with manual shaking at 10 min intervals, 0·3 ml of sodium acetate-acetic acid buffer (pH 4·5) 0·1M, followed by 0·2 ml of macrophage homogenate, were added to each tube; total volume 0·6 ml, final concentration of TG 0·16 mM. One of the tubes without surfactant received 1·9 ml of chloroform-methanol (C-M, 1 : 2) immediately, and was brought to 4° C, to prevent hydrolysis (' negative control '); all the other tubes (including the second tube without surfactant —the ' standard ') were reincubated at 37° C for 20–24 h except where otherwise stated, without shaking. Hydrolysis was then stopped with 1·9 ml of C-M (1 : 2).

The concentration of Intralipid used (giving 0·16 mM TG) and the long incubation period were necessary to show inhibitory or enhancing effects of the different surfactants on lipase activity. The pH of 4·5 was adopted after finding that in the above system buffered at pH 2·7–8·5, macrophage homogenate gave a single peak at pH 4·0–4·5 for optimum TG-splitting activity, judged by FA release after 4 and 18 h; there was no activity at pH 8·5.

The acidic pH optimum was consistent with a lysosomal hydrolase. The finding that the macrophage homogenate split 2-naphthyl acetate over a broad pH range—activity being greater at pH 6·0, 7·2 and 8·5 than at 4·5—indicated that the lipolytic enzymes studied were not simple esterases.

Thin-layer chromatography

The lipids were extracted from the reaction mixture according to Fowler & de Duve (1969). The solvents were made up to a known volume and an aliquot placed on a thin layer of Kieselgel G (Merck). The neutral lipids were separated by the solvent systems of Manners, Kidder & Parsons (1969), namely, petroleum ether (40°–60°)-diethyl ether-glacial acetic acid (35 : 65 : 0·5, v/v) to 14 cm, followed by petroleum ether-diethyl ether (47 : 3, v/v) to 19 cm. The plates were charred with 50% H_2SO_4 (v/v) at 160° C, and the lipids were scanned by photodensitometry according to Payne (1964). Activity was measured as FA and monoglyceride (MG) produced, percent of total neutral lipid.

Since lipase activity was assayed by measuring FA and MG spots by photodensitometry, the possible influence of factors other than lipase activity were considered. Further surfactant-free controls were examined, namely, with heat-inactiviated enzyme, with enzyme omitted, with enzyme but without added substrate, and with substrate added terminally; also surfactant-containing controls without enzyme, or with substrate added terminally. The additional controls without active enzyme showed negligible amounts of hydrolysis products by thin-layer chromatography. The enzyme preparations showed small quantities of TG and cholesterol from the macrophages in a chromatographic system separating neutral lipids;

in the amounts used in the incubation mixture the TG content contributed only about 6% of the total TG. As a further check, each surfactant was extracted with C-M and submitted to thin-layer chromatography. All showed traces of spots in the TG and FA positions. It was calculated that these would have the effect of underestimating inhibition of lipase activity and of slightly overestimating enhancement, as judged by FA production.

Quantitation by densitometry of free fatty acids produced in the system is justified as although the enzyme preparations contribute small quantities of lipid to the substrate and the phospholipid present in the Intralipid a further 6%, nearly 90% of the lipid available for lipase activity is the TG of soy bean oil.

Results

Effect of surfactants on triglyceride-splitting activity of macrophage homogenates

The lipase activity of macrophage homogenate in the presence of the various surfactants was compared with that in similar incubation mixtures without surfactant ('standard'), tested concurrently. The results for the HOC series are shown in Fig. 1. The standard shows an increase in FA over the negative control, as well

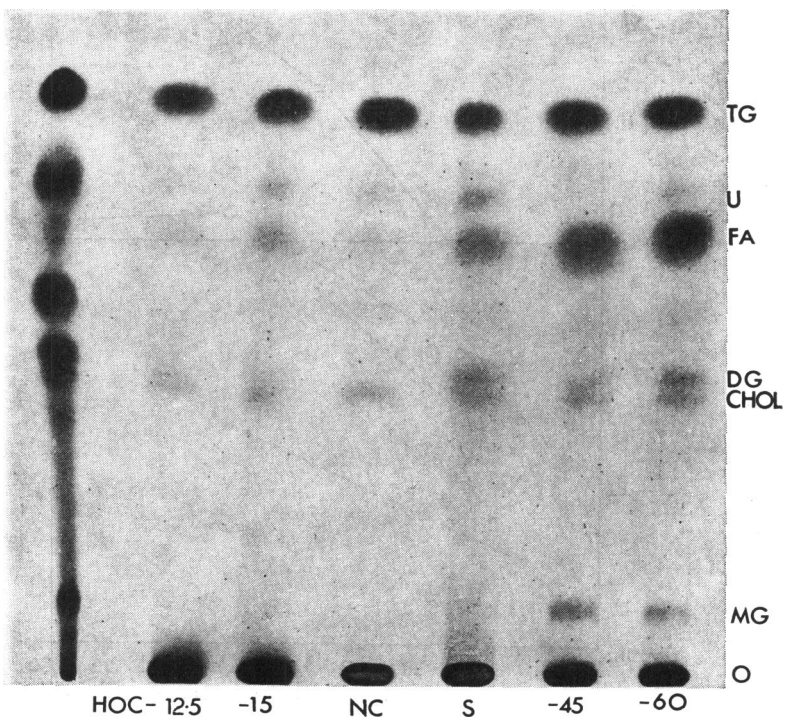

FIG. 1. Effect of the HOC series of surfactants on lipase activity of mouse peritoneal macrophages. Incubation mixture: final concentration of triglyceride 0·16 mM, of sodium acetate-acetic acid buffer (pH 4·5) 0·05 M of surfactant 0·2%, and of macrophage homogenate 33%. Incubation time 22 h at 37° C. The standard (S) was the incubation mixture without surfactant; the negative control (NC) was the complete mixture but without incubation. In the thin-layer chromatogram TG refers to triglyceride, DG to diglyceride, MG to monoglyceride, FA to fatty acid, CHOL to cholesterol, U to unidentified spot, O to origin (at which surfactants remain).

as diglyceride (DG) and a trace of MG. In the presence of the compounds with shorter polyoxyethylene chain lengths (HOC-12·5 and HOC-15), FA is reduced as compared with the standard, and no DG or MG is seen. With the compounds that have longer chain lengths (HOC-45 and HOC-60), FA and MG are increased, and there is some DG; the accumulated MG was shown, by comparison with a standard equilibrium mixture, to be composed of both 1- and 2-MG, the latter in the greater concentration. These differences within the series are interpreted as inhibition and enhancement, respectively, of TG-splitting lipase activity.

In Fig. 2 the FA production is plotted against the number of ethylene oxide units of surfactants from the six series. A correlation is evident between poloxyethylene chain length and fatty acid release. The compounds with short chains inhibited the release more than those with long chains. In the HOC and D_2 series the compounds with long chains enhanced the lipase activity. The single compounds OPD-64 and WR-1339 fit the pattern. The standard in these experiments usually showed only trace amounts of MG after the 20–24 h incubation (after 4 h small quantities were seen, which presumably were further hydrolysed to FA later). The compounds with short chains showed no MG, but those with long chains showed an accumulation (3%–12% of total neutral lipid); an exception was the long-chained LOC-60.

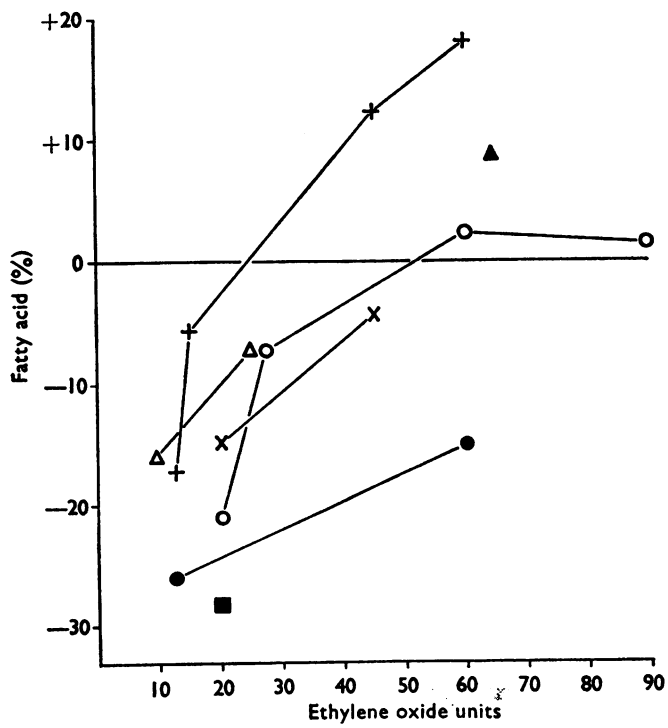

FIG. 2. Effect of surfactants on release of fatty acids from triglyceride by macrophage homogenates, in relation to polyoxyethylene chain length. Six series, designated by chemical nucleus: HOC (+——+); D_2 (○——○); OPD (▲); HHC (△——△); DoD_2 (×——×); LOC (●——●); and Triton WR-1339 (■) in addition. Incubation mixture: concentrations of ingredients as for Fig. 1; pH 4·5; incubation time 20–24 hours. Abcissa: average number of ethylene oxide units per nuclear group. Ordinate: difference in FA (FA expressed as percentage of total neutral lipid) at end of incubation in presence of surfactant from that in the standard mixture without surfactant (shown as 'O').

Discussion

The present results extend the observation that Triton WR-1339 inhibits the activity of lipases. We have shown, with a number of analogous surfactant series, that by altering the structural design so as to change the lipophilic-hydrophilic balance of the molecule, the inhibitory effect on trigylceride-splitting macrophage lipase activity decreases with increasing polyoxyethylene chain length and can even be reversed. Although this inhibition and enhancement may not be part of the same mechanism, an obvious possible basis is a varying complexing of substrate with surfactant which impedes or facilitates contact or reaction between substrate and enzyme. The accumulation of monoglyceride in the presence of the long-chained compounds might be due to enhanced degradation of triglyceride, together with inhibited isomerization of 2- to 1-monoglyceride.

The correlation of chain length of these compounds with their effects on lipase activity is similar to that previously reported for chain length and effect on experimental tuberculosis (see above). Figure 3 shows this correlation for the compounds used in the present study. Inhibition and enhancement of tuberculous infection are evident for the seven short and seven long-chained compounds respectively.

Evidence on the mechanism of the enhancing and suppressive actions of these surfactants on tuberculous infection have so far depended on correlations with their membrane-permeability effects *in vitro*, particularly on lipids of red blood cells and on artificial lipid membranes (see Hart, 1968). The most striking is in the lysis of human red cells after sudden cooling, the antituberculous agents decreasing, but the protuberculous increasing, cell sensitivity to this shock (Lovelock & Rees, 1955).

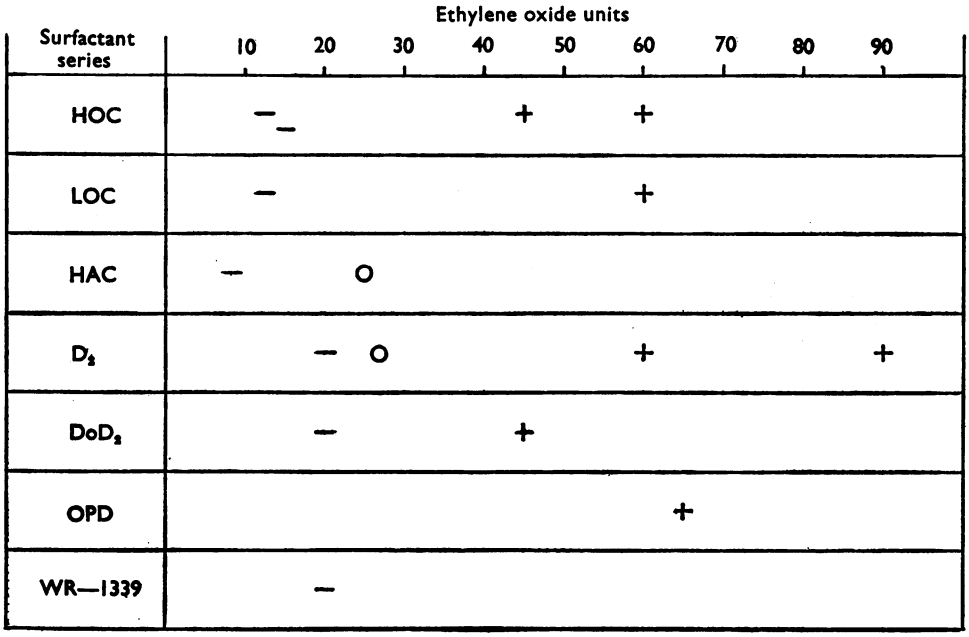

FIG. 3. Effect of surfactants on experimental tuberculous infection in relation to polyoxyethylene chain length. Six series, designated by chemical nucleus (HOC, D_2, etc.), and Triton WR-1339 in addition. Abcissa: average number of ethylene oxide units per nuclear group. —, Suppressive; +, enhancing; O, inactive; the centre of each symbol indicates the number of ethylene oxide units. (Data from Cornforth *et al.*, 1955; Hart, 1968.)

Our finding that chemical structure is correlated with the effect on lipase activity as well as on tuberculous infection may contribute to an understanding of the mechanism of the latter relationship. The system studied contains acidic macrophage lipase, and neutral lipids that are also known to be present in macrophages and to accumulate in their lysosomes when they are treated with some of the surfactants (J. A. Armstrong & P. 'Darcy Hart, unpublished observation, 1970); from this situation the products of enzymic degradation or, alternatively, the retention of lipid could influence intracellular multiplication of the tubercle bacilli.

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