

THE ROLE OF SERUM IN THE NUTRITION OF *ASTEROCOCCUS MYCOIDES*

By A. W. RODWELL*

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Summary

For growth of *Asterococcus mycoides* in a complex liquid medium, serum could be replaced by balanced mixtures of a heat-stable protein fraction, cholesterol, and oleate, when a suitable osmotic pressure was provided. Oleate could be replaced by linoleate or by linolenate, and the protein fraction by bovine serum albumin or by starch. Both the protein fraction and cholesterol inhibited the rapid lysis brought about by low concentrations of oleate or other surface-active agents, anionic and non-ionic. It was concluded that serum provides a non-toxic source of oleate, probably as a molecular association with protein and cholesterol, but the evidence did not indicate whether or not cholesterol is also required as a nutrient.

I. INTRODUCTION

The causal organism of bovine pleuropneumonia, *Asterococcus mycoides*, like all strains of the pleuropneumonia group of microorganisms except those isolated from sewage (Laidlaw and Elford 1936) requires for growth in complex media factors which may be supplied by serum or ascitic fluid. Serum may be autoclaved with the medium without destruction of its growth-promoting properties (Priestley and White 1952), and it may be replaced by egg yolk (Nakamura, Futamura, and Watanuki 1926).

Preliminary work in this Laboratory indicated that lipids were involved in the growth-promoting activity of serum for *A. mycoides*. Serum extracted by the methods of Hardy and Gardiner (1910), McFarlane (1942), or of Sprince and Kupferberg (1947) would no longer support growth, but growth was restored in the presence of both the lipid extract emulsified in "Tween 80" as dispersing agent and of the lipid-extracted residue. When human plasma was fractionated by method 10 of Cohn *et al.* (1950), the activity was quantitatively recovered in the crude fractions III (o), IV (i), and V (i.e. the α - and β_1 -lipoproteins and the albumin fraction). A lipoprotein prepared from horse serum (Macheboeuf and Rebeyrotte 1949) also had some activity in replacing serum.

Edward and Fitzgerald (1951) and Edward (1953) showed that both lipid and protein fractions of serum were required, and that the lipid fraction could be replaced by cholesterol. Serum could be replaced for a number of strains by a mixture of cholesterol and either starch or serum albumin. Growth was improved by the further addition of egg-yolk phospholipids. Cholesterol could be replaced by cholestanol or stigmasterol. Cholesterol hydrogen phthalate replaced cholesterol for some strains, but the oleate, stearate, and acetate esters were inactive for all strains. Smith and Morton (1951, 1952) described an electrophoretically pure, lipid-free, low molecular

* Division of Animal Health and Production, C.S.I.R.O., Animal Health Research Laboratory, Parkville, Vic.

weight protein from serum which replaced serum for a number of strains of human origin.

These discordant findings have now been reconciled by a paper of Smith, Lecce, and Lynn (1954), in which the protein of Smith and Morton (1952) was shown to contain bound lipid, including cholesterol. After methanol extraction of both basal medium and protein, a requirement for cholesterol was found. Phospholipids in low concentrations increased the growth obtained in the presence of cholesterol and methanol-extracted protein. The inactivity of the oleate, acetate, and stearate esters was confirmed, but cholesteryl laurate was superior to cholesterol. Smith *et al.* (1954) postulated that the activity of the protein was due to its high cholesterol-binding capacity. It could be replaced by higher concentrations of lipid-extracted serum albumin or β -lactoglobulin.

Our investigations have been concerned with a strain of *A. mycoides*. This species was not studied by Smith *et al.* (1954). Although Edward (1953) found this species to be more fastidious in serum replacement experiments than other members of the genus, it grows readily in liquid media, and growth may therefore be evaluated quantitatively.

II. METHODS

(a) Cultural Methods

- (i) *The Organism*.—Strain "V5" (Campbell 1938) was used.
(ii) *Basal Medium*.—The composition (per litre) of the basal medium was:

"Vitamin-free", acid-hydrolysed casein	≡ 2.5 g casein
L-Cystine	5 mg
L-Tryptophan	5 mg
"Difco" yeast extract	10 g
Na ₂ HPO ₄ -KH ₂ PO ₄ buffer, pH 7.4	0.04 mole.

Glucose (1 per cent. w/v) was added from a sterile 50 per cent. w/v solution after autoclaving the medium at 10 lb for 20 min. Tubes of medium having a final volume of 10 ml were capped with aluminium caps.

(iii) *Growth Conditions*.—The inoculum was prepared from a 3.4 day culture in BVF-OS medium (Turner, Campbell, and Dick 1935), containing between 10^{10} and 10^{11} viable particles per ml. The culture was centrifuged, the deposit was suspended in twice the original volume of saline buffered with 0.01M phosphate, and 1 drop was used to inoculate 10 ml of medium.

The tubes were incubated in air at 37°C for 3 or 4 days.

(iv) *Estimation of Growth*.—Two methods were used: optical density and titratable acidity. Optical density was measured using a filter transmitting maximally at 660 m μ , and with uninoculated medium as blank; titratable acidity was estimated by titrating both cultures and uninoculated medium in a stream of carbon dioxide-free nitrogen, to pH 8.0 as measured by a glass electrode. The two methods gave results in agreement.

(b) Fractionation of Horse Serum

(i) *Fraction A*.—Serum diluted with an equal volume of water was heated to 75°C, cooled, and its pH brought to 5.6 with N acetic acid; it was then heated to 80°C, cooled, and filtered on a Büchner funnel. Filtrate $\equiv$ fraction A.

(ii) *Fraction A₁*.—Impermeate after dialysis of fraction A.

(iii) *Fraction A₂*.—Combined permeates from dialysis of fraction A, concentrated at reduced pressure, and neutralized.

(iv) *Fraction B*.—Fraction A (pH 6.4) was saturated with ammonium sulphate, the precipitate was dissolved in water (approximately one-twentieth of the original volume of serum), and the solution was dialysed.

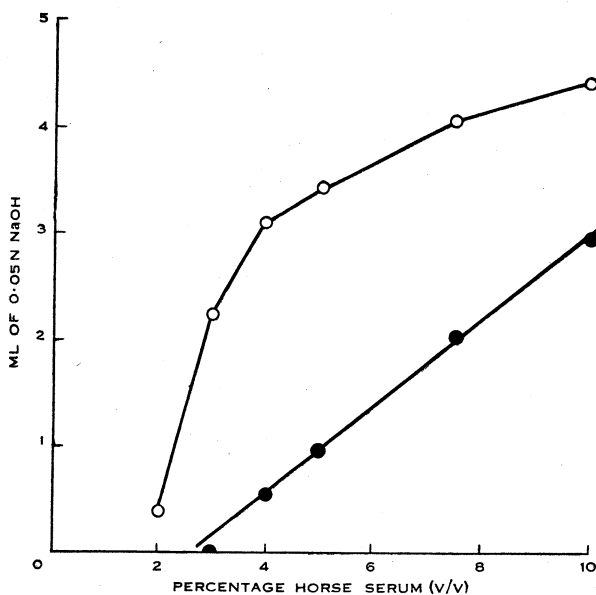


Fig. 1.—Effect of yeast extract concentration on serum requirement for growth of *A. mycoides*. ● 0.5 per cent. “Difco” yeast extract. ○ 1.0 per cent. “Difco” yeast extract.

(v) *Fraction C*.—Fraction B (protein content approximately 3 per cent. w/v) was chilled and added slowly to 10 volumes of acetone at -5°C , 1 ml of M acetate buffer pH 4.5 was added per litre of mixture, and the mixture was allowed to stand for 30 min at -5°C . The precipitate was washed successively with acetone, acetone-ether mixture, and ether, and extracted with ether for 6-10 hr in a Soxhlet extractor.

(c) Lipids

(i) *Reagents*.—Cholesterol was recrystallized twice from ethanol. Cholesteryl monosuccinate was prepared by the method of Cataline *et al.* (1944). Polyoxyalkalene sorbitan oleate (“Tween 80”) was purified by ether extraction as described by Davis (1947), and kept as a 20 per cent. w/v solution at -80°C .

The samples of linoleic and linolenic acid were the *trans*-isomers. They were kept *in vacuo* at -80°C . The oleic acid was a commercial sample of redistilled oleic acid. It was used without further purification.

(ii) *Addition of Reagents to Medium*.—Unsaturated fatty acids were added to the medium either as soaps, or as ethanolic solutions. In the latter case the amount of solution added did not exceed 1 per cent. (v/v).

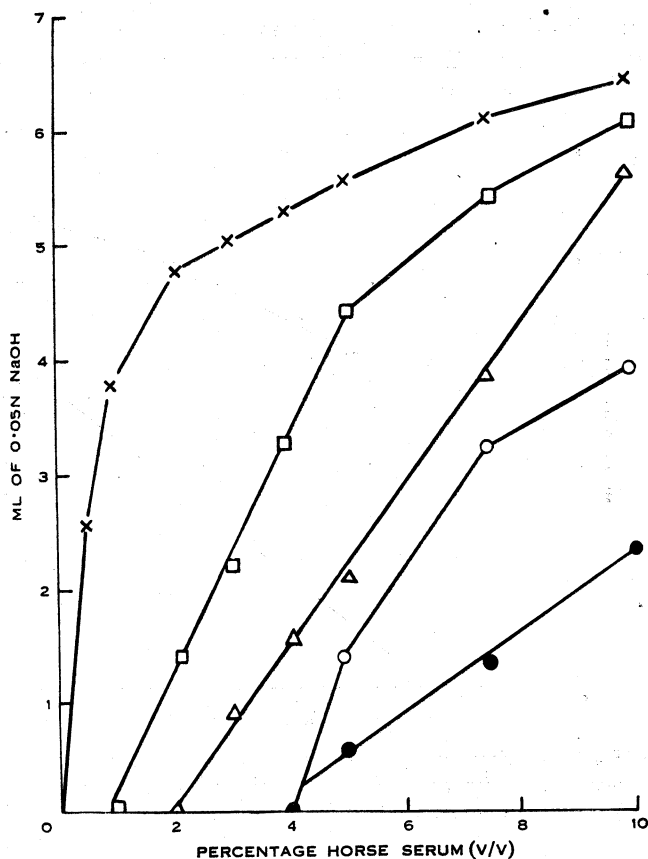


Fig. 2.—Effect of sodium acetate and of “Tween 80” on serum requirement for growth of *A. mycoides*. Yeast extract concentration 0.5 per cent. w/v. ● No additions. ○ “Tween 80”, 2.0 mg/10 ml; cholesterol, 0.5 mg/10 ml. △ Sodium acetate, 50 mg/10 ml. □ “Tween 80”, 2.0 mg/10 ml. × Sodium acetate, 50 mg/10 ml; “Tween 80”, 2.0 mg/10 ml.

Cholesterol dispersion in “Tween 80” was prepared by adding 0.5 ml of an ethanolic solution at 60°C in a fine stream to 10 ml of “Tween 80” solution at 60°C . When cholesterol or its succinate was to be added without “Tween 80”, a dispersion in water was prepared analogous to that in “Tween 80” solution. Faintly opalescent dispersions were obtained.

(iii) *Estimation of Phospholipid Content of Fraction C.*—Fraction C, (0.5 g) was refluxed four times with 20 ml portions of ethanol-ether (3:1) mixture. The combined extracts were evaporated to a small volume, and the phospholipids were precipitated with acetone containing a few drops of ethanolic solution of magnesium chloride. The acetone precipitate was dissolved in ether, and the total phosphorus of the solution was determined. The total phospholipid was calculated by multiplying the phospholipid phosphorus by the factor 25.

(iv) *Determination of Cholesterol Content of Fraction C.*—Cholesterol was determined in the supernatant, after acetone precipitation of the phospholipids, by the method of Sperry and Brand (1943).

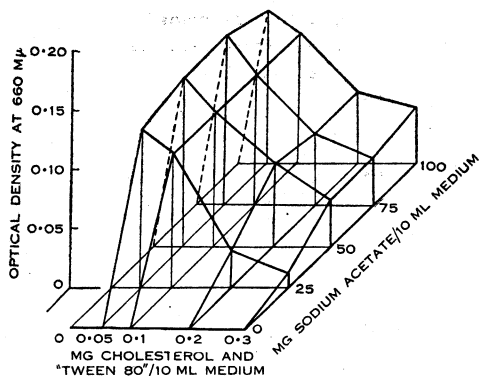


Fig. 3

Fig. 3.—Relationship between growth of *A. mycoides* and concentrations of cholesterol in "Tween 80" dispersion and concentrations of sodium acetate. Fraction C, 1.0 mg/10 ml medium.

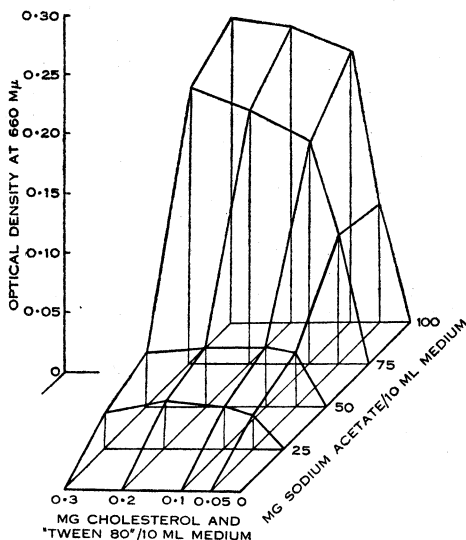


Fig. 4

Fig. 4.—Relationship between growth of *A. mycoides* and concentrations of cholesterol in "Tween 80" dispersion and concentrations of sodium acetate. Fraction C, 4.0 mg/10 ml medium.

(d) Protein Estimation

Protein was estimated by the biuret reaction as described by Weichselbaum (1946), with Armour's "Bovine Albumin Fraction V" as the standard.

III. EXPERIMENTAL

(a) Growth Experiments

(i) *Substances Sparing the Requirement for Serum.*—Acetate, "Tween 80", and yeast extract all had a pronounced effect on the quantitative requirement for serum. The effect of the yeast extract concentration is shown in Figure 1, and that of acetate and of "Tween 80" in Figure 2. Cholesterol, in the presence of whole serum, reversed the effect of "Tween 80" (Fig. 2). With ether-extracted serum, however, both cholesterol and "Tween 80" were essential for growth. A concentrate of α -lipoic acid did not spare the requirement for serum.

(ii) *Results of Horse Serum Fractionation.*—When serum was fractionated by the method set out in Section II (b), it was found that about half of the total activity of serum was recovered in fraction A, with approximately a four-fold purification. No growth was obtained when this fraction was autoclaved with the basal medium, although this did not cause any visible precipitate. It was necessary to add fraction A as a sterile Seitz filtrate after autoclaving the basal medium.

The activity of fraction A was lost after dialysis (fraction A₁), but was restored by the addition of the concentrated neutralized permeates (fraction A₂). Fraction A₂ contained the acetate used in the preparation of fraction A, and it was found that its activity could be totally accounted for by its acetate content.

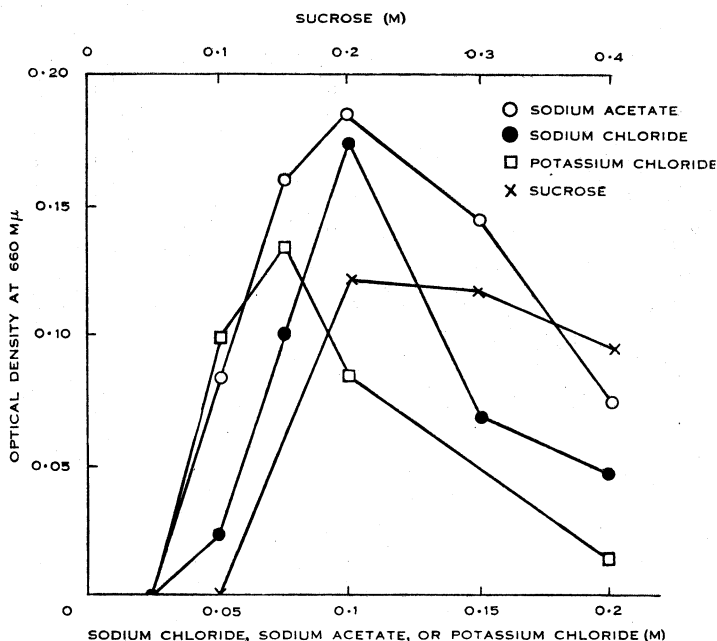


Fig. 5.—Replacement of sodium acetate by sodium chloride, potassium chloride, or sucrose for the growth of *A. mycoides*. Cholesterol, 0.5 μ mole; sodium oleate, 0.15 μ mole; fraction C, 5.0 mg/10 ml medium.

The activity of fraction A₁ was quantitatively recovered in the saturated ammonium sulphate precipitate (fraction B). In the presence of fraction C (i.e. fraction B after acetone-ether extraction) cholesterol, "Tween 80", and acetate were essential. The activity of fraction C, unlike that of fraction B, was not impaired by autoclaving. The optimum concentrations of cholesterol dispersion in "Tween 80", of fraction C, and of acetate were interrelated. This is illustrated in Figures 3 and 4.

(iii) *Specificity of Components.*—The specificity of the requirement for each of the components sodium acetate, "Tween 80", cholesterol, and the protein fraction was investigated.

(1) *Acetate*.—Sodium acetate could be replaced by approximately equimolar concentrations of other uni-univalent salts (e.g. sodium or potassium chloride), or by sucrose in twice these molar concentrations (Fig. 5).

(2) "*Tween 80*".—"Tween 80" was replaceable by oleate. In order to decrease the amount of growth in the absence of added oleate, the yeast extract used for the preparation of the basal medium was extracted with ether in a continuous extractor for 20 hr at pH 2.5. The growth responses to varying concentrations of oleate, cholesterol, and fraction C are illustrated in Figures 6 and 7. For 1 mg of fraction C per 10 ml (Fig. 6), the optimum oleate and cholesterol concentrations were 0.05 and 0.1 μ mole per ml respectively. At this concentration of fraction C, an oleate concen-

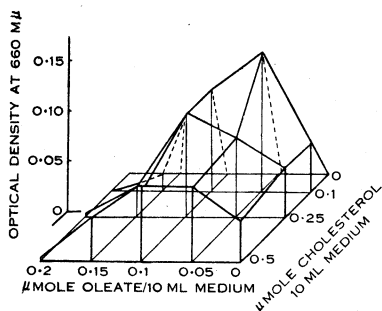


Fig. 6.

Fig. 6.—Relationship between growth of *A. mycoides* and concentrations of oleate and of cholesterol. Fraction C, 1.0 mg/10 ml medium; acetate 0.1M.

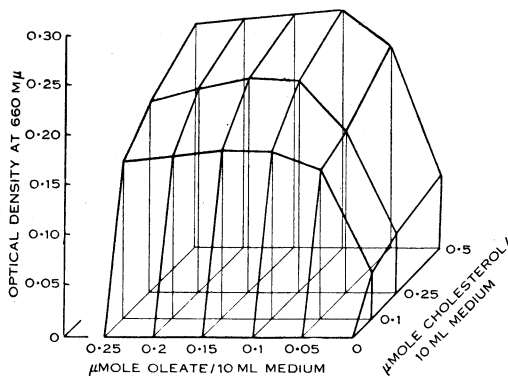


Fig. 7.

Fig. 7.—Relationship between growth of *A. mycoides* and concentrations of oleate and of cholesterol. Fraction C, 5.0 mg/10 ml; acetate 0.1M.

tration of 0.2 μ mole per 10 ml completely suppressed growth at all cholesterol concentrations. For 5 mg of fraction C per 10 ml (Fig. 7), the optimum oleate and cholesterol concentrations were 0.1 and 0.5 μ mole per 10 ml respectively, and growth was only slightly inhibited by oleate concentrations as high as 0.25 μ mole per 10 ml. Oleate could be replaced by linoleate and linolenate (Fig. 8), although these acids became inhibitory at lower concentrations than oleate.

(3) *Cholesterol*.—It may be seen from Figures 6 and 7 that the optimum cholesterol concentration varied with the concentration of fraction C. The effect of varying the cholesterol concentration for a single concentration of fraction C and of oleate is illustrated in Figure 9. Cholesterol could not be replaced for growth by cholesteryl monosuccinate. No other esters or sterols were tested.

(4) *Fraction C*.—The activity of fraction C was destroyed by treatment with pepsin or pancreatin, but not by takadiastase; it was reduced by boiling for 10 min at pH 11, but it was unaffected by boiling for 10 min at pH 2.5. Paper electrophoresis in veronal buffer at pH 8.6 showed the presence of at least three protein components. One of the components, comprising approximately one-third of the protein, was insoluble at pH 4.8. The proteins soluble and insoluble at pH 4.8

were, however, of approximately equal activity. The activity and recovery of the protein insoluble at pH 4.8 remained unchanged after precipitation was repeated five times. It was evident that the activity of fraction C was not associated with a single protein component. Therefore the separation of the proteins in fraction C was not pursued.

Fraction C contained 3.8 per cent. (w/v) total phospholipid and 0.03 per cent. (w/v) of esterified cholesterol; its peptide content as measured by the biuret reaction, against the "Bovine Albumin Fraction V" standard, was 97 per cent. The presence of phospholipid in fraction C was not essential to its activity, since the residue after hot ethanol-ether extraction was still active in supporting growth. The optimum cholesterol concentration was decreased from 0.5 μ mole per 10 ml to 0.1 μ mole per 10 ml after ethanol-ether extraction, in a system containing 0.15 μ mole oleate, and 5 mg of fraction C per 10 ml.

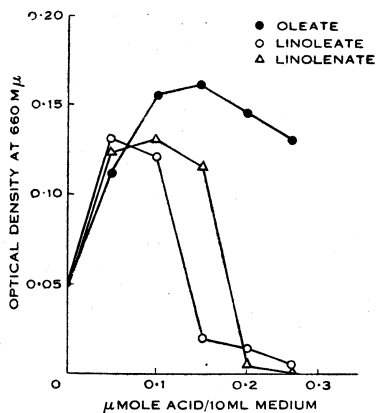


Fig. 8.

Fig. 8.—Replacement of oleate by linoleate and by linolenate for growth of *A. mycoides*. Fraction C, 5 mg/10 ml medium; cholesterol, 0.5 μ mole/10 ml medium; acetate 0.1M.

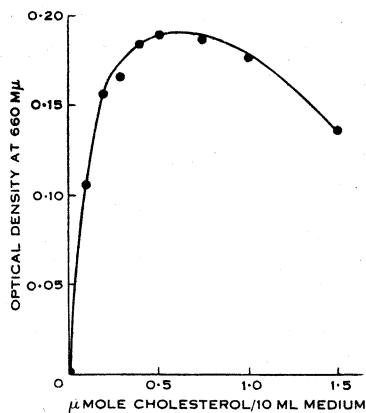


Fig. 9.

Fig. 9.—Effect of cholesterol concentration on growth of *A. mycoides*. Oleate, 0.15 μ mole; fraction C, 5 mg/10 ml medium; acetate 0.1M.

Fraction C was replaceable to a limited extent by 0.25 per cent. (w/v) of soluble starch, or by 0.25 per cent. (w/v) of "Bovine Serum Albumin Fraction V" after treatment with ethanol and ether. The optimum cholesterol concentration was much lower, and the amount of growth was only about one-quarter of that obtained with fraction C.

(b) Lysis of *A. mycoides*

(i) *Effect of Tonicity of the Medium.*—When cells of *A. mycoides* were suspended in dilute buffer solution (0.01M phosphate buffer pH 7.4), the optical density of the suspension measured at 660 μ m was lower than that of the culture from which it had been prepared. The decrease in optical density varied for different cultures from 20 to 30 per cent., and was accompanied by the release of 260 μ m-absorbing material

from the cells into the suspending medium. The decrease of optical density was prevented by suspending cells in isotonic buffered saline, or in Krebs-Ringer-phosphate solution. Removal of calcium ions by including "Versene" (ethylenediaminetetra-acetic acid) in the suspending medium had no effect on the stability of suspensions.

(ii) *Lysis by Surface-active Substances*.—In these experiments, sodium chloride (0.15M) in 0.01M phosphate buffer (pH 7.4) was used as the suspending medium. Cells of *A. mycoides* are known to be rapidly lysed by surface-active substances such

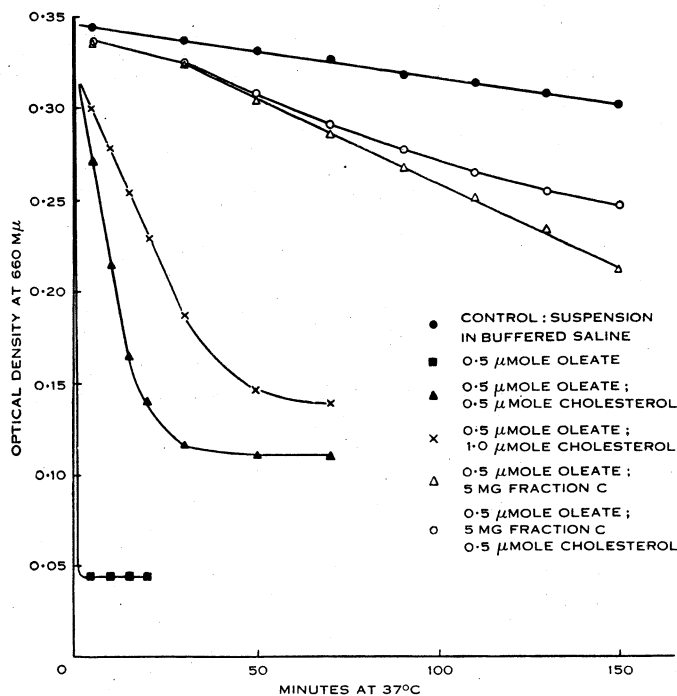


Fig. 10.—Inhibition of oleate lysis of *A. mycoides* by cholesterol and by fraction C. Mixtures of oleate and inhibitors incubated at 37°C for 15 min before system completed by addition of cells. Additions per 10 ml medium indicated in legend on figure.

as the saponins and bile salts. Since the decrease in optical density of suspensions in the presence of surface-active agents was proportional to the amount of 260 mμ-absorbing material released, optical density changes were used as a measure of lysis. The effect of a number of surface-active substances on the optical density of cell suspensions is shown in Table 1.

(iii) *Absence of Lysis by Lecithinase C*.—Since cells of *A. mycoides* were susceptible to lysis by surface-active haemolytic agents, it was of interest to see whether they were susceptible to lysis by lecithinase C. It may be seen from Table 1 that *Clostridium welchii* lecithinase C did not cause lysis of cells of *A. mycoides*.

(iv) *Absence of Lysis by Cl. welchii θ-Haemolysin*.—The —SH-dependent *Cl. welchii* θ-haemolysin did not lyse cells of *A. mycoides* (Table 1).

(v) *Inhibition of Lysis Caused by Oleate*.—Cholesterol and fraction C both inhibited lysis by oleate. Both inhibitors together were more effective than either alone. This is illustrated in Figure 10. Approximately twice the molar concentration of cholesteryl monosuccinate as of the unesterified sterol was required to reverse the effect of the same amount of oleate.

TABLE 1

LYSIS OF CELLS OF *A. MYCOIDES* BY SURFACE-ACTIVE SUBSTANCES

Cultures in BVF-OS centrifuged at 18,000 *g* for 10 min, deposit washed once, and resuspended to a density of approximately 2×10^{10} cells per ml. Optical density of suspensions plotted against period of incubation at 37°C

Suspending Media	Compound	Nature	Concentration ($\mu\text{g/ml}$)	Incubation Time (min)	Decrease in Optical Density (%)
NaCl, 0.154M; phosphate buffer, 0.01M, pH 7.4 " " " " " " " " " " " " " " "	Oleic acid	Anionic	5	10	12
			10	10	80
	Sodium lauryl sulphate	Anionic	30	10	8
			60	10	92
	Sodium deoxycholate	Anionic	200	10	11
			300	10	85
	"Tween 80"	Non-ionic	200	10	34
			400	10	64
" " "	Saponin	Non-ionic	20	10	19
			50	10	73
NaCl, 0.154M; tris (hydroxymethyl)-aminomethane-HCl buffer, pH 7.4; 0.005M CaCl_2	<i>Cl. welchii</i> α -toxin	Lecithinase C	90 HU*/ml	30	0
NaCl, 0.154M; phosphate buffer, 0.01M; sodium thioglycollate, 0.1% w/v	<i>Cl. welchii</i> θ -toxin	Oxygen-labile haemolysin	333 HU*/ml	30	0

* Haemolytic unit—amount of toxin liberating 50% of haemoglobin from sheep red cell suspension containing 7.5 mg haemoglobin per ml in 30 min at 37°C.

IV. DISCUSSION

The results reported in this paper are in general agreement with those of Edward and Fitzgerald (1951) and of Smith *et al.* (1954) but showed a requirement by *A. mycoides* for an unsaturated fatty acid in addition to cholesterol and a protein fraction. The experiments also showed that the optimum concentrations of these three factors were interrelated.

Cells of *A. mycoides* were extremely sensitive to lysis by surface-active agents, particularly by oleate. In this respect they resembled erythrocytes, but differed in

their complete resistance to the lecithinase (α -toxin) and the —SH-dependent θ -toxin of *Cl. welchii*. Fraction C and cholesterol inhibited lysis by oleate, and a mixture of both was more effective than either inhibitor alone.

These results are explained most simply by assuming that a molecular association is formed amongst protein, cholesterol, and fatty acid, and that this provides a non-toxic source of the acid. Cholesterol may also be required as a nutrient, as postulated by Edward and Fitzgerald (1951) and by Smith *et al.* (1954).

Both Edward and Fitzgerald (1951) and Smith *et al.* (1954) found that the addition of phospholipids increased the growth obtained in the presence of cholesterol and protein. Phospholipids are known to reverse the inhibitory effects of unsaturated fatty acids on the growth of *Lactobacillus casei* (Kodicek 1949), and to enhance the antihaemolytic activity of cholesterol (Lee and Tsai 1942; Ponder 1946).

The lipid requirements can be finally assessed only when the complete nutritional requirements are known. Attempts to replace yeast extract by known growth factors have so far been unsuccessful. Yeast extract and other natural materials have been shown to contain substances inhibiting the growth of the L form of *Proteus* (Medill and O'Kane 1954).

When serum was replaced by an unsaturated fatty acid, cholesterol, and fraction C, the importance of the tonicity of the medium became apparent. This recalls the finding of Koser, Breslove, and Dorfman (1942) that a suitable salt concentration is necessary for the growth of strains of the genus *Brucella*. When cells of *A. mycoides* were suspended in dilute buffer solution, approximately 30 per cent. of the total amount of 260 m μ -absorbing material was released into the medium. Osmotic effects on cells of *A. mycoides* have been noted by Freundt (1952), who showed that the pH of the culture medium in which the cells were grown influenced their osmotic resistance. If unsaturated fatty acids are involved in membrane permeability (Kodicek 1949), it might be expected that they would also effect the sensitivity of cells to osmotic effects. The lack of a rigid cell wall, and the sensitivity to osmotic effects and to lysis by surface-active substances, are probably the most outstanding features distinguishing the pleuropneumonia group of organisms, and perhaps also the L forms, from other forms of bacteria.

V. ACKNOWLEDGMENTS

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