The Identification of the Antimicrobial Factors of the Stomach Contents of Sucking Rabbits

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1. A procedure for the extraction and purification of antimicrobial factors from the stomach contents of sucking rabbits is described. 2. The fatty acid composition of the hydrogenated 'rabbit stomach oil' is given. 3. The most active factors isolated were identified as free *n*-decanoic acid and *n*-octanoic acid. 4. The antimicrobial activities of some fatty acids and that of 'rabbit stomach oil' are compared.

In most domestic mammals the alimentary tract becomes heavily populated with bacteria within a few hours of birth, and this state persists throughout the life of these animals. Recent studies (Smith, 1965, 1966) showed that baby rabbits were exceptional in this respect, for during the suckling period their stomachs and small intestines were virtually sterile. Cultures of organisms given by mouth to such rabbits were rendered sterile and the same effect was observed *in vitro* when organisms were added to the stomach contents from the rabbits. The antimicrobial activity of the stomach contents, which was not associated with secreted hydrochloric acid, was much greater against organisms such as *Staphylococcus aureus*, *Candida albicans*, *Lactobacillus acidophilus* and *Clostridium welchii* than against *Escherichia coli* and *Streptococcus faecalis* and was contained in the chloroform-soluble fraction of the stomach contents. The antimicrobial factor was not present either in the milk, as shown by the lack of inhibition by milk extracts of different cultures of organisms, or in the stomach contents of weaned rabbits. A significant fact emerged from experiments *in vitro* (Smith, 1966). Rabbit milk fat is inactive against micro-organisms, as were homogenized baby rabbits' stomachs, but when both were incubated together an antimicrobial factor appeared. Homogenized small-intestine tissues incubated with rabbit-milk fatty fraction did not produce the antimicrobial effect.

These results indicated the presence of a substrate in the fatty portion of rabbit milk that was transformed into an antimicrobial factor by the enzymes in the stomach walls of the rabbit. The present paper deals with the isolation and chemical identification of the antimicrobial factors.

MATERIALS AND METHODS

Preparation of chloroform extracts from the stomach contents. Freshly removed stomach contents (free from extraneous materials other than clotted milk) of sucking New Zealand White rabbits, aged about 10–14 days, were pooled, dried over P_2O_5 under vacuum and then extracted twice with chloroform (10ml./g. of solid) at room temperature. The extracts were combined and filtered, and the chloroform was removed by evaporation under reduced pressure. The residual oil, referred to below as 'rabbit stomach oil', was stored in the refrigerator under N_2. Its percentage composition varied according to the stage of digestion the rabbit's milk reached in the stomach.

Analytical data on 'rabbit stomach oil'. These were as follows. (*a*) Elementary analysis: Found: C, 68·4; H, 10·3; P, 1·3; Cl, 3·6%; N, nil; S, nil; ash, nil. (*b*) Saponification value: 246·5. (*c*) Iodine value: 41·4. (*d*) Free acids: the percentage by weight of free fatty acids was determined by neutralizing 5g. of rabbit stomach oil dissolved in aq. 50% (v/v) ethanol (100ml.) with 0·1N-$Ba(OH)_2$ by using phenolphthalein as indicator. The mixture was evaporated to dryness and extracted with ether (4×25ml.), and the solid barium soaps were dried *in vacuo* over P_2O_5 to constant weight. The ethereal extracts were evaporated to dryness and the residual oil, dried over P_2O_5 to constant weight, gave the percentage of glycerides and unsaponifiable material in the sample. The barium soaps were treated with 2N-H_2SO_4, and the precipitated $BaSO_4$ was washed with water (3×10ml.), ignited in a crucible and its weight recorded. From this weight and that of the barium soaps the total percentage of free fatty acids was calculated to be 14%. (*e*) Infrared spectrum (in chloroform): 3500–3000cm.$^{-1}$ (broad) (acid), 2880 and 2950cm.$^{-1}$ (CH_3 and CH_2), 1725 and 1760cm.$^{-1}$ (acid and ester). (*f*) Fluorescence spectrum (in carbon tetrachloride): activation max., 350mμ; fluorescence max., 440mμ.

Positive colour spot tests with 'rabbit stomach oil' (spray reagents). Unsaturation was detected by alkaline $KMnO_4$ and iodine–starch reagents (Hais & Macek, 1963). Free

acids were shown to be present with bromocresol blue [0·04% solution in aq. 80% (v/v) ethanol]; free fatty acids were detected with cupric acetate–$K_3Fe(CN)_6$, mercuric acetate–diphenylcarbazone (Buchanan, 1959) or phosphomolybdic acid (Kaufmann & Makus, 1960). The last-named when used in thin-layer chromatograms, also gave an indication of the degree of unsaturation. The presence of diols was shown with sodium metaperiodate–benzidine reagent (Hais & Macek, 1963) and that of phospholipids with $HClO_4$–ammonium molybdate reagent (Hais & Macek, 1963). Cholesterol esters were detected with $SbCl_3$ (Hais & Macek, 1963). Protoporphyrin (Sulya & Smith, 1960), Sudan black (Amelung & Böhm, 1954), Nile blue (Hais & Macek, 1963) and Rhodamine B (Kaufmann & Makus, 1960) reagents were used on paper and thin-layer chromatograms to detect lipids.

Preparation of methyl esters. A freshly distilled ethereal solution of diazomethane cooled at 0° (threefold excess) was added to an ethereal solution of the saturated fatty acids. The mixture was kept for 20min. at room temperature. The excess of diazomethane and ether was removed on the water bath. The methyl esters were stored in methanolic solution at 0°.

Paper chromatography. Descending chromatography on Whatman no. 1 paper was carried out in the following solvents: solvent *A*, carbon tetrachloride–methanol–aq. ammonia (sp. gr. 0·88) (81:18:1, by vol.) (Holasek & Winsaher, 1954); solvent *B*, aq. 85% (v/v) methanol with small amounts of mN-HCl (Ashley & Westphal, 1955). Ascending chromatography was performed in the following solvents: solvent *C*, light petroleum (b.p. 60–70°) or diisopropyl ether (Hammarberg & Wickberg, 1960); solvent *D*, acetone–acetic acid–water (8:1:2, by vol.) (Kaufmann & Mohr, 1958). Two-dimensional chromatograms were obtained with Whatman no. 3 paper in the following solvents (*E*): acetic acid–water (93:7, v/v) and 85% formic acid–acetic acid–water (10:10:1, by vol.) (Michalec, 1958).

Thin-layer chromatography. The following systems were used: solvent *F*, acetic acid–water (24:1, v/v) with undecane-impregnated silica gel G (Kaufmann & Makus, 1960); solvent *G*, acetic acid–formic acid–water (2:2:1, by vol.) or acetic acid–water (3:1 or 17:3, v/v) (Malins & Mangold, 1960) or acetic acid–acetonitrile–water (2:14:5, by vol.) with silicone-impregnated silica gel G.

Gas–liquid chromatography. A Pye Argon chromatograph instrument was used with the following columns: column *J* (10% polyethylene glycol adipate on 100–120-mesh Celite); column *K* (15% polyethylene glycol adipate on 100–120-mesh Celite); column *L* (10% Reoplex 400–Embacel).

Assay of antimicrobial activity. A series of solutions of the test substance were made in 0·5ml. amounts of nutrient broth, pH 6·5, each half the concentration of the previous one in the series. If the pH of the primary dilution was below 5·5, it was adjusted to approx. pH 5·5 with NaOH. Approx. 10^8 viable *Staph. aureus* cells and 10^6 viable *Cand. albicans* cells were added to each tube, and after 2hr. at room temperature one loopful of the contents of each tube was inoculated on to blood agar, 10% salt agar and Sabaraud's agar to determine whether or not the organisms had been killed (Smith, 1966). The lowest concentration in which no *Staph. aureus* or *Cand. albicans* was found was recorded as the minimum lethal concentration. With few exceptions, the relative activity of all the materials tested was similar, irrespective of whether the results were assessed by means of *Staph. aureus* or *Cand. albicans* destruction. 'Rabbit stomach oil' was included in all tests so that comparison could be made.

Purification of 'rabbit stomach oil' (oil I). The crude oil (oil I) (87g.) was treated with light petroleum (b.p. 30–40°) until no further precipitation of solid took place. The flocculent solid was recovered by centrifuging and dried *in vacuo* to yield 1·19g. of solid flakes (solid I). This solid contained phosphate (positive colour reaction with $HClO_4$–ammonium molybdate reagent; Hais & Macek, 1963) and had no activity in the microbiological tests, and was rejected. The light-petroleum supernatant was evaporated *in vacuo* and the clear oil (oil II) that was recovered was emulsified in a blender with water (2 × 100ml.) for 45min. The mixture was centrifuged at 5600*g* for 30min. The aqueous phase was extracted with light petroleum (b.p. 30–40°) (2 × 25ml.) and evaporated *in vacuo* to give a white semi-solid material (solid II, 1·70g.) that gave a positive test for phosphate (Hais & Macek, 1963). The antimicrobial activity of this fraction was less than one-eighth of that of the original oil and was not further investigated. The oily layer was dissolved in 250ml. of light petroleum (b.p. 30–40°), dried over anhydrous Na_2SO_4 and evaporated *in vacuo* to yield a clear oil (III) that had the same antimicrobial activity as the original oil.

Hydrogenation experiments. The partially purified oil III (0·5g.), dissolved in acetic acid (10ml.), was added to a prehydrogenated catalyst (10% palladium on charcoal, 0·1g.) in acetic acid (15ml.). Hydrogenation was carried out at room temperature and uptake (21·5ml. of H_2 at 762·5mm. Hg and 10°) was complete after 15min. When ethanol was used as a solvent identical results were achieved. A white waxy solid (solid III, 0·5g.) was obtained that showed the same biological activity as the original oil. Hence hydrogenation did not alter the biological activity of the oil III.

Separation of neutral and acidic fraction from oil III. Solid III (3g.), in ether–ethanol (25:1, v/v) (7–8ml.), was shaken with aq. 10% (w/v) $KHCO_3$ (50ml.) for 1hr. in an emulsifier. The ethereal layer was re-extracted in the same way, dried over anhydrous Na_2SO_4 and evaporated *in vacuo* to give 2·6g. (86% yield) of an oil ('neutral fraction'). The aqueous layers were combined, extracted once with ether (25ml.) and acidified to pH 2 with HCl, and the precipitated acids were extracted into ether (3 × 50ml.). The ethereal solution was washed with water (25ml.), dried over anhydrous Na_2SO_4 and evaporated to give 0·42g. of an oil ('acidic fraction'). Microbiological assays showed that the activity of the acidic fraction was 40–80 times that of the neutral one.

Examination of the 'acidic fraction'. Paper chromatography and thin-layer chromatography in solvent systems *A*, *B*, *C*, *D*, *F* and *G* showed the presence of hexanoic acid, octanoic acid, decanoic acid, traces of lauric acid, palmitic acid and behenic acid. Octanoic acid was the major component. Examination of the mixture by gas–liquid chromatography either as acids or as methyl esters (columns *J*, *K* and *L*) and comparison with authentic specimens confirmed the presence of the above-mentioned acids and showed, in addition, the presence of two small peaks that corresponded approximately to C_{15} and C_{22} acids.

Integration of the areas covered by the different peaks gave the percentages of these acids in the mixtures as: *n*-hexanoic acid, 2·7; *n*-octanoic acid, 77·5; *n*-decanoic acid,

12·5; palmitic acid, 2·4; behenic acid, 2·3; C_{15} (?) acid, 2·6; lauric acid, traces. The *n*-octanoic acid/*n*-decanoic acid ratio was thus 6·2:1.

Examination of the 'neutral fraction'. The neutral fraction (2·6g.) was saponified with NaOH (10g.) in aq. 70% (v/v) ethanol (75ml.) under reflux for 4hr. with exclusion of atmospheric CO_2. The solvent was evaporated *in vacuo*, the residue was taken up in water (20ml.) and extracted with ether, and the unsaponifiable extracts were rejected as they were devoid of antimicrobial activity. The aqueous layer was acidified (pH 2) and extracted with ether (2 × 50ml.), and the ethereal solution was dried over anhydrous Na_2SO_4 and evaporated to give a mixture of acids that had 6–8 times the activity of the original oil (III). This activity was practically lost when the acids were converted into their methyl esters. Examination of the mixture of acids (or methyl esters) from this 'neutral fraction' by thin-layer, paper and gas–liquid chromatography as described for the 'acidic fraction' gave the following acids and percentages: *n*-octanoic acid, 19·2; *n*-decanoic acid, 47·7; lauric acid 5·1; myristic acid, 1·0; palmitic acid, 5·5; C_{18} and C_{22} acids, 9·1; lignoceric acid, 7·4; cerotic acid (?), 5·0%.

The percentage fatty acid composition of hydrogenated 'rabbit stomach oil' was then as follows: *n*-hexanoic acid 0·4; *n*-octanoic acid, 20·7; *n*-decanoic acid, 27·1; lauric acid, 2·7; myristic acid, 0·6; C_{15} (?) acid, 0·2; palmitic acid, 3·3; mixture of C_{18}, C_{20} and C_{22} acids, 5·1; lignoceric acid, 3·9; cerotic acid, 2·7%.

Separation and identification of steam-volatile acids. Oil III (64g.) was hydrogenated in ethanol (500ml.) with 10% palladium on charcoal catalyst (10g.), saponified and acidified to pH 2 to give a mixture of fatty acids (oil IV, 44·2g.). This quantity corresponds to 69% (by wt.) of oil III. Oil IV was fractionated into steam-volatile and non-volatile acids (Popják & Beeckmans, 1950). The distillate was collected in $Ba(OH)_2$ solution. The barium soaps were recovered by evaporation *in vacuo* of the aqueous suspension. The acids were obtained by acidifying with 2N-H_2SO_4, saturating the solution with Na_2SO_4 and extracting with ether.

Neither the mixture of non-volatile acids nor that of their methyl esters displayed any significant antimicrobial activity and they were shown by gas–liquid chromatography to consist of lauric acid, myristic acid, palmitic acid, stearic acid, behenic acid, lignoceric acid and cerotic acid.

The mixture of steam-volatile acids, which was 6–8 times as active as oil III, was converted into their methyl esters and fractionated by distillation. Pure methyl *n*-hexanoate, methyl *n*-octanoate, methyl *n*-decanoate and methyl laurate were obtained. The following solid derivatives were prepared from them for identification purposes: *n*-hexanoic acid: amide, m.p. 101°, and anilide, m.p. 95–96°; *n*-octanoic acid: amide, m.p. 109–110°, and hydrazide, m.p. 88–89°; *n*-decanoic acid: hydrazide, m.p. 98–99°, toluidide, m.p. 76–78°, and hydroxamic acid, m.p. 86–87°; lauric acid: toluidide, m.p. 86–87°.

Assay of the antimicrobial activity of different fatty acids. 'Rabbit stomach oil' was used as standard in the microbiological assays carried out under identical conditions of pH, concentration, temperature etc. Table 1 illustrates the activities of different fractions of 'rabbit stomach oil' and also those of several fatty acids in relation to 'rabbit stomach oil'.

In a final assay of the activities of *n*-decanoic acid and *n*-octanoic acid, *E. coli*, *Strep. faecalis*, *L. acidophilus* and *Cl. welchii* were used as test organisms as well as *Staph. aureus* and *Cand. albicans*. The use of all these organisms except *E. coli* and *Strep. faecalis* confirmed that *n*-decanoic acid had an antimicrobial activity about 8 times, and *n*-octanoic acid 4 times, that of 'rabbit stomach oil'. The activity of *n*-decanoic acid against *E. coli* and *Strep. faecalis* was, however, much less than that of 'rabbit stomach oil'

Table 1. *Comparison of the antimicrobial activities of different fatty acids with 'rabbit stomach oil'*

The test organisms used were *Staph. aureus*, *Cand. albicans* and *Cl. welchii*. The antimicrobial activities are expressed as percentages of that obtained with 'rabbit stomach oil'.

Acid	Activity (%)	Acid	Activity (%)	'Rabbit stomach oil' fraction	Activity (%)
Saturated acids		Hydroxy acids			
Hexanoic	25	DL-α-Hydroxyisohexanoic	<6	Solid I	6
Octanoic	400	DL-α-Hydroxyoctanoic	<6	Oil II	100
Nonanoic	400	α-Hydroxyisobutyric	<6	Solid II	6
Decanoic	800–1000	ω-Hydroxypentadecanoic	<6	Oil III	100
Undecanoic	800	Unsaturated acids		Solid III	100
Dodecanoic	6	Hex-2-enoic	25	'Neutral fraction'	6–12
		Hex-3-enoic	25		
Tetradecanoic	6	Hept-2-enoic	50	'Acidic fraction'	400
Pentadecanoic	6	Oct-2-enoic	100	Free acids of 'neutral fraction'	400–500
Hexadecanoic	6	Oct-3-enoic	100		
Octadecanoic	<6	Non-2-enoic	100–200	Oil IV	400–500
Docosanoic	<6	Dec-2-enoic	200–400	Steam-volatile acids (oil IV)	600–800
		Undec-10-enoic	200–400	Non-volatile acids (oil IV)	6–12
Branched-chain acids		Linoleic	6*	Non-volatile acids, methyl esters (oil IV)	6–12
3-Methylpentanoic	6	Linolenic	6*		
4-Methylpentanoic	6	Ricinoleic	6	Tri-*n*-decanoyl glyceride	12–25

* Activity 800% when *Staph. aureus* was the test organism.

itself, and that of *n*-octanoic acid was sufficient to account for the slight activity of 'rabbit stomach oil' against these two organisms, on a quantitative basis.

DISCUSSION

Fatty acids have been known for some time to possess antifungal and antibacterial properties. Tetsumoto (1933) reported the bactericidal action on *Salmonella typhi* and *Vibrio cholerae* of *n*-octanoic acid, *n*-nonanoic acid and *n*-decanoic acid among other fatty acids assayed. Inhibition of the tubercle bacillus by fatty acids (*n*-octanoic acid, *n*-nonanoic acid, *n*-decanoic acid) has been the subject of many publications since the 1930s (Hailer, 1938; Katsura, Tamura, Halori & Maeda, 1948; Dubos, 1950; Hukuhara, 1954). Sodium decanoate and sodium undecanoate were found by Keeney, Ajello & Lankford (1944, 1945) to be the most active of all the sodium salts of fatty acids that were tested *in vitro* against broth cultures of various strains of bacteria. The fungicidal properties of fatty acids are also well documented: decanoic acid possessed the highest fungicidal properties over a wide pH range (Hoffman, Schweitzer & Dalby, 1939; Keeney *et al.* 1944).

Rabbit's milk contains approx. 13% of fatty substances, and 90 ml. of the contents of sucking rabbits' stomachs yielded about 20 ml. of the 'rabbit stomach oil'. It appears that it is the combination of the high fat concentration in the milk, the composition of the fat and the unique enzymic system of the rabbit's stomach that renders sterile the young rabbit's stomach, and subsequently its small intestine. Thus, when other sucking mammal species were fed with rabbit's milk, the antimicrobial properties of the stomach contents after ingestion were slight in comparison with those in the rabbit (Smith, 1966). It is therefore postulated that the presence of free fatty acids, and especially of *n*-decanoic acid and *n*-octanoic acid, in the stomach of young rabbits is responsible for the virtual sterility of the gastrointestinal tract and of its contents. The sterile intestinal contents show *in vitro* a lack of antimicrobial activity, irrespective of differences in the pH of the medium, which may be due to the absorption of the active principles by the small intestine.

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