Studies on the Fatty Acid Composition of some *Salmonellas*

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SUMMARY

The available data on the fatty acid composition of *Salmonella* organisms is meagre and incomplete; the present work indicates that the three organisms examined have fatty acids ranging from C₁₂ to C₂₂ in their lipids. Changes in the growth medium may be responsible not only for quantitative differences but also for qualitative changes in the fatty acid spectrum of neutral compounds as well as phospholipids. The organisms can become avirulent possibly through fatty acid alterations of an endotoxin molecule.

INTRODUCTION

The development of our knowledge of bacterial lipids began with the work of R. J. Anderson on the tubercle bacilli in 1920. This work was continued for over 30 years and was concerned with the isolation and characterization of the bacterial fatty acids (including the complex mycolic acid), phosphatides and glycolipids. It is now known that the infective unit or endotoxin in many bacterial species has lipid as one of its constituents. In the early days, researches were mainly directed towards the study of the lipids of Gram-positive organisms which contain more than 15% lipid. Gram-negative organisms having lesser amounts of lipid (below 10%) are now studied to achieve a certain evaluation of the taxonomic approach towards the differences between Gram-positive and Gram-negative organisms. Some results have been obtained in that the Gram-negative organisms have a high proportion of even-numbered saturated and unsaturated acids and odd-numbered cyclic acids; while the Gram-positive organisms have high amounts of the saturated odd-numbered branch-chain acids and relatively low amounts of the straight-chain saturate acids. The occurrence of some unusual fatty acids in the microbial lipids has also stimulated efforts to correlate these lipid molecules with virulence and disease (O'Leary, 1962; Neilson, 1966). Several organisms have been investigated for their fatty acid and lipid composition (Kates, 1964, 1966). With the availability of thin-layer and gas-liquid chromatography, rapid and sensitive techniques have become available for otherwise cumbersome lipid work. Although gas chromatographic methods alone cannot be used for the identification of these components, tentative identifications can be made and used as taxonomic criteria (Abel, Deschmertz & Peterson, 1963; Asselineau, 1961; Henis, Goult & Alexander, 1966; Steinhauer, Flentge & Lechowich, 1967). Differences in the cultural conditions have been shown to be responsible for the changes in the fatty acid composition by many workers (Kates, Kuchner & Janes, 1962; Marr & Ingraham, 1962; Gavin & Umbreit, 1965; Divakaran & Modak, 1968). As regards *Salmonella*, not much information on the lipid composition is available

except for a few reports by Cmelik (1952, 1953, 1954, 1955), MacFarlane (1962) and Gray (1962). The present work was to study the lipid composition of three strains of *Salmonella* and to see the variations, if any. A fatty-acid analyses of the phospholipid and neutral lipid fractions after separation on kieselgel-G has also been included.

METHODS

The cultures of *Salmonella typhi* (TY 2 strain), *S. paratyphi A* and *S. paratyphi B* were supplied by the Bacteriology Department of this Institute (these strains are used for the production of TAB vaccine). The bacteria were grown in 250 ml. Erlenmeyer flasks containing 50 ml. nutrient broth or defined medium (Modak, Nair & Venkataraman, 1968) on a rotary shaker (100 rev./min.) for 18 hr. The cell-mass after centrifugation at 10,000 rev./min. in the cold was washed with cold saline and dried in vacuum over KOH.

Lipid extraction. Weighed amounts of the dried bacteria (about 500 mg.) were suspended in 30 ml. of chloroform + methanol in a 50 ml. beaker and subjected to ultrasonic disintegration for 30 min. at maximum power (MSE ultrasonic disintegrator). The bacterial residue after centrifugation was once again extracted overnight with the solvent mixture at 30°. The combined extracts were filtered through a micro-sinter glass funnel and the non-lipid contaminants and methanol removed by saline washing as described by Folch, Lees & Sloane-Stanley (1957). The chloroform + methanol was distilled off under a gentle stream of nitrogen and the % lipid on dry weight basis calculated. The lipid extracts were divided into two parts. One part was used for the gross fatty acid pattern, while the other portion was applied on kieselgel-G for the separation of phospholipid and neutral lipid fraction. The kieselgel-G (E. Merck) was activated by heating to 105° for 18 hr before use (for adsorption). Neutral lipids were first eluted with chloroform, while the phospholipid was eluted with methanol. The individual fractions of these lipids were concentrated under nitrogen and transmethylated as described below.

Preparation of methyl esters. Fatty acids from total lipid, neutral lipid and phospholipid were transesterified directly as described by Peterson, Deschmertz & Abel (1962) and Morrison & Smith (1964) by boiling the lipids for 5 min. in 5 ml. of 10% boron trifluoride in methanol (Applied Science Laboratories) followed by the addition of 3 ml. normal saline and extraction with ether. The solvent phase was evaporated under nitrogen.

Hydrogenation and bromination of unsaturated methyl esters. An apparatus like that described by Brian & Gardner (1968) was used. About 5 mg. samples of methyl esters of fatty acids were taken up in 20 ml. of ethyl acetate into a 50 ml. Quick-Fit flask and platinum catalyst (200 mg. of 5% platinum oxide on charcoal) added. The flask was connected to the hydrogen source (a cylinder) and the contents kept stirred with a magnetic stirrer, after flushing with hydrogen. When hydrogen uptake ceased (60 min.) the catalyst was filtered off and the methyl esters dried under nitrogen. A portion of this hydrogenated sample was used for gas-liquid chromatographic analysis while the rest was used for bromination at 50° under nitrogen (1:5 bromine solution in ether) as described by Brian & Gardner (1968). Bromination was necessary to break the cyclic acids which were converted to the straight chain variety.

Gas-liquid chromatography (g.l.c.). All g.l.c. work was done with a Panchromatograph

Table 1. *Fatty acid composition of salmonellas (gross pattern)*

Fatty acids	Organism and growth medium					
	<i>S. typhi</i>		<i>S. paratyphi A</i>		<i>S. paratyphi B</i>	
	Nutrient broth	Defined medium	Nutrient broth	Defined medium	Nutrient broth	Defined medium
Total lipid (%)	5.8	5.4	5.6	4.9	5.8	5.2
12:0*	1.5	7.6	1.6	4.6	11.7	3.3
14:0	9.0	9.0	11.5	9.2	10.3	8.1
15:0	0.6	0.65	0.65	0.7	13.9	0.12
16:0	49.5	60.7	56.5	52.2	30.5	25.0
16:1	0.6	0.7	1.6	0.9	4.8	0.13
16:2	—	—	—	—	—	0.76
17:0	Trace	Trace	Trace	Trace	Trace	Trace
17:0 (Cyc.)	0.4	1.0	2.0	1.7	0.37	0.27
18:0	0.28	0.53	0.3	0.25	0.95	Trace
18:1	4.4	2.3	6.1	7.1	9.5	1.3
18:2	11.9	6.0	7.2	6.8	7.15	Trace
19:0	—	—	—	—	—	29.9
19:0 (Cyc.)	14.4	9.2	4.3	3.8	5.28	18.9
20:0	2.3	—	—	—	—	—
20:4	Trace	0.9	0.85	0.9	Trace	2.3
21:0	14.4	0.9	7.8	11.05	5.05	5.3
22:0	4.7	0.7	0.9	0.85	0.5	4.7

* The number preceding the colon designates the number of carbons; the number following indicates the unsaturation.

Table 2. *Fatty acid composition of neutral lipid of salmonellas*

Fatty acids	Organism and growth medium					
	<i>S. typhi</i>		<i>S. paratyphi A</i>		<i>S. paratyphi B</i>	
	Nutrient broth	Defined medium	Nutrient broth	Defined medium	Nutrient broth	Defined medium
Neutral lipid (% of the total lipid)	37.6	42.3	38.4	44.2	35.2	40.3
12:0*	2.02	1.57	2.5	2.4	1.03	1.08
14:0	5.95	11.0	11.3	10.2	8.95	10.4
15:0	0.2	0.7	1.38	0.9	0.4	1.1
16:0	62.5	37.5	23.8	30.5	37.4	22.2
16:1	4.2	0.1	8.95	2.3	Trace	26.5
17:0	Trace	Trace	0.1	—	0.4	0.7
17:0 (Cyc.)	1.68	0.2	2.65	3.2	3.75	0.99
18:0	0.13	3.9	0.07	0.9	0.26	0.69
18:1	8.75	0.9	28.0	22.0	0.26	24.4
18:2	—	17.6	—	10.0	6.25	—
19:0 (Cyc.)	5.7	—	9.65	10.2	15.5	3.4
20:0	5.9	16.2	6.4	2.4	—	—
20:4	—	—	2.57	1.6	—	—
21:0	2.5	6.35	3.4	11.2	3.55	1.79
22:0	0.37	0.47	1.54	0.9	3.1	0.5

* The number preceding the colon designates the number of carbons; the number following indicates the unsaturation.

equipped with ^{90}Sr ionizing detector. The g.l.c. conditions were: column packing, 15% ethylene glycol succinate (EGS) polyester on chromosorb W (80 to 100 mesh); column length 5 ft. (118 cm.) $\times$ 4 mm. internal diameter; column temperature 184° ; detector temperature 210° ; argon flow rate 30 to 60 ml./min.

Identification of the g.l.c. peaks. Identification of the g.l.c. peaks was done by: (i) comparing the retention time with standard compounds (Sigma) of known purity; (ii) analysis of g.l.c. results before and after hydrogenation to fix the identity of unsaturated fatty acid methyl esters; (iii) plotting retention times against chain length relative to methyl palmitate and determining the chain length of the unknown peak from the curve, (iv) comparing the retention times relative to methyl palmitate and stearate to those reported in literature (Brian & Gardner, 1968). No differentiation was attempted as regards the position of the double bonds. The percentage composition was calculated by the triangulation method.

Table 3. *Fatty acid composition of phospholipid of salmonellas*

Fatty acids	Organism and growth medium					
	<i>S. typhi</i>		<i>S. paratyphi A</i>		<i>S. paratyphi B</i>	
	Nutrient broth	Defined medium	Nutrient broth	Defined medium	Nutrient broth	Defined medium
Phospholipid (% of the total lipid)	50.0	45.6	49.8	44.2	52.7	40.2
10:0*	—	—	—	—	—	3.75
12:0	0.33	1.19	6.0	5.8	2.27	5.25
14:0	6.6	7.15	12.0	13.2	7.72	5.25
15:0	1.39	5.6	5.0	6.1	27.2	61.0
16:0	56.5	51.0	68.0	62.0	38.5	0.05
16:1	2.38	—	—	0.9	7.1	0.1
16:2	0.69	0.67	—	—	—	1.2
17:0	1.66	Trace	—	—	—	1.67
17:0 (Cyc.)	Trace	Trace	Trace	Trace	Trace	Trace
18:0	12.3	6.12	2.3	0.9	1.79	4.46
18:1	6.72	9.35	4.1	3.2	3.0	5.25
18:2	3.57	1.52	2.5	—	—	—
19:0	—	—	—	—	4.3	—
19:0 (Cyc.)	Trace	Trace	Trace	10.8	Trace	7.2
20:0	Trace	Trace	Trace	Trace	1.57	4.8
20:4	—	5.6	—	Trace	1.5	Trace
21:0	—	7.6	Trace	Trace	3.0	4.2
22:0	1.49	4.24	Trace	Trace	—	4.8

* The number preceding the colon designates the number of carbons; the number following indicates the unsaturation.

RESULTS

Gross fatty acid composition. The results of the gross fatty acid analyses are given in Table 1. More than 60% of the total fatty acids were C_{16} (palmitic) and the lower acids, palmitic acid being maximum in all the cases. Though there were quantitative differences, all the three organisms showed more or less similar spectra of fatty acids ranging from C_{12} to C_{22} . The change in the growth medium did not affect the spectrum, except in the case of *Salmonella paratyphi B* grown in defined medium where C_{19} acid

appeared in appreciable amounts. This acid was absent in *S. typhi* and *S. paratyphi A* and also in *S. paratyphi B* grown in nutrient broth. Similarly, C₂₀ acid was present only in *S. typhi* grown in nutrient broth. The major acids present included C₁₄, C₁₆, C₂₁ normal saturated, C₁₈ and C_{18:2} normal unsaturated and C₁₉ cyclic.

Fatty acid composition of neutral lipids. The neutral lipid fractions of the three organisms were qualitatively similar except for the absence of C₂₀ from *Salmonella paratyphi B*. Major acids are C₁₆, C_{16:1}, C_{18:1}, C₁₉ cyclic and C₂₀. In addition, detectable amounts of C₁₇ cyclic and C₂₁ were also noted (Table 2).

Fatty acids of phospholipids. The major acid in all the three organisms was palmitic acid; high amounts of C₁₅ acid were noted in the phospholipids of *Salmonella paratyphi B*. C₁₉ cyclic acid was absent (Table 3). A peculiar observation of these analyses was that in *S. typhi* grown on defined medium, C_{20:4} and C₂₁ were present to the extent of 5%, though these acids were not noted in the phospholipids of *S. typhi* grown in nutrient broth. C₁₇ acid was detected in traces. More than 80% of the fatty acids of the total phospholipids of *S. paratyphi A* were palmitic (C₁₆) and lower.

DISCUSSION

Cmelik (1952, 1953, 1954, 1955) investigated the lipids of several members of the Enterobacteriaceae. The total lipid content ranged from 1.5 to 10% of which 50 to 90% was neutral lipid. Fatty acids were reported as being mainly palmitic and C₁₈ monoenoic acids. As against this report, MacFarlane (1962) found a lipid content of 5.6% in *Salmonella typhimurium* of which only 14% was neutral lipid while the rest was phosphatidyl ethanolamine. The fatty acids consisted mainly of palmitic (C₁₆), odd-numbered C₁₇ and C₁₉ cyclopropane, C₁₆ and C₁₈ monoenoic acids with small amounts of myristic and stearic acids (Macfarlane, 1962; Gray, 1962). Our results agree with those of Cmelik in that the neutral lipid content of the three *Salmonella* strains examined in the present study was around 40% and palmitic acid was the major fatty acid. Our values of total lipid are in good agreement with those of MacFarlane, for *S. typhimurium*. The fatty acid spectrum we found differs from MacFarlane's report in having lesser amounts of monoenoic hexadecanoic (C_{16:1}) and cyclic C₁₇ acids. Stearic acid was below 1% while myristic acid was in quite appreciable quantities (about 10%). Amounts of C_{18:2} and C₂₁ have been noted in this study which were not detected by earlier workers.

Data on the fatty acid composition of neutral and phospholipids are not available in the literature for comparison. The neutral lipid we found, showed a similar spectrum of fatty acids to that of total lipid but seemed to be much affected by the change in the medium for growth. This effect is seen more prominently for *Salmonella paratyphi B*. The values for palmitic, palmito-oleic (C_{16:1}) and monoenoic-octadecanoic (C_{18:1}) may be cited as an example of this; with *S. typhi* and *S. paratyphi A* this effect was seen in C₁₆, C_{18:1} and C₂₀ acids. *Salmonella paratyphi A* contained higher amounts of monoenoic stearic acid as compared with the other two strains.

When the fatty acid composition of the phospholipid was examined, it was seen that *Salmonella paratyphi A* contained the highest concentration of palmitic acid (62 to 68%). Noticeable amounts of stearic acid were detected in this fraction. Cyclic and straight chain C₁₈ were absent. Arachidonic (C_{20:4}) acid was present in *S. typhi* (5.4%) and *S. paratyphi B* (1.5%) and was absent in *S. paratyphi A*. The phospho-

lipid isolated from *S. paratyphi B* grown in defined medium showed an unusually high content of C₁₅ (61 %) and an extremely small amount of palmitic acid.

Thus, though fatty acid composition of the total, neutral and phospholipids of the three organisms were examined quantitatively and in some instances qualitatively, nothing definite could be inferred about variations observed in the same organism when subjected to different growth medium. It is known that temperature, growth medium and growth conditions can alter the fatty acid composition (Kates, 1964). Hence such quantitative alterations in the fatty acid composition of the bacteria grown in the defined medium and nutrient broth were to be expected. However, the changes observed were not only quantitative but also qualitative, i.e. the introduction or elimination of one or more fatty acids in the original profiles. On the basis of the above observation, it may be suggested that the loss of virulence in the *Salmonella* or other such pathogenic species when grown on different media or transferred from natural medium to defined medium might be the result of the fatty acid alterations.

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REFERENCES

- ABEL, K., DESCHMERTZING, H. & PETERSON, J. I. (1963). Classification of micro-organisms by analysis of chemical composition. I. Feasibility of utilizing gas chromatography. *Journal of Bacteriology* **85**, 1039.
- ASSELINEAU, J. (1961). Sur quelques applications de la chromatographie en phase gazeuse à l'étude d'acides gras bactériens. *Annales de l'Institut Pasteur, Paris* **100**, 109.
- BRIAN, B. L. & GARDNER, E. W. (1968). Fatty acids from *Vibrio cholerae* lipids. *Journal of Infectious Diseases* **118**, 47.
- CMELIK, S. (1952). Bacterial lipids. I. The lipids of *Salmonella typhosa*. *Hoppe-Seyler's Zeitschrift für Physiologische Chemie* **290**, 146.
- CMELIK, S. (1953). Bacterial lipids. II. The lipids of *Salmonella ballerup*. *Hoppe-Seyler's Zeitschrift für Physiologische Chemie* **293**, 222.
- CMELIK, S. (1954). Lipids and virulence in typhoid bacteria. *Experientia* **10**, 372.
- CMELIK, S. (1955). Bacterial lipids. V. Lipids of typhoid bacteria. *Hoppe-Seyler's Zeitschrift für Physiologische Chemie* **299**, 227.
- DIVAKARAN, P. & MODAK, M. J. (1968). Fatty acid composition of mycelium of *Penicillium chrysogenum* grown in different carbohydrates as a sole source of carbon. *Experientia* **24**, 1102.
- FOLCH, J., LEES, M. & SLOANE-STANLEY, G. H. (1957). A simple method for the isolation and purification of total lipids from animal tissues. *Journal of Biological Chemistry* **226**, 497.
- GAVIN, J. J. & UMBREIT, W. W. (1965). Effect of biotin on fatty acid distribution in *Escherichia coli*. *Journal of Bacteriology* **89**, 437.
- GRAY, G. M. (1962). The cyclopropane ring fatty acids of *Salmonella typhimurium*. *Biochimica et Biophysica Acta* **65**, 135.
- HENIS, Y., GOULT, J. R. & ALEXANDER, M. (1966). Detection and identification of bacteria by gas chromatography. *Applied Microbiology* **14**, 513.
- KASAI, N. (1966). Chemical studies on the lipid component of endotoxin with special emphasis on its relation to biologic activity. *Annals of the New York Academy of Sciences* **133**, 486.
- KATES, M. (1964). Bacterial lipids. *Advances in Lipid Research* **2**, 17.
- KATES, M. (1966). Biosynthesis of lipids in micro-organisms. *Annual Review of Microbiology* **20**, 13.
- KATES, M., KUCHNER, P. J. & JANES, A. T. (1962). The lipid composition of *Bacillus cereus* as influenced by the presence of alcohol in the culture medium. *Canadian Journal of Biochemistry and Physiology* **40**, 83.

- MACFARLANE, M. G. (1962). Lipid component of *Staphylococcus aureus* and *Salmonella typhimurium*. *Biochemical Journal* **92**, 40 p.
- MARR, A. G. & INGRAHAM, J. M. (1962). Effect of temperature on the composition of fatty acids in *Escherichia coli*. *Journal of Bacteriology* **84**, 1260.
- MODAK, M. J., NAIR, S. & VENKATARAMAN, A. (1968). Biochemistry of the Salmonella. IV. Metabolism of [$1-^{14}\text{C}$] xylose by *S. typhi*, *S. paratyphi A* and *S. paratyphi B* under stationary conditions of their growth. *Indian Journal of Pathology and Bacteriology* **11**, 24.
- MORRISON, W. R. & SMITH, L. M. (1964). Preparation of fatty acid methyl esters and dimethylacetals from lipids with boron fluoride-methanol. *Journal of Lipid Research* **5**, 600.
- NEILSON, H. S. JUN. (1966). Variation in lipid content of strains of *Histoplasma capsulatum* exhibiting different virulent properties for mice. *Journal of Bacteriology* **91**, 273.
- O'LEARY, W. M. (1962). Fatty acids of bacteria. *Bacteriological Reviews* **26**, 429.
- PETERSON, J. I., DESCHMERTZING, H. & ABEL, K. (1962). Transesterification of lipids with boron trichloride. *Proceedings of the 142nd Meeting of the American Chemical Society* p. 21B.
- STEINHAEUER, J. E., FLENTGE, R. L. & LECHOWICH, R. V. (1967). Lipid pattern of selected micro-organisms as determined by gas-liquid chromatography. *Applied Microbiology* **15**, 826.