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Free Fatty Acids from the Intracellular-Thermostable Hemolytic Fraction of *Vibrio parahaemolyticus* and their Hemolytic Effect

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Abstract

The hemolytic agents of intracellular-thermostable hemolytic fraction (ITHF) were studied using two strains of the food poisoning bacteria, *Vibrio parahaemolyticus*. Each ITHF preparation was separated into seven portions by lipid extraction and column and thin-layer chromatography. The resultant free fatty acid fraction was further analysed for acid composition by gas-liquid chromatography. The assessment of hemolytic activities of samples obtained and of fatty acids experimentally found was done quantitatively and qualitatively. Most of the lytic activity was detected in the free fatty acid fraction rather than in the other lipids. The acid fraction similarly was largely composed of C16:1 acid (more than 80%) and contained smaller proportions of C18:1, C16:0, C14:0 and C12:0 acids. On titrating the corresponding authentic standards, almost all of these acids were verified to be hemolytic so high were the C16:0 acid contents of the ITHF preparations. Consequently, the hemolytic activity of ITHF was concluded to be greatly ascribed to the surface activity of the C16:1 acid, which was contained abundantly in phosphatidyl ethanolamine of this organism.

Sakazaki[1] found by mild alkaline hydrolysis that an intracellular thermostable hemolytic fraction (ITHF) was derived from cells of *Vibrio parahaemolyticus*, regardless of the positive and negative Kanagawa reaction. As the washed cells of this organism had no hemolytic activity,[2),3)] the activity of ITHF proved to originate from the alkaline treatment. Preliminary isolation trials for the active agents indicated, either that they were lipo-soluble[4)] or that the hemolytic agents of ITHF were low in molecular weight because their activities decreased or were lost by dialysis against water.[2)] As to the major phospholipid of this organism, Misaki et al.[5)] described the phosphatidyl ethanolamine as having C16:1 acid making up as much as 50% of fatty acid composition.

The present paper deals with the results and implications for the characterization of a representative hemolytic agent of ITHF, C16:1 fatty acid, detected by experiments with two of its strains.

Materials and Methods

Strains tested: Two strains of *V. parahaemolyticus* were tested. Strain 128–71 (O3:K6), Kanagawa positive, was isolated from human diarrheal stools.

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Strain 156–71 (O6: K46), Kanagawa negative, was isolated from marine fish. They were maintained by the gelatin disc method of Stamp.[6] Just before use one of the discs was inoculated into 5 ml of 3% NaCl broth. After incubation at 37°C, a loopful of the culture was placed in BTB-teepol agar in order to purify and further incubate it.

Bacterial cultivation and harvest: The 3% NaCl broth was also used for the preparation of inocula and bacterial cells. The medium, 400 ml in content, inoculated with a drop of the 16–18 hr culture, was incubated at 37°C in a rotary shaker. The cells were harvested at the latter log phase of growth, 16–17 hr after commencement of culture, and washed twice with 3% NaCl aqueous solution containing 0.01 M $MgSO_4$.

Preparation of ITHF: After the procedure developed by Sakazaki[1], an aliquot of washed cells was added with an equal volume of 0.1 N NaOH and kept at 100°C for 20 min in a steam bath. To the product was added 1N HCl, dropwise, stirring to bring it to neutrality, and the resultant sediment was removed by centrifugation. From the supernatant fluid, ITHF preparation was sedimented by adjusting the pH of the liquid to 4.5 with 1N HCl while stirring.

Lipid extraction: ITHF preparations were extracted with a solvent system of chloroform/methanol/water, 1:2:0.8, according to the Bligh and Dyer method.[7] After removal of the residue, the aqueous methanol and chloroform layers were separated from each other and then evaporated to dryness at 35°C or less; from those the non-lipid fraction and total lipids were obtained, respectively. The samples were weighed and dissolved in a small volume of solvent; total lipids in chloroform, and the non-lipid fraction in water. They were held at –20°C under N_2 gas until use. The extracted residue was dried over concentrated H_2SO_4, in a vacuum desiccator at –20°C, ground and dried again until it reached a constant weight under the same conditions as mentioned above; its yield was obtained as the difference in weight between the ITHF preparation and the sum of the total lipids and the non-lipid fraction.

Separation of lipids: For the fractionation of major classes, portions of total lipids were applied to a column (2 by 8 cm) of activated silicic acid (Mallinckrodt Chemical Co., St. Louis, Mo.). Elution was carried out successively with 150 ml of chloroform, 100 ml of acetone, and 300 ml or more of methanol to separate the neutral, glyco- and phospholipids. Aliquot samples of the neutral lipids were further fractionated on thin-layer chromatographic plates coated with 0.25 mm thick layers of Wakogel B-5 (Wako Pure Chemical Ind., Ltd.) by using a solvent system containing petroleum ether/diethyl ether/acetic acid, 82:18:1. Bands of neutral lipids were located by the spraying of Rhodamine B (0.05%, in 95% ethanol). The individual lipids were identified by comparing their mobilities with those of standard specimens such as trioleoylglycerol, linoleic acid, cholesterol, dilauroyl- and monooleoylglycerols run on the same plate. The separated band of free fatty acids alone was scraped off, and the acids were eluted with chloroform. After weighing, these lipid samples were dissolved in chloroform and stored as described above.

Dry weight determination: For hydrous samples such as ITHF preparations and extracted residues, dry weight percentages were calculated gravimetrically after drying at 105°C for 4 hr.

Estimation of hemolytic activity: The defibrinated red cells of rabbit or horse were used for this test. They were washed three times with the phosphate-buffered saline (PBS) and suspended in PBS to yield a 2% suspension. The suspension was standardized for hemoglobin content in a spectrophotometer in order that the 50% hemolysis tube might show an optical density of 0.29 at 550 nm. The materials to be tested were also dissolved or suspended in PBS; suspensible materials had been stirred previously with the known volume of PBS at 4°C while being saturated with N_2, until homogenous suspension occurred. To 4 ml of each of the dilutions was added 4 ml of the red cell suspension. The mixtures were placed in a 37°C water bath for 30 min and shaken, and the unhemolysed cells were centrifuged down. The color of the supernatant fluid obtained from the twofold dilution-test tubes was compared visually with that of the standard tubes, which included the dilutions for 50% hemolysis and for no hemolysis; thus the approximate hemolytic activity of each test materials was estimated. Subsequently, a quantitative analysis was done by using the test tubes with the more closely spaced dilution in the following way. The amount of hemoglobin released from the red cells into the fluid was measured spectrophotometrically at 550 nm. The dilution showing just 50% hemolysis was then determined by the three tubes, comprising 50% hemolysis standard, and the last positive and negative test dilutions determined by interpolation; dry weight in mg equivalent to a hemolytic unit (HU) was read immediately. Thus HU value for each hemolytic agent was obtained as the reciprocal of the mg value just described.[4]

In Table 2, the hemolytic potency of test materials was judged the presence of liberated hemoglobin in the 1st tube of the twofold dilution mentioned above, and classified as positive (+) or negative (−) for hemolysis. In the case of the results given in Table 4, the rates of optical density of every test tubes were calculated relative to that of the 100% hemolysis standard tube, and the activity was denoted in percent hemolysis by the following marks; ⧺ (100%), ⧺ (75% to less than 100%), +(25% to less than 75%), ±(less than 25%), and − (no hemolysis).

Qualitative analyses of lipids and hemolytic agents: Aliquot lipid samples were subjected to thin-layer chromatographic separation as described above. The following solvent systems were used: for neutral lipids and glycolipids petroleum ether/diethyl ether/acetic acid, 82:18:1 (A) or 90:10:1 (B); for glycolipids chloroform/methanol/ acetic acid/water, 75:20:1:2 (C) and chloroform/ methanol/ acetic acid, 70:40:4 (D); and for phospholipids chloroform/ methanol/ 28% ammonia, 65:25:5 (E), in addition to (C) and (D). The lipids were commonly located either by staining with a Rhodamine B reagent or by charring with a chromic-sulfuric acid. The ferric chloride reagent of Lowry[8] and the $SbCl_3$ solutions given by Noda[9] were used for the detection of cholesterol or sterols. The anthrone reagent of Patton and Thomas[10] was used for the glycolipids; these lipids were also, together with the phospholipids, detected by Skidmore and Entenman reagents[11] for esters, and subsequently by the periodate-Schiff reagents[12] for vicinal groups. The esterified phosphate was detected with the Vaskovsky and Kostetsky reagent,[13] choline with a modified Dragendorff reagent[14] and amino groups with ninhydrin. In order to compare the hemolytic activity of the suspensible and

nonsuspensible lipid samples, a qualitative hemolysis test was performed based on a modification of the Watanabe and Seaman method.[15] The thin-layer chromatogram was applied on a blood agar plate made from the Wagatsuma agar containing 5% rabbit red blood cells, though the use of this medium may be a special one. After preserving at 37°C for 20–24 hr, the agar plate was inspected for hemolysis.

Ligated gut loop tests in rabbits: This was achieved according to a modification of De-Chatterjee method[16] reported by Sakazaki et al.[17] by inoculating 1 ml quantity of appropriate dilutions of certain lipid samples.

Gas-liquid chromatography: Methyl esters of the fatty acids were prepared by the BF_3-methanol method of Metcalfe and Schmitz.[18] The separation of methyl esters was carried out by gas-liquid chromatography using a Hitachi K53–0130 type instrument equipped with a flame ionization detector with a glass column (0.3 by 200 cm) containing 20% DEGS on 60–80 mesh Uniport KA. The operating conditions employed were as follows: column temperature, 185°C; nitrogen pressure, 0.7 Kg/cm^2; hydrogen pressure, 0.6 Kg/cm^2; air pressure, 2.0 Kg/cm^2. The esters were identified by comparing their retention times with those of known standards in mixtures. The peak areas represented by component esters were calculated by multiplying the peak heights by the widths at half the heights, and the rate of each peak was expressed as a percentage of the total area of all peaks.

Chemicals: All organic solvents were redistilled before use. Commercial sources of standard lipid specimens were: lauric, myristic, palmitic, stearic and oleic acids and cholesterol (Wako Pure Chemical Ind.); plamitoleic and linoleic acids, monooleoyl-, dilauroyl- and trioleoylglycerols (Nakarai Chemicals, Ltd.). Phosphatidyl ethanolamine was the generous gift of the Laboratory of Food Chemistry 1, Faculty of Fisheries, Hokkaido University, and from this specimen lysophosphatidyl ethanolamine was obtained enzymically,[19][20] using *Crotalus adamanteus* venom (Sigma Chemical Co., St. Louis, Mo.).

Results

Quantitative hemolytic activity determination of suspensible or soluble samples

The yields in per cent value of the derived fractions from ITHF preparations and from the hemolytic activity of those samples are given in Tables 1 and 2 respectively; and in Figure 1 the relative total activity is given, namely the percentages of products of the above values, yields in percentage and hemolytic unit in HU, to that of ITHF itself. These data show that the greater part of the hemolytic activity present in ITHF appears first in the total lipids, next in the neutral lipids and last in the free fatty acid fractions, whereas non-lipid fractions and extracted residues seemed not to show much activity of the ITHF preparations.

Qualitative analyses for composition and hemolysis of three lipid samples

The quantitative hemolytic ability of glyco- and phospholipids was not shown, due to very low solubility of these samples for PBS. Therefore, the qualitative hemolysis test was applied to them. In this process, fatty acid fractions were used as a standard for comparison of lytic activity. Figures 2a, b, c, and d show thin-layer chromatograms of the three samples from the ITHF preparation of

Table 1. *Yields in percentage of ITHF preparations and the separated fractions.*

Sample	ITHF from strain 128–71		ITHF from strain 156–71	
	Average	Range	Average	Range
ITHF	100, {42.3 (39.6-45.3)}		100, {43.9 (41.7-46.1)}	
Total lipids	5.6	(5.1 - 6.2)	5.5	(5.1 - 6.0)
Neutral lipids	3.8	(3.6 - 4.2)	4.0	(3.6 - 4.4)
Free fatty acids	3.8	(3.6 - 4.0)	4.0	(3.6 - 4.4)
Glycolipids	0.10	(0.09- 0.11)	0.13	(0.12- 0.14)
Phospholipids	1.2	(1.1 - 1.3)	0.9	(0.8 - 1.0)
Non-lipid fraction	2.8	(2.5 - 3.2)	2.4	(2.3 - 2.5)
Extracted residue	91.6	(90.6 -92.5)	91.8	(91.0 -92.6)

Figures in braces show percentages of the ratio of ITHF to dried cells, and the rest show the relative percentages to ITHF.

Table 2. *Hemolytic activity of ITHF preparations and the separated fractions on rabbit red cells.*

Sample	ITHF from strain 128–71		ITHF from strain 156–71	
	Hemolysis	Hemolytic unit HU	Hemolysis	Hemolytic unit HU
ITHF	+	0.36	+	0.34
Total lipids	+	9.56	+	7.84
Neutral lipids	+	12.96	+	12.94
Free fatty acids	+	13.02	+	13.44
Non-lipid fraction	+	0.15	+	0.07
Extracted residue	−	N.C.	−	N.C.

The values are the averages of two separate determinations.
N.C.: Not calculated.

strain 128–71 and the blood agars indicating the hemolytic characteristics of the samples; the figures exemplify the general aspect of lipid components and hemolytic activities for the lipid samples.

Free fatty acids appeared to occupy a big proportion of neutral lipids, which accounted for the quantitative data for these fractions (Table 1). The chromatograms of glycolipids in solvent systems C and A exhibited six spots that were ester-positive (spots 1, 2, 3, 4, 5 and 6 in Fig. 2b) and some contaminators from the neutral lipid fraction. The use of the solvent systems A, B and C did not permit a desirable separation of components, but the yield of these fractions was so low that further characterization was precluded. The chromatogram of phospholipids in solvent system E revealed the presence of seven esterified phosphates corresponding to spots 2, 3, 4, 5, 6, 7 and 8 in Fig. 2d, and they were periodate-Schiff- and Dragendorff- negative. With spots 3 and 5, they showed a positive ninhydrin test and were respectively presumed to be lysophosphatidyl

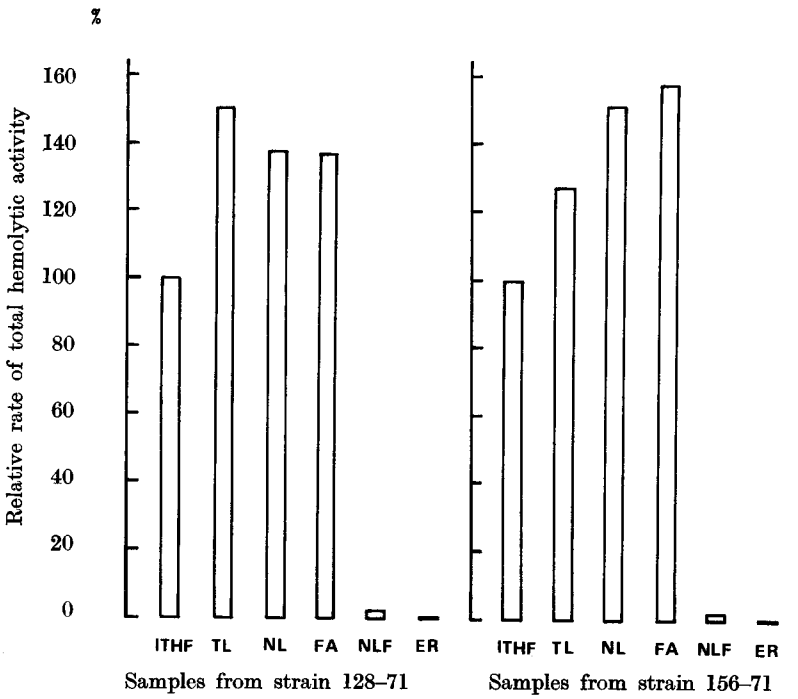


Fig. 1. Relative rate of total hemolytic activity of ITHF preparations and the separated fractions on rabbit red cells. Abbreviations: TL, total lipids; NL, neutral lipids; FA, free fatty acids; NLF, non-lipid fraction; ER, extracted residue.

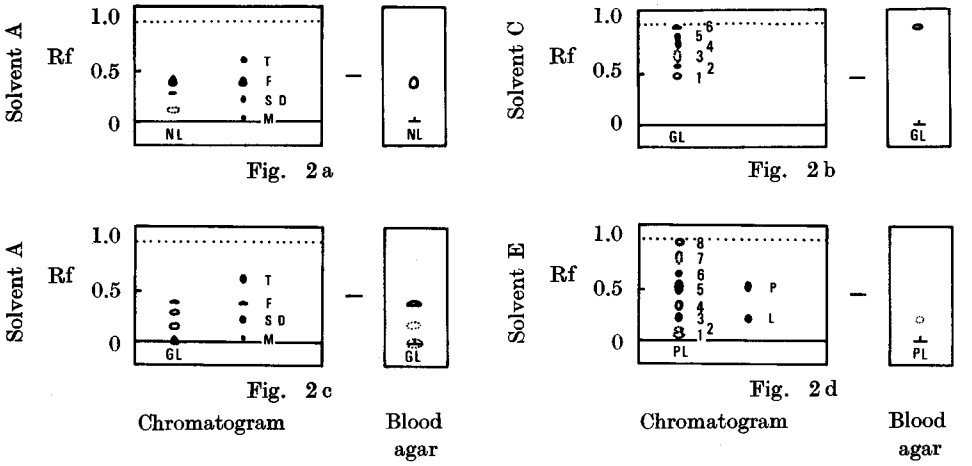
Table 3. *Major free fatty acids occurring in ITHF preparations.*

Fatty acid	% acid fraction		% ITHF dry weight	
	ITHF from 128–71	ITHF from 156–71	ITHF from 128–71	ITHF from 156–71
C 12:0	2.3	2.0	0.09	0.08
C 14:0	2.5	1.3	0.10	0.05
C 16:0	6.1	5.0	0.23	0.20
C 16:1	80.8	82.6	3.06	3.29
C 18:1	6.4	8.3	0.24	0.33

The values are the average of duplicate determinations, and all acids comprising less than 0.6% of the acid fraction are omitted.

ethanolamine and phosphatidyl ethanolamine, as reported on *V. parahaemolyticus* by Oliver and Colwell[21] and Siervo and Reynolds.[22]

As regards the patterns obtained by the qualitative hemolysis test, the neutral lipids and free fatty acids alone turned out to be positive and always behaved as good lysins (acting at low concentrations). The glycolipid and phospholipid fractions were found to show little activity; only obscurely hemolytic activity was observed in spite of high concentrations of test materials, possibly owing to the low solubility of those lipids in the medium.



Figs. 2a, b, c, and d. Thin-layer chromatography of three lipid fractions obtained from ITHF of strain 128–71 and hemolysis findings. Sample: NL as neutral lipids, GL as glycolipids, and PL as phospholipids. Standard: T as triglyceride (trioleoylglycerol), F as fatty acid (linoleic acid), S as sterol (cholesterol), D as diglyceride (dilauroylglycerol), M as monoglyceride (monooleoylglycerol), P as phosphatidyl ethanolamine, and L as lysophosphatidyl ethanolamine. Solvent systems: For abbreviations *see* p. 156. Detection of lipid classes: Chromic-sulfuric acid followed by charring; ●, very distinct; ○, distinct; ◌, faint. Survey for hemolysis; ○, very distinct; ◌, faint.

Table 4. *Hemolytic activity of five fatty acid specimens on rabbit red cells.*

Specimen	Hemolytic activity						
	Concentration of specimen (mM)						
	1/1	1/2	1/4	1/8	1/16	1/32	1/64
Lauric acid	##	−	−	−	−	−	−
Myristic acid	##	##	##	±	−	−	−
Palmitic acid	±	−	−	−	−	−	−
Palmitoleic acid	##	##	##	##	±	−	−
Oleic acid	##	##	##	##	##	+	−

Thus, the lytic action of ITHF preparations proved more attributable to the hemolytic activity of free fatty acids than to that of any other lipid.

Components of the free fatty acid fraction and their hemolytic activity

The data on principal component acids of the acid fraction are given in Table 3. The C16:1 acid was found to be the most abundant component, occurring at concentrations of more than 80% of the acid composition. The next most common members were the C18:1 and C16:0. They emerged, however, only at concentrations of less than 9% in the acids. Next were low percentages (below 3%) of the C12:0 and C14:0 acids. Consequently, it was revealed that the free fatty acids of

ITHF preparations comprise 80% or more of the monounsaturated compounds, irrespective of the strains tested; this corresponds to reports by Misaki et al.,[5] Kojima[23] and Beuchat and Worthington[24] on the cellular lipid composition of this organism.

Then the titration of *in vitro* hemolytic activity was performed with the authentic fatty acid standards corresponding to the acids just mentioned. As indicated in Table 4, three kinds of standards show considerable degrees of individual lytic activity in this order: oleic>palmitoleic>myristic acids. Lauric and palmitic acids showed relatively low hemolysis among the specimens analysed. Although a full analysis for stearic acid was excluded due to its low suspensibility to PBS, this specimen was shown to be negative for hemolysis, according to the analysis in a 0.25 mM suspension.

Lipid composition of heat-treated washed cells

The lipid composition of heat-treated washed cells are shown in Table 5; the cellular materials were obtained by preservation of washed cells in a steam bath for 30 min in order to inactivate their autolytic enzymes. The cells contained relatively few neutral lipids and proportionately more phospholipids, whereas the reverse was the case with ITHF (Table 1). A large proportion of C12:0 acid and a small proportion of C16:1 also present compared to the acid composition for ITHF (Table 3), although a small amount of neutral lipids would have resulted in creating a poverty of free fatty acids.

Table 5. *Yields in percentage of component lipids of heat-treated washed cells.*

Sample	Strain	
	128–71	156–71
Total lipids	6.3	5.9
Neutral lipids	0.23	0.23
Free fatty acids	0.14	0.16
C12:0	(60)	(110)
C16:0	(10)	(10)
C16:1	(40)	(20)
C18:1	(10)	(10)
Glycolipids	0.7	0.7
Phospholipids	5.4	5.1

The values are calculated on the basis of dry weights of samples resulting from a single determination.

Figures in parentheses show the values multiplied by 1000.

Susceptibility composition of erythrocytes

Miyamoto et al.[25] necessitated the present authors to compare erythrocytes of rabbit and horse, which data are given in Table 6. The values appear as fractions of the hemolytic activity of rabbit red cells. The proportions for horse cells are

Table 6. *Relative susceptibility of rabbit and horse red cells to hemolysis resulting from two separate determinations.*

Source of red cells	Ratio of HU value for horse red cells to that for rabbit red cells									
	Samples from strain 128–71				Samples from strain 156–71				Standard specimens	
	ITHF	Total lipids	Neutral lipids	Free fatty acids	ITHF	Total lipids	Neutral lipids	Free fatty acids	Palmit-oleic acid	Oleic acid
Rabbit	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
Horse	0.52	0.71	0.80	0.80	0.54	0.84	0.83	0.80	0.86	0.84

less than 1 in each case and account for the lower susceptibility of horse red cells in relation to that of the rabbit cells.

Ligated gut tests in rabbits

The ileal loop reactivity for ITHF preparations was observed as negative. Nor did any inoculation into gut loops, with 10 mg of the total and neutral lipid and free fatty acid fractions, produced any changes in the intestine.

Discussion

Hemolysis produced by long-chain fatty acids has been reported by several researchers.[26)27)28)29)30)] Based on Schulman's assumption[29)] that reactivities of a series of the long-chain compounds with an identical head group commence at about C8 and reach a maximum at about C18, the acid fraction reported here seems to be rich in surface active fatty acids. During this work, we found that ITHF preparations lysed the erythrocytes at non-linear, as is usual with surface active compounds. Our data, along with the other descriptions, strongly suggest that the *in vitro* hemolytic activity of ITHF is caused by the surface activity of fatty acids, especially C16:1 acid. Considering the fatty acid composition of cellular lipids of this organism,[5)] it is likely that these active fatty acids in the ITHF preparation would be enriched greatly by the alkaline hydrolysis of the main constituent phosphatidyl ethanolamine. The differences in lipid composition between ITHF preparation and bacterial cells (Tables 3 and 5) give evidence in support of this.

The degree to which the C16:1 acid can participate in the hemolysis of ITHF, the activity of palmitoleic acid specimen, was further assessed; i.e. *cis*-9-*hexadecenoic acid* contrasted as a corresponding standard. Table 7 shows that the amount of C16:1 acid in the ITHF dilutions needed to produce 50% hemolysis is slightly larger than the corresponding amount of standard dilution. Furthermore, when the activity for preparation of ITHF was assumed to be attributed to the C16:1 acid alone, bearing the titer equivalent to that of palmitoleic acid, the tentative hemolytic titers of ITHF could be calculated. As shown in Table 8, the calculated titers are close to, but greater than those experimentally found. These data suggest the coexistence of some factor which is responsible for the decrease in the lytic activity of C16:1 acid. With our present knowledge, however,

Table 7. *Comparative data of hemolytic activity on rabbit red cells.*

Sample	Amount of C16:1 acid in the ITHF dilution showing 50% hemolysis 10^{-3} m mol/100 ml		Amount of palmitoleic acid in the dilution of the acid showing 50% hemolysis 10^{-3} m mol/100 ml	
	Average	Range	Average	Range
ITHF from strain 128–71	8.5	(7.7- 9.5)	7.6	(7.6-7.6)
ITHF from strain 156–71	9.8	(8.4-11.3)		

Table 8. *Actual and tentative hemolytic activity of ITHF preparations on rabbit red cells.*

Sample	Actual hemolytic unit HU		Tentative hemolytic unit HU	
	Average	Range	Average	Range
ITHF from strain 128–71	0.36	(0.32-0.40)	0.45	(0.45-0.45)
ITHF from strain 156–71	0.34	(0.29-0.39)	0.48	(0.48-0.48)

whether or not the fatty acids participate in the Kanagawa reaction is uncertain.

Verzár and Kúthy,[30][31] and Schulman et al.[28][29] described the specific interaction of long-chain fatty acids with bile salts by considering the fat respiration mechanism of the intestine. Verzár and Kúthy[30] demonstrated that the interaction of acids such as palmitic, stearic and oleic acids with glycocholic acid brings about a remarkable increase in the surface activity of the resultant compounds in a physiologically significant pH range of 6–8. Pethica et al.[28][32] showed the surface activity increment for the solution to consist of the micelle-forming substance, sodium taurocholate, and some surface active materials. These also indicate the possibility of permeability promotion of the intestinal wall by some long-chain fatty acids under conditions such as the compounds produced. Although the preparations of ITHF and the derived lipid fractions give negative results in ligated gut loop tests in rabbits, the results of this work suggest that further investigation should be done on the hemolytic activity originating from bacterial fatty acids.

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