ROLE OF FATTY ACIDS IN THE GROWTH STIMULATION OF *SARCINA* SPECIES BY VITAMIN-FREE CASEIN DIGESTS

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Received for publication June 1, 1959

In most studies on microbial nutrition today, commercial vitamin-free casein digests are used as sources of amino acids. In many cases, the workers have reported the inability of amino acid mixtures to replace the hydrolyzates and have concluded that peptides were required. In the present investigation a similar observation was made. However, with further study, we were able to demonstrate that the activity of the digest was due, not to peptides, but to long chain fatty acids.

MATERIALS AND METHODS

Identification of organism. The culture isolated in our laboratory was identified by the procedures of the Society of American Bacteriologists, Committee on Bacteriological Technic (1957), i. e., fermentations, indole production (Gnezda and Kovacs tests), gelatin liquefaction, starch hydrolysis, action on litmus milk, and utilization of $NH_4H_2PO_4$ as sole source of nitrogen; and the methods recommended by Smith *et al.* (1952), i. e., gelatin hydrolysis, production of urease, production of nitrite from nitrate, production of acetylmethylcarbinol, and casein hydrolysis. Catalase was detected by gas evolution upon addition of 3 per cent (v/v) H_2O_2 to a slant culture.

Medium. The basal medium employed for the nutritional studies is shown in table 1. Glass distilled water was used throughout the study. Lyophile tubes were used to preserve the culture. Fresh tubes were opened monthly, the cells were diluted in 0.02 M K_2HPO_4 and were spread on enough nutrient agar slants for one week's use. Each week during the month a fresh batch of slants was made from a slant of the previous series. Slants were stored at 5 C.

The basal medium was prepared in double strength. Five ml were added to each 20 by 175 mm colorimeter tube followed by a calculated amount of water. Morton stainless steel closures (Bellco Glass Company, Vineland, New Jersey) were used to cover the tubes. The tubes were autoclaved at 120 C for 15 min and cooled to room temperature. Autoclaved additives were added aseptically to bring the volume to 10 ml. Inoculum was prepared by washing the cells from a slant culture once in a dilute mineral salts solution,[1] suspending them in the same menstruum and diluting to an optical density (OD) of 0.01 to 0.12, using the Lumetron colorimeter (model 402-E) fitted with a 660 mμ filter. One drop (about 0.05 ml) of such a suspension was used to inoculate each tube. Incubation was carried out for approximately 10 hr at 37 C on a shaking machine imparting a rotary motion of 220 rpm. Duplicate tubes were used ln all cases.

Estimation of growth. Growth was measured with the Lumetron colorimeter, using uninoculated medium to set the instrument to an OD of zero. When the OD was greater than 0.4, the tube contents were diluted with water and the density was reread. The figures given represent the product of the observed optical density and the dilution factor.

Compounds. Water-soluble compounds were dissolved in water and sterilized by autoclaving. Fatty acids were dissolved in ethanol at a concentration of 1 mg per ml, then diluted to a level of 10 μg per ml with water and autoclaved.

The oleic acid used had an iodine number of 87.2, a boiling point of 171 to 174 C at 0.2 mm and was composed of 95.3 per cent oleic acid, 0.7 per cent linoleic acid, and 4 per cent saturated acids. The linoleic acid was 95 per cent pure. The linolenic acid was purchased from Mann Laboratories, had an iodine value (Wijs) of 273.0 and was of Mann Assayed quality. The arachidonic acid was 65 per cent pure and the lactobacillic acid was at least 95 per cent pure. Erucic

[1] The salts solution was composed of those mineral salts present in the basal medium at 10 per cent of the concentration shown with the exception of the phosphates which were used at full strength.

TABLE 1

Composition of basal medium

Component	Amt per L	Component	Amt per L
Glucose	10 g	Ca-pantothenate	1 mg
L-Glutamic acid	2.6 g	Riboflavin	1 mg
DL-Valine	790 mg	Pyridoxine	1 mg
DL-Aspartic acid	600 mg	Pyridoxamine	1 mg
DL-Leucine	480 mg	Pyridoxal	1 mg
DL-Isoleucine	480 mg	Nicotinic Acid	1 mg
DL-Serine	480 mg	Thiamine	1 mg
L-Proline	440 mg	*p*-Aminobenzoic acid	100 μg
DL-Threonine	400 mg	Pantetheine	100 μg
DL-Phenylalanine	390 mg	Biotin	10 μg
DL-Methionine	330 mg	Folic acid	10 μg
L-Tyrosine	330 mg	Lipoic acid	10 μg
L-Lysine·HCl·H_2O	320 mg	Leucovorin	10 μg
L-Asparagine	200 mg	B_{12}	2 μg
L-Glutamine	200 mg	NH_4Cl	5 g
DL-Alanine	190 mg	K_2HPO_4	3 g
L-Arginine·HCl	190 mg	NH_4NO_3	1 g
L-Histidine·HCl·H_2O	120 mg	KH_2PO_4	1 g
DL-Tryptophan	120 mg	Na_2SO_4	1 g
Glycine	25 mg	$MgSO_4 \cdot 7H_2O$	100 mg
L-Cystine	20 mg	$MnSO_4 \cdot 4H_2O$	10 mg
L-Hydroxyproline	10 mg	$FeSO_4 \cdot 7H_2O$	10 mg
Inositol	2 mg	$CaCl_2$	5 mg

pH adjusted to 6.8 to 7.0.

acid was obtained from Matheson Chemical Company (practical grade) and was recrystallized before use. Tween 80 was purchased from the Atlas Powder Company.

RESULTS

Identification studies. Microscopic examination of the growth from 24-hr nutrient agar slants showed gram-positive, nonsporeforming, nonmotile cocci occurring singly, in pairs, tetrads, and packets. The aerobic nature of the organism was shown by its more rapid growth under shaking conditions than in static culture.

Fermentation tests revealed that acid was produced from lactose, sucrose, fructose, glucose, maltose, galactose, and glycerol. Rhamnose, xylose, mannose, arabinose, sorbitol, and mannitol were not fermented. No gas was produced.

The culture produced catalase but not urease, indole, or acetylmethylcarbinol. Starch and casein were not hydrolyzed. Gelatin was neither liquefied nor hydrolyzed. Nitrite was produced from nitrate. The organism did not act on litmus milk during a week of incubation. Only after 1 month incubation was there a change from the orginal blue pink to a pink color. No growth occurred with $NH_4H_2PO_4$ as the sole source of nitrogen.

Consideration of the above characteristics indicates that the culture is a member of the genus *Sarcina* as defined in *Bergey's Manual of Determinative Bacteriology* (Breed *et al.*, 1957), although it cannot be placed in any of the described species. On the other hand, the classification of Shaw *et al.* (1951) would include it in their subgroup 3, i. e., *Staphylococcus lactis*. In view of the confusion surrounding the classification of this group, we have chosen to designate our culture as *Sarcina* sp.

Nutrition studies. (1) Effect of casein hydrolyzates:—When *Sarcina* sp. was inoculated into the basal medium described above, any of the following commercial digests of casein were capable of shortening its lag phase of growth: trypticase (BBL), casamino acids and "vitamin-free" casamino acids (Difco), vitamin-free casein hydrolyzate, acid, and Vitamin-free casein hydrolyzate, enzyme (Nutritional Biochemicals Corp.). On the other hand, Vitamin-free casein

(Nutritional Biochemicals Corp.) failed to stimulate growth. When the casein was refluxed with 7 N H_2SO_4 for 24 hr, it became active as shown in figure 1. Time course experiments on hydrolysis showed that most of the activity was liberated after one hour.

Since many of the growth-promoting effects of casein digests on bacteria have been traced to peptides, we immediately tested high levels of single amino acids (0.5 to 3 mg per ml) which usually replace peptides (Peters *et al.*, 1953; Demain and Hendlin, 1958). However, none showed activity. Furthermore, the following peptides were tested and also were inactive: glutathione (0.2 to 50 μg per ml), histidyl histidine (0.2 to 50 μg per ml), cysteinyl glycine (0.2 to 50 μg per ml), and carnosine (1 to 10 μg per ml).

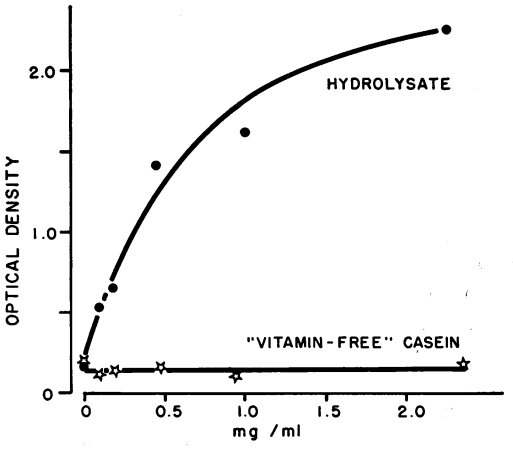

Figure 1. Response of *Sarcina* sp. to increasing concentrations of vitamin-free casein and its acid hydrolyzate.

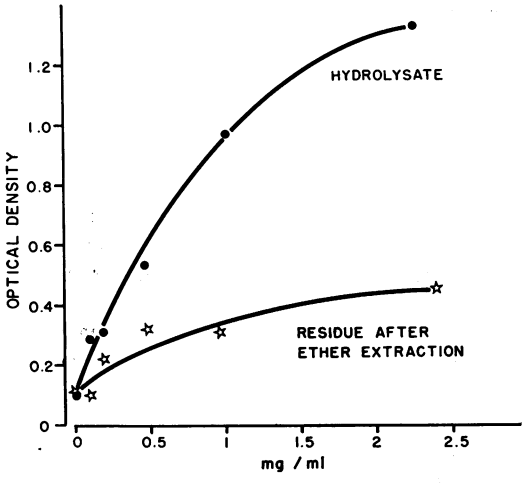

Figure 2. Response of *Sarcina* sp. to increasing concentrations of the acid hydrolyzate of vitamin-free casein and its residue after ether extraction.

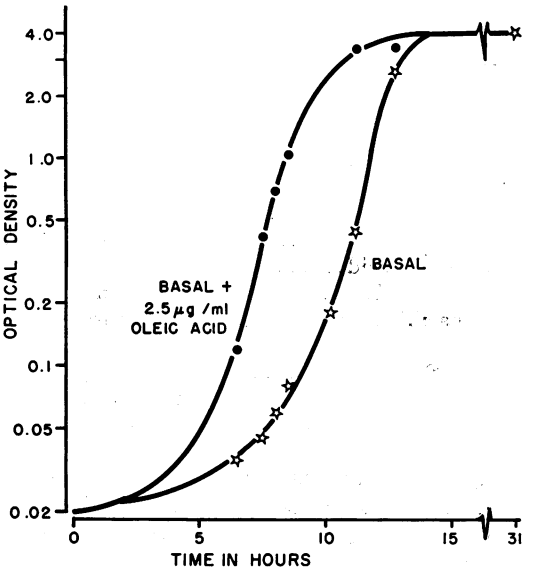

Figure 3. Growth curve of *Sarcina* sp. in the presence and absence of oleic acid.

(2) Activity of fatty acids:—It was next found that most of the activity of the 24 hr hydrolyzate could be removed by a continuous ether extraction at low pH (see figure 2). The extract, after the ether was removed by evaporation, was taken up in absolute ethanol and was found to be highly active. Thus, a fatty acid requirement was indicated.

Oleic acid was tested and was highly potent, allowing half-maximal growth at 130 mμg per ml. Figure 3 shows the effect of oleic acid on the lag phase of the organism. Since this was the first indication of a fatty acid requirement in the genus *Sarcina*, we studied the nutrition of the organism in some detail. We were first interested in finding out whether the need for oleic acid was connected in any way with a biotin requirement as it is with some other microorganisms (Nieman, 1954). These experiments showed that the organism has no biotin requirement either in the presence or absence of oleate and that the vitamin had no effect on the oleate stimulation. This was also shown by the lack of inhibition by avidin,

TABLE 2

Active fatty acids

Compound	Type	Total No. Expt.	Amt for ½ Max Growth	
			Range	Avg
			μg/ml	μg/ml
Oleic acid	C18, *mono*-unsaturated	8	0.03–0.29	0.13
Linoleic acid	C18, *di*-unsaturated	5	0.02–0.41	0.16
Arachidonic acid	C20, *tetra*-unsaturated	2	0.07–0.36	0.22
Linolenic acid	C18, *tri*-unsaturated	2	0.15–0.47	0.32
Lactobacillic acid	C19, saturated, with cyclopropane ring	2	0.2–1	0.6
Tween 80	Polyoxyethylene sorbitan monooleate	2	4–7	5.5

an agent known to inactivate biotin (Eakin *et al.*, 1941), in both the presence and absence of oleate. We did note, however, that *Sarcina* sp. had an absolute requirement for some other component of our vitamin mixture. Elimination experiments showed that nicotinic acid was required. The requirements for nicotinic acid was completely independent of the oleate stimulation.

Other unsaturated fatty acids were capable of substituting for oleate as shown in table 2. Also quite potent was lactobacillic acid, a C19 saturated compound with a cyclopropane ring (Hofmann *et al.*, 1958). Only those acids which were tested more than once are listed in the table. Single experiments were conducted with several other compounds. It was found that elaidic acid, the *trans* form corresponding to oleic acid was much less active than the latter, requiring several μg per ml. A similar result was obtained with erucic acid (C22, *mono*-unsaturated). The phospholipids, lecithin, and cephalin were not as active as oleic acid but were still quite potent, allowing half-maximal growth at levels below 1 μg per ml. Triolein had only about 1 per cent of the activity of oleic acid. The only inactive unsaturated acid tested was the C11 *mono*-unsaturated undecylenic acid.

All the saturated acids tested other than lactobacillic were inactive. The following acids were examined at various concentrations ranging in general from one to 100 μg per ml: acetic, butyric, isobutyric, *n*-valeric, caproic, *n*-heptanoic, caprylic, nonanoic, capric, undecanoic, lauric, tridecanoic, myristic, palmitic, margaric, stearic, arachidic, and behenic. Other inactive compounds related to lipids included Tweens 20 and 40, cholesterol, squalene, mevalonic acid, *o*-phosphorylethanolamine, and vitamins D_2, D_3, E, K, and K_1.

DISCUSSION

Sarcina sp. resembles most of the previously described microorganisms with fatty acid requirements (Nieman, 1954) in its stimulation by unsaturated acids and the lack of activity of the saturated forms. The activity of the cyclopropane fatty acid, lactobacillic acid, was not surprising in view of the work of Hofmann and Panos (1954) with the lactic acid organisms.

The present investigation demonstrates the presence of stimulatory fatty acids in commercial casein digests rendered vitamin-free. Thus, we encountered a nutritional situation which had many of the superficial symptoms of a peptide requirement but which turned out to be something quite different. Our results should point up the danger of assuming peptide requirements on the basis of a limited amount of evidence, i. e., activity of hydrolyzates and inactivity of pure amino acid mixtures.

Years ago, several investigators (Cohen *et al.*, 1941; Hutner, 1942; Feeney *et al.*, 1943) pointed out that the traces of milk fat present in crude casein digests were stimulatory to various microorganism with fatty acid requirements. Since then a large number of studies have been published on the fatty acid requirements of microorganisms, especially the lactic acid organisms. In these investigations, the source of amino acids was usually a vitamin-free casein digest. In view of our results, we wondered how fatty acid requirements could be demonstrated and studied in such media. The answer lies in the larger quantities of unsaturated fatty acids required by these

organisms in contrast to *Sarcina* sp. Whereas our culture shows optimum growth below 1 μg per ml (and inhibition at higher levels) most of the previously studied species have been reported to require at least several μg per ml. In our own laboratory we have found the following concentrations to be required for half-maximal growth of *Lactobacillus acidophilus* strain ATCC 4963 in a medium containing vitamin-free acid-hydrolyzed casein (per ml): oleic 2.5 μg, lactobacillic 2.5 μg, linolenic 42 μg, arachidonic > 50 μg, and linoleic > 100 μg (Demain, Hendlin, and Newkirk, *unpublished data*). If one compares these figures to the much lower ones in table 2, it is understandable why casein digest media do not allow significant growth of many lactobacilli without further fatty acid supplementation. The present study, however, illustrates the danger of using such natural materials as sources of amino acids in examining the fatty acid nutrition of previously unstudied microorganisms.

ACKNOWLEDGMENTS

The authors are indebted to Dr. A. C. Page and Mr. Edward Rickes for chemical help and their useful suggestions during these studies. We would also like to thank Dr. Klaus Hofmann of the University of Pittsburgh for the lactobacillic acid and Drs. I. Schoenwaldt and D. Bosshardt of the Merck Sharp and Dohme Research Laboratories for the samples of oleic, linoleic, and arachidonic acids.

SUMMARY

An organism, identified as a *Sarcina* species, showed superficial symptoms of a peptide requirement for the shortening of its lag phase of growth. Activity was present in commercial vitamin-free casein only after hydrolysis. Further study showed the activity to be due to fatty acids, not peptides. This appears to be the first report of fatty acid stimulation in sarcina. The most potent compounds were long chain unsaturated acids. Of many saturated acids studied, only lactobacillic acid was active. The data indicate that the use of vitamin-free casein digests as sources of amino acids would be unwise in studying fatty acid requirements of previously unstudied microorganisms.

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