THE RELATION OF CALCIUM AND OTHER CONSTITUENTS OF A DEFINED MEDIUM TO PROLIFERATION OF LACTIC STREPTOCOCCUS BACTERIOPHAGE[1]

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During a study of certain aspects of the relationships between bacteriophage and *Streptococcus lactis* and *Streptococcus cremoris*, proliferation of the bacteriophage on organisms growing in media of known chemical composition became desirable. So far as could be determined, proliferation of the lactic streptococcus bacteriophages on organisms growing in completely defined media has not been reported.

METHODS

Defined basal media. The medium of Niven (1944) was modified by omitting NaCl and also by substituting some amino acids of L-configuration for those of DL-configuration; when such substitutions were made, the quantities of the acids in question were decreased by half. These modifications were found to have no influence on the growth of the test bacteria. This medium was modified further for some of the test cultures by adding 0.2 per cent sodium acetate and 0.2 per cent sorbitan monooleate, as suggested by Collins, Nelson, and Parmelee (1950). Glutamine and asparagine, sterilized by filtration, were added aseptically after the media, sterilized by autoclaving, had cooled to room temperature. These basal media were modified as outlined in the subsequent portions of this study.

Cultures and preparation of inocula. Cultures were separated into the *S. lactis* and the *S. cremoris* types, using growth at 40 C and production of ammonia from arginine as bases of differentiation. Three strains of the *S. lactis* type (565, W2, and 712), seven strains of the *S. cremoris* type, and homologous bacteriophages for each of these ten strains of streptococci were used. Cultures were propagated in litmus milk and in defined medium. Inocula for test media were prepared by sedimenting twice by centrifugation cultures grown 12 hours in defined medium, resuspending each, and adjusting the washed cells with 0.9 per cent NaCl to a turbidity reading of 40 with a Klett-Summerson photoelectric colorimeter using filter number 54. Inocula prepared in this manner gave plate counts of approximately 1×10^8 per ml. One per cent inoculum was used for all test media.

Bacteriophage filtrates. All bacteriophage strains used in this study, except F54 and F24, the ones active against *S. lactis* strains 565 and W2, respectively, were carried as whey filtrates. Bacteriophage strains F54 and F24, the only two

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that multiplied when the test organisms were grown in the unsupplemented defined medium of Niven, were carried as filtrates from Niven's medium. In preparing these filtrates, phage-lysed cultures were adjusted colorimetrically to approximately pH 6.7 for *S. lactis* strain W2 and to approximately pH 7.0 for *S. lactis* strain 565 and immediately filtered through sterile Selas microporous procelain filters (porosity no. 03) to remove bacterial cells. When a culture in defined medium was not neutralized prior to filtration, the resulting filtrate usually contained comparatively few bacteriophage particles. This was especially true when the majority of the bacterial cells had not been lysed and the culture was turbid. The various test media were inoculated with 1 per cent of a distilled water dilution of filtrate containing about 1×10^5 phage particles per milliliter.

Estimation of numbers of bacteriophage particles. Numbers of bacteriophage particles were estimated in litmus milk by the limiting dilution technique of Nelson, Harriman, and Hammer (1939). This method was chosen because plaques formed by most of the combinations used were extremely small and frequently very difficult to count. Three tubes of each dilution were made, and the probable number of bacteriophage particles per milliliter was computed using probability tables (Buchanan and Fulmer, 1928). Each tube was inoculated with one drop of a 1:8 dilution in litmus milk of a 24-hour litmus milk culture of bacteria.

Estimation of bacterial populations. Bacterial populations were estimated by turbidity measurements, using a Klett-Summerson photoelectric colorimeter with filter number 54. The colorimeter was adjusted to 0 with an uninoculated tube of medium. Plate counts on tomato juice agar were made for confirmation in some experiments.

Testing for bacteriophage proliferation. Test media were prepared and dispensed aseptically in quantities of 10 ml into 16-by-125-mm screw cap culture tubes. Two tubes of each medium were used for each combination, one as an index of organism growth in the absence of bacteriophage and the other for determination of bacteriophage multiplication. The medium was adjusted to 32 C in a water bath, and 0.1 ml of resuspended cells was added to each of the two tubes. Twenty minutes later 0.1 ml of diluted filtrate containing bacteriophage was added to the tube used for the determination of bacteriophage proliferation, and 5 minutes later the number of bacteriophage particles was estimated. Bacteriophage estimations and turbidity measurements were made at the indicated times during the course of the experiment, with the time of first estimation of bacteriophage numbers as zero time.

RESULTS

Bacteriophages F54 and F24 multiplied on cultures of their respective homologous strains of *S. lactis*, 564 and W2, in the defined medium of Niven. In various runs, the probable number of bacteriophage particles increased from about 1×10^3 to about 1×10^6 per ml during a 7-hour incubation period. The effect of the omission of individual amino acids, vitamins, and purine and pyrimidine bases on the populations of bacteria and bacteriophages was de-

termined for these two combinations. Representative data are given in table 1 for combination 565-F54. When the omission of an individual component from the medium caused a decrease in the rate of bacteriophage proliferation, a similar

TABLE 1

Effect of the omission of individual nutrients from the medium of Niven upon the multiplication of Streptococcus lactis 565 and bacteriophage F54

NUTRIENT OMITTED	NO. OF BACTERIOPHAGE PARTICLES (7 HR)*	TURBIDITY OF CONTROL (7 HR)
Nicotinic acid	1.5×10^2	7
DL-Leucine	2.0×10^2	6
L-Glutamine	4.5×10^2	10
DL-Valine	9.5×10^2	14
Thiamine	2.5×10^3	13
DL-Methionine	2.5×10^3	12
L-Arginine	4.5×10^3	22
DL-Isoleucine	1.5×10^4	11
Riboflavin	4.5×10^4	22
DL-Serine	4.5×10^4	17
Biotin	9.5×10^4	15
Uracil	9.5×10^4	17
L-Histidine	9.5×10^4	9
Adenine	4.0×10^5	18
L-Hydroxyproline	9.5×10^5	15
DL-Phenylalanine	4.5×10^6	15
DL-Alanine	9.5×10^6	20
L-Lysine	2.5×10^7	21
DL-Threonine	2.5×10^7	72
Ca-pantothenate	4.0×10^7	23
Pyridoxine	9.5×10^7	27
Guanine	9.5×10^7	61
L-Tyrosine	1.5×10^8	59
Asparagine	2.5×10^8	50
Glycine	4.5×10^8	91
DL-Aspartic acid	4.5×10^8	100
Xanthine	4.5×10^8	82
L-Cystine	9.5×10^8	77
L-Glutamic acid	9.5×10^8	85
DL-Tryptophan	9.5×10^8	117
L-Proline	2.5×10^9	96
DL-Norleucine	2.5×10^9	93
None (control)	1.5×10^8	90

* Number of bacteriophage particles was 9.5×10^2 at 0 hour.

decrease in the rate of bacterial multiplication usually was encountered, indicating a definite dependence of the former on the latter. Although mass lysis occurred in milk, this was not always the case in the defined medium with bacteriophage F54 or F24. A clear and foamy culture at 6 or 7 hours always was indicative of large numbers of bacteriophage particles, but in many cases

in which these characteristics were not present the numbers of bacteriophage particles were the same as those of the clear and foamy cultures.

Although the defined medium of Collins, Nelson, and Parmelee, containing sodium acetate and sorbitan monooleate, supported the growth of all organisms used, it was inadequate for the proliferation of bacteriophage on *S. lactis* culture 712 and on the seven *S. cremoris* cultures (table 2). Only culture 712 could be considered as growing well in this medium, but bacteriophage proliferation has been observed in other instances in cultures with turbidity levels of the same range as those encountered in this phase of the study. Several attempts were made to secure bacteriophage proliferation by supplementing the medium. During most of this study only two representative combinations, 712-F56 and E8-F63, were used. Bacteriophage proliferation was not supported when a mixture containing 20 g *p*-aminobenzoic acid, 1 g folic acid, 0.5 mg thymine, 0.1 mg pyridoxamine, 0.1 mg pyridoxal, 0.1 mg nicotinamide, 0.1 mg inositol, and 0.04 g vitamin B_{12}

TABLE 2

Multiplication of bacteriophages on bacteria growing in the defined medium of Collins, Nelson, and Parmelee

ORGANISM STRAIN	BACTERIOPHAGE STRAIN	NO. OF BACTERIOPHAGE PARTICLES PER ML			TURBIDITY OF CONTROL (7 HR)
		0 hr	3 hr	6 hr	
712	F56	2.5×10^3	2.5×10^5	3.0×10^1	140
FH8	F65	9.5×10^3	2.5×10^4	9.5×10^3	4
318B/27	F74	2.0×10^3	2.5×10^5	2.0×10^3	23
E8	F63	4.5×10^2	4.5×10^2	7.0×10^1	27
IP5	F68	7.5×10^2	3.0×10^2	9.0×10^1	21
HP	F59	2.0×10^2	4.5×10^3	9.5×10^2	8
459	F52	4.5×10^3	9.5×10^2	7.0×10^1	16
122-1	F76	2.5×10^3	2.0×10^2	9.5×10^3	11

was added to 100 ml of the basal defined medium. Addition of such complex mixtures as yeast extract, tomato juice, proteose peptone, reticulogen (a commercial liver extract), peptonized milk, and hydrolyzed casein did not permit bacteriophage multiplication, although these additions did stimulate organism growth.

To improve the anaerobic conditions of the medium, 10 ml thioglycolic acid and 10 mg cysteine were added per 100 ml of medium, and cultures were grown in an atmosphere consisting of about 10 per cent carbon dioxide and 90 per cent hydrogen, using the anaerobic technique of Weiss and Spaulding (1937). These modifications of the medium and culture conditions had little effect on organism growth and did not result in bacteriophage proliferation.

Since the medium was not highly buffered, the K_2HPO_4 content was increased to maintain a pH level more nearly that of milk, which was considered optimum for bacteriophage multiplication. Increases in the K_2HPO_4 content of 1 or 2 per cent improved organism growth, but did not permit multiplication of bacteriophage.

Addition of 5 per cent dried skim milk before sterilization of the basal medium containing 1 per cent phosphate permitted increases in bacteriophage populations in 7 hours from 1.5×10^3 to 4.5×10^5 in the case of 712-F56 and from 9.5×10^2 to 2.5×10^3 in the case of E8-F63. These results were incompatible with the fact that liquid skim milk commonly is used in this laboratory for the propagation of bacteriophages active against the lactic group of streptococci. In a trial using unsupplemented reconstituted 5 per cent dried skim milk, the bacteriophage population increased in 7 hours from 1.5×10^3 to 4.5×10^7 for combination 712-F56 and from 9.5×10^2 to 2.5×10^9 for combination E8-F63. Plate counts on the control cultures revealed that the bacteria growing in the absence of bacteriophage did not reach as high concentrations in the reconstituted skim milk as in the basal medium supplemented with dried skim milk.

TABLE 3

Effect of the omission of nutrients from defined medium containing 1.0 per cent K_2HPO_4, 5.0 per cent dried skim milk, 0.2 per cent sodium acetate, and 0.2 per cent sorbitan monooleate on the multiplication of S. cremoris E8 and bacteriophage F63

NUTRIENTS OMITTED	NO. OF BACTERIOPHAGE PARTICLES PER ML AT 6 HR*
None	1.5×10^2
Amino acids	7.0×10^1
Purine and pyrimidine bases	1.5×10^2
Sodium thioglycolate and sorbitan monooleate	2.5×10^2
Vitamins, glutamine, and asparagine	2.5×10^2
Minerals, sodium acetate, glucose, and phosphate	2.5×10^9
Magnesium sulfate	9.0×10^1
Manganous chloride	4.0×10^1
Ferrous sulfate	4.0×10^1
Sodium acetate	1.5×10^2
Glucose	4.0×10^1
Phosphate	2.5×10^9

* Number of bacteriophage particles was 9.5×10^2 at 0 hour.

Since the medium that failed to support appreciable multiplication of the bacteriophage apparently was the more nutritive medium, some constituent of the defined medium apparently was inhibitory to bacteriophage action.

When all constituents of the normal medium except K_2HPO_4 were included in the defined medium supplemented with 5 per cent dried skim milk, both bacteriophages developed very rapidly. Results using combination E8-F63 are given in table 3; similar results were obtained using combination 712-F56. Further study showed that addition of 0.5 or 1.0 per cent K_2HPO_4 to the medium before sterilization greatly inhibited bacteriophage multiplication.

Use of 0.1 per cent K_2HPO_4 permitted bacteriophage increases from 9.5×10^2 to 2.5×10^9 per ml for both combinations 712-F56 and E8-F63. Ammonium phosphate and sodium citrate also prevented bacteriophage proliferation when added to the defined medium containing dried skim milk, whereas the presence

of sodium acetate seemed to have no appreciable effect. This inhibition of bacteriophage increase by phosphate or citrate possibly was the result of the unavailability of some nutrient, probably calcium, essential for bacteriophage proliferation.

Addition of 0.1 to 0.4 per cent $CaCl_2 \cdot 2H_2O$ and 0.5 per cent K_2HPO_4 to the reconstituted skim milk prior to autoclaving did not make the medium to which the milk was added adequate for bacteriophage multiplication, but similar additions after autoclaving gave small and consistent increases in bacteriophage during 6 hours. Reducing the phosphate to 0.1 per cent without added calcium chloride gave excellent bacteriophage proliferation in the defined medium supplemented with skim milk, even though all constituents were combined before autoclaving.

When the ash of 2 g dried skim milk was added per 100 ml of medium supplemented with 5 per cent "vitamin-free" casein hydrolyzate but not supplemented

TABLE 4

Effect of method of sterilization and K_2HPO_4 content of supplemented defined base medium containing the ash of 2 g dried skim milk per 100 ml upon bacteriophage multiplication*

BACTERIOPHAGE	METHOD OF STERILIZATION	K_2HPO_4 CONC. (G PER 100 ML)	NO. OF BACTERIOPHAGE PARTICLES PER ML		TURBIDITY OF CONTROL (6 HR)
			0 hr	6 hr	
F56	Autoclaved	0.1	4.5×10^2	1×10^4	Precipitated
	Filtered	0.1	4.5×10^2	4.0×10^7	52
	Filtered	0.4	4.5×10^2	1×10^4	54
F63	Autoclaved	0.1	4.5×10^2	1×10^4	Precipitated
	Filtered	0.1	4.5×10^2	2.5×10^8	55
	Filtered	0.4	4.5×10^2	1×10^4	55

* Ten per cent casein hydrolyzate, 5 ml per 100 ml.

with calcium other than that in the milk ash, a precipitate was formed during autoclaving and the bacteriophages did not multiply on organisms growing in the medium (table 4). However, when this medium was sterilized by filtration rather than autoclaving, the bacteriophages multiplied. If the K_2HPO_4 content of the basal medium was increased to 0.4 per cent, the resulting medium was unsuitable for bacteriophage increase, even when sterilized by filtration.

When the basal defined medium was supplemented with 5 per cent casein hydrolyzate plus a number of vitamins later proved unessential and then sterilized by filtration, the addition of 0.2 per cent $CaCl_2 \cdot 2H_2O$ resulted in increases in number of bacteriophage particles per milliliter during 6 hours from the original 4.5×10^2 to 2.5×10^9 for combination E8-F63 and from 4.5×10^2 to 2.5×10^7 for combination 712-F56. The addition of 0.1 per cent $CaCl_2 \cdot 2H_2O$ gave similar results. When $CaCl_2$ was not added, the bacteriophages did not multiply. Autoclaving the phosphate solution and the medium containing $CaCl_2$ separately and mixing the two after cooling gave a medium that was as adequate for the production of bacteriophages as a medium sterilized by filtration. Neither $MnCl_2$ nor $MgSO_4$

duplicated the effect of $CaCl_2$ in promoting bacteriophage multiplication. A slight stimulation, which was much less than that of $CaCl_2$ for bacteriophages F56 and F63, resulted from the use of 0.2 per cent $MgSO_4$.

Bacteriophage did not increase in the unsupplemented defined medium when the K_2HPO_4 content was decreased to 0.01 per cent; complete elimination of phosphate prevented organism growth.

When the eight bacteriophages that did not proliferate in the absence of soluble calcium were inoculated, along with their susceptible host cells, into basal defined medium containing 0.1 per cent K_2HPO_4 and supplemented with sodium acetate, sorbitan monooleate, and 0.2 per cent $CaCl_2 \cdot 2H_2O$, bacteriophage proliferation occurred in each case (table 5). The increase in numbers of bacteriophage particles for each combination was as great as, or greater than, would be predicted from the development of the bacteria as determined by turbidity measurements on control tubes containing only susceptible organisms.

TABLE 5

The effect on bacteriophage proliferation of 0.2 per cent $CaCl_2 \cdot 2H_2O$ in the defined medium of Collins, Nelson, and Parmelee*

ORGANISM STRAIN	BACTERIOPHAGE STRAIN	NO. OF BACTERIOPHAGE PARTICLES PER ML			TURBIDITY	
		0 hr	7 hr	12 hr	7 hr	12 hr
712	F56	4.5×10^2	4.5×10^7	—	68	—
FH8	F65	4.5×10^2	4.5×10^6	2.4×10^7	9	17
318B/27	F74	2.5×10^2	2.5×10^7†	2.5×10^8	22	46
E8	F63	4.5×10^2	9.5×10^4	—	9	—
IP5	F68	4.5×10^2	2.5×10^7†	2.5×10^8	7	18
HP	F59	2.0×10^2	2.5×10^7†	4.5×10^7	6	19
459	F52	2.5×10^3	4.5×10^5	2.5×10^7	8	17
122-1	F76	2.5×10^2	4.5×10^5	2.5×10^8	5	15

* Sterilized by filtration.

† Highest dilution made.

Two attempts were made to demonstrate whether calcium was necessary for the adsorption of bacteriophage. Decreases in the numbers of bacteriophage particles remaining in the centrifugally obtained supernatants in which no calcium was present were as great as, or greater than, they were in those in which calcium was present. Although the precision of estimation was not such as to justify definite conclusions, the results indicate that possibly calcium was not necessary for the adsorption of bacteriophages F56 and F63.

DISCUSSION

With two organism-bacteriophage combinations, 565-F54 and W2-F24, the bacteriophages multiplied in the presence of host cells growing in the chemically defined medium of Niven (1944). When individual nutrients were omitted from the defined medium, a decrease in bacteriophage proliferation was accompanied by a corresponding decrease in multiplication of the host bacteria. This does not

eliminate the possibility that a similar study with more refined procedures for bacteriophage enumeration might reveal slight variations from this close relationship. Elimination of groups of nutrients, rather than only single nutrients, also might lead to different results, but the number of permutations and combinations possible precluded embarking on a program of that type at the time this work was in progress. Data on the effect of the omission of nutrients other than calcium and phosphate from the defined medium were not obtained with the eight strains requiring calcium; these eight strains might be more specific in their requirements of other medium constituents for proliferation.

The ability of an organism to grow in a particular medium does not ensure that a bacteriophage to which the organism is sensitive will proliferate on the organism in that medium. All ten strains of lactic streptococci used in these studies could grow in a defined medium without calcium supplementation, but eight of the ten homologous bacteriophages required the addition of soluble calcium for proliferation. Similar calcium requirements have been demonstrated for *Shigella* bacteriophages (Stassano and De Beaufort, 1925; Bordet and Renaux, 1928), for staphylococcal bacteriophages (Rountree, 1947; Smith, 1948), for single strains of *Escherichia coli* and *Shigella* bacteriophage by Wahl (1946), for a mutant of *E. coli* bacteriophage T4 (Delbrück, 1948), and for *coli* bacteriophage T5 (Adams, 1949). Shew (1949) reported that eight strains of bacteriophage active against *S. lactis* all needed calcium for maximum development, as judged by plaque formation on solid media and clearing in broths. The media used contained yeast extract or yeast infusion, in addition to other complex nutrients, some of which possibly contributed calcium, so that the optimum concentration of calcium varied considerably, with some stimulation resulting from as little as 0.001 M. Reiter (1949) used 0.01 M $CaCl_2$ in his agar medium for demonstrating plaque production by bacteriophages from three strains of lactic streptococci. Cherry and Watson (1949) reported 0.005 to 0.05 M $CaCl_2$ was stimulatory to proliferation of the one strain of bacteriophage studied on a yeast extract tryptone glucose medium without buffer. Even at low levels oxalate permitted no lysis in the medium and citrate was inhibitory.

The requirement of calcium for proliferation by some lactic bacteriophage strains used in this work and not by others is interesting in view of the observation by Burnet (1933) that lysis by members of 4 of 11 serological groups of dysentery-coli bacteriophages was inhibited completely by 0.5 per cent citrate; 1 per cent was inhibitory for 1 group, and 6 groups were unaffected by 1.5 per cent citrate. Adams (1949) observed that, whereas T5 *E. coli* phage required at least 10^{-4} M calcium ion for multiplication and T1 phage was slightly less sensitive, the host cells and five other *E. coli* phages had little or no requirement for soluble calcium. Establishment of minimum levels of calcium requirement for a variety of lactic streptococcus bacteriophages would be desirable, as the variability in several characteristics that has been encountered with this type of bacteriophage indicates the possibility of a considerable range in the requirements of the various strains. Data available at present do not indicate whether all of the variability encountered is associated with the bacteriophage or whether

some may be associated with the host bacteria, although Wahl (1946) has reported that a bacteriophage strain sensitive to citrate when acting on one host was citrate-sensitive on all hosts.

Available information on the role played by soluble calcium in bacteriophage proliferation leaves many questions unanswered. Andrews and Elford (1932) demonstrated that 0.75 per cent added citrate permitted *E. coli* bacteriophage to "kill" the cells but would not permit lysis. Addition of citrate as long as 40 minutes after mixture of the organism and the bacteriophage prevented lysis, and addition at 60 minutes slowed lysis slightly. Wahl (1946) has reported that calcium is necessary for the multiplication of bacteriophage, but adsorption of the bacteriophage by the sensitive cell occurs in the absence of calcium. Delbrück (1948) reported that coli phage T4,12 required both tryptophan, or an analogue, and calcium ion for "invasion" or adsorption. Cherry and Watson (1949) reported the greatest adsorption of bacteriophage by cells when the calcium concentration was optimum for lysis. The results of the present study are not conclusive, but they do support the conclusion that bacteriophage adsorption by the cells occurred when calcium was unavailable. Adams (1949) reported that calcium "seems to be required for some stage of the infectious process subsequent to adsorption of virus to the host cell." Rountree (1947) has ascribed the effects of calcium removal on bacteriophage multiplication to an effect upon the activity of certain enzymes that may be necessary for bacteriophage multiplication. Certainly the function of calcium in the proliferation of bacteriophage deserves additional study.

That calcium is necessary for the multiplication of many bacteriophages active against strains of lactic streptococci may be of significance to the dairy industry. Precipitation of calcium to prevent the lysis of bacteria growing in milk probably would not be feasible. However, by using chemically defined nutrients containing no calcium or by using complex nutrients low in calcium content and supplemented with sufficient phosphate or other calcium-binding ion, a medium might be designed for the carrying of many strains of the lactic group of streptococci with lessened danger of bacteriophage action. The ability of bacteriophage strains F54 and F24, active on cultures 565 and W2, respectively, to proliferate in the absence of appreciable amounts of soluble calcium indicates a probable limitation of the application of this possible control technique. However, if the procedure could be used for carrying even a limited group of organisms in mother cultures without bacteriophage difficulties, it might prove useful.

SUMMARY

With two *Streptococcus lactis*-bacteriophage combinations, multiplication of bacteriophages and organisms was affected similarly by the omission of individual components from the defined medium of Niven (1944). Bacteriophage multiplication seemed to be closely associated with organism multiplication for these two combinations.

Eight other bacteriophage strains tested proliferated on their susceptible host

cells growing in a completely defined medium containing sodium acetate and sorbitan monooleate when soluble calcium was available in the medium. Under these conditions, the close relationship between bacteriophage proliferation and organism multiplication which had been noted for other strains was evident also for these more exacting strains.

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