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Relationship of the nutrition of Streptococcus lactis to bacteriophage proliferation

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NOTE TO USERS

RELATIONSHIP OF THE NUTRITION OF *STREPTOCOCCUS LACTIS* TO BACTERIOPHAGE PROLIFERATION

by

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INTRODUCTION

Bacteriophage problems pertaining to the dairy industry have been approached to date primarily by taking precautionary steps to prevent cultures from coming in contact with bacteriophages and by establishing practices which tend to reduce to a minimum the numbers of particles of bacteriophages that may gain entrance to dairy products and multiply therein. Such practices consist of the selection and rotation of cultures, special precautions for the preparation of mother and bulk starters, and the maintenance of strict sanitation of plant equipment and surroundings.

It is believed that a consideration of the more fundamental bacteriophage-organism relationships may result in partial elucidation of some of the factors affecting multiplication of both bacteria and bacteriophages in milk. Therefore, a study of the effect of the nutrition of Streptococcus lactis upon the proliferation of the homologous bacteriophages was initiated.

STATEMENT OF PROBLEM

The purpose of the work reported herein was to study the relation of the nutrition of Streptococcus lactis to bacteriophage proliferation.

REVIEW OF LITERATURE

Since Twort (1915) reported a transmissible lytic agent active against staphylococcus organisms isolated from calf vaccines, much evidence has accumulated to show that bacteriophages active against a wide variety of bacteria can be demonstrated. A tremendous amount of work has been done in an effort to elucidate the mechanism of bacteriophage action, and many excellent reviews are available which summarize known data and theorize on the debatable aspects. Some of the more recent reviews are those of Delbrück (1946), Anderson (1946), Price (1948a), and Cohen (1949).

Several investigators (Burnett, 1929; Krueger and Northrup, 1930; Clifton and Morrow, 1936; and Hunter, 1943) have shown that the proliferation of bacteriophages is conditioned by bacterial reproduction and that whenever physical or physicochemical conditions are altered in such a way as to interfere with cell division, bacteriophage production is reduced. In predominantly the more recent studies it has been shown that bacteriophage reproduction, although related very closely to bacterial reproduction, may be influenced separately under certain nutritional conditions. For purposes of review, the effect of electrolytes upon bacteriophages will first be taken up, followed by the effect of various other growth factors.

Scribner and Krueger (1937) and Krueger and Strietmann

(1938) noted the effects of sodium chloride and sodium sulfate, respectively, while growing a Staphylococcus aureus strain S_3K-bacteriophage combination in nutrient broth and determining multiplication of the bacteriophage by time of lysis under controlled conditions. Scribner and Krueger found that the presence of M/4 sodium chloride in the medium gave a slightly increased total bacteriophage production, while Krueger and Strietmann obtained the same result upon the addition of M/8 sodium sulfate. In neither case was the growth of the susceptible host detectably affected, and the augmented end titers of the bacteriophage were attributed to increased lytic thresholds, i.e., increased ratios of bacteriophage to bacteria requisite for lysis. They reasoned that because of increased organism resistance, production of bacteriophage continued longer, accounting for the eventual increases in bacteriophage yields. Later Fong and Krueger (1949), using Staph. aureus bacteriophage strain K and two methods, lysis time and plaque counts, for the enumeration of bacteriophage in a tryptose phosphate broth, found that a mixture of bacteriophage and organism maintained at 42° C. failed to show an increase in bacteriophage and actually registered a considerable loss. However, the inclusion of M sodium chloride in the medium prevented the loss by preventing adsorption of bacteriophage by bacterial cells, as was shown by an adsorption study at 36° C. It seems that these findings might help to further explain the results of Scribner and Krueger (1937) and Krueger

and Strietmann (1938). The increased bacteriophage yields found by these workers could have been a result of decreased adsorption rates due to the high concentrations of sodium ions, which would have allowed greater concentrations of cells to develop prior to mass lysis and hence greater total bacteriophage yields.

Gest (1943) found that plaques formed by a strain of bacteriophage active against Escherichia coli became increasingly turbid with rising concentration of monovalent cationic salts. Plaque turbidity was barely noticeable at 0.009M, and plaques were barely distinguishable at 0.36M. With rising concentration of divalent cationic salts, plaque counts remained normal until a critical concentration was reached, about 0.005M, above which an increase in salt concentration by a factor of two prevented the appearance of any visible plaques. Gest attributed these effects to an increased development of resistant organisms which, in the presence of high salt concentrations, masked the effect of lysis.

Krueger and West (1935) found that minute concentrations of manganous ions, when added to beef infusion broth, accelerated lysis of a strain of Staph. aureus by bacteriophage without detectably affecting organism growth. Determining numbers of bacteriophage particles by the time required for lysis under controlled conditions, they reasoned that lysis was accelerated by a depression of the lytic threshold, which accounted for a corresponding decrease in the total quantity

of bacteriophage produced. Spizizen (1943b) determined plaque counts upon cells of E. coli which had been infected with bacteriophage in nutrient broth, sedimented, and transferred to low concentrations of glycine anhydride containing various test substances. He found that the addition of ferrous, ferric, manganous, and magnesium ions stimulated bacteriophage production. Since Spizizen (1943a) had found that infected cells suspended in glycine anhydride did not multiply, cell multiplication was not determined in these experiments.

It was noticed by Stassano and de Beaufort (1925) that sodium citrate inhibited the multiplication of some strains of bacteriophages active against species of Shigella and that this action could be counteracted by using calcium chloride. Bordet and Renaux (1928), also using species of Shigella, confirmed the work of Stassano and de Beaufort, finding that sodium oxalate had the same effect, and considered that the effect was due to the removal of calcium ions which were necessary for the multiplication of the bacteriophages. This finding was extended by Andrews and Elford (1932) to include a strain of E. coli. They found that nutrient agar containing 0.75 to 1 per cent sodium citrate gave almost normal growth of E. coli, but in the presence of sodium citrate the homologous bacteriophage did not form plaques. When an excess of bacteriophage was added to citrate agar plates containing susceptible organisms, no colonies or plaques appeared, which indicated that while the presence of citrate prevented bacteriophage

multiplication it did not prevent bacteriophage adsorption and the consequent inactivation of bacterial cells. Andrews and Elford did not try to counteract the inhibition caused by citrate.

Wahl (1946), working with four organism-bacteriophage combinations and a synthetic medium, demonstrated with plaque counts that bacteriophages C36, active against a strain of E. coli, and S13, active against a species of Shigella, required calcium for multiplication. An adsorption study disclosed that bacteriophage C36 was adsorbed by either dead or living cells in the absence of calcium. Although plaque counts indicated bacteriophage multiplication when calcium was added to the synthetic medium, no lysis or clearing was evident, and no plaques were formed on a solid medium which contained the synthetic medium, calcium, and gelatin. The addition of thiamin to this calcium-containing medium caused lysis by C36 with what seemed to be no difference in final bacteriophage concentration, but thiamin seemed insufficient to cause lysis by S13. While typing staphylococci with bacteriophages, Smith (1943) encountered three batches of nutrient agar that were inhibitory to bacteriophage action. After discarding one batch of the media, he added 0.01M calcium chloride to the other two batches and found that this addition made the media completely satisfactory for the demonstration of bacteriophage activity. Following Anderson's work with L-tryptophan as an adsorption cofactor (1945 and 1948), Delbrück, upon comparing plaque

counts on synthetic medium and nutrient agar followed by an adsorption study, reported finding three clearly distinct mutants of bacteriophage strain T4, active against E. coli strain B; these were (1) a type requiring no cofactor; (2) a type requiring tryptophan or similar substances and (3) a type requiring tryptophan or similar substances and in addition requiring calcium ions. The activity of calcium as an adsorption cofactor in the synthetic medium could not be replaced by sodium, potassium and magnesium ions.

Quantitative studies between the relationships of bacteriophages and bacteria have depended usually upon either the serial dilution method or the plaque count for the enumeration of particles of bacteriophages. Each of these methods has definite limitations, and neither is completely satisfactory for analysis of a system in which concentrations are changing rapidly. Methods for the determination of organism growth, usually plate counts or turbidity measurements, also have definite limitations. Thus one should proceed with caution when drawing conclusions from results which in at least some cases border on experimental variations and which were completed by different investigators under different experimental conditions. However, it seems obvious that changes in the inorganic constituents of media do affect the multiplication and/or adsorption of bacteriophages in some cases to a greater degree than such changes affect the multiplication of suscep-

tible host cells. Especially is this true of calcium which seems to play a very important role in either the adsorption or the multiplication of several bacteriophages. It appears that various strains of bacteriophages differ quite markedly in their response to various changes in the inorganic constituents of media. At the present these differences appear to be related primarily to differences in bacteriophages rather than to differences in host cells.

Several studies have shown that other growth factors may play important roles in the adsorption and/or multiplication of bacteriophages. While attempting to use a synthetic medium for the assay of the T bacteriophages, active against E. coli strain B, Anderson (1945) noticed that T4 and T6 gave fewer plaques on the synthetic medium than upon nutrient agar. The numbers of plaques formed on the synthetic medium by T4 and T6 were brought almost to normal upon the addition of minute amounts of L-tryptophan. Further experiments revealed that L-tryptophan, acting as a cofactor, enhanced the activities of these two bacteriophages by increasing their rates of adsorption on the host cells. Later Anderson (1948) found that at 15° C., E. coli grown on synthetic medium, was resistant to some of the stocks and picked clones of bacteriophage T4 which were active at 37° C., while there were no differences in the numbers of plaques formed on nutrient agar plates incubated at the two temperatures. Since in all cases the efficiency of plaque formation by bacteriophage T4 on

the synthetic medium at 15° C. was brought to unity by the addition of 20 γ/ml. of L-tryptophan, Anderson suggested that the metabolism of the bacteria in minimal medium furnished sufficient cofactor for the activation and adsorption of those strains of T4 which were efficient in producing plaques on the synthetic medium at 37° C., but that these amounts of cofactor in many cases were not sufficient at 15° C. A survey of the other bacteriophages of the T group disclosed similar decreases in the numbers of plaques formed at 15° C. by T1 and T7. Preliminary tests indicated increases in the numbers of plaques formed upon the addition of isoleucine, methionine, or norleucine to the synthetic medium for T1 and isoleucine, leucine, methionine, or norleucine to the synthetic medium for T7. As already has been mentioned, Delbrück (1948) found that mutant types of T4 required cofactor or cofactors, while others did not.

Price (1947) reported that niacin, when added to a dilute nutrient broth medium containing 2.5×10^8 Staphylococcus muscae cells per ml. and homologous bacteriophage, caused an increase in bacteriophage as determined by plaque counts without an increase in bacteria as shown by turbidity readings. Several other nutrients tested were without effect on bacteriophage formation in this system. Price also reported that Fildes' synthetic medium did not support bacteriophage proliferation unless broth or yeast extract were added. A solution containing 19 different growth factors and a strepogenin

concentrate could not replace broth or yeast extract. Using complete lysis as an index of bacteriophage multiplication, Price (1948b) reported that acid digests of vitamin-free casein, when added to Fildes' synthetic medium, promoted bacteriophage proliferation. A total of 21 different amino acids failed to replace the casein digest; however, the active substance did not appear to be a peptide since it was stable to very strong acid and alkali. Later Price (1949), using one-step-growth experiments, found that a non-dialyzable fraction from baker's yeast increased the yield of Staph. muscae bacteriophage from cells growing in the logarithmic phase of multiplication in synthetic medium. Turbidity measurements indicated that there was no effect on organism growth.

Ellis and Spizizen (1940), upon infecting E. coli cells in nutrient broth, sedimenting, resuspending in distilled water, and adding the infected cells to solutions containing various nutrients, found that a solution of glycine supported an increase in bacteriophage, as determined by plaque counts. Spizizen (1943) added infected E. coli cells, prepared by the method of Ellis and Spizizen (1940), to various test substances suspended in phosphate buffer at pH 6.9. He found that low concentrations of glycine, glycine anhydride, and hippuric acid supported bacteriophage multiplication without bacterial growth, as determined by plate counts.

Fowler and Cohen (1948) infected E. coli strain B cells with bacteriophage T2 and determined the effect of adding various nutrients to a synthetic medium upon latent period and burst size. They obtained stimulation, reflected in diminished latent period or increased burst size, or both, with L-arginine, L-aspartic acid, L-isoleucine, L-lysine, L-phenylalanine, L-proline and L-glutamic acid, the last producing the greatest effect. L-histidine, L-tyrosine and L-valine gave variable results, while L-cystine, L-leucine and L-serine were inhibitory to bacteriophage in the synthetic medium. Cohen and Fowler (1948), used the same procedures, except that various nutrients were omitted from a chemically defined medium of approximately the bacteriophage growth-promoting properties of nutrient broth (Fowler and Cohen, 1948) and the course of desoxyribosenucleic acid synthesis was followed. They found that L-histidine, L-isoleucine, L-leucine, L-methionine, L-phenylalanine, L-tryptophan, L-tyrosine, L-valine, L-glutamic acid and adenine should be present in the medium for maximal production of bacteriophage. Fowler and Cohen (1948) and Cohen and Fowler (1948), following the evidence of Cohen and Anderson (1946) that E. coli cells do not multiply after infection and assuming that diluting cultures 1:10,000 after a five minute adsorption period prevented adsorption during the experiment, did not assay to determine the effect of addition or of omission of nutrients upon bacterial multiplication.

These investigations indicate that the growth factor requirements of bacteriophages for multiplication and/or adsorption are many and that they are quite difficult to separate from the requirements of the host bacteria.

EXPERIMENTAL METHODS

Basal Synthetic Medium

It is highly desirable in this type of study to use a chemically-defined medium on which host cells and bacteriophages will proliferate. Niven (1944) reported a synthetic medium upon which all 21 of the strains of S. lactis which he used could be transferred serially and invariably develop turbidity within 24 hours. This synthetic medium autoclaved 11 minutes at 15 lb. pressure and with minor alterations was used in this work as a basal medium from which nutrients were omitted or to which nutrients were added. While Niven used 1 mg. of each amino acids per 10 ml. of medium (Niven and Sherman, 1944), in the present work where amino acid with L configuration were used only 0.5 mg. of an amino acid was included per 10 ml. of medium. Since Gest (1943) reported that high concentrations of monovalent cations promoted secondary growth, the NaCl was left out, leaving present in the medium only the NaCl formed during neutralization of the medium. This change was made after some preliminary studies upon organism growth showed no demonstrable effect was produced by this modification. All other modifications of the basal medium are reported in the appropriate section of this thesis.

In table 1 are listed the various complex supplements used in determining the growth requirements of those strains of the lactic group which were found not to grow on the medium of Niven. All of the complex supplements used in attempts to secure multiplication of bacteriophages other than F54 and F24 also are included.

Bacteriophage multiplication was checked at different initial pH values between pH 6.0 and pH 7.0 for combinations 565-F54 and W2-F24. Bacteriophage F54 was found to reach a slightly higher concentration when the initial pH of the synthetic medium was 6.7, while an initial pH value of 6.4 was found to give a slightly higher final concentration of bacteriophage F24. Subsequently, all media used for bacteriophage studies with combination W2-F24 were adjusted to pH 6.4. All other media used in these investigations were adjusted to pH 6.7. A Leeds and Northrup glass electrode system was used for the adjustment of the pH of all media.

Stock solutions for making the synthetic medium were kept in a refrigerator at 3 to 5° C., in brown bottles, and under a thin layer of toluene.

TABLE 1

List of Supplements

Supplement number	Nutrients (/100 ml. medium)
I	Reticulogen[a], 20 microl.
II	Sodium acetate, 0.2 g.; Twen 80[b], 0.2 g.; reticulogen, 20 microl.; p-aminobenzoic acid, 20 γ; folic acid, 1 γ; thymine, 0.5 mg.; pyridoxamine, 0.1 mg.; pyridoxal, 0.1 mg.; nicotinamide, 0.1 mg.; inositol, 0.1 mg. and cysteine, 10 mg.
III	Supplement II minus reticulogen.
IV	Supplement II minus nicotinamide, inositol, cysteine and reticulogen.
V	Supplement II minus pyridoxamine, pyridoxal and reticulogen.
VI	Supplement II minus p-aminobenzoic acid, folic acid, thymine and reticulogen.
VII	Supplement II minus sodium acetate, Tween 80 and reticulogen.
VIII	Supplement II minus sodium acetate and reticulogen.
IX	Supplement II minus Tween 80 and reticulogen.
X	Supplement IX with sodium acetate increased to 0.5 g.
XI	Sodium acetate, 0.2 g. and Tween 80, 0.2 g.
XII	$MgSO_4 \cdot 7HOH$, 8 mg.; $FeSO_4 \cdot 7HOH$, 0.4 mg. and $MnCl_2 \cdot 4HOH$, 0.12 mg.
XIII	$MgSO_4 \cdot 7HOH$, 16 mg. and $MnCl_2 \cdot 4HOH$, 0.24 mg.
XIV	Supplement II plus casein hydrolysate, 5 ml. and vitamin B_{12}, 0.04 γ.
XV	$MgSO_4 \cdot 7HOH$, 16 mg.; $FeSO_4 \cdot 7HOH$, 0.8 mg. and $MnCl_2 \cdot 4HOH$, 0.24 mg.

[a] A commercial liver extract.

[b] A polyoxyethylene derivative of sorbitan monooleate.

Designations and Sources of Bacteriophage-Organism Combinations

Bacteriophage-organism combinations used for this study were obtained from the collection maintained in the Dairy Bacteriology Laboratories at Iowa State College. In table 2 are presented the designations and sources of all combinations used.

Combinations 565-F54, W2-F24 and 712-F56 were purified prior to use in this study. Purification of the S. lactis strains was accomplished by plating each strain on tryptone glucose extract milk agar (American Public Health Association, 1948) and making transfers from well isolated colonies to litmus milk. After incubating the purified cultures at 32° C. until coagulated, the cultures were again purified in the same manner.

Purification of the bacteriophage strains was accomplished by pouring plaque plates, using the method of Turner (1948), and transferring bacteriophages from isolated plaques to litmus milk. After inoculating with susceptible organisms and allowing the cultures to incubate overnight, whey filtrates were prepared using 1 per cent of this mixture as bacteriophage inoculum. Only purified S. lactis strains were used in purifying bacteriophages to increase the probability of having homologous strains of bacteriophages after purification.

The other bacteriophage-organism combinations used in

TABLE 2

Designations and sources of bacteriophage-organism combinations

Bacteriophage designation	S. lactis designation	Source of combination
F54	565	United Dairies, London, England.
F24	W2	Iowa State College.
F56	712	United Dairies, London, England.
F65	FH8	Dairy Research Institute, Palmerston North, N. Z.
F74	318B/27	National Institute for Research in Dairying, Univ. of Reading, England.
F25	M1	Iowa State College.
F63	E8	Dairy Research Institute, Palmerston North, N. Z.
F68	IP5	National Institute for Research in Dairying, Univ. of Reading, England.
F59	HP	Dairy Research Institute, Palmerston North, N. Z.
F52	459	United Dairies, London, England.
F21	W4	Iowa State College.
F76	122-1	Iowa State College.

this work were obtained from the laboratory stock collection without further purification.

Propagation of Cultures and Preparation of Inocula

S. lactis cultures 565, W2 and 712 were carried on litmus milk and in the basal synthetic medium. The other S. lactis cultures used for the investigations were carried in litmus milk and in the basal synthetic medium supplemented with sodium acetate and Tween 80. All cultures were subcultured daily during actual use and every third day when not being used. After 14 to 16 hr. incubation, cultures were kept in a mechanical refrigerator maintained at 3 to 5° C. Prior to use cultures always were transferred daily for two or three successive transfers in order to have the organisms in a physiologically active state. Since cultures carried in synthetic medium soon lost their ability to coagulate milk rapidly, cultures carried in synthetic medium were used only for the inoculation of test media, and litmus milk cultures were used in all cases for the assay of bacteriophages.

Inocula were prepared by sedimenting twice by centrifugation cultures grown 12 hr. in synthetic medium, resuspending each time in 0.9 per cent NaCl 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. It was found, using cultures 565, W2 and 712, that

inoculum prepared in this manner contained approximately 10^8 bacteria per ml. One per cent inoculum was used for all test media.

Propagation and Storage of Bacteriophage Filtrates

All bacteriophage strains used in this study except F54 and F24, bacteriophages active against S. lactis strains 565 and W2, respectively, were carried in whey filtrates. Bacteriophages F54 and F24, the only two strains which were found to multiply in the basal medium, were carried in basal synthetic medium filtrates.

To prepare filtrates, 1 per cent litmus milk culture or 1 per cent synthetic medium culture was added to 100 ml. sterile skim milk or 100 ml. sterile synthetic medium, as the case might be, along with 1 per cent bacteriophage filtrate, diluted to contain approximately 10^5 bacteriophage particles, and incubated in a water bath at 32° C. for 6 or 7 hr. The skim milk cultures were coagulated by adding 3.6 ml. of sterile 10 per cent lactic acid, filtered through coarse filter paper to remove the coagulated proteins, and finally filtered through a sterile Selas microporous porcelain filter (porosity #03) to remove bacterial cells. Cultures in synthetic medium were adjusted colorimetrically to approximately pH 6.7 for S. lactis strain W2 and approximately pH 7.0 for S. lactis strain

565 and immediately filtered through sterile Selas filters. By these procedures, filtrates containing 10^8 or 10^9 bacteriophage particles per ml. generally were obtained.

When a culture in synthetic medium was not neutralized prior to filtration, the resulting filtrate usually had a very low bacteriophage concentration. This was especially true when the majority of the bacterial cells had not been lysed and the culture was turbid. A pH of 6.5 or 6.6 seemed to be sufficient neutralization for filtrates from W2-F24, while neutralization to pH 6.8 or 6.9 seemed better for filtrates from 565-F54.

Bacteriophage filtrates were stored under refrigeration at 3-5° C. until needed for use.

For inoculum, a filtrate was diluted with distilled water to contain approximately 10^5 bacteriophage particles per ml., and 1 per cent of the diluted filtrate was used for all test media. Fresh dilutions were made each day of testing.

Determination of Numbers of Bacteriophage Particles

Quantitative estimation of the numbers of bacteriophage particles was made by the limiting dilution technique used by Harriman (1934) and by Nelson (1936). This method was chosen because plaques formed by most of the combinations used were extremely small and very difficult to count. Moreover,

Turner (1948) in comparing the plaque count method with the limiting dilution method found little or no significant difference between the two methods. Turner used undiluted S. lactis culture as inoculum when using the limiting dilution method. In preliminary trials for this work it was found that many questionably coagulated tubes were eliminated by diluting the S. lactis culture used as inoculum 1:8 in litmus milk.

The limiting dilution method as used in this study consisted of making successive 100-fold dilutions in sterile distilled water of a medium containing the unknown number of bacteriophage particles. One ml. and 0.1 ml. quantities of each successive dilution were transferred in triplicate to tubes containing about 8 ml. of litmus milk. This gave the overall effect of successive 10-fold dilutions in litmus milk. All litmus milk tubes containing diluted sample and one control tube were inoculated with 1 drop of susceptible S. lactis culture, using a 1:8 dilution of the organism in litmus milk, and incubated 14 to 16 hr. at 32° C. Tubes of litmus milk then were compared to the coagulated control tube, and those in which normal coagulation had not taken place were considered to have contained at least one bacteriophage particle. The "most probable number" of bacteriophage particles was determined using the probability table (Buchanan and Fulmer, 1928) for three tubes of each dilution.

Determination of Bacterial Populations

Plate counts of bacteria were made using tryptone glucose extract milk agar (American Public Health Association, 1948) and incubating plates 48 hr. at 32° C.

A Klett-Summerson photoelectric colorimeter with filter number 54 was used for turbidity measurements which served as indices of bacterial populations. The colorimeter was adjusted to 0 using an uninoculated tube of medium.

EXPERIMENTAL RESULTS

Growth of the Lactic Group of Streptococci on Synthetic Media

Two quantities of media were prepared according to the formulae given by Niven (1944), one containing casein hydrolysate and the other containing amino acids. Forty cultures, most of which have been carried in litmus milk for some time in the stock collection of this laboratory and for which this laboratory has bacteriophage filtrates, were transferred to the two media using as inoculum one loop of litmus milk culture per 5 ml. tube. In the medium containing casein hydrolysate, after an incubation period of 24 hr. at 32° C., fifteen cultures were quite turbid and eight cultures were slightly turbid. After 48 hr. these eight cultures and seven others in which growth was questionable at 24 hours were quite turbid. In the medium containing amino acids, five cultures were quite turbid at 24 hours., and three other cultures were slightly turbid at 48 hours; these last three cultures were lost upon subsequent transferring (incubation for as long as 72 hours did not result in growth of these cultures), leaving only five cultures, 712, 565, W2, W8 and H, which grew readily on the synthetic medium. Since there is considerable doubt that W8 and W2 are different strains of S. lactis, and since

the bacteriophage filtrate for H was found to be inactive, W8 and H were eliminated, leaving three strains of S. lactis which grew readily on the synthetic medium and with which a study of the bacteriophage-organism relationships could be made under rigidly defined conditions with respect to cell nutrition.

Upon finding that the omission of NaCl produced no demonstrable effect upon organism growth, only the NaCl of neutralization was included in the medium from this point on in these investigations.

Niven (1944) noted that if glutamine and asparagine were autoclaved for 15 minutes at 15 lb. pressure in the medium, erratic growth resulted. He avoided erratic growth by sterilizing glutamine and asparagine by filtration and adding them to the autoclaved medium after it was cool. To find out whether erratic growth could be avoided by a different procedure, six quantities of basal medium were prepared and subjected to the sterilization treatments indicated in table 3.

These media were dispensed aseptically into test tubes, 5 ml./tube, and inoculated with basal medium cultures of S. lactis strains 565, W2 and 712. Ten tubes of a medium were used for each organism, and one drop of culture per tube was used as inoculum. Turbidity readings were made after 22 hours incubation at 32° C.

When glutamine and asparagine were autoclaved 11 minutes separately from the medium, most cultures of strain 565 did

TABLE 3

Effect of different sterilization treatments for glutamine and asparagine on the growth of S. lactis in the synthetic basal medium

S. lactis strain	Heat treatment Time (min.)	Heat treatment Temp. (°C.)	Turbidity reading (22 hours) 1	2	3	4	5	6	7	8	9	10
565	11	121	84	79	86	87	87	87	88	85	88	87
	13	121	79	80	67	77	84	74	83	68	65	78
	15	121	71	76	20	77	68	69	74	6	74	73
	11	a	4	0	1	0	0	4	10	1	0	0
	3	b	88	93	93	84	80	97	92	87	71	95
	0	c	105	91	107	108	102	105	104	102	--	--
W2	11	121	94	90	91	90	88	87	91	87	92	92
	13	121	87	83	83	84	84	84	86	88	93	90
	15	121	12	82	83	82	64	80	76	77	38	85
	11	a	115	118	120	120	123	118	119	120	118	114
	3	b	117	116	121	118	122	122	118	118	115	113
	0	c	119	132	135	136	135	135	134	132	--	--
712	11	121	86	86	85	85	82	82	85	83	83	82
	13	121	79	78	78	75	72	72	77	77	78	82
	15	121	82	81	81	78	79	82	82	81	80	79
	11	a	100	101	101	103	--	--	--	--	--	--
	3	b	111	109	--	--	--	--	--	--	--	--
	0	c	127	127	128	126	126	124	126	126	125	--

[a] Glutamine and asparagine autoclaved separately from medium at 121° C.

[b] Glutamine and asparagine heated to about 90° C. in Arnold steamer.

[c] Glutamine and asparagine sterilized by filtration.

not initiate growth during 22 hours, while the turbidities developed by cultures of strains W2 and 712 were only slightly less than those developed when glutamine and asparagine were sterilized by filtration. Erratic growth was the result with two cultures of strain 565 and three cultures of strain W2 when glutamine and asparagine were autoclaved 15 minutes with the medium, while there was decreased growth with three cultures of 565 when the time of autoclaving glutamine and asparagine with the medium was decreased to 13 minutes. Erratic growth apparently may be avoided by autoclaving the medium containing glutamine and asparagine only 11 minutes; however, by so doing the growth of each of these three strains of S. lactis was slightly below that in the medium containing unheated glutamine and asparagine.

Other than for carrying stock cultures of 565, W2 and 712, glutamine and asparagine were sterilized by filtration for all media used in these investigations, unless stated otherwise in a particular section of this thesis.

After the synthetic basal medium was shown to be sufficient for multiplication of bacteriophages active against S. lactis strains 565 and W2 and insufficient for the bacteriophage active against S. lactis strain 712, it was desirable to know if other bacteriophages would multiply in the basal medium supplemented so as to allow growth of their respective host cells. Hence, a study was conducted to determine what nutrients when added to

the basal medium would permit growth of other strains of the lactic group of streptococci.

While little work has been conducted on the nutrition of S. lactis and S. cremoris, an extremely large amount of research has been conducted during the last decade on the nutrition of other organisms producing lactic acid. This work was reviewed by Snell (1946)(1948), Woods (1947) and Koser (1948). Supplement II(table 1) containing several nutrients which have been shown necessary for various lactic acid organisms was made up and added to the basal medium in an effort to supply the necessary growth factors for several strains of S. lactis and S. cremoris. This supplement was composed of sodium acetate (Guirard, Snell, and Williams, 1946), 2 mg./ml.; Tween 80, a polyoxyethylene derivative of sorbitan monooleate, (Williams, Proquist, and Snell, 1947) 2 mg./ml.; reticulogen, a commercial liver extract, (Baumann and Sauberlick, 1948), 0.2 microl./ml.; p-aminobenzoic acid (Lampen and Jones, 1947), 0.2 γ/ml.; folic acid (Mitchell, Snell, and Williams, 1941), 0.01 γ/ml.; thymine (Snell and Mitchell, 1941), 5 γ/ml.; pyridoxamine (Snell, 1946), 1 γ/ml.; pyridoxal (Snell, 1946), 1 γ/ml.; nicotinamide (Snell, 1946), 1 γ/ml.; and inositol, 1 γ/ml. Although the latter was not required by any lactic acid organism used by Shankman, Camien, Block, Merrifield, and Dunn (1946), it was included in a medium used by Dunn, Shankman, Camien, and Block (1946). Cysteine, 0.1 mg./ml., also was included to increase the anaerobic conditions of the medium.

Results obtained by adding supplement II and various modifications of this supplement to the basal medium for 22 laboratory stock cultures, none of which grew at 40° C. or produced ammonia from L-arginine, are given in table 4. Results using 20 cultures just isolated for the study, 8 cultures isolated in December, 1945, and 3 stock cultures, all 31 of which grew at 40° C. and produced ammonia from L-arginine, are given in table 5. All cultures were transferred from litmus milk to basal medium to which supplement II had been added. After three serial transfers in the supplemented basal medium, cultures were transferred in duplicate to tubes of basal medium containing the indicated modifications of Supplement II. Turbidity readings were made after 24 hours incubation at 32° C. on the second serial transfer in a medium in which turbidity developed during the first 24 hour incubation period. One drop of inoculum was used in all cases.

The important functions of sodium acetate and unsaturated fatty acids in the growth of various lactic acid bacteria have been reported by several workers. Guirard, Snell, and Williams (1946), finding that acetate could be replaced by several fatty acids, keto acids, and sterols for a few lactic acid bacteria, indicated that acetate might serve in the synthesis of cellular lipoidal materials. That the presence of high molecular weight unsaturated fatty acids eliminated the requirement of one strain of L. casei for biotin (Williams and Fieger, 1946 and 1949)

TABLE 4

Multiplication of strains of *S. cremoris* on the synthetic basal medium supplemented[a] as indicated

(av. of duplicate determinations)

	Turbidity readings at 24 hr. with supplement:											
Culture	None	I	II	III	IV	V	VI	VII	VIII	IX	X	XI
FH8	0	34	74	19	93	55	50	0	0	5	2	86
#6	1	98	129	130	125	116	130	0	0	0	0	141
318B/27	3	104	144	125	140	138	138	0	0	112	133	110
497	0	82	131	107	134	114	119	0	0	0	0	104
144F	0	78	108	68	100	95	91	0	0	25	46	108
M1	0	16	86	16	88	89	82	8	0	60	76	110
DL	2	107	138	96	115	111	100	7	53	108	127	147
H1-2	0	45	74	2	0	0	0	0	--	--	--	--
H1-10	1	73	83	123	115	111	107	0	0	21	88	127
ML1	0	46	94	35	36	58	50	0	0	28	28	70
Q	0	75	114	50	80	70	68	0	0	37	33	108
E8	1	60	90	65	98	105	105	0	0	5	10	90
799/11	1	93	143	116	125	124	122	0	84	120	143	85
IP5	0	94	117	93	135	119	118	0	0	15	23	118
K_2	0	54	86	67	99	92	91	0	0	79	108	74
HP	0	90	80	64	70	55	56	0	0	0	0	89
K.H.	0	70	105	72	103	92	96	0	10	50	93	126
459	0	78	101	55	78	81	75	0	0	39	63	105
573	0	84	75	56	58	59	59	0	0	37	80	105
799	1	77	100	93	90	105	95	0	0	49	60	61
W4	0	110	139	100	120	113	113	0	81	31	49	115
122-1	0	103	122	73	92	89	95	0	105	71	88	79

[a] See table 1

TABLE 5

Multiplication of strains of S. lactis on the synthetic basal medium supplemented as indicated

(av. of duplicate determinations)

Culture	Turbidity readings at 24 hr. with supplement: No supplement	Supplement XI[a]
1	60	---
2	1	127
3	52	---
4	132	---
5	0	131
6	1	126
7	88	---
8	92	---
9	81	---
10	108	---
11	110	---
12	100	---
13	82	---
14	0	136
15	0	103
16	120	---
17	94	---
18	96	---
19	143	---
20	110	---
1,1	80	95
2,1	125	155
3,1	113	183
4,1	0	150
1,8	0	155
4,8	32	123
2,15	0	170
3,15	0	200
712	136	187
565	128	185
W2	132	182

[a] See table 1

indicated that biotin functions as a coenzyme in this system (Williams, Broquist, and Snell, 1947). Guirard, Snell, and Williams (1946) found that while several saturated and unsaturated fatty acids could replace sodium acetate for L. arabinosus and while the unsaturated acids had some activity in replacing sodium acetate for L. acidophilus, none of the fatty acids used, including oleic acid, was active in replacing sodium acetate for the strain of L. casei used. Three lactobacillus organisms and one streptococcus organism were reported by Williams, Broquist, and Snell (1947) and one strain of L. arabinosus was reported by Whitehill, Oleson and SubbaRow (1947) for which the oleic acid or high molecular weight unsaturated fatty acid requirement could not be modified by using acetate, biotin and other growth factors.

In the nutrition of many strains of S. cremoris and S. lactis, as shown in tables 4 and 5, both acetate and oleic acid play important roles. All 22 strains of S. cremoris and 9 of 31 strains of S. lactis failed to become turbid on the basal medium within 24 hours until supplemented with either sodium acetate plus Tween 80 or reticulogen. Even when the basal medium was supplemented with the entire mixture of growth factors minus reticulogen, sodium acetate, and Tween 80 (supplement VII) all strains of S. cremoris failed to become turbid, which indicates that the requirement by these cultures for either reticulogen or sodium acetate and Tween 80 was not eliminated

by any nutrient or combination of nutrients used in the supplement. When sodium acetate and reticulogen were left out of the supplemented medium (supplement VIII) only one strain, 122-1, developed normally, while strains DL, 799/11, KH and W4 developed turbidity at a rate somewhat less than on the more complex medium. Upon including sodium acetate and leaving out Tween 80 (supplement IX) there was an increase in the number of cultures which developed turbidity. However, only cultures 318B/27, DL, 799/11, K2 and 122-1 developed turbidity somewhat normally. The stimulation caused by an increase in the sodium acetate content from 2 to 5 mg./ml (supplement X) was not as great as that caused by the addition of 2 mg./ml. of Tween 80 (supplement III). In the basal medium supplemented only with reticulogen (Supplement I), turbidities in general were slightly less than when the basal medium was supplemented with sodium acetate and Tween 80 (supplement XI). However, turbidities developed by strains 318B/27, 799/11, HP, 799 and W4 were about equal to those developed when sodium acetate and Tween 80 was the supplement, and strains H1-2 and 122-1 actually became more turbid when the supplement was reticulogen. Strain H1-2, an extremely slow culture even in milk, usually became turbid between 36 and 48 hours in the basal medium supplemented with sodium acetate and Tween 80. Cultures 318B/27, 497, ML1, 799/11, 799, W4 and 122-1 developed slightly higher turbidities with the

entire mixture of growth factors (supplement II) than with only sodium acetate and Tween 80 (supplement XI), while cultures M1, H1-10, KH and 573 developed slightly lower turbidities when the entire mixture of growth factors was added. Thus the adding of reticulogen and the several other growth factors provided little stimulation for most cultures growing in the basal medium supplemented with sodium acetate and Tween 80. Those cultures of S. lactis which did not require sodium acetate and Tween 80 were stimulated by the addition of these ingredients in every case checked (table 5).

Vitamin B_{12} was used in an effort to satisfy the growth requirement of 12 strains of S. cremoris for sodium acetate and Tween 80, but the addition of 0.0004 γ/ml. did not cause any of the cultures to become visibly turbid within 24 hours.

Though the original classification and the time of isolation for most of the 22 cultures which were classified as S. cremoris is not available, it is interesting that all of these cultures have been carried in laboratories for, as far as is known, many years. The 20 cultures isolated for this study, 8 cultures which were isolated in December, 1947, and W2 which was isolated in 1945, all were classified as S. lactis, along with 712 and 565, both of which were obtained from United Dairies, London, England. Nichols and Hoyle (1948) found that of 277 strains isolated from commercial starters all were S. cremoris and that, of 72 wild strains suitable for use as starters and isolated from samples

of sour milk, 70 were S. lactis while 2 were doubtful S. cremoris.

Multiplication of Bacteriophages on Susceptible Host Cells in the Basal Medium

In order to give direction to the study, it was necessary to know whether or not bacteriophages would multiply on susceptible host cells while growing in the basal synthetic medium. Results from such work would determine whether the investigation was to proceed by the omission or the addition of nutrients.

Preliminary investigation showed that with combinations 565-F54 and W2-F24 the bacteriophages did multiply in the basal medium; however, with the other combinations used, the bacteriophages did not multiply.

After carrying bacteriophage strains F54 and F24 serially through four transfers on their respective host cells in the basal medium, making a new filtrate after each transfer, comparisons were made between the multiplication of these bacteriophages in sterile skim milk and in the basal synthetic medium. For these comparisons the basal medium and the sterile skim milk were made up in quantities of 100 ml. and stored in the refrigerator overnight. Glutamine and asparagine were autoclaved in the basal medium. After allowing time for the media to reach 32° C. in a water bath, at 0 hr. minus 25 minutes, they were inoculated with susceptible cells; and, at 0 hr. minus 5 minutes, the homologous bacteriophages were added. After allowing 5 min. for agitation

and adsorption, plate counts were made and the most probable numbers of bacteriophages were determined at 0 hour and as indicated. Tables 6 and 7 show these comparisons with combinations 565-F54 and W2-F24, respectively.

Tables 6 and 7 indicate that the development of bacteriophage caused mass lysis in milk, but not in synthetic medium. Actually only slight drops in the number of organisms occurred in the synthetic medium. That the large numbers of viable cells present at 10 hours in the synthetic medium did not result from the multiplication of resistant organisms seemed evident upon finding that these organisms did not form colonies when 1 ml. of bacteriophage filtrate containing 10^8 or 10^9 bacteriophage particles was added to each plate in making survival counts. Evidently other factors were involved which made the organisms temporarily resistant to bacteriophage action. Delbruck (1948) noted such a phenomenon with E. coli, which produced a substance, probably indole, from tryptophan which inhibited the adsorption of T4 bacteriophage to cells. Clearing did not always occur in the synthetic medium, even when the concentration of bacteriophage was as high as 10^8 bacteriophage particles per ml.

While bacteriophages active against S. lactis strains 565 and W2 multiply on the basal medium, the rate of multiplication is somewhat less rapid than in milk. While no comparisons were made between milk and the basal medium containing filtered

TABLE 6

Comparison of synthetic basal medium with milk as a growth medium for S. lactis (565) and bacteriophage (F54)

Incubation time (hr.)	Total plate count/ml. Synthetic basal medium		Milk	No. of bacteriophage particles/ml.	
	Control bacteria	Bacteria with bacteriophage	Bacteria with bacteriophage	Synthetic basal medium	Milk
0	5.5×10^5	1.1×10^6	2.5×10^6	2.5×10^3	2.5×10^3
1	--	--	1.0×10^7	--	2.5×10^4
2	4.7×10^6	8.6×10^6	1.7×10^7	9.5×10^3	2.5×10^5
3	--	--	1.0×10^5	--	2.0×10^6
4	1.3×10^7	2.5×10^7	1.0×10^1	2.5×10^5	4.5×10^6
5	--	--	1.0×10^2	--	9.5×10^6
6	6.0×10^7	2.8×10^7	1.1×10^2	4.5×10^6	4.5×10^6
7	--	--	--	--	--
8	1.2×10^8	2.9×10^6	--	4.5×10^6	--
9	--	--	--	--	--
10	1.8×10^8	--	--	4.5×10^6	--
11	--	--	--	--	--
12	2.5×10^8	1.2×10^6	--	4.5×10^6	--

Note:

Total plate count of bacteria growing in synthetic medium with bacteriophage at 10 hr. with 1 ml. filtrate added per plate was 0 colony on duplicate 10^6 plates.

TABLE 7

Comparison of synthetic basal medium with milk as a growth medium for S. lactis (W2) and bacteriophage (F24)

Incubation time (hr.)	Total plate count/ml.			No. of bacteriophage particles/ml.	
	Synthetic basal medium		Milk		
	Control bacteria	Bacteria with bacteriophage	Bacteria with bacteriophage	Synthetic basal medium	Milk
0	2.5×10^5	3.9×10^5	9.2×10^5	9.5×10^1	9.5×10^1
1	--	--	4.5×10^6	--	9.5×10^2
2	5.1×10^6	3.7×10^6	1.1×10^7	2.5×10^3	1.5×10^4
3	--	--	3.0×10^7	--	2.5×10^5
4	3.0×10^7	2.5×10^7	4.7×10^6	2.5×10^5	2.5×10^7
5	--	--	5.0×10^1	--	2.5×10^7
6	5.0×10^7	3.1×10^7	2.0×10^1	4.5×10^6	2.0×10^6
7	--	--	--	--	--
8	1.3×10^8	1.8×10^6	--	9.5×10^6	--
9	--	--	--	--	--
10	1.0×10^8	5.9×10^6	--	4.5×10^7	--
11	--	--	--	--	--
12	--	2.0×10^7	--	2.5×10^7	--

Note:

Total plate count of bacteria growing in synthetic medium with bacteriophage at 10 hr. with 1 ml. filtrate added per plate was 0 colony on duplicate 10^6 plate.

glutamine and asparagine, a comparison between heated and unheated glutamine and asparagine indicated that higher numbers of bacteriophages were present at 7 hours in cases in which the glutamine and asparagine were sterilized by filtration. This is shown in the section concerned with the omission of nutrients.

While S. lactis strain 712 rapidly produced turbidity in the synthetic basal medium, all attempts to obtain multiplication of its homologous bacteriophage, F56, failed. The number of demonstrable bacteriophage particles declined after 2 to 3 hours incubation at 32° C. Adjusting the initial pH of the medium to pH 6.0, pH 7.0 and to various intervals between these two values had no detectable effect upon the multiplication of bacteriophage F56 in the synthetic basal medium.

An experiment was run to determine whether nine other bacteriophages would multiply on their respective host cells in the synthetic medium. For this experiment the synthetic basal medium was supplemented with sodium acetate and Tween 80 so that the susceptible cultures would multiply, and the glutamine and asparagine were sterilized by filtration. The synthetic medium was found inadequate for the multiplication of these bacteriophages (table 8) and actually permitted comparatively little organism growth in most cases during the 7 hour period.

It was evident at this stage of the investigation that two courses of study were open. First, it was desirable to know the effect of the omission of nutrients from the synthetic basal medium upon the two bacteriophages which were found to multiply

TABLE 8

Multiplication of bacteriophage on the synthetic basal medium supplemented with 2 mg./ml. sodium acetate and 2 mg./ml. Tween 80

Organism strain	Bacterio-phage strain	No. of bacteriophage particles			Turbidity of control (7 hr.)
		0 hr.	3 hr.	6 hr.	
712	F56	2.5×10^3	2.5×10^3	3×10^1	140
FH8	F65	9.5×10^3	2.5×10^4	9.5×10^3	4
318B/27	F74	2.0×10^3	2.5×10^3	2.0×10^3	23
M1	F25	4.5×10^2	2.5×10^2	4.5×10^2	16
E8	F63	4.5×10^2	4.5×10^2	7×10^1	27
IP5	F68	7.5×10^2	3.0×10^2	9×10^1	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	7×10^1	16
W4	F21	2.5×10^3	2.5×10^3	2.5×10^3	46
122-1	F76	2.5×10^3	2.0×10^3	9.5×10^3	11

on their respective host cells when grown in the synthetic basal medium; and secondly, it was desirable to know what changes in or additions to the supplemented basal medium were necessary to make it suitable for the proliferation of the several other strains of bacteriophage.

Effect of Omission of Nutrients Upon the Multiplication of Bacteriophages and Organisms of Combinations 565-F54 and W-2F24

In this study slightly different procedures were followed for the different trials. In the first trials, 100 ml. quantities of media were placed in 6 oz. screw cap bottles, and plate counts were made at 0 hr. and 3 hr. to give an indication of organism growth. The end of 3 hours was chosen as the time for checking organism growth because it allowed sufficient time for differences in organism growth to develop and because there was little danger of a decline in cell count due to bacteriophage action until after three hours. A separate control culture was not used because several investigators have shown that during the early stages of growth the survival and normal curves of organism growth are quite similar. Also, since it had been found that autoclaving the glutamine and asparagine with a medium 11 minutes caused only slightly decreased growth with S. lactis strains 565, W2 and 712, glutamine and asparagine were autoclaved in the media for the first trials.

In the second trials media were prepared in quantities of 25 ml. and immediately after being inoculated with susceptible host cells, each lot of medium was divided into one 5 ml. and two 10 ml. quantities and placed in 16 x 125 mm. screw-cap culture tubes. To one 10 ml. quantity, 0.1 ml. of diluted bacteriophage filtrate was added. To the second 10 ml. quantity was added 0.1 ml. of heated diluted bacteriophage filtrate, and the 5 ml. quantity was used as a control on normal organism growth. Turbidity readings were made at 7 hours on the control and the control plus heated filtrate. With both trials media were allowed to reach 32° C. in a water bath, and susceptible organisms were added at 0 minus 25 minutes. At 0 minus 5 minutes the homologous bacteriophages were added.

The nutrients (tables 9,10,11 and 12) are listed in order of increasing concentration of bacteriophage for the second trial with combination 565-F54 (table 10). Such an arrangement of nutrients automatically arranged, with a few exceptions, the turbidity measurements in order of increasing organism growth (tables 10 and 12). This was shown to a lesser extent by plate counts at 3 hours (tables 9 and 11). If the absence of a nutrient decreased the growth of an organism, it also decreased the rate of multiplication of bacteriophage growing in combination with that organism and vice versa.

The omission of certain nutrients from the basal medium, namely, DL-phenylalanine, L-lysine, Ca pantothenate, pyridoxine,

TABLE 9

Effect of the absence of nutrients upon the multiplication of *Streptococcus lactis* (565) and bacteriophage (F54) (first trial)

Nutrient omitted	No. of bacteriophage particles		Total plate count	
	0 hr.	7 hr.[a]	0 hr.	3 hr.
Nicotinic acid	9.5×10^2	2.5×10^2	1.7×10^6	4.8×10^6
DL-leucine	9.5×10^2	4.5×10^2	1.5×10^6	1.7×10^6
L-glutamine	9.5×10^2	9.5×10^2	1.6×10^6	1.3×10^6
DL-valine	9.5×10^2	1.5×10^2	1.7×10^6	1.7×10^7
Thiamine	9.5×10^2	2.5×10^4	1.8×10	1.7×10
DL-methionine	9.5×10^2	9.5×10^2	1.5×10^6	4.0×10^6
L-arginine	9.5×10^2	9.5×10^4	1.3×10^6	1.3×10^7
DL-isoleucine	9.5×10^2	2.5×10^4	1.5×10^6	2.9×10^6
Riboflavin	9.5×10^2	4.5×10^5	1.8×10^6	8.4×10^6
DL-serine	9.5×10^2	2.5×10^5	1.7×10^6	5.0×10^6
Biotin	9.5×10^2	9.5×10^3	1.7×10^6	5.8×10^6
Uracil	9.5×10^2	9.5×10^4	1.6×10^6	1.4×10^6
L-histidine	9.5×10^2	7.5×10^4	1.3×10^6	4.3×10^6
Adenine	9.5×10^2	2.5×10^3	1.5×10^6	4.4×10^6
L-hydroxyproline	9.5×10^2	4.5×10^5	1.8×10^6	9.4×10^6
DL-phenylalanine	9.5×10^2	9.5×10^5	1.5×10^6	2.2×10^6
DL-alanine	9.5×10^2	7.5×10^5	1.5×10^6	7.8×10^6
L-lysine	9.5×10^2	2.5×10^7	1.5×10^6	8.4×10^6
DL-threonine	9.5×10^2	2.5×10^4	1.5×10^6	5.0×10^6
Ca pantothenate	9.5×10^2	4.5×10^4	1.5×10^6	9.4×10
Pyridoxine	9.5×10^2	2.5×10^7	1.5×10^6	1.6×10^7
Guanine	9.5×10^2	2.5×10^7	1.8×10^6	8.5×10^6
L-tyrosine	9.5×10^2	2.5×10^7	1.4×10^6	2.2×10^7
Asparagine	9.5×10^2	2.5×10^7	1.5×10^6	9.0×10^7
Glycine	9.5×10^2	2.5×10^7	1.5×10^6	1.1×10^7
DL-aspartic acid	9.5×10^2	2.5×10^7	1.3×10^6	1.6×10^7
Xanthine	9.5×10^2	2.5×10^7	1.4×10^6	1.4×10^7
L-cystine	9.5×10^2	7.5×10^5	1.5×10^6	3.4×10^7
L-glutamic acid	9.5×10^2	2.5×10^5	1.5×10^6	1.5×10^7
DL-tryptophane	9.5×10^2	2.5×10^5	1.7×10^6	2.5×10^7
L-proline	9.5×10^2	2.5×10^7	1.6×10^6	1.5×10^7
DL-norleucine	9.5×10^2	9.5×10^7	1.4×10^6	1.1×10^7
None	9.5×10^2	2.5×10^7	1.4×10^6	1.6×10^7

[a] Dilution 7 was highest dilution made for results in this column.

TABLE 10

Effect of the absence of nutrients upon the multiplication of *Streptococcus lactis* (565) and bacteriophage (F54) (second trial)

Nutrient omitted	No. of bacteriophage particles		Turbidity (7 hr.)	
	0 hr.	7 hr.	Control plus heated filtrate	Control
Nicotinic acid	4.5×10^{2}	1.5×10^{2}	6	7
DL-leucine	1.5×10^{3}	2.0×10^{2}	5	6
L-glutamine	4.5×10^{2}	4.5×10^{2}	9	10
DL-valine	1.5×10^{3}	9.5×10^{2}	13	14
Thiamine	4.5×10^{3}	2.5×10^{3}	13	13
DL-methionine	1.5×10^{3}	2.5×10^{3}	12	12
L-arginine	1.5×10^{3}	4.5×10^{4}	21	22
DL-isoleucine	1.5×10^{3}	1.5×10^{4}	11	11
Riboflavin	4.5×10^{2}	4.5×10^{4}	23	22
DL-serine	4.5×10^{2}	4.5×10^{4}	18	17
Biotin	4.5×10^{2}	9.5×10^{4}	16	15
Uracil	4.5×10^{2}	9.5×10^{4}	17	17
L-histidine	1.5×10^{3}	9.5×10^{5}	10	9
Adenine	1.5×10^{3}	4.0×10^{5}	18	18
L-hydroxyproline	4.5×10^{2}	9.5×10^{5}	15	15
DL-phenylalanine	1.5×10^{3}	4.5×10^{6}	13	15
DL-alanine	1.5×10^{3}	9.5×10^{6}	25	20
L-lysine	1.5×10^{3}	2.5×10^{7}	22	21
DL-threonine	1.5×10^{3}	2.5×10^{7}	75	72
Ca pantothenate	1.5×10^{3}	4.0×10	22	23
Pyridoxine	4.5×10^{2}	9.5×10^{7}	27	27
Guanine	4.5×10^{2}	9.5×10^{7}	80	61
L-tyrosine	1.5×10^{3}	1.5×10^{8}	59	59
Asparagine	1.5×10^{3}	2.5×10^{8}	51	50
Glycine	1.5×10^{2}	4.5×10^{8}	87	91
DL-aspartic acid	4.5×10^{2}	4.5×10^{8}	94	100
Xanthine	4.5×10^{2}	4.5×10^{8}	75	82
L-cystine	1.5×10^{3}	9.5×10^{8}	77	77
L-glutamic acid	1.5×10^{3}	9.5×10^{8}	70	85
DL-tryptophane	1.5×10^{3}	9.5×10^{8}	110	117
L-proline	1.5×10^{3}	2.5×10^{9}	97	96
DL-norleucine	1.5×10^{2}	2.5×10^{8}	92	93
None	4.5×10^{2}	1.5×10^{8}	84	90

TABLE 11

Effect of the absence of nutrients upon the growth of *Streptococcus lactis* (W2) and bacteriophage (F24) (first trial)

Nutrients omitted	No. of bacteriophage particles		Total plate count	
	0 hr.	7 hr.[a]	0 hr.	3 hr.
Nicotinic acid	9.5×10^2	2.0×10^2	2.4×10^6	4.3×10^6
DL-leucine	9.5×10^2	0×10^2	2.4×10^6	2.2×10^6
L-glutamine	9.5×10^2	9.5×10^3	2.1×10^6	3.3×10^6
DL-valine	9.5×10^2	0×10^2	1.6×10^6	1.7×10^6
Thiamine	9.5×10^2	2.5×10^5	2.2×10^6	2.1×10^7
DL-methionine	9.5×10^2	1.5×10^5	1.8×10^6	1.9×10^7
L-arginine	9.5×10^2	1.5×10^5	1.6×10^6	1.3×10^6
DL-isoleucine	9.5×10^2	4.5×10^7	2.1×10^6	4.4×10^7
Riboflavin	9.5×10^2	2.5×10^7	2.0×10^6	1.0×10^7
DL-serine	9.5×10^2	2.5×10^7	2.5×10^6	2.1×10^7
Biotin	9.5×10^2	9.5×10^3	2.2×10^6	1.4×10^7
Uracil	9.5×10^2	1.5×10^6	1.6×10^6	8.7×10^6
L-histidine	9.5×10^2	9.5×10^7	1.6×10^6	4.0×10^7
Adenine	9.5×10^2	2.5×10^7	2.4×10^6	1.9×10^7
L-hydroxyproline	9.5×10^2	2.5×10^4	2.3×10^6	1.6×10^6
DL-phenylalanine	9.5×10^2	1.5×10^4	1.9×10^6	7.4×10^6
DL-alanine	9.5×10^2	7.5×10^5	2.3×10^6	1.7×10^7
L-lysine	9.5×10^2	2.5×10^5	1.8×10^6	1.6×10^6
DL-threonine	9.5×10^2	4.0×10^4	1.8×10^6	7.0×10^6
Ca pantothenate	9.5×10^2	2.5×10^3	2.3×10^6	9.2×10^6
Pyridoxine	9.5×10^2	2.5×10^7	2.6×10^6	1.8×10^7
Guanine	9.5×10^2	2.5×10^6	2.0×10^6	2.1×10^7
L-tyrosine	9.5×10^2	9.5×10^6	2.2×10^6	2.0×10^7
Asparagine	9.5×10^2	4.5×10^5	1.5×10^6	2.0×10^7
Glycine	9.5×10^2	2.5×10^7	2.2×10^6	1.6×10^7
DL-aspartic acid	9.5×10^2	2.5×10^7	2.4×10^6	2.3×10^7
Xanthine	9.5×10^2	2.5×10^7	2.6×10^6	3.1×10^7
L-cystine	9.5×10^2	9.5×10^6	1.9×10^6	1.8×10^7
L-glutamic acid	9.5×10^2	2.5×10^6	2.0×10^6	1.7×10^7
DL-tryptophane	9.5×10^2	4.5×10^4	1.8×10^6	1.0×10^7
L-proline	9.5×10^2	2.5×10^6	2.3×10^6	2.0×10^7
Dl-norleucine	9.5×10^2	2.5×10^5	2.4×10^6	2.6×10^7
None	9.5×10^2	9.5×10^6	2.1×10^6	2.0×10^7

[a] Dilution 7 was highest dilution made for results in this column.

TABLE 12

Effect of the absence of nutrients upon the growth of Streptococcus lactis (W2) and bacteriophage (F24) (second trial)

Nutrient omitted	No. of bacteriophage particles		Turbidity (7 hr.)	
	0 hr.	7 hr.	Control plus heated filtrate	Control
Nicotinic acid	1.5×10^3	9.5×10^5	11	13
DL-leucine	2.5×10^3	9.5×10^4	11	11
L-glutamine	1.5×10^3	2.0×10^4	11	14
DL-valine	2.5×10^3	9.5×10^2	3	5
Thiamine	1.5×10^3	4.5×10^8	55	55
DL-methionine	2.5×10^3	2.5×10^7	6	9
L-arginine	2.5×10^3	4.0×10^6	20	21
DL-isoleucine	2.5×10^3	2.5×10^6	9	9
Riboflavin	1.5×10^3	9.5×10^8	62	62
DL-serine	1.5×10^3	4.5×10^8	28	27
Biotin	2.5×10^3	4.5×10^8	19	20
Uracil	2.5×10^3	4.5×10^5	35	51
L-histidine	2.5×10^3	2.5×10^5	4	4
Adenine	1.5×10^3	9.5×10^8	60	59
L-hydroxyproline	1.5×10^3	1.5×10^8	60	60
DL-phenylalanine	2.5×10^3	2.5×10^8	50	45
DL-alanine	2.5×10^3	2.5×10^8	20	24
L-lysine	2.5×10^3	2.5×10^8	58	60
DL-threonine	2.5×10^3	2.5×10^8	60	60
Ca pentothenate	2.5×10^3	2.5×10^4	40	35
Pyridoxine	1.5×10^3	1.5×10^9	54	56
Guanine	1.5×10^3	9.5×10^7	64	66
L-tyrosine	2.5×10^3	9.5×10^8	53	59
Asparagine	1.5×10^3	4.5×10^8	63	67
Glycine	2.5×10^3	4.5×10^7	51	52
DL-aspartic acid	1.5×10^3	1.5×10^8	61	65
Xanthine	1.5×10^3	1.5×10^9	79	79
L-cystine	2.5×10^3	4.5×10^8	45	57
L-glutamic acid	2.5×10^3	4.5×10^8	55	55
DL-tryptophane	2.5×10^3	9.5×10^8	54	54
L-proline	2.5×10^3	2.5×10^8	54	54
DL-norleucine	2.5×10^3	9.5×10^7	45	48
None	1.5×10^3	4.5×10^7	43	44
		4.5×10^8	54	58

L-tyrosine and asparagine with combination 565-F54 (table 10), and DL-methionine, DL-isoleucine, DL-serine, biotin and DL-alanine with combination W2-F24 (table 12) seemed to have greater detrimental effect upon the development of turbidity by the host organisms during the 7 hour period than upon the multiplication of the homologous bacteriophages. Upon rechecking this point (tables 13 and 14), such results seemed to arise from variations in the activity of the organisms and bacteriophages during the 7 hour period; however, this effect was still pronounced when either DL-serine or DL-alanine was omitted from the medium for combination W2-F24. This point was investigated further by checking the growth of W2 and F24 at various intervals as shown in table 15. Omitting DL-serine from the basal medium had only a slight effect upon the growth of W2. There also was a slight decrease in the rate of bacteriophage proliferation. The large differences in turbidity at 7 hours seem not to have resulted from differences in the numbers of organisms. This information further indicated that bacteriophage multiplication was very closely associated with the rate of organism multiplication.

The absence of Ca pantothenate from the basal medium for combination W2-F24 seemed to be more harmful to bacteriophage proliferation than to the multiplication of susceptible host cells (table 12); but, this effect was not evident when the experiment was repeated (table 14), and it did not appear to be demonstrated in the first trial (table 11).

TABLE 13

Effect of the absence of nutrients upon the multiplication of Streptococcus lactis (565) and bacteriophage (F54) (third trial)

Nutrient omitted	No. of bacteriophage particles		Turbidity (7 hr.)	
	0 hr.	7 hr.	Control plus heated filtrate	Control
DL-phenylalanine	9.5×10^2	4.5×10^5	19	18
L-lysine	9.5×10^2	9.5×10^6	45	40
Pantothenic acid	9.5×10^2	2.5×10^3	20	20
Pyridoxine	9.5×10^2	9.5×10^8	130	129
L-tyrosine	9.5×10^2	9.5×10^8	132	136
Asparagine	9.5×10^2	1.5×10^9	144	139
None	9.5×10^2	9.5×10^8	158	154

TABLE 14

Effect of the absence of nutrients upon the multiplication of Streptococcus lactis (W2) and bacteriophage (F24) (third trial)

Nutrient omitted	No. of bacteriophage particles		Turbidity (7 hr.)	
	0 hr.	7 hr.	Control plus heated filtrate	Control
DL-methionine	1.5×10^3	2.5×10^5	13	14
DL-isoleucine	1.5×10^3	9.5×10^5	18	20
DL-serine	1.5×10^3	2.5×10^9	43	44
Biotin	1.5×10^3	9.5×10^6	30	34
DL-alanine	1.5×10^3	4.5×10^8	25	28
Ca pantothenate	1.5×10^3	2.5×10^5	19	17
None	1.5×10^3	2.5×10^8	93	93

TABLE 15

Effect of the absence of serine upon the multiplication of *Streptococcus lactis* (W2) and bacteriophage (F24)

Incubation time (hr.)	No. of bacteriophage particles		Total plate count		Direct microscopic count	
	Control	DL-serine omitted	Control	DL-serine omitted	Control	DL-serine omitted
0	2.5×10^3	2.5×10^3	3.12×10^6	2.90×10^6	4.9×10^6	6.4×10^6
1 1/2	9.5×10^3	7.5×10^3	1.36×10^7	1.08×10^7	2.0×10^7	2.3×10^7
3 1/4	9.5×10^5	1.5×10^5	4.35×10^7	4.30×10^7	3.5×10^7	7.0×10^7
5	9.5×10^7	2.5×10^7	1.15×10^8	9.3×10^7	2.0×10^8	1.8×10^8
6	4.5×10^8	4.5×10^7	2.08×10^8	1.18×10^8	2.5×10^8	1.9×10^8
7	2.5×10^7	1.5×10^8	2.46×10^8	1.36×10^8	2.1×10^8	2.8×10^8
8 1/4	9.5×10^7	4.5×10^7	2.30×10^8	1.66×10^8	4.2×10^8	5.6×10^8
10	4.5×10^7	2.5×10^6	2.00×10^8	1.95×10^8	--	--

Note:

Ten ml. of culture was removed from each test bottle after inoculating with organism. At 7 hr. turbidity readings of these cultures were: control 63 and DL-serine omitted 26. There was very little difference in the observed turbidity of these cultures at 10 hr. Total plate counts on plates containing 1 ml. filtrate/plate were 0 colony on duplicate 10^6 plates at 10 hr.

S. lactis strain 565 is more fastidious in its nutritional requirements than strain W2. While strain W2 (tables 11 and 12) developed just as readily in the absence of any one of the following nutrients: thiamine, riboflavin, pyridoxine, DL-phenylalanine, L-lysine, L-hydroxyproline, and adenine, the omission of any one of these nutrients from the basal medium reduced very markedly the growth of strain 565 (table 10). S. lactis strain W2 differed from the 21 strains reported by Niven (1944) in that all 21 of those strains required pyridoxine for the development of normal turbidity.

When glutamine and asparagine were autoclaved in the medium (table 9 and 11), bacteriophage multiplication was generally less than when filtered glutamine and asparagine were added to the medium (tables 10 and 12). This possibly was due to decreased organism growth.

Effect of the Addition of Nutrients Upon the Multiplication of Bacteriophages and Organisms of Combinations Other Than 565-F54 and W-2-F24

A study was undertaken to determine what alteration or supplementation of the synthetic medium was necessary to make it suitable for the proliferation of bacteriophage strains other than F54 and F24 which have been shown to multiply in the basal medium.

During most of this study only two combinations, 712-F56 and E8-F63, were used because a more informative study could

be made while working with only two bacteriophage-organism combinations and because these were representative of a large number of bacteriophage-organism combinations. S. lactis strain 712 grew very rapidly on the basal medium, while S. cremoris strain E8 represented a larger number of S. cremoris strains which grew on the basal medium only when it was supplemented with sodium acetate and Tween 80 or with a more complex supplement, and even then growth during the first few hours was considerably slower than was the growth of S. lactis strain 712 during the same period.

A standard procedure was used throughout this work. The various media indicated were prepared in quantities of 50 ml. and aseptically dispensed in quantities of 10 ml. into 16 x 125 mm. screw cap culture tubes. This gave two tubes of a medium for each combination, one to be used as an index of organism growth and the other to be used as an index of bacteriophage proliferation. The fifth tube of medium was used to standardize the Klett-Summerson photoelectric colorimeter. After allowing the medium to reach 32° C. in a water bath, at 0 minus 25 minutes, 0.1 ml. of resuspended cells was added to each of the two tubes; and at 0 minus 5 minutes, 0.1 ml. of diluted filtrate was added to the tube used for the determination of bacteriophage proliferation. Determinations of bacteriophage concentration and turbidity measurements were made as indicated.

Since the synthetic medium seemed to lack some growth

factor or factors necessary for the adsorption or multiplication of the bacteriophages, several attempts were made to secure bacteriophage multiplication by supplementing the medium. The addition of supplement II, which made the synthetic medium relatively complete with known growth factors important in bacterial nutrition and also included reticulogen, caused a slight increase in F56, but seemed completely inadequate for F63 (tables 16 and 17). While the addition of reticulogen did not cause bacteriophage multiplication, the growth of S. cremoris (E8) was stimulated during the 7 hour experimental period. The addition of such complex substances as yeast extract, hydrolyzed casein, tomato juice, peptonized milk and dried skim milk in small quantities, while supporting considerable growth of organisms, did not produce any significant effect upon the multiplication of bacteriophages F56 and F63.

To improve the anaerobic conditions of the medium small quantities of thioglycollic acid and cysteine were added and cultures were grown in an atmosphere consisting of about 10 per cent carbon dioxide and 90 per cent hydrogen (table 18). These modifications of the medium had little effect on organism growth and did not result in bacteriophage proliferation. The addition of casein hydrolysate and extra K_2HPO_4 in addition to the thioglycollic acid and cysteine stimulated organism growth, but did not cause increases in bacteriophage.

Since the synthetic medium was not highly buffered, the K_2HPO_4 content was increased to maintain a pH level more nearly

TABLE 16

Multiplication of S. lactis (712) and bacteriophage (F56) on the synthetic basal medium supplemented with 2 mg./ml. sodium acetate, 2 mg./ml. Tween 80 and as indicated

Supplement (/100 ml. medium)	No. of bacteriophage particles					Turbidity of control 7 hr.
	0 hr.	3 hr.	4 hr.	6 hr.	7 hr.	
None	1.5×10^2	--	$< 1 \times 10^2$	--	$< 1 \times 10^2$	147
Reticulogen (20 microl.)	1.5×10^2	--	$< 1 \times 10^2$	--	$< 1 \times 10^2$	152
Supplement II[a]	4.5×10^2	$< 1 \times 10^2$	--	4.0×10^5	--	142
Yeast extract (0.5 g.)	4.5×10^2	$< 1 \times 10^2$	--	$< 1 \times 10^2$	--	135
Whey (10 ml.)	4.5×10^2	3×10^1	--	$< 1 \times 10^2$	--	102
Casein hydrolysate (5 ml.)	4.5×10^2	3×10^1	--	7.5×10^5	--	162
Casein hydrolysate (5 ml.)	1.5×10^2	--	$< 1 \times 10^2$	--	9.5×10^2	177
Trypticase[b] (0.5 g.)	1.5×10^2	--	4.0×10^1	--	2.5×10^3	181
Dried Skim milk (1.0 g.)	1.5×10^2	--	1.5×10^2	--	4.5×10^5	--

[a] See table 1

[b] A pancreatic digest of casein

TABLE 17

Multiplication of *S. cremoris* (E8) and bacteriophage (F63) on the synthetic basal medium supplemented with 2 mg./ml. sodium acetate, 2 mg./ml. Tween 80 and as indicated

Supplement (/100 ml. medium)	No. of bacteriophage particles					Turbidity of control 7 hr.
	0 hr.	3 hr.	4 hr.	6 hr.	7 hr.	
None	9.5×10^2	--	9.5×10^2	--	2.5×10^2	18
Reticulogen (20 microl.)	9.5×10^2	--	4.5×10^2	--	7.0×10^1	56
Supplement II[a]	9.5×10^2	2.5×10^3	--	1.5×10^2	--	70
Yeast extract (0.5 g.)	9.5×10^2	9.5×10^2	--	4.0×10^1	--	60
Whey (10 ml.)	9.5×10^2	9.5×10^2	--	9.0×10^1	--	13
Casein hydrolysate (5 ml.)	9.5×10^2	4.5×10^2	--	4.5×10^3	--	86
Casein hydrolysate (5 ml.)	9.5×10^2	--	9.5×10^3	--	2.0×10^4	100
Trypticase (0.5 g.)	9.5×10^2	--	1.5×10^3	--	7.0×10^1	85
Dried skim milk (1.0 g.)	9.5×10^2	--	4.5×10^2	--	9.0×10^1	--

[a] See table 1

TABLE 18

Multiplication of organism and bacteriophage in anaerobic jar on the synthetic basal medium supplemented with 2 mg./ml. sodium acetate, 2 mg./ml. Tween 80 and as indicated

Organism-bacteriophage combination	Supplement (/100 ml. medium)	No. of bacteriophage particles		Turbidity of control (7 hr.)
		0 hr.	7 hr.	
712-F56	Thioglycollic acid, 10 microl.; cysteine, 10 mg.	9.5×10^3	7.5×10^2	136
	Thioglycollic acid, 10 microl.; cysteine, 10 mg.; casein hydrolysate, 5 ml.	9.5×10^3	$< 1 \times 10^2$	183
	Thioglycollic acid, 10 microl.; cysteine, 10 mg.; K_2HPO_4 increased to 1 g.	9.5×10^3	4.0×10^1	180
E8-F63	Thioglycollic acid, 10 microl.; cysteine, 10 mg.	4.5×10^2	7.0×10^1	9
	Thioglycollic acid, 10 microl.; cysteine, 10 mg.; casein hydrolysate, 5 mg.	4.5×10^2	9.0×10^1	95
	Thioglycollate acid, 10 microl.; cysteine, 10 mg.; K_2HPO_4 increased to 1 g.	4.5×10^2	9.0×10^1	42

like that of milk, which was considered optimum for bacteriophage multiplication (tables 19 and 20). When enough phosphate was included to buffer the lactic acid formed sufficiently to keep the pH of the medium above pH 6.0, organism growth was greatly decreased. An increase in the K_2HPO_4 content to 1 per cent improved organism growth, but it did not cause multiplication of bacteriophages. With the K_2HPO_4 content of the medium increased to 1 per cent, tomato juice, proteose peptone and peptonized milk were again added to the medium; but, the medium was still inadequate for bacteriophage multiplication (tables 19 and 20). Actually the concentrations of demonstrable bacteriophages decreased.

Upon finding that 5 per cent dried skim milk when added to the supplemented basal medium caused only a slight proliferation of F56 and no detectible effect upon F63 (tables 19 and 20), which is incompatible with the fact that liquid skim milk is used routinely in laboratories for the propagation of bacteriophages active against the lactic group of streptococci, a trial was made using reconstituted 5 per cent dried skim milk. The reconstituted skim milk allowed rapid proliferation of both bacteriophages (table 21), while plate counts on the control cultures revealed that the susceptible organisms growing in the absence of bacteriophages did not reach as high concentrations in the reconstituted skim milk as in the basal medium supplemented with dried skim milk (tables 19 and 20). While the multiplication

TABLE 19

Effect of phosphate concentration on multiplication of *S. lactis* (712) and bacteriophage (F56) in basal synthetic medium supplemented with 2 mg./ml. sodium acetate, 2 mg./ml. Tween 80 and as indicated

Phosphate concentration (g./100 ml. medium)	Supplement (/100 ml. medium)	No. of bacteriophage particles			Turbidity of control (7 hr.)	Remarks
		0 hr.	4 hr.	7 hr.		
K_2HPO_4, 3.3 g. KH_2PO_4, 2.5 g.	None	2.5×10^2	4.0×10^1	$< 1 \times 10^2$	Sl. turbid	M.P.N. at 10 hr. was $< 1 \times 10^2$
K_2HPO_4, 3.3 g. KH_2PO_4, 2.5 g.	Casein hydrolysate, 5 ml.	2.5×10^2	$< 1 \times 10^2$	$< 1 \times 10^2$	Sl. turbid	M.P.N. at 10 hr. was $< 1 \times 10^2$
K_2HPO_4, 1.1 g. KH_2PO_4, 0.9 g.	None	9.5×10^3	9.5×10^2	2.5×10^3	214	--
K_2HPO_4, 1.1 g. KH_2PO_4, 0.9 g.	Casein hydrolysate, 5 ml.	9.5×10^3	9.5×10^3	1.5×10^4	186	--
K_2HPO_4, 1.0 g.	None	1.5×10^3	9.5×10^2	4.5×10^4	208	pH at 7 hr. was 5.6
No phosphate	None	9.5×10^2	--	9.5×10^2	No visible growth	--
K_2HPO_4, 1.0 g.	Tomato juice, 40 ml.; proteose peptone; #3, lg. peptonized milk, lg.	1.5×10^3	2.5×10^2	$< 1 \times 10^2$	305	pH at 7 hr. was 4.7
K_2HPO_4, 1.0 g.	Dried skim milk, 5 g.	1.5×10^3	1.5×10^4	4.5×10^5	a	pH at 7 hr. was 5.30

[a] Actual plate count at 7 hr. was 1.8×10^9

TABLE 20

Effect of phosphate concentration on multiplication of S.cremoris (E8) and bacteriophage (F63) in basal synthetic medium supplemented with 2 mg./ml. sodium acetate, 2 mg./ml. Tween 80 and as indicated

Phosphate concentration (g./100 ml. medium)	Supplement (/100 ml. medium)	No. of bacteriophage particles			Turbidity of control (7 hr.)	Remarks
		0 hr.	4 hr.	7 hr.		
K_2HPO_4, 3.3 g. KH_2PO_4, 2.5 g.	None	9.5×10^2	9.5×10^2	2.5×10^2	No visible growth	--
K_2HPO_4, 3.3 g. KH_2PO_4, 2.5 g.	Casein hydrolysate, 5 ml.	9.5×10^2	4.5×10^2	2.5×10^2	No visible growth	--
K_2HPO_4, 1.1 g. KH_2PO_4, 0.9 g.	None	4.5×10^2	9.5×10^2	2.5×10^2	46	--
K_2HPO_4, 1.1 g. KH_2PO_4, 0.9 g.	Casein hydrolysate, 5 ml.	4.5×10^2	4.5×10^2	$< 1 \times 10^2$	82	--
K_2HPO_4, 1.0 g.	None	9.5×10^2	7.5×10^2	1.5×10^2	46	pH at 7 hr. was 6.4
No phosphate	None	9.5×10^2	--	4.5×10^2	No visible growth	--
K_2HPO_4, 1.0 g.	Tomato juice, 40 ml.; proteose peptone #3, 1.0 g.; peptonized milk, 1.0 g.	9.5×10^2	9.5×10^2	$< 1 \times 10^2$	246	--
K_2HPO_4, 1.0 g.	Dried skim milk, 5 g.	9.5×10^2	2.5×10^3	2.5×10^3	a	pH at 7 hr. was 5.85

[a] Actual plate count at 7 hr. was 1.9×10^8

TABLE 21

Multiplication of bacteriophage-organism combinations on reconstituted 5% dried skim milk

Organism	Bacteriophage	No. of bacteriophage particles				pH 7 hr.	Plate count of control 7 hr.
		0 hr.	4 hr.	6 hr.	7 hr.		
712	F56	1.5×10^3	4.5×10^6	--	4.5×10^7	6.55	3.3×10^7
712	F56	9.5×10^2	--	2.5×10^7	--	6.55	1.2×10^7
E8	F63	9.5×10^2	2.5×10^{7a}		2.5×10^{9a}	6.50	8.2×10^7
E8	F63	9.5×10^2	--	2.5×10^{9a}	--	6.55	7.0×10^7

[a] Highest dilution made.

of organisms was expected to be greater in the medium consisting of the synthetic medium, dried skim milk and 1 per cent K_2HPO_4 than in reconstituted skim milk, it was surprising that significant bacteriophage multiplication occurred only in the reconstituted skim milk. Since the medium which supported no appreciable multiplication of the bacteriophages was the more nutritive medium, it seemed that some constituent of the synthetic medium might be inhibitory to bacteriophage action.

Upon omitting various nutrients from the synthetic medium supplemented with 5 per cent dried skim milk (tables 22 and 23), it was found that when all constituents of the medium except K_2HPO_4 were included both bacteriophages developed very rapidly. However, when K_2HPO_4 was included in the medium, bacteriophage multiplication either did not take place or was very much slower than in the medium containing no K_2HPO_4.

Ammonium phosphate and sodium citrate also were found to prevent bacteriophage proliferation when added to the synthetic medium containing dried skim milk, while the presence of sodium acetate seemed to have no appreciable effect (table 24). Thus it seemed that the phosphate ion was inhibiting bacteriophage action, possibly by making unavailable some nutrient which was essential for bacteriophage proliferation. That ammonium phosphate and sodium citrate inhibited bacteriophage multiplication while sodium acetate did not was not conclusive proof that the encountered inhibition of bacteriophage multiplication was

TABLE 22

Effect of omission of nutrients from synthetic medium containing K_2HPO_4, 1 g./100 ml.; dried skim milk, 5 g./100 ml. and supplement XI[a], on the multiplication of S. lactis (712) and bacteriophage (F56)

Nutrients omitted	No. of bacteriophage particles	
	0 hr.	6 hr.
None	9.5×10^2	4.0×10^1
Amino acids	9.5×10^2	1.5×10^2
Purine and pyrimidine bases	9.5×10^2	9.0×10^1
Sodium thioglycollate and Tween 80	9.5×10^2	0×10^2
Vitamins, glutamine and asparagine	9.5×10^2	4.0×10^1
Minerals, sodium acetate, glucose, K_2HPO_4	9.5×10^2	9.5×10^8
$MgSO_4 \cdot 7$ HOH	9.5×10^2	9.5×10^4
$MnCl_2 \cdot 4$ HOH	9.5×10^2	4.0×10^1
$FeSO_4 \cdot 7$ HOH	9.5×10^2	0×10^2
Glucose	9.5×10^2	0×10^2
Sodium acetate	9.5×10^2	4.5×10^2
K_2HPO_4	9.5×10^2	9.5×10^8

[a] See table 1

TABLE 23

Effect of omission of nutrients from synthetic medium containing K_2HPO_4, 1 g./100 ml.; dried skim milk, 5 g./100 ml. and supplement XI[a], on the multiplication of S. cremoris (E8) and bacteriophage (F63)

Nutrient omitted	No. of bacteriophage particles	
	0 hr.	6 hr.
None	9.5×10^2	1.5×10^2
Amino acids	9.5×10^2	7.0×10^1
Purine and pyrimidine bases	9.5×10^2	1.5×10^2
Sodium thioglycollate and Tween 80	9.5×10^2	2.5×10^2
Vitamins, glutamine and asparagine	9.5×10^2	2.5×10^2
Minerals, sodium acetate, glucose, K_2HPO_4	9.5×10^2	2.5×10^9[b]
$MgSO_4 \cdot 7$ HOH	4.5×10^2	9.0×10^1
$MnCl_2 \cdot 4$ HOH	4.5×10^2	4.0×10^1
$FeSO_4 \cdot 7$ HOH	4.5×10^2	4.0×10^1
Glucose	4.5×10^2	4.0×10^1
Sodium acetate	4.5×10^2	1.5×10^2
K_2HPO_4	4.5×10^2	2.5×10^9[b]

[a] See table 1

[b] Dilution 9 was highest dilution made

TABLE 24

Effect of buffers on multiplication of bacteriophages in reconstituted 5 per cent dried skim milk

Bacteriophage	Buffer (1 per cent)	No. of bacteriophage particles	
		0 hr.	6 hr.
F56	Ammonium phosphate, dibasic	9.5×10^2	4.5×10^2
	Sodium acetate	9.5×10^2	2.5×10^7
	Sodium citrate	9.5×10^2	3.0×10^1
F63	Ammonium phosphate, dibasic	9.5×10^2	2.5×10^2
	Sodium acetate	9.5×10^2	4.5×10^7
	Sodium citrate	9.5×10^2	4.0×10^1

caused by the phosphate ion rather than by the potassium ion; however, it seemed adequate as a basis for further experimentation. This explanation seemed likely in that the autoclaving of media containing dried skim milk and K_2HPO_4 caused a slightly brown color and a slight precipitation.

Believing that by adding $CaCl_2$, Stassano and de Beaufort (1925) and Bordet and Renaux (1928) had solved similar situations, efforts were made to overcome with $CaCl_2 \cdot 2$ HOH in reconstituted 5 per cent dried skim milk the effect of 0.5 per cent K_2HPO_4 by mixing the ingredients prior to autoclaving (table 25) and by sterilizing all ingredients separately and mixing them after cooling (table 27). The addition of $CaCl_2 \cdot 2$ HOH and K_2HPO_4 to the reconstituted skim milk prior to autoclaving did not make the medium adequate for bacteriophage multiplication; but, with bacteriophage F56, increases were realized in several cases where the components of the medium were autoclaved separately. The addition of small amounts of $MgSO_4 \cdot 7$ HOH, $FeSO_4 \cdot 7$ HOH and MnCl2 • 4 HOH did not increase the effect of calcium chloride. In no case was the inhibition of 0.5 per cent K_2 HPO_4 or 0.5 per cent sodium citrate completely overcome.

An experiment was run to determine how much K_2HPO_4 could be added to the synthetic medium supplemented with 5 per

TABLE 25

Effect of $CaCl_2 \cdot 2HOH$ added before autoclaving in counteracting the effect of K_2HPO_4 (0.5 g./100 ml.) on bacteriophage multiplication in reconstituted 5 per cent dried skim milk

Bacteriophage	$CaCl_2 \cdot 2HOH$ conc. (g./100 ml.)	No. of bacteriophage particles	
		0 hr.	6 hr.
F56	None	9.5×10^2	4.5×10^2
	0.4 g.	Coagulated upon heating	
	0.2 g.	9.5×10^2	1.5×10^3
	0.1 g.	9.5×10^2	2.5×10^3
F63	None	9.5×10^2	1.1×10^2
	0.4 g.	Coagulated upon heating	
	0.2 g.	9.5×10^2	4.0×10^1
	0.1 g.	9.5×10^2	4.0×10^1

TABLE 26

Effect of $CaCl_2 \cdot 2HOH$ in counteracting the effect of K_2HPO_4 (0.5 g./100 ml.) on bacteriophage (F56) multiplication in reconstituted 5 per cent dried skim milk

(All ingredients were neutralized to pH 6.7, autoclaved and mixed after cooling.)

$CaCl_2 \cdot 2HOH$ conc. (g./100 ml.)	Additional salt supplement	No. of bacteriophage particles	
		0 hr.	6 hr.
None	None	9.5×10^2	9.5×10^3
0.5 g.	Supplement XV[a]	9.5×10^2	9.5×10^4
0.4 g.	None	9.5×10^2	2.5×10^5
0.2 g.	None	9.5×10^2	9.5×10^4
0.2 g.	Supplement XV[a]	9.5×10^2	9.5×10^4
0.1 g.	None	9.5×10^2	4.5×10^5
0.05 g.	None	9.5×10^2	2.5×10^5

[a] See table 1

TABLE 27

Effect of $CaCl_2 \cdot 2HOH$ in counteracting the effect of sodium citrate (0.5 g./100 ml.) on bacteriophage (F56) multiplication in reconstituted 5 per cent dried skim milk

(All ingredients were neutralized to pH 6.7, autoclaved and mixed after cooling.)

$CaCl_2 \cdot 2HOH$ conc. (g./100 ml.)	Additional salt supplement	No. of bacteriophage particles	
		0 hr.	6 hr.
None	None	9.5×10^2	2.5×10^3
0.4 g.	None	9.5×10^2	9.5×10^5
0.2 g.	None	9.5×10^2	1.5×10^6
0.2 g.	Supplement XV[a]	9.5×10^2	4.5×10^5
0.1 g.	None	9.5×10^2	4.5×10^5
0.1 g.	None	9.5×10^2	2.5×10^5

[a] See table 1

cent dried skim milk without affecting bacteriophage multiplication. The concentration of K_2HPO_4 in the basal synthetic medium was to be decreased to this concentration before making additional efforts to obtain bacteriophage multiplication in the synthetic medium. The addition of 1.0 or 0.5 per cent K_2HPO_4 (table 28) prevented bacteriophage multiplication in reconstituted 5 per cent skim milk, whereas the addition of 0.1 per cent K_2HPO_4 did not prevent normal multiplication of bacteriophages F56 and F63. The adding of 1 per cent sterile K_2HPO_4 to the reconstituted skim milk after autoclaving seemed much less detrimental to the proliferation of bacteriophage F56 than autoclaving the K_2HPO_4 in the medium, but this difference in effect was not noted for bacteriophage F63.

In the basal medium supplemented with supplement XIV, bacteriophages did not multiply when the K_2HPO_4 content was decreased from 0.4 per cent to 0.1, 0.05 and 0.01 per cent (tables 29 and 30). The glucose content was also decreased to keep down the production of acid by the bacteria.

In cases in which the glucose content was decreased, determinations of pH using a quinhydrone potentiometric system revealed that at 7 hr. the pH values were higher than that of combination W2-F24 in synthetic medium and that of combination 712-F56 in synthetic medium containing no K_2HPO_4 and supplemented with small quantities of dried skim milk. While no pH values have been given for combination W2-F24 in synthetic medium or combination 712-F56 in the supplemented medium, in both cases the

TABLE 28

Effect of K_2HPO_4 on multiplication of bacteriophage on susceptible host cells in synthetic medium supplemented with 5 per cent dried skim milk

Bacteriophage	K_2HPO_4 conc. (g./100 ml.)	No. of bacteriophage particles	
		0 hr.	6 hr.
F56	1.0 g.	9.5×10^2	4.0×10^1
	0.5 g.	9.5×10^2	2.5×10^2
	0.1 g.	9.5×10^2	2.5×10^{9}[a]
	0.0 g.	9.5×10^2	2.5×10^{9}[a]
	1.0 g.[b]	9.5×10^2	2.5×10^5
F63	1.0 g.	9.5×10^2	1.5×10^2
	0.5 g.	9.5×10^2	9.0×10^1
	0.1 g.	9.5×10^2	2.5×10^{9}[a]
	0.0 g.	9.5×10^2	9.5×10^8
	1.0 g.[b]	9.5×10^2	7.0×10^1

[a] Dilution 9 was the highest dilution made.

[b] K_2HPO_4 autoclaved separately and added after cooling.

TABLE 29

Effect of K_2HPO_4 concentration on multiplication of *S. lactis* (712) and bacteriophage (F56) in basal synthetic medium supplemented with supplement XIV[a]

K_2HPO_4 conc. (g./100 ml.)	Glucose conc. (g./100 ml.)	No. of bacteriophage particles		pH (7 hr.)	pH of uninoculated medium	Turbidity of control (7 hr.)
		0 hr.	7 hr.			
0.4 g.	0.5 g.	4.5×10^2	$< 1 \times 10^2$	4.90	6.65	166
0.1 g.	0.5 g.	4.5×10^2	4.5×10^3	4.65	6.60	149
0.05 g.	0.1 g.	4.5×10^2	$< 1 \times 10^4$	5.20	6.60	88
0.01 g.	0.1 g.	4.5×10^2	$< 1 \times 10^4$	5.05	6.60	85
None	0.1 g.	4.5×10^2	$< 1 \times 10^4$	6.40	6.55	2
0.04 g.	None[b]	4.5×10^2	$< 1 \times 10^4$	6.30	6.60	4

[a] See table 1

[b] Lactose, 0.2 g., was substituted for glucose.

TABLE 30

Effect of K_2HPO_4 concentration on multiplication of S. cremoris (E8) and bacteriophage (F63) in basal synthetic medium supplemented with supplement XIV[a]

K_2HPO_4 conc. (g./100 ml.)	Glucose conc. (g./100 ml.)	No. of bacteriophage particles		pH (7 hr.)	pH of uninoculated medium	Turbidity of control (7 hr.)
		0 hr.	7 hr.			
0.4 g.	0.5 g.	4.5×10^2	$<1 \times 10^2$	6.30	6.65	44
0.1 g.	0.5 g.	4.5×10^2	4×10^1	5.75	6.60	52
0.05 g.	0.1 g.	4.5×10^2	$<1 \times 10^4$	5.70	6.60	25
0.01 g.	0.1 g.	4.5×10^2	$<1 \times 10^4$	5.60	6.60	21
None	0.1 g.	4.5×10^2	$<1 \times 10^4$	6.50	6.55	2
0.04 g.	None[b]	4.5×10^2	$<1 \times 10^4$	6.10	6.60	15

[a] See table 1

[b] Lactose, 0.2 g., was substituted for glucose.

pH values were generally below pH 5.0 at 7 hours. This indicated that the failure of bacteriophages to multiply in the synthetic medium containing only small amounts of K_2HPO_4 was not due to the pH of the medium.

The substitution of lactose (tables 29 and 30) for glucose was to no avail other than to point out that S. lactis strain 712 no longer was able to ferment lactose rapidly.

With cases in which the K_2HPO_4 content was decreased below 0.1 per cent (tables 29 and 30) a decrease in organism growth resulted, and with cases in which K_2HPO_4 was omitted the organisms did not develop detectable turbidity during the 7 hour experimental period. This reduction in organism growth due to decreased phosphate content, in conjunction with the fact that very high concentrations of bacteriophages were obtained in synthetic medium supplemented with dried skim milk and 0.1 per cent K_2HPO_4 (table 28), further indicated that the failure of bacteriophages to multiply, rather than being due to the presence of the phosphate ion, was due to the absence of some nutrient in the synthetic medium. This nutrient, though present in dried skim milk, was made unavailable in a medium containing dried skim milk by concentrations of K_2HPO_4 greater than 0.1 per cent.

Thus, with the K_2HPO_4 and glucose contents decreased to 0.1 per cent, other attempts were made (tables 31 and 32) to secure bacteriophage proliferation by addition of small quantities of $CaCl_2 \cdot 2HOH$ and by increasing the salts already present in a

TABLE 31

Effect of salt supplements on the multiplication of S. lactis (712) and bacteriophage (F56) in the basal medium supplemented with supplement XIV[a]

Salt supplement (g./100 ml.)	K_2HPO_4 conc. (g./100 ml.)	Glucose conc. (g./100 ml.)	No. of bacteriophage particles		Turbidity of control (7 hr.)
			0 hr.	7 hr.	
$CaCl_2 \cdot 2HOH$, 0.2 g.	0.4 g.	0.5 g.	4.5×10^2	$< 1 \times 10^2$	69
$CaCl_2 \cdot 2HOH$, 5 mg.	0.05 g.	0.1 g.	4.5×10^2	$< 1 \times 10^4$	84
Supplement XII[a] $CaCl_2 \cdot 2HOH$, 5 mg.	0.05 g.	0.1 g.	4.5×10^2	4.5×10^5	82
Supplement XII[a] $CaCl_2 \cdot 2HOH$, 5 mg.	0.05 g.	0.1 g.	4.5×10^2	$< 1 \times 10^4$	83
Supplement XII[a] $CaCl_2 \cdot 2HOH$, 5 mg. sodium acetate, 0.5 g.	0.01 g.	0.1 g.	4.5×10^2	$< 1 \times 10^4$	71
Supplement XII[a]	0.05 g.	0.1 g.	4.5×10^2	$< 1 \times 10^4$	76
Supplement XIII[a] $CaCl_2 \cdot 2HOH$, 10 mg.	0.05 g.	0.1 g.	4.5×10^2	$< 1 \times 10^4$	80

[a] See table 1

TABLE 32

Effect of salt supplements on the multiplication of S. cremoris (E8) and bacteriophage (F63) in the basal medium supplemented with supplement XIV[a]

Salt supplement (g./100 ml.)	K_2HPO_4 conc. (g./100 ml.)	Glucose conc. (g./100 ml.)	No. of bacteriophage particles		Turbidity of control (7 hr.)
			0 hr.	7 hr.	
$CaCl_2 \cdot 2HOH$, 0.2 g.	0.4 g.	0.5 g.	4.5×10^2	9×10^1	80
$CaCl_2 \cdot 2HOH$, 5 mg.	0.05 g.	0.1 g.	4.5×10^2	$< 1 \times 10^4$	39
Supplement XII[a] $CaCl_2 \cdot 2HOH$, 5 mg.	0.05 g.	0.1 g.	4.5×10^2	$< 1 \times 10^4$	25
Supplement XII[a] $CaCl_2 \cdot 2HOH$, 5 mg.	0.05 g.	0.1 g.	4.5×10^2	$< 1 \times 10^4$	44
Supplement XII[a] $CaCl_2 \cdot 2HOH$, 5 mg. sodium acetate, 0.5 g.	0.01 g.	0.1 g.	4.5×10^2	$< 1 \times 10^4$	8
Supplement XII[a]	0.05 g.	0.1 g.	4.5×10^2	$< 1 \times 10^4$	36
Supplement XIII[a] $CaCl_2 \cdot 2HOH$ 10 mg.	0.05 g.	0.1 g.	4.5×10^2	$< 1 \times 10^4$	48

[a] See table 1

medium which was also supplemented with the complex supplement XIV. In one medium containing 5 mg. calcium chloride per 100 ml. bacteriophage F56 increased slightly (table 31), but this result was not duplicated in a second trial. The K_2HPO_4 content was reduced to 0.05 and 0.01 per cent to no avail. There seemed to be considerable doubt that the essential nutrient had been added and the use of more complex nutrients again was employed.

While trypticase, a pancreatic digest of casein, caused a slight increase in bacteriophages F56 and F63 (table 33), dried skim milk again was the only supplement which produced any great effect upon the multiplication of bacteriophage. The addition of 1.5 per cent dried skim milk caused normal multiplication of F56 and F63. Thus, it seemed necessary to determine the constituent of milk which was necessary for bacteriophage proliferation. Some dried skim milk was ashed, and the ash was added to the medium at the rate of the ash of 2 g. dried skim milk per 100 ml. A precipitate was formed during autoclaving, and the bacteriophages did not multiply in the medium (table 34). However, when a medium containing the ash of 2 g. dried skim milk per 100 ml. was sterilized by filtration, the bacteriophages multiplied. If the K_2HPO_4 content was increased from 0.1 to 0.4 per cent, the resulting medium was inactive for the multiplication of bacteriophages. A mineral mixture of the approximate composition of milk ash was made up, and a quantity of this mixture approximately equal

TABLE 33

Effect of complex supplements on multiplication of bacteriophage on host in half-strength basal medium minus K_2HPO_4 and containing only 0.05 g./100 ml. glucose

Bacteriophage	Supplement (/100 ml.)	No. of bacteriophage particles	
		0 hr.	7 hr.
F56	Dried skim milk, 3 g.	4.5×10^2	4.5×10^8
	Dried skim milk, 1.5 g.	4.5×10^2	2.5×10^8
	Dried skim milk, 0.75 g.	4.5×10^2	4.5×10^8
	Tomato juice, 40 ml.	4.5×10^2	$< 1 \times 10^4$
	Tomato juice, 20 ml.	4.5×10^2	$< 1 \times 10^4$
	Trypticase, 4 g.	4.5×10^2	4.0×10^5
F63	Dried skim milk, 3 g.	4.5×10^2	4.5×10^8
	Dried skim milk, 1.5 g.	4.5×10^2	2.5×10^8
	Dried skim milk, 0.75 g.	4.5×10^2	2.5×10^7
	Tomato juice, 40 ml.	4.5×10^2	$< 1 \times 10^4$
	Tomato juice, 20 ml.	4.5×10^2	$< 1 \times 10^4$
	Trypticase, 4 g.	4.5×10^2	4.0×10^5

TABLE 34

Effect of method of sterilization and K_2HPO_4 content of the supplemented[a] basal medium containing the ash of 2 g. dried skim milk/100 ml. upon bacteriophage multiplication on susceptible host cells

Bacteriophage	Method of sterilization	K_2HPO_4 conc. (g./100 ml.)	No. of bacteriophage particles[c] (6 hr.)	Turbidity of control (6 hr.)	pH Test	pH Control	pH Uninoculated medium
F56	Autoclaved	0.1 g.	$<1x10^4$	Precipitated	5.35	--	5.95
	Filtered	0.1 g.	$4.0x10^7$	52	5.95	5.80	6.60
	Filtered	0.4 g.	$<1x10^4$	54	6.20	6.15	--
F63	Autoclaved	0.1 g.	$<1x10^4$	Precipitated	5.30	--	5.95
	Filtered	0.1 g.	$2.5x10^8$	55	5.90	6.00	6.60
	Filtered	0.4 g.	$<1x10^4$	55	6.35	6.35	--

[a] Medium contained only 0.1 g. glucose/100 ml. and supplements XII[b] and XIV[b].

[b] See table 1

[c] Number of bacteriophage particles at 0 hr. was $4.5x10^2$

to the ash of 2 g. dried skim milk was substituted for the milk ash. This mineral mixture also caused bacteriophage proliferation (tables 35 and 36), and when the synthetic medium was not supplemented neither F56 nor F63 multiplied.

Since the major mineral constituent of milk ash other than phosphorus and potassium is calcium, another attempt was made to cause the multiplication of the bacteriophages by adding $CaCl_2 \cdot 2HOH$. This time comparatively large quantities of $CaCl_2 \cdot 2HOH$ were added, and the medium was sterilized by filtration. The addition of either 0.2 or 0.1 per cent $CaCl_2 \cdot 2HOH$ resulted in rapid multiplication of bacteriophages (tables 35 and 36). Autoclaving the medium and the K_2HPO_4 separately and adding the two after cooling gave a medium which was as active for the production of bacteriophages as a medium sterilized by filtration. Neither $MnCl_2 \cdot 4HOH$ nor $MgSO_4 \cdot 7HOH$ duplicated the effect of $CaCl_2 \cdot {}^2HOH$ in promoting bacteriophage multiplication (tables 35 and 36). A slight stimulation which was much less than that of calcium chloride for bacteriophages F56 and F63, resulted from the use of $MgSO_4 \cdot 7HOH$.

It seemed that calcium was the nutrient which, when available in the chemically defined medium, would make it sufficient for the proliferation of those bacteriophages which in the absence of available calcium did not multiply. A quantity of the basal synthetic medium containing only 0.1 per cent K_2HPO_4 and supplemented with sodium acetate, Tween 80 and 0.2 per cent $CaCl_2 \cdot 2HOH$ was made up. The effect of this medium in promoting the growth

TABLE 35

Substitution of salts for milk ash for bacteriophage (F63) in basal medium containing 0.1 g./100 ml. K_2HPO_4 and 0.1 g./100 ml. glucose, supplemented with supplements XII[a] and XIV[a] and sterilized by filtration

Salt substituted (g./100 ml.)	No. of bacteriophage particles		Turbidity of control (6 hr.)
	0 hr.	6 hr.	
None	4.5×10^2	$< 1 \times 10^4$	24
Mineral mixture[b]	4.5×10^2	7.5×10^7	54
Mineral mixture[b,c]	4.5×10^2	4.5×10^8	54
$CaCl_2 \cdot 2HOH$, 0.2 g.	4.5×10^2	2.5×10^9	49
$CaCl_2 \cdot 2HOH$, 0.1 g.	4.5×10^2	2.5×10^9	54
$CaCl_2 \cdot 2HOH$, 0.1 g.[d]	4.5×10^2	2.5×10^9	58
$CaCl_2 \cdot 2HOH$, 20 mg.	4.5×10^2	4.5×10^7	69
$MgSO_4 \cdot 7HOH$, 0.2 g.	4.5×10^2	7.5×10^4	60
$MgSO_4 \cdot 7HOH$, 0.1 g.	4.5×10^2	$< 1 \times 10^4$	78
$MgSO_4 \cdot 7HOH$, 0.2 g. $CaCl_2 \cdot 2HOH$, 20 mg.	4.5×10^2	2.5×10^7	62
$MnCl_2 \cdot 4HOH$, 0.1 g.	4.5×10^2	$< 1 \times 10^4$	44

[a] See table 1

[b] Contained $CaCl_2 \cdot 2HOH$, 66 mg.; $MgSO_4 \cdot 7HOH$, 25 mg.; $FeSO_4 \cdot 7HOH$, 1.1 mg.; NaCl, 13 mg.; K_2HPO_4, 75 mg. and Na_2HPO_4, 2 mg.

[c] Contained no K_2HPO_4 except that in mineral mixture.

[d] K_2HPO_4 autoclaved separately and added to the autoclaved mixture.

TABLE 36

Substitution of salts for milk ash for bacteriophage (F56) in basal medium containing 0.1 g./100 ml. K_2HPO_4 and 0.1 g./100 ml. glucose, supplemented with supplements XII[a] and XIV[a] and sterilized by filtration

Salt substituted (g./100 ml.)	No. of bacteriophage particles		Turbidity of control (6 hr.)
	0 hr.	6 hr.	
None	4.5×10^2	$< 1 \times 10^4$	47
Mineral mixture[b]	4.5×10^2	2.5×10^7	54
Mineral mixture[b,c]	4.5×10^2	1.5×10^6	66
$CaCl_2 \cdot 2HOH$, 0.2 g.	4.5×10^2	2.5×10^7	60
$CaCl_2 \cdot 2HOH$, 0.1 g.	4.5×10^2	4.5×10^7	63
$CaCl_2 \cdot 2HOH$, 0.1 g.[d]	4.5×10^2	4.5×10^8	80
$CaCl_2 \cdot 2HOH$, 20 mg.	4.5×10^2	2.5×10^6	61
$MgSO_4 \cdot 7HOH$, 0.2 g.	4.5×10^2	7.5×10^5	84
$MgSO_4 \cdot 7HOH$, 0.1 g.	4.5×10^2	2.5×10^5	95
$MgSO_4 \cdot 7HOH$, 0.2 g. $CaCl_2 \cdot 2HOH$, 20 mg.	4.5×10^2	2.5×10^6	60
$MnCl_2 \cdot 4HOH$, 0.1 g.	4.5×10^2	$< 1 \times 10^4$	71

[a] See table 1

[b] Contained $CaCl_2 \cdot 2HOH$, 66 mg.; $MgSO_4 \cdot 7HOH$, 25 mg.; $FeSO_4 \cdot 7HOH$, 1.1 g.; NaCl, 13 mg.; K_2HPO_4, 75 mg. and Na_2HPO_4, 2 mg.

[c] Contained no K_2HPO_4 except that in mineral mixture.

[d] K_2HPO_4 autoclaved separately and added to the autoclaved medium.

of six bacteriophages which did not multiply in the absence of available calcium is shown in table 37. In each case the multiplication of bacteriophage was as great as or greater than would be predicted from the development of turbidity. Thus, when calcium was available in the chemically defined medium, the close relationship between the multiplication of organism and bacteriophage was again evident.

One-tenth of 1 per cent $CaCl_2 \cdot 2HOH$ seemed optimum in a synthetic medium containing 0.1 per cent K_2HPO_4. In cases in which the K_2HPO_4 content was increased or the medium was autoclaved in the presence of the phosphate, the calcium seemed to be removed from solution, possibly in the form of tri-calcium phosphate. When sterilization was accomplished by filtration, all ingredients were added prior to filtration. A very slight precipitate usually was formed when a medium was prepared using the proportions of $CaCl_2 \cdot 2HOH$ and K_2HPO_4 indicated and adjusted to pH 6.7. The removal of this precipitate by filtration seemed not to harm the medium.

Two attempts were made to determine whether calcium was necessary for the adsorption of bacteriophage to cells or for the multiplication of bacteriophage after adsorption. Two quantities of basal medium were prepared, one of which contained 0.1 per cent $CaCl_2 \cdot 2HOH$. The K_2HPO_4 content was 0.1 per cent in each case. The media were dispensed in quantities of 9 ml. and placed in a 32° C. water bath. Each tube of medium was inoculated with

TABLE 37

The effect of $CaCl_2 \cdot 2HOH$, 0.2 g./100 ml., in promoting multiplication of bacteriophages in synthetic medium containing sodium acetate, 2 mg./ml., and Tween 80, 2 mg./ml. and sterilized by filtration

S. lactis strain	Bacteriophage strain	No. of bacteriophage particles			Turbidity	
		0 hr.	7 hr.[a]	12 hr.[b]	7 hr.	12 hr.
FH8	F65a	4.5×10^2	4.5×10^6	2.5×10^7	9	17
318B/27	F74	2.5×10^2	2.5×10^7	2.5×10^8	22	46
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

[a] Dilution 7 was highest dilution made at 7 hr.

[b] Dilution 9 was highest dilution made at 12 hr.

1 ml. of washed cells containing about 10^8 bacteria per ml. Thirty minutes later 1 ml. of diluted bacteriophage filtrate, containing about 10^5 bacteriophage particles per ml., was added per tube, and the bacteriophage concentrations were determined. At the indicated time intervals tubes were removed from the water bath, centrifuged 5 minutes at 4,000 r.p.m. to sediment the bacterial cells, and assayed for bacteriophage remaining in the supernatant. The results of these experiments are given in table 38.

The results in almost every instance were within the experimental error of the methods used, and the precision of estimation attained in enumerating the bacteriophage particles does not justify any definite conclusions. However, the decrease in bacteriophage remaining in the supernatant where no calcium was present was as great as or greater than in cases where calcium was present. This seemed to indicate that possibly calcium was not necessary for the adsorption of bacteriophages F56 or F63.

Cohen and Anderson (1946), upon studying the effect of the adsorption of T2 bacteriophage by E. coli in a Warburg respirometer, found that after 1 to 3 hours there was a gradual decrease in O_2 consumption. They attributed the decrease in O_2 consumption to the dying of infected cells. It has been noticed throughout this work that usually in cases in which there was no increase in bacteriophage particles during a 6 or 7 hour test

TABLE 38

Effect of $CaCl_2 \cdot 2HOH$ upon the adsorption of bacteriophages F56 and F63

Bacteriophage (strain)	Organism (strain)	$CaCl_2 \cdot 2HOH$ (g./100 ml.)	No. of bacteriophage particles in supernatant after time (min.) allowed for adsorption			
			0	10	20	30
		0	7.5×10^4	4.5×10^4	--	--
		0.1	4.5×10^4	2.5×10^4	--	--
F56	712	0	4.5×10^4	9.5×10^3	4.5×10^3	3.0×10^3
		0.1	9.5×10^4	2.5×10^4	4.5×10^4	2.5×10^4
		0	4.5×10^4	4.5×10^4	--	--
		0.1	2.5×10^4	7.5×10^4	--	--
F63	E8	0	2.5×10^5	4.5×10^4	2.5×10^4	2.5×10^4
		0.1	1.5×10^4	2.5×10^4	4.5×10^4	4.5×10^4

period there was a decrease. In table 8 this is evident for several bacteriophages. If bacteriophages are adsorbed to living cells, bacteriophage multiplication would commence when such infected cells are transferred to litmus milk, and the bacteriophages would be demonstrated. If bacteriophages are not adsorbed to cells in the test medium, adsorption would take place in the litmus milk. However, if bacteriophages are adsorbed to dead cells, multiplication of the adsorbed bacteriophages would not take place in litmus milk, and such bacteriophages would not be demonstrated. Since it is unlikely that bacteriophages would die off during a 6 or 7 hour test period, the observed decreases in numbers of demonstrable bacteriophages in this work may be considered to have resulted from the inactivity of infected cells. This could be considered additional evidence that several of the bacteriophages used in this work were adsorbed to cells in the absence of calcium, and that the calcium was necessary for some other function in bacteriophage proliferation.

Inhibition of Lysis by Adding K_2HPO_4 to Litmus Milk

Since it was found that concentrations of K_2HPO_4 of 0.5 per cent or greater inhibited bacteriophage multiplication in solutions of 0.5 per cent dried skim milk, possibly by making unavailable the calcium ion, it was desirable to know if this

phenomenon would be exhibited as well in liquid skim milk. For this purpose two different experiments were run. In the first experiment the K_2HPO_4 in concentrations indicated was added to and autoclaved with the liquid skim milk, while in the second trial a neutralized solution of K_2HPO_4 was autoclaved separately and added to the sterile skim milk in sufficient amounts to give the desired concentrations.

In all cases the litmus milk was dispensed aseptically in quantities of 10 ml. into test tubes, plugged with cotton and incubated at 32° C. for 16 to 18 hours, at which time visual observations were recorded. For each tube one drop of fresh litmus milk culture was used as organism inoculum and one drop of filtrate diluted 1:100 in sterile distilled water was used as bacteriophage inoculum. Although this procedure did not standardize the filtrates used as inocula for the different combinations, the quantity of inoculum used for each tube of a combination was standard.

The addition of 0.5 per cent K_2HPO_4 to skim milk prior to autoclaving (table 39) enabled most of the organisms tested to bring about coagulation in the presence of their respective bacteriophages, while 0.2 per cent K_2HPO_4 did not prevent lysis in any case. With combinations W2-F24, FH8-F65, and HP-F59 there was evidence of bacteriophage activity, in that, while coagulation did take place, the curd was very soft. Coagulation did not take place with the control cultures of combinations

TABLE 39

Inhibition of lysis by K_2HPO_4 added to litmus milk prior to autoclaving

S. lactis strain	Bacteriophage strain	Concentration of K_2HPO_4		
		0.5% K_2HPO_4		0.2% K_2HPO_4[a]
		No bacteriophage	Containing bacteriophage	No bacteriophage
712	F56	Coagulated	Coagulated	Coagulated
W2	F24	Coagulated	Coagulated, very soft curd	Coagulated
E8	F63	Reduced	Reduced	Reduced
FH8	F65	Coagulated	Coagulated, very soft curd	Coagulated
318B/27	F74	Reduced	Reduced	Reduced
M1	F25	Coagulated	Coagulated	Coagulated
IP5	F68	Coagulated	Coagulated	Coagulated
H.P.	F59	Coagulated	Coagulated very soft curd	Coagulated
459	F52	Coagulated	Coagulated	Coagulated
W4	F21	Coagulated	Coagulated	Coagulated
122-1	F76	Reduced	Reduced	Reduced

[a] Litmus milk tubes containing 0.2 per cent K_2HPO_4 and bacteriophage were lysed[b] in all cases.

[b] Lysed indicates that there was little or not visible change in the litmus milk.

E8-F63, 318B/27-F74, and 122-1-F76 during the experimental period, which possibly can be explained upon the basis that greater quantities of lactic acid are required to coagulate skim milk in the presence of K_2HPO_4. Upon the addition of K_2HPO_4 to skim milk after autoclaving (table 40), there was a marked difference in the activity of different strains of bacteriophage. The addition of 1 per cent K_2HPO_4 was necessary to prevent bacteriophage action, and even this quantity prevented the action of only two bacteriophages, F56 and F25. Whether these differences in the effect of K_2HPO_4 in preventing lysis by different strains of bacteriophage were due to differences in the amounts of calcium required by these bacteriophages or to some other reason is not clear. As was the case in a synthetic medium, the ability of K_2HPO_4 to prevent lysis, possibly by making calcium unavailable for use by the bacteriophage, seemed much more effective when the K_2HPO_4 was added prior to autoclaving.

TABLE 40

Inhibition of lysis by K_2HPO_4 added to litmus milk after autocleving

S. lactis strain	Bacteriophage strain	Concentration of K_2HPO_4			
		1.0% K_2HPO_4		0.5% K_2HPO_4	
		No bacteriophage	Containing bacteriophage	No bacteriophage	Containing bacteriophage
712	F56	Coagulated	Coagulated	Coagulated	Lysed[a]
W2	F24	Coagulated	Lysed	Coaguled	Lysed
E8	F63	Reduced	Reduced	Reduced	Reduced
FH8	F65	Coagulated	Lysed	Coagulated	Lysed
318B/27	F74	Reduced	Reduced	Reduced	Lysed
M1	F25	Coagulated	Coagulated	Coagulated	Lysed
IP5	F68	Coagulated	Lysed	Coagulated	Lysed
H.P.	F59	Coagulated	Lysed	Coagulated	Lysed
459	F52	Coagulated	Lysed	Coagulated	Lysed
W4	F21	Coagulated	Coagulated, very soft curd	Coagulated	Coagulated, very soft curd
122-1	F76	Reduced	Reduced	Reduced	Reduced
H-1,2	PF2	Reduced	Reduced	Reduced	Reduced
ML1	F69	Coagulated	Acid, coagulated in bottom of tube	Coagulated	Lysed
565	F54	Coagulated	Lysed	Coagulated	Lysed

[a] Indicates that there was little or no visible change in the litmus milk.

Note:

In a control experiment using litmus milk with no K_2HPO_4 added, all tubes containing no bacteriophage were coagulated and all tubes containing bacteriophage were lysed.

DISCUSSION

The requirement of strains of the lactic group of streptococci for sodium acetate and a source of oleic acid apparently is much more general among the strains of bacteria in the Streptococcus lactis group than has been reported for other lactic acid bacteria. No strain of S. cremoris tested was able to develop turbidity in the completely synthetic medium in the absence of both sodium acetate and Tween 80 unless a complex nutrient was included in the medium. Tween 80 alone was found to permit growth of only one of 22 cultures tested, and, although sodium acetate gave better results than Tween 80 alone, both of these nutrients seemed necessary for the rapid development of turbidity within 24 hours by most cultures. Although Tween 80 could not be substituted for sodium acetate, the stimulation provided by its addition to the medium seems to agree with the report (Guirard, Snell, and Williams, 1946) of a nutritional relationship between acetate and the synthesis of cell lipoidal materials in bacteria. Sodium acetate seems either to perform an additional function in the metabolism of these organisms or it is transformed by the organism into some substance which is essential for growth. The substitution of very small quantities of the liver extract, reticulogen, for sodium acetate and Tween 80 may indicate the addition of

a growth factor or factors which may serve in the formation of acetate or in a metabolic scheme in which some substance formed from acetate also is active. Baumann and Sauberlich (1948), upon finding that small quantities of reticulogen promoted growth of Leuconostoc citrovorum 8081 in a synthetic medium, attributed its growth promoting quality to vitamin B_{12} which was not available at the time of their work. Vitamin B_{12} was used in an effort to promote the growth of 12 strains of S. cremoris in this work and found ineffective in all cases.

The species "cremoris" is often considered to be a select group containing the more fastidious strains of the species "lactis". That the 29 most recently isolated organisms used in this study classified as S. lactis, while those cultures which have been carried on litmus milk for many years all classified as S. cremoris, is presumptive evidence that pure cultures of S. lactis organisms may become more fastidious upon being carried for a long period of time in heated milk and finally are classified as S. cremoris. Only 9 of 31 strains of S. lactis tested required sodium acetate and Tween 80, while all 22 strains of S. cremoris required this supplement. This indicates the more fastidious nature of S. cremoris organisms in this one requirement.

The growth of strains of the lactic group of streptococci on chemically defined media seems to present a nutritional gradation reaching from those organisms such as strains W2, 565 and 712 which developed turbidity very rapidly on the unsupplemented medium of Niven (1944) to strain H 1-2 which developed

turbidity very slowly in a medium supplemented with a long list of nutrients. All cultures that required sodium acetate and Tween 80 for growth developed turbidity rather slowly during the first 7 hours. When the defined medium was supplemented with such complex mixtures as tomato juice, casein hydrolysate, dried skim milk or yeast extract for S. cremoris strain E8, there was considerable stimulation of growth during the first seven hours, which seemed to indicate these complex mixtures contain other nutrients necessary for the rapid initiation of growth.

With two bacteriophage-organism combinations, 565-F54 and W2-F24, the bacteriophages were found to multiply in the presence of host cells growing in the chemically defined basal medium; however, no bacteriophage multiplication in the basal medium supplemented with sodium acetate and Tween 80 was evident with ten other combinations. While mass lysis occurred in milk, this was not always the case in the basal medium with bacteriophages F54 or F24. Although a clear and foamy culture at 6 or 7 hours always was indicative of high numbers of bacteriophage, in many cases in which these characteristics were not present, the numbers of bacteriophage were the same as those of the clear and foamy cultures. Clearing of a medium caused by the action of bacteriophage, appears to be the result of the associated action of several factors which may or may not allow the bacteriophages to reach a sufficient concentration to cause mass lysis prior to

the time when the bacterial cells become temporarily resistant to bacteriophage action. The rate of bacteriophage multiplication appears to be determined to a great extent by the rate of cell multiplication. Factors which allow the organism opportunity to become temporarily resistant possibly include slight physical and physico-chemical changes and changes in the physiological state of host and bacteriophage which favor bacterial multiplication to a greater extent than bacteriophage multiplication. The temporary resistant state of bacterial cells may result from changes in the pH of the medium and other changes due to bacterial growth such as the production of metabolic by-product(s) inhibitory to bacteriophage action.

The studies of omission of nutrients from the synthetic basal medium with combinations 565-F54 and W2-F24 seemed to emphasize the close relationship between the proliferation of bacteriophages and susceptible host cells. All efforts to obtain bacterial growth without bacteriophage proliferation failed. When a method for detecting smaller differences in concentrations of bacteriophages active against strains of the lactic group of streptococci is available, a similar study may reveal slight variations from this close relationship. However, such variations would seem to be of little value to the dairy industry.

When calcium was not included in the medium, the usual relationship between bacteriophage multiplication and organism multiplication seemed to be completely altered for all bacteriophage-organism combinations used except 565-F54 and W2-F24.

While the addition of available calcium did not detectably affect the growth of these organisms, it seemed to play a very important role in the prdiferation of the homologous b cterio-phages. In cases in which calcium was not available in the synthetic medium, the bacteriophages did not multiply; in the presence of calcium, bacteriophage multiplication occurred and the close relationship between multiplication of organism and bacteriophage proliferation again was apparent.

That calcium is necessary for the multiplication of many bacteriophages in a chemically defined medium may be of great significance to the dairy industry. The prevention of lysis of bacteria growing in milk requires the addition of rather large quantities of K_2HPO_4 which may result in heat coagulation; therefore, this practice does not seem feasible. However, it would seem that by using chemically defined nutrients containing no calcium or by using complex nutrients low in calcium content and supplemented with K_2HPO_4, a medium may be designed for the carrying of many strains of the lactic group of streptococci with little or no danger of bacteriophage action. Where the purpose of carrying such cultures is that they might eventually be used in milk or milk products, lactose would be used as a source of carbohydrate to prevent a loss of ability to coagulate milk rapidly.

SUMMARY AND CONCLUSIONS

A chemically defined medium made by adding sodium acetate and Tween 80 (a source of oleic acid) to the medium of Niven (1944) permitted the growth of all strains of the lactic group of streptococci which did not grow on the unsupplemented medium. The addition of sodium acetate and Tween 80 was necessary for the growth of 22 strains of S. cremoris and 9 of 31 strains of S. lactis. Reticulogen, a commercial liver extract, could be substituted in somewhat smaller quantities for sodium acetate and Tween 80 and also permitted rapid growth of one strain of S. cremoris which did not show detectable growth until after 24 hours in the medium supplemented with sodium acetate and Tween 80.

Using ammonia formation from arginine and growth at 40° C. as the basis for separation, all strains of the lactic group which had been in the laboratory for considerable time were found to be S. cremoris, while all recently isolated strains were found to be S. lactis.

With two S. lactis-bacteriophage combinations, multiplication of bacteriophages and organisms were affected similarly by the omission of individual components from the unsupplemented synthetic medium of Niven (1944). Bacteriophage multiplication seems to be closely associated with organism multiplication for these two combinations.

When calcium was available in the medium, eight bacteriophage strains tested were found to multiply on their susceptible host cells in a completely synthetic medium which did not permit these bacteriophage strains to proliferate without the addition of $CaCl_2 \cdot 2HOH$. When calcium was available in the medium and these bacteriophages multiplied, the close relationship between bacteriophage multiplication and organism multiplication seemed evident for these strains also. Calcium was rendered unavailable in a medium containing 0.1 per cent $CaCl_2 \cdot 2HOH$ by either autoclaving the entire medium or by increasing the K_2HPO_4 content above 0.1 per cent.

A close relationship seems to exist between the nutrition or organisms of the S. lactis group and multiplication of their homologous bacteriophages. However, calcium, while of no detectable importance to organism growth, seems to be required for the multiplication of many bacteriophages.

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