THE EFFECT OF GROWTH STIMULATING SUBSTANCES IN THE MEDIUM ON THE PRODUCTION OF BACTERIOPHAGE

THESIS FOR THE DEGREE OF M. S.

Beatrice Leora Kelly

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Bacteriology - Culture media

Bacteriology

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THESIS

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Introduction

The experiments hereinafter described were undertaken with the view of learning certain facts concerning the availability of various types of mediums for the production of bacteriophage.

The phenomenon of bacteriophagy was first described by d'Herelle (8) in 1917. His investigations on the subject continued over a period of years and brought him to the conclusion that the bacteriophage was a living principle, particulate in nature. This autonomous virus theory is supported by much evidence but today has few adherents. A considerable number of hypotheses on the nature of bacteriophage have been advanced from various sources.

Kabeshima (11) suggests that a catalytic agent, arising in the tissues of an infected animal, activates a proferment contained within the bacterial bodies; the activated ferment lyses the bacterium from which it arises, is then liberated and passed on to other bacteria to liberate the proferment which they contain.

Bordet and Ciuca (3) believe that a nutritional disorder of bacteria brings about autolysis and the liberation of an agent capable of transmitting the affection to other bacteria.

Bail (2) says that the bacteriophagous agent, (splitter) arises from the bacteria themselves.

Hadley (7) suggests that phage is a filterable state in the cyclogeny of organisms.

These theories vary widely one from the other and so far none seems to be backed by conclusive proof. The net result of these conflicting findings, interpretations, and views is that knowledge of the nature of bacteriophagy is in a somewhat confused state. It is not possible at the present time to make a final decision concerning the nature and source of the bacteriophagous agent.

In spite of these uncertainties, a great deal of progress has been made in the application of bacteriophage to various bacteriological and clinical problems, among which its use in the field of clinical medicine has received marked attention and has resulted in a literature of literally thousands of articles from which one can only conclude that the possibilities of

its use in the field of clinical medicine are limitless. With the development of bacteriophage for clinical use there has arisen a necessity for a clear understanding of the mechanism of its action both in the body and in the test tube in order that the principle may be suitably administered and that it may be prepared in its most potent and readily available form. Neither problem has been solved, but it has been recognized for a long time that the medium exercises a profound influence upon the development of a bacteriophage. An analysis has shown that of the various constituents of the medium, peptones are probably the most significant. This is of interest not only because of the influence of peptone upon the production of the principle, but also because when bacteriophage is applied through inoculations as a therapeutic agent, the presence of peptone is undesirable in general. Hence, various investigators have attempted to produce bacteriophage in the absence of peptone, especially when the product was to be administered intravenously. It has been observed by Larkum and others (personal communication), that a bacteriophage of weak

potency only resulted from the use of mediums poor in peptone, and most workers in this field have failed to achieve the results reported by MacNeal(loc.cit.) and Applebaum through the use of a snythetic medium containing no peptone.

Although the general observation had been made that peptones influence the development of bacteriophage probably because of their influence on the rate of growth of bacteria which has a direct relation to the development of bacteriophage, no attempts have been made to quantitatively determine just what concentration of peptone represents the optimum in a given medium. It was in order to shed some light on this question that the first group of experiments to be reported in this paper was undertaken.

A chance observation on the development of a growth stimulating substance for bacteria by Hughes (9) suggested the possibility that the objectionable peptone might be avoided through the incorporation in a synthetic medium of this growth stimulating substance which would have the further advantage of reducing the total nitrogen content of the medium without inhibiting the effective growth of bacteria. These experiments constitute the second portion of this study.

PART I. Work with Various Peptones

Section A. The Effect of Peptone on the Rate and Degree of Lysis.

General Procedure:

The general procedure for the following experiments consisted in the preparation of mediums containing variable concentrations of the selected peptones and making a study of the rate of lysis of Staphylococcus aureus after the seeding of the staphylococcus cultures with a constant amount of staphylococcus bacteriophage. In all cases the medium was aerated to expedite growth and bacterial dissolution. The rate of lysis was determined by a comparison of the turbidity of these broth cultures at stated intervals with a set of turbidity standards containing known numbers of bacteria. In this manner it was possible to get a fairly accurate measure of both the rate of growth and the rate of lysis of the organisms. The medium used was a beef extract broth with the following formula in which, obviously, the concentration of peptone varied:

Dist. water	1000 g.
Beef extract (Bacto)	3 g.
Peptone	
NaCl	5 g.

The standard turbidity suspensions were prepared for each experiment by adding graduated amounts of culture suspension to broth mediums containing peptone in the amount used in the experiments and formalin sufficient to prevent multiplication of the bacteria. The approximate number of bacteria added to each tube was determined by previous centrifugation of a portion of the suspension in a calibrated centrifuge tube. The details of this method are described by Kreuger. (13) After incubation of the flasks for 24 hours at room temperature, their contents was passed through S cm. Seitz filters and the filtrates titrated in order to determine the quantity of the phage produced in these various concentrations of peptone. Titrations were performed according to the method first described by Applemans (1) which consists of serial dilutions of bacteriophage prepared by the addition of .1cc of filtrate to 9.9 cc of broth and the transference of .1cc of this mixture to a second tube containing 9.9 cc of broth. This causes an initial dilution of 10^{-2} of bacteriophage and increasing dilutions of 10^{-4}, 10^{-6} etc. Culture suspension is added to each tube and the degree of lysis read after 24 and 48 hours incubation at room temperature.

Experiment 1.

Three peptones were used in the first experiment two of which were products of the Difco Labofatories, Bacto-Peptone and Bacto Proteose Peptone, the other was the well known Witte's Peptone. Published analyses (6) (14) of these peptones reveals the fact that they are distinguished by the variation in non-protein nitrogen with lesser variation in other nitrogen compounds. The total nitrogen does not vary so markedly.

Bacto-Peptone:

Total N - about	14.0%
Non-protein N "	13.0%
Polypeptid N "	.8%
Van Slyke Amino N "	8.2%
Free Ammonia	almost negligible

Proteose-Peptone:

Total N	12.5%
Non-protein N	6.9%
Protein N.	5.5%
Polypeptid N	2.3%
Van Slyke Amino N.	4.8%
Free Ammonia	almost negligible

Witte's Peptone:

Total N	15.0%
Non-protein N	6.0%
Protein N.	8.0%
Polypeptid N.	2.5%
Van Slyke Amino N.	8.0%
Free Ammonia	almost negligible

Turbidity standards were prepared for each concentration of peptone to be used, containing the following approximate numbers of bacteria per cc:

1. None
2. 1073×10^4
3. 2146×10^4
4. 4292×10^4
5. 8584×10^4
6. 1073×10^5
7. 2146×10^5
8. 4292×10^5
9. 7438×10^5
10. 8584×10^6

12 flasks were prepared containing:

100.00 cc Peptone broth medium, pH 7.6. 4 flasks each of Bacto, proteose, and Witte's peptone with concentrations of .5%, 1.0%, 2.0%, and 3.5%.

1.00 cc Staphylococcus phage in each flask

.50 cc Culture suspension made by washing a 24 hour Staph. aureus culture (Lab. No.1771) grown in a Blake bottle (beef infusion agar) with 20 cc of saline in each bottle.

The flasks were aerated by suction in order to keep the liquid agitated. At irregular intervals about 2cc of the content of each flask was removed and the turbidity determined by comparison with the standards.

In Table I is found an analysis of the results of experiment 1.

In Table II is found a graphic representation of the results of this first experiment. In this graph the log. of the concentration of bacteria per cc forms the ordinate and the time in hours the abscissa.

Table I.

Logs. of Maximum Bacterial Growth

	0.5%	1.0%	2.0%	3.5%
Bacto	7.93369	7.63266	7.93369	7.93369
Proteose	7.93369	8.33161	8.33161	8.33161
Witte	7.93369	7.33161	7.93369	8.63266

Time Required for Maximum Growth of Bacteria

Bacto	4 hrs.	4 hrs.	5 hrs.	5.5 hrs.
Proteose	5	5	5	5.5
Witte	5	5.5	8	8.0

Time Required for First Appearance of Lysis

Bacto	5. hrs.	5. hrs.	5.5 hrs.	6. hrs.
Proteose	5.5	5.5	5.5	6.
Witte	5.5	6.0	none in 8 hrs	6.

Time Required for Maximum Lysis

Bacto	5.5 hrs.	5.75 hrs.	6.0 hrs.	7.0 hrs.
Proteose	6.5	6.5	7.5	6.0
Witte	6.75	6.25	none in 8 hrs.	6.0

Lysates from the preceding were filtered at 24 hrs. and filtrates titrated.

Titration Readings at 48 hrs.

	10^{-2}	10^{-4}	10^{-6}	10^{-8}	10^{-10}	10^{-12}
* Bacto .5%	++++	++++	++++	-	-	-
Proteose .5%	++++	++++	++++	++++	-	-
Witte .5%	++++	++++	++++	++++	-	-
Bacto 1%	++++	++++	++++	-	-	-
Proteose 1%	++++	++++	++++	++++	-	-
Witte 1%	++++	++++	++++	-	-	-
Bacto 2%	++++	++++	++++	++++	++++	-
Proteose 2%	++++	++++	++++	+++	-	-
Witte 2%	++++	-	-	-	-	-
Bacto 3.5%	++++	++++	++++	++++	++++	-
Proteose 3.5%	++++	++++	++++	-	-	-
Witte 3.5%	++++	++++	-	-	-	-

Table II

Ordinate = Logs. of approx. numbers of bacteria present per cc.

Abscissa = Progress of lysis by hours

Note: 10×10^4 bacteria per cc is here considered apparent lysis, since it is definitely below the lowest visible conc. of organisms of this size.

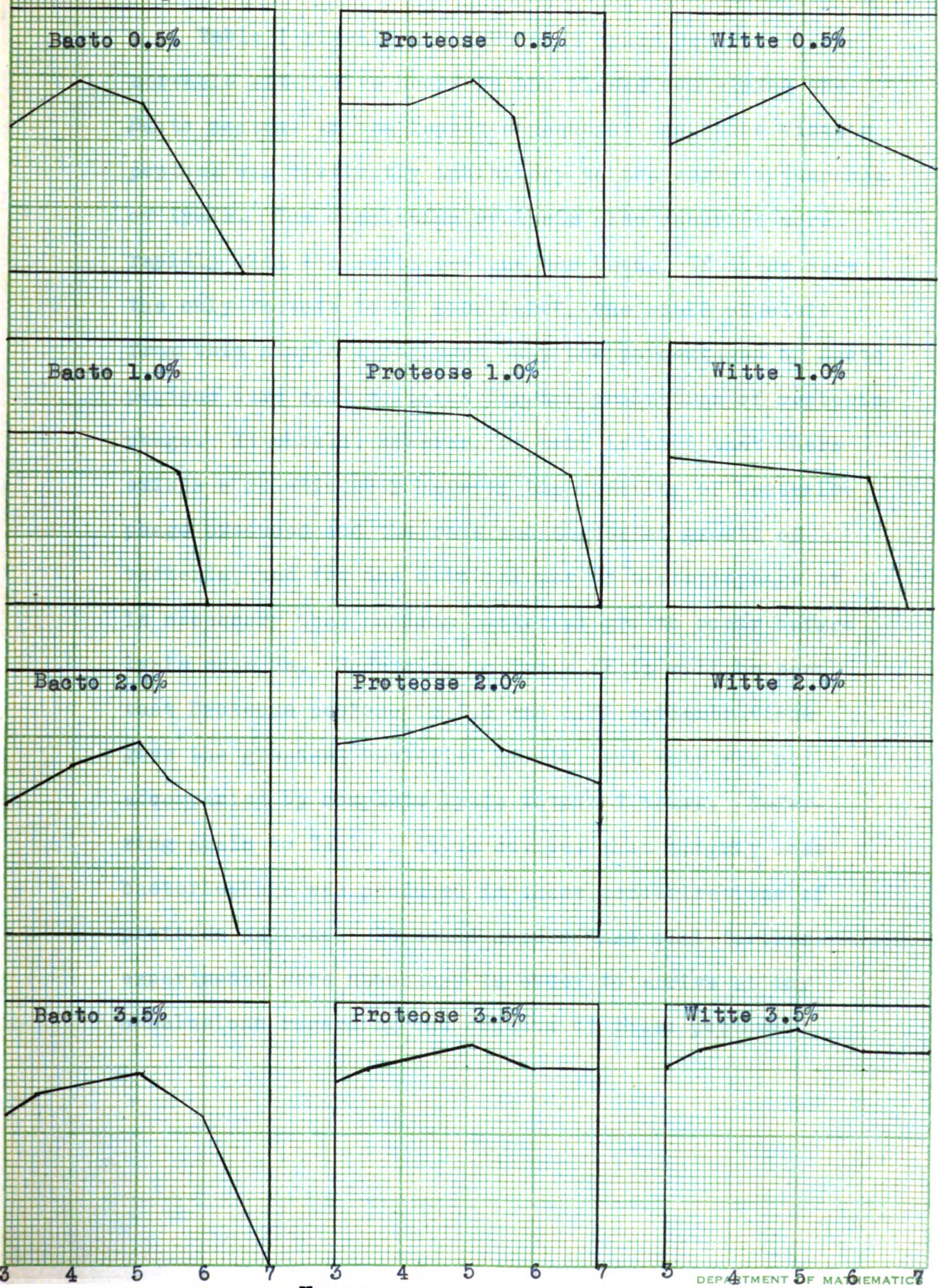

Table I reveals the fact that lysis was first observable after five hours in the flasks containing the Bacto peptone in the lowest concentrations. The Witte's peptone was distinctly inhibitory to the early appearance of lysis. The completion of lysis also occurred more rapidly when the Bacto peptone was used. If these were the only factors to be considered, one would conclude from this single experiment that low percentages of Bacto peptone were most suitable for the production of bacteriophage. It should, however, be born in mind that there exists a relationship between the quantity of bacteriophage produced and the rate and degree of growth of bacteria, and for the clinical use of bacteriophage it is necessary that maximum bacterial growth occur before lysis sets in. Referring to Table II it will be seen that Proteose peptone in 2.0 and 3.5% concentrations gave the greatest bacterial growth, but this was not accompanied by complete lysis within 7 hours. Referring again to Table I it is obvious that slightly less bacteriophage was produced in these concentrations of proteose peptone. It would appear then that further experiments were necessary in order to arrive at any conclusions regarding the effects of these peptones.

Experiment 2:

This experiment is essentially a repetition of experiment 1 with the exceptions that Parke Davis peptone with a total nitrogen content of about 14 % was substituted for the Witte's peptone and the concentrations of peptones changed to .1%, 1.0%, 5.0% and 10.0%.

Turbidity standards were prepared for each concentration as before.

The results are tabulated in Tables III and IV, and are comparable to Tables I and II with respect to the information given.

Table III.

Logs. of Maximum Bacterial Growth

	0.1%	1.0%	5.0%	10.0%
Bacto	8.52956	8.30771	8.13162	7.83059
Proteose	7.83059	8.52956	8.52956	8.83059*
Parke Davis	8.30771	8.30771	8.13162	8.30771*

Time Required for Maximum Growth of Bacteria

Bacto	4.0 hrs.	4.0 hrs.	4.0 hrs.	1.0 hrs.
Proteose	2.0	3.5	3.5	5.5
Parke Davis	4.0	4.0	4.5	7.25

Time Required for First Appearance of Lysis

Bacto	4.75 hrs.	4.5 hrs.	6.0 hrs.	6.25 hrs.
Proteose	6.0	5.5	6.5	5.25
Parke Davis	5.0	4.5	6.0	5.75

Time Required for Maximum Lysis

Bacto	6.0 hrs.	5.5 hrs.	7175 hrs.	7.75 hrs.
Proteose	6.25	6.25	6.5	6.75
Parke Davis	6.0	6.0	7.75	6.25

Lysates from preceding were filtered at 24 hrs. and filtrate titrated.

Titration Readings at 48 Hrs.

	10^{-2}	10^{-4}	10^{-6}	10^{-8}	10^{-10}	10^{-12}
Bacto .1%	++++	++++	++++	++++	++++	-
Proteose .1%	++++	++++	++++	++++	++++	++++
Pk.Davis .1%	++++	++++	++++	++++	++++	+
Bacto 1.%	++++	++++	++++	++++	++++	-
Proteose 1.%	++++	++++	-	-	-	-
Pk.Davis 1.%	++++	++++	++++	++++	++++	++++
Bacto 5.%	++++	++++	++++	++++	++	-
Proteose 5.%	++++	++++	++++	++++	++++	-
PK.Davis 5.%	++++	++++	++++	++++	++++	+
Bacto 10%	++++	++++	++++	++++	++++	+
Proteose 10%	++++	++++	++++	++++	++++	++++
Pk.Davis 10%	++++	++++	++++	++++	+++	-

*A secondary growth occurred, more profuse than the first.

Table IV.

Ordinate = Logs. of approx. numbers of bacteria present per cc.

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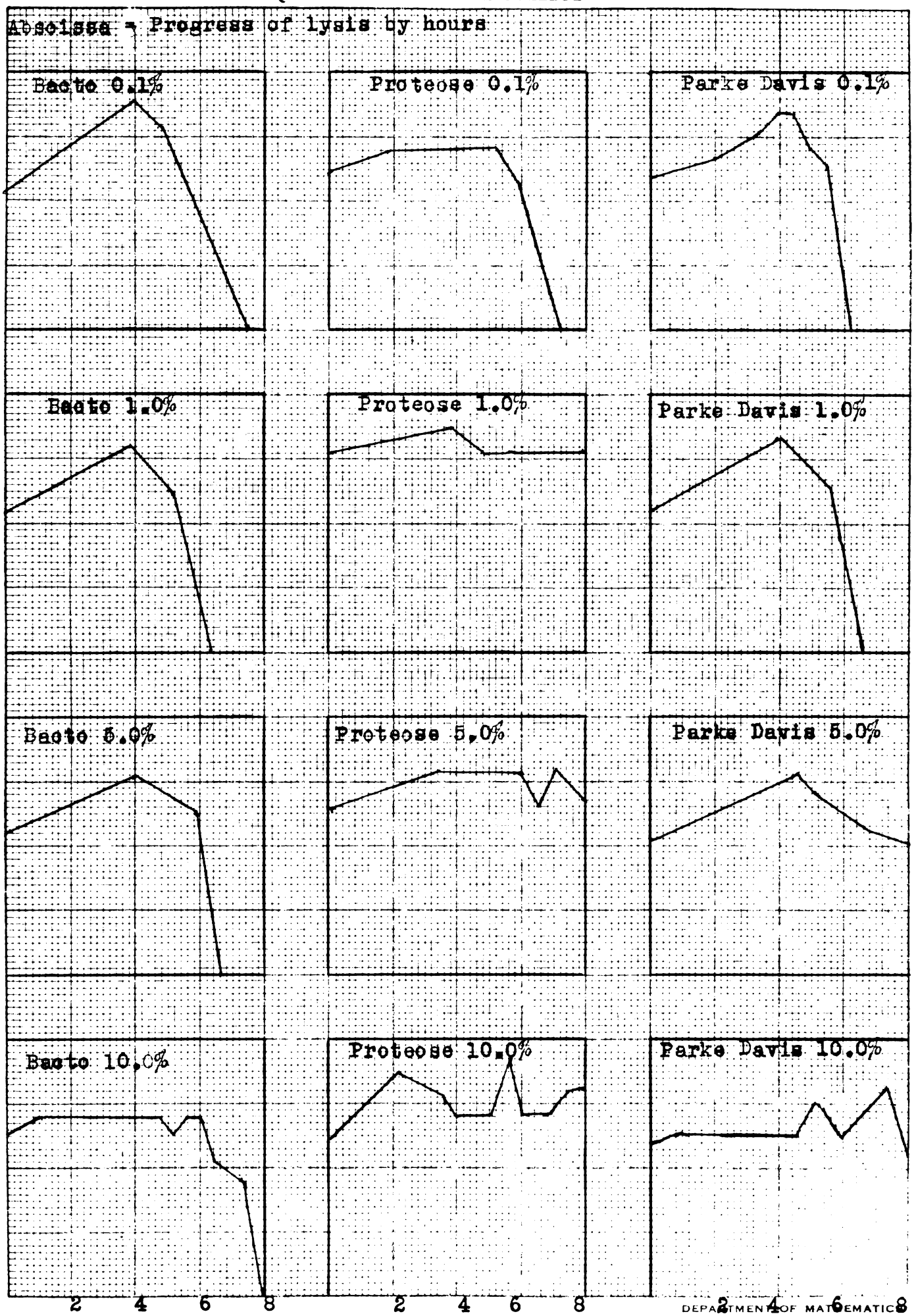

As in the preceding experiment, the first appearance of lysis occurred with the 1% Bacto peptone although the Parke Davis peptone gave a similar result. Completion of lysis was also first manifest in the 1.0% Bacto peptone broth. Maximum growth again was present with the Proteose peptone although the differences were slight in all cases. The amount of bacteriophage produced was comparable throughout the series.

Experiment 3.

In experiment 3 the same peptones were used and an attempt made to narrow the range of percentages, and also to determine the effects of complete absence of peptone. The results are tabulated in Tables V and VI.

Table V.

Logs. of Maximum Bacterial Growth

	No peptone (7.83059)	.5%	2.5%
Bacto		7.83059	7.52956
Proteose		7.83059	7.83059
Parke Davis		8.13162	8.13162

Time Required for Maximum Growth of Bacteria

	(4. hrs)		
Bacto		4.0 hrs.	4.5 hrs.
Proteose		5.0	4.0
Parke Davis		4.5	4.0

Time Required for First Appearance of Lysis

	(5 hrs.)		
Bacto		5.0 hrs.	5.75 hrs.
Proteose		5.25	5.5
Parke Davis		5.0	5.0

Time Required for Maximum Lysis

	(5.25 hrs.)		
Bacto		5.25 hrs.	6.0 hrs.
Proteose		5.5	7.5
Parke Davis		5.5	6.0

The lysates from the preceding were filtered at 24 hrs. and the filtrates titrated.

Titration Readings at 48 hrs.

	10^{-2}	10^{-4}	10^{-6}	10^{-8}	10^{-10}	10^{-12}
No peptone	+	+	++	++++	++++	-
Bacto .5%	++++	++++	++++	++++	++++	-
Proteose.5%	++++	++++	++++	++++	++++	-
Pk.Davis.5%	++++	++++	++++	++++	++++	++++
Bacto 2.5%	++++	++++	++++	++++	-	-
Proteose 2.5%	++	++	++++	++++	-	-
Pk.Davis 2.5%	++++	++++	++++	++++	++	-

Table VI

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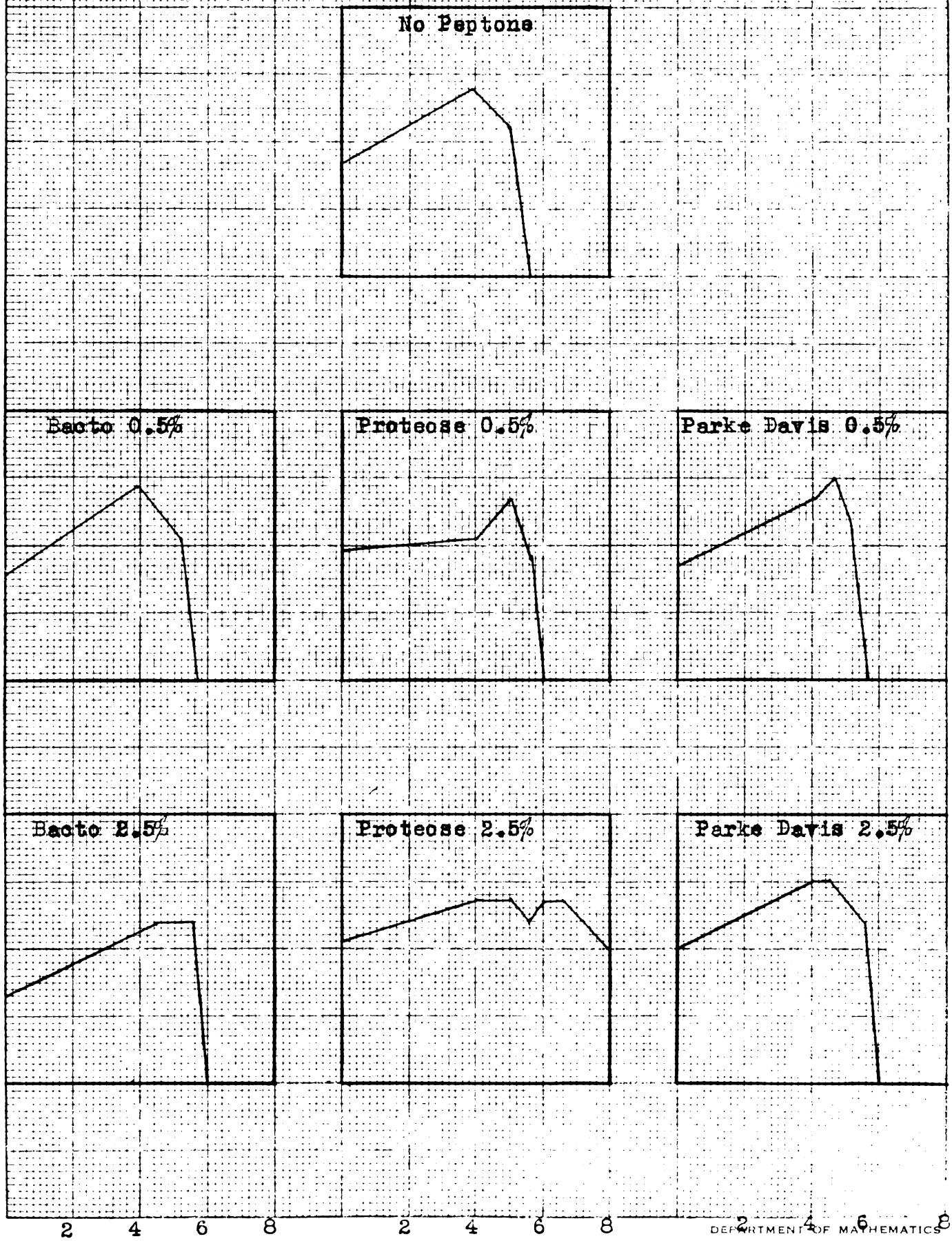
Ordinate = logs. of approx. numbers of bacteria present per cc.
Abscissa = Progress of lysis by hours.



In Table VI we again find that the first appearance of lysis and the shortest time required for complete lysis occurred with the least concentration of peptone, and that there is little difference with respect to the different peptones used when low concentrations are employed. Titrations again reveal that there is relatively little difference in the amount of bacteriophage produced under these conditions. In this instance the Parke Davis peptone gave a slightly greater concentration of bacteriophage.

Experiment 4.

Experiment 4 is in the nature of a check on previous experiments. The procedure was identical. The results are shown in Tables VII and VIII.

In Table VII we again find that the first appearance of lysis occurs in the flasks containing the least amounts of peptone. In the complete absence of peptone eight hours were required for complete lysis as compared to seven hours with the 1.0 and 2.0% peptone broths. The titrations show relatively little difference in the quantity of bacteriophage produced.

Table VII.

Logs. of Maximum Bacterial Growth

	No Peptone	1%	2%	5%
	(7.62325)			
Bacto		7.40140	7.22531	7.62325
Proteose		7.40140	7.92428	7.92428
Park Davis		7.40140	7.62325	7.62325

Time Required for Maximum Growth of Bacteria

	(4. hrs.)		
Bacto	5.0 hrs.	4.0 hrs.	5 hrs.
Proteose	5.5	5.0	5
Park Davis	4.0	5.0	5

Time Required for First Appearance of Lysis

	(5 hrs.)		
Bacto	5.5 hrs.	5.0 hrs.	6 hrs.
Proteose	6.0	6.5	8
Park Davis	5	5.5	6

Time Required for Maximum Lysis

	(8 hrs.)		
Bacto	7.0 hrs.	7.0 hrs.	7.5 hrs.
Proteose	7.5	7.5	8.0
Park Davis	7.5	8.0	8.0

The lysates from the preceding were filtered at 24 hrs. and the filtrates titrated.

	Titration Readings at 48 hrs.					
	10^{-2}	10^{-4}	10^{-6}	10^{-8}	10^{-10}	10^{-12}
No peptone	++++	++++	+++	+++	-	-
Bacto 1%	+++	-	-	-	-	-
Proteose 1%	++++	++++	++++	+++	-	-
Pk. Davis 1%	++++	++++	+++	+++	+	-
Bacto 2%	++++	++++	+++	+++	-	-
Proteose 2%	++++	++++	++++	++++	-	-
Pk.Davis 2%	++++	++++	+	+	-	-
Bacto 5%	++++	+++	-	+++	-	-
Proteose 5%	++++	++++	++++	+++	-	-
Pk.Davis 5%	++++	++++	+++	+++	-	-

Table VIII.

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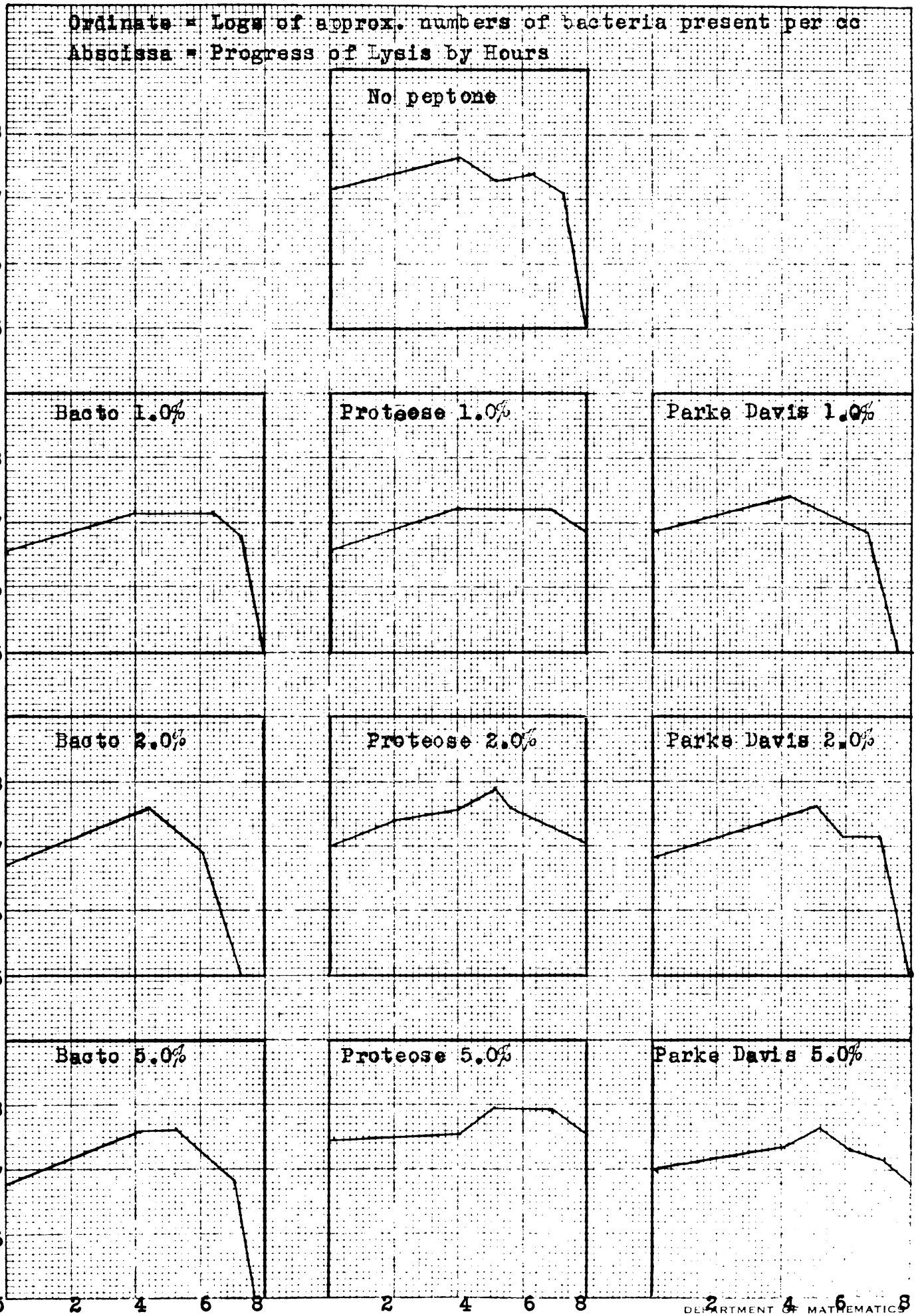

Summarizing the results obtained in the preceding four experiments, it is obvious that there is considerable variation even when a single experiment is repeated. Thus in the absence of peptone we found in experiment 3 that complete lysis occurred in five and a quarter hours,whereas in experiment 4 under similar conditions eight hours were required. Nevertheless, within rather wide limits the results with the different peptones in any one experiment are roughly comparable. There is no question but that high concentrations of peptone retard the progress of lysis under the influence of bacteriophage. This apparently is a result of a slower growth of bacteria, maximum concentrations of bacteria in all cases reaching an apparent critical point numerically before lysis occurred. Our experiments shed no light on the quality of phage produced through the use of different peptones. The quantity was fairly uniform regardless of the type or concentration of peptone used. It would appear then that one is justified in using any peptone available in the preparation of bacteriophage, and if,as in the case when the product is to be used for clinical purposes,peptone is undesirable, it may be omitted altogether.

But the problem of preparation of bacteriophage involves more than the quantity produced. In order to carry on successfully the propagation of this principle the quality must be taken into consideration, and the particular quality enabling the principle to maintain its virulence throughout a number of serial passages is highly important. If peptones exert no marked influence on the quantity of bacteriophage produced, it remains to be determined to what extent they influence the quality of the product. In Section B are found experiments bearing upon this question.

Section B. Effect of Peptone on Serial Transmission of Bacteriophage.

General Procedure:

The experiments reported here followed a materially different method than the preceding. Tubes were prepared containing 10 cc of medium. In one set was beef extract broth containing no peptone, and in the others Bacto, Proteose, and Parke Davis peptones in concentrations ranging from .1% to 10%. (Specific concentrations are listed on record of each experiment.) To each of these were added 2 drops of culture suspension obtained by washing one beef extract agar slant bearing a 24 hour growth of organisms desired, with 10 cc of saline; also .5cc of phage. After 48 hours the lysates were filtered through 3cm. Seitz filters and .5cc of the filtrate added to a new tube containing the same peptone concentration, and the series continued. These dilutions were carried through as many contacts as results dictated. The incubation was at room temperature. In all cases the degree of lysis was checked by comparison with controls containing the same amount of culture suspension, but containing no bacteriophage.

A record of the results of individual experiments appears on the following pages.

Experiment 1.

In experiment 1, Staphylococcus aureus, culture # 1771 which was shown to be susceptible to the Staphylococcus phage was employed. Broth was used containing Bacto, Proteose, and Parke Davis peptone in concentrations of 0.1, 1.0, 5.0 and 10.0%. The results are found in Table IX. After the seventh contact had occurred, a portion of the filtrate was titrated in order to determine the quantity of bacteriophage produced.

Even a cursory examination of this table indicates that the quality of the bacteriophage was altered in the low concentrations of peptone to such an extent that it failed to propagate itself successfully after a few contacts. Differences resulting from the brand of peptone used were insignificant.

Staph. aureus, # 1771 Table IX.

	No Peptone	.1% B.	.1% P.	.1% P.D.	1.0% B.	1.0% P.	1.0% P.D.	5.0% B.	5.0% P.	5.0% P.D.	10.0% B.	10.0% P.	10.0% P.D.
1 Contact													
24 hrs.	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++
48 "	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++
2 Contact													
24 hrs.	+	-	-	-	-	-	-	++++	-	++++	-	+	++++
48 hrs.	++++	-	-	+++	-	-	-	++++	++++	++++	-	++++	++++
3 Contact													
24 hrs.	-	-	-	-	-	-	-	-	-	++++	-	-	++++
48 hrs.	-	-	-	++++	-	-	-	+++	++++	++++	-	++++	++++
4 Contact													
24 hrs.	-	0*	0	-	0	0	0	+++	++++	++++	0	+++	++++
48 hrs.	-	0	0	+++	0	0	0	++++	++++	++++	0	++++	++++
5 Contact													
24 hrs.	0	0	0	+	0	0	0	+	+++	+++	0	++	+++
48 hrs.	0	0	0	+	0	0	0	++++	++++	+++	0	+++	++++
6 Contact													
24 hrs.	0	0	0	-	0	0	0	++++	-	++	0	+	+++
48 hrs.	0	0	0	-	0	0	0	++++	-	++++	0	+	++++
7 Contact													
24 hrs.	0	0	0	±	0	0	0	±	-	++++	0	-	++
48 hrs.	0	0	0	±	0	0	0	++++	++	++++	0	+	++++

* 0 = discontinued

Table IX - continued

	No Peptone	.1% B.	.1% P.	.1% P.D.	1.0% B.	1.0% P.	1.0% P.D.	5.0% B.	5.0% P.	5.0% P.D.	10.0% B.	10.0% P.	10.0% P.D.
8 Contact													
24 hrs.	0	0	0	-	0	0	0	++++	+++	++++	0	+	++++
48 hrs.	0	0	0	-	0	0	0	++++	++++	++++	0	+	++++
9 Contact													
24 hrs.	0	0	0	0	0	0	0	++	++	++++	0	+++	++++
48 hrs.	0	0	0	0	0	0	0	++++	++++	++++	0	++++	++++
10 Contact													
24 hrs.	0	0	0	0	0	0	0	+	+	++	0	+	+
48 hrs.	0	0	0	0	0	0	0	++++	++++	++++	0	++++	++++
11 Contact													
24 hrs.	0	0	0	0	0	0	0	++++	++++	++++	0	++++	++++
48 hrs.	0	0	0	0	0	0	0	++++	++++	++++	0	++++	++++

Titration of filtrate from 7th Contact

	10^{-2}	10^{-4}	10^{-6}	10^{-8}	10^{-10}	10^{-12}
Bacto 5%	++++	++++	++++	++	-	-
Proteose 5%	++++	++++	++++	++	++++	-
Pk.Davis 5%	++++	++++	++++	++++	-	-
Pk.Davis 10%	++++	++++	++	++	-	-

Experiment 2.

In order to provide a basis for a little more generalization, an entirely different bacteriophage (Colon bacillus) and its susceptible organism were employed in a procedure the same as with Experiment 1. Table X shows that the results here are likewise clear cut in that they indicate that the low percentages of peptone are less satisfactory for the serial transmission of bacteriophage. No further discussion of these results is indicated at this time.

B. coli, # 20c

Table X.

	No Peptone	.1%			1.0%			5.0%			10.0%		
		B.	P.	P.D.	B.	P.	P.D.	B.	P.	P.D.	B.	P.	P.D.
1 Contact													
24 hrs.	++++	++++	++++	++++	++++	+++	++++	++++	++++	++++	++++	++++	++++
48 hrs.	++++	+++	++	++	++++	+	++	++++	++	++++	+++	+++	++++
2 Contact													
24 hrs.	-	++++	-	++	+++	+	++	++++	++	++++	++++	++++	++++
48 hrs.	-	++++	-	+	+++	++	++	++++	+++	+++	++++	++++	+++
3 Contact													
24 hrs.	-	++++	-	-	+++	++	+++	++++	++	+++	+++	++	+++
48 hrs.	+++	++++	-	-	+++	+++	+++	++++	+++	++++	++++	+++	++++
4 Contact													
24 hrs.	++++	++++	++++	++++	++++	++++	++++	++++	+	++++	++++	++++	++++
48 hrs.	++++	++++	+	++++	++++	++	++++	++++	+	++++	++++	++	++++
5 Contact													
24 hrs.	+++	++++	+	++	++++	++	++++	++++	+	++++	++++	+	++++
48 hrs.	+++	+++	+	++++	++++	++	++++	++++	++	++++	++++	++++	++++
6 Contact													
24 hrs.	+++	+	-	+	+++	+	+++	++++	+	++++	++++	++++	++++
48 hrs.	+++	+	-	+	++++	++	++++	++++	+	++++	++++	++++	++++
7 Contact													
24 hrs.	++	+	++	++	+++	++	++++	++++	+++	++++	++++	++++	++++
48 hrs.	++	+	+	+	+++	++	++++	++++	+++	++++	++++	+++	+++

Table X - continued

	No Peptone	.1% B.	.1% P.	.1% P.D.	1.0% B.	1.0% P.	1.0% P.D.	5.0% B.	5.0% P.	5.0% P.D.	10.0% B.	10.0% P.	10.0% P.D.
8 Contact													
24 hrs.	++++	++	++++	+	++++	+++	++++	++++	++++	++++	++++	++++	++++
48 hrs.	++++	++	++++	-	++++	++	++++	++++	++	++++	++++	++	++++
9 Contact													
24 hrs.	+	++	+++	±	++++	++++	++++	++++	++	++++	++++	+++	++++
48 hrs.	-	+	+++	-	++++	+++	+++	++++	+++	++++	++++	+++	++++
10 Contact													
24 hrs.	-	-	+++	-	++++	++++	++++	++++	++++	++++	++++	++++	++++
48 hrs.	-	-	+++	-	++++	++++	++++	++++	++++	++++	++++	++++	++++
11 Contact													
24 hrs.	-	+	++++	+++	++++	++++	++++	++++	+++	++++	++++	++++	++++
48 hrs.	-	-	++++	++	++++	++++	++++	++++	++++	++++	++++	++++	++++
12 Contact													
24 hrs.	-	-	+++	+++	0*	0	0	0	0	0	0	0	0
48 hrs.	-	-	++	++	0	0	0	0	0	0	0	0	0
13 Contact													
24 hrs.	-	-	++	-	0	0	0	0	0	0	0	0	0
48 hrs.	-	-	+++	-	0	0	0	0	0	0	0	0	0
14 Contact													
24 hrs.	+	+	++	-	0	0	0	0	0	0	0	0	0
48 hrs.	-	-	+++	-	0	0	0	0	0	0	0	0	0

* 0= Discontinued

Table X - continued

Titration of filtrates from 8 contact

	10^{-2}	10^{-4}	10^{-6}	10^{-8}	10^{-10}	10^{-12}	10^{-14}
No Peptone	++++	+++	+++	+	-	-	-
Bacto .1%	++++	++++	++	+	-	-	-
Proteose .1%	++++	+++	+++	++	-	-	-
Pk.Davis .1%	++	++	+	-	-	-	-
Bacto 1.0%	++++	++++	++++	++++	+	-	-
Proteose 1.0%	++	++	+	-	-	-	-
Pk.Davis 1.0%	++++	++	++	+	-	-	-
Bacto 5.0%	++++	++++	++++	+++	++	+	-
Proteose 5.0%	++++	++++	+++	+	-	-	-
Pk.Davis 5.0%	++++	++++	+++	+++	-	-	-
Bacto 10.0%	++++	++++	+++	+++	+	-	-
Proteose10.0%	++++	++++	+++	+++	-	-	-
Pk.Davis10.0%	++++	++++	+++	+	+	-	-

PART II. Work with Synthetic Medium

Section A. Rate and Degree of Lysis in synthetic medium.

In as much as high concentrations of peptone seemed desirable for the propagation of bacteriophage, and since they are undesirable in a product to be used for clinical application, it would be of material assistance to find some substitute for the peptone which might enable one to carry on the propagation of phage in mediums of low nitrogen content. A recent publication by Hughes (9) concerning a growth stimulating substance found in meat extract suggested a solution to the problem. It was considered that the addition of this growth stimulating substance to a synthetic medium might provide the elements necessary for the development of an active bacteriophage capable of successful propagation.

Several investigators have reported the use of synthetic mediums for the preparation of bacteriophage for therapeutic use. Caldwell (5) found that a medium described by Ayers gave satisfactory results with the Colon bacillus, but that Uschinsky's medium

was not satisfactory. MacNeal prepared a synthetic medium which he found suitable.

In view of these results it seemed advisable to select one which gave the best growth of the organism to be used in the following experiments. Accordingly, four mediums, Uschinsky's glycerol ammonium lactate solution, Uschinsky's asparagin salt solution, Jordan's snythetic medium, MacNeal's medium, (10) (15), and after inoculation with Staphylococcus and with Colon bacillus, Uschinsky's Asparagin Salt solution was selected. This medium has the following formula:

Dist. water	1000 cc
Asparagin	3.4 g
NaCl	5.0 g
$MgSO_4$	.2 g
$CaCl_2$	.1 g
KH_2PO_4	1.0 g
$FeSO_4$	Trace

The growth stimulating substance was contained in the acetone soluble portion of beef extract, which was added to the synthetic medium.

Kjehldal nitrogen determination showed:

Uschinsky's asparagin salt solution plus activator contains.....84.50 mgs. N per 100 cc.

Beef extract broth (used as control).....218.16 mgs. N per 100 cc.

Section A. Rate and Degree of Lysis in Synthetic Medium containing growth stimulating substance.

Experiment 1.

The experimental methods employed were identical with those described under Section A of Part I; that is, noting the progress of lysis in mechanically agitated media, upon introduction of phage and susceptible culture.

Culture: Staph. aureus # 1963
Phage: #017873A Staphylococcus
Mediums: Beef Extract broth
Uschinsky's asparagin Salt sol. plus growth stimulating substance.

Turbidity standards were prepared as usual.

The results appear in Tables XI and XII.

Table XI.

Logs. of Maximum Bacterial Growth

Beef Extract Broth	Synthetic Medium*
7.50772	7.50772

Time Required for Maximum Growth of Bacteria

1.5 hrs.	3.0 hrs.

Time Required for First Appearance of Lysis

7.25 hrs.	None at 10.5 hrs. Complete at 24 hrs.

Time Required for Maximum Lysis

8.0 hrs.	10.5 - 24 hrs.

The lysates from the preceding were filtered at 24 hours and the filtrates titrated.

Titration Readings at 48 hrs.

	10^{-2}	10^{-4}	10^{-6}	10^{-7}	10^{-8}	10^{-9}	10^{-11}
Beef Ext.Broth	++++	++++	++++	-	-	-	-
Synthetic Media	++++	++++	++++	++	-	-	-

* The term synthetic mediumrefers to Uschinsky's asparagin salt solution containing a constant amount of activator.throughout the remainder of experiments.

Table XII.

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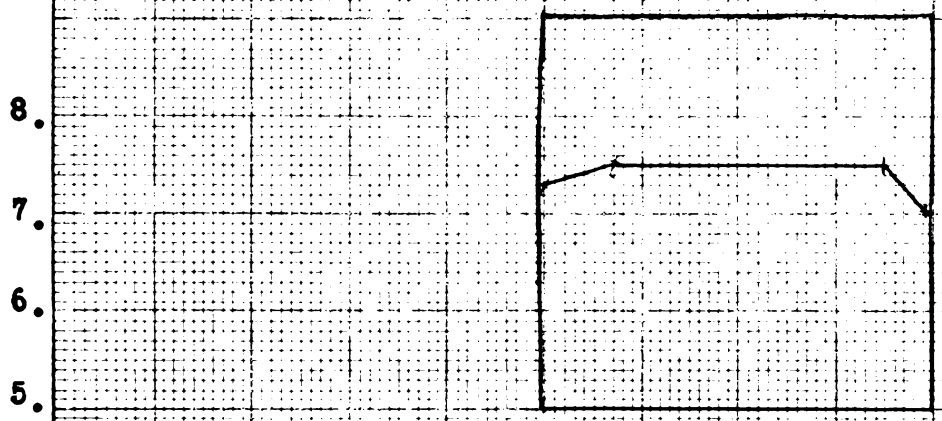
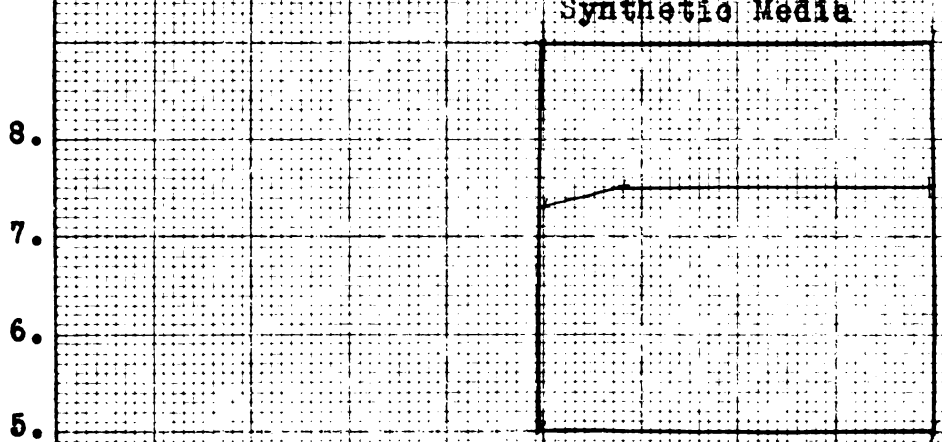
Ordinate = logs. of approx. numbers of bacteria present / cc.

Abscissa = progress of lysis by hours.

Beef Extract Broth



Synthetic Media



Note: Both these cultures stood unobserved for sixteen hours after the completion of the above readings. At the expiration of that time, lysis was apparently complete. (See titration Readings preceding page.)

2 4 6 8

Table XI shows that the progress of lysis was slow in both mediums. However, the quantity of bacteriophage produced was essentially the same as is shown by the titration results.

Experiment 2.

Experiment two is a repetition of experiment 1 with the exception of the fact that two different cultures of Staphylococcus were applied. Results are shown in Tables XIII and XIV.

It is quite obvious in this case that the beef extract broth permitted more rapid lysis and that little difference resulted in respect to the organism employed. The titrations were essentially identical.

Table XIII.

Logs. of Maximum Bacterial Growth

	Beef Ext.Broth	Synthetic Medium
Staph. aureus #1963	7.50772	7.50772
Staph. aureus # 1961	7.50772	7.50772

Time Required for Maximum Growth of Bacteria

Staph. aureus #1963	5.5 hrs.	5.5 hrs.
Staph. aureus #1961	5.5 hrs.	5.5 hrs.

Time Required for First Appearance of Lysis

Staph. aureus #1963	6.0 hrs.	6.5 hrs.
Staph. aureus #1961	6.0 hrs.	6.5 hrs.

Time Required for Maximum Lysis

Staph. aureus #1963	10. hrs.	14.0 hrs.
Staph. aureus #1961	8.5 hrs.	10.0 hrs.

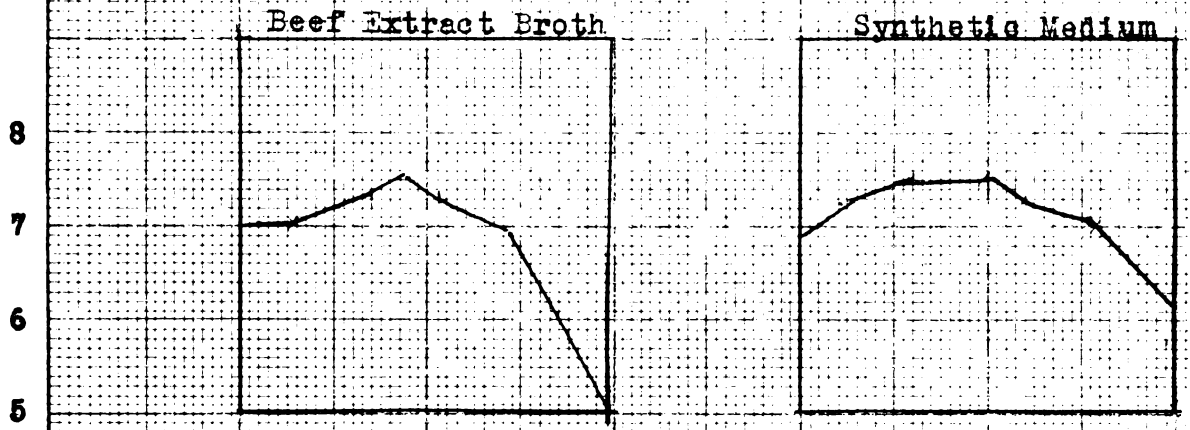
The lysates from the preceding were filtered at 24 hours and the filtrates titrated.

Titration Readings at 48 hrs.

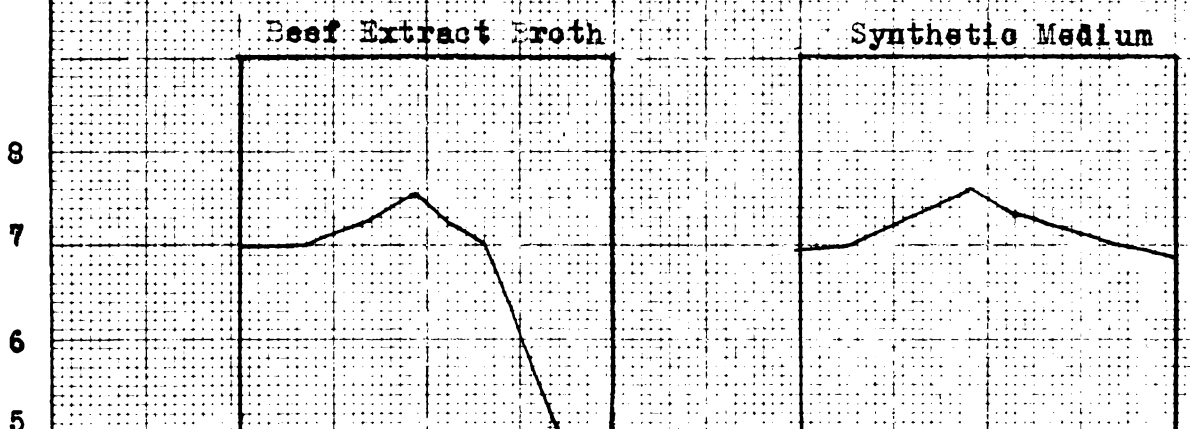
	10^{-2}	10^{-4}	10^{-6}	10^{-7}	10^{-8}	10^{-9}
Staph #1963						
Beef Ext.Broth	++++	++++	+++	-	-	-
Synthetic Medium	++++	++++	+++	-	-	-
Staph #1963				-	-	-
Beef Ext. Broth	++++	++++	++++	-	-	-
Synthetic Medium	++++	++++	++++	-	-	-

Ordinate = logs. of approx. numbers of bacteria present / cc.
Abscissa = progress of lysis by hours.

Staph. aureus # 1963



Staph. aureus # 1961



Note: In both cases the cultures in the synthetic media showed apparently complete lysis at the end of 24 hours. (See titration results on preceding page)

Experiment 3.

Colon bacillus with two different bacteriophage strains were used instead of the Staphylococcus employed in the previous two experiment. Tables XV and XVI show no distinct differences with the two methods employed.

Table X V.

Logs. of Maximum Bacterial Growth

	Beef Ext.Broth	Synthetic Medium
E.coli (phage M.K.13)	6.68124	6.68124
E.coli (" " 16)	6.68124	6.68124
Time Required for Maximum Growth of Bacteria		
E.coli (phage M.K.13)	2 hrs.	2 hrs.
E.coli (" M.K.16	2 hrs.	2 hrs.
Time Required for First Appearance of Lysis		
E. coli (phageM.K.13)	3 hrs.	3 hrs.
E. coli (" " 16)	3 hrs.	3 hrs.
Time Required for Maximum Lysis		
E.coli (phage M.K.13)	3 hrs.	3 hrs.
E.coli (phage M.K.16)	3 hrs.	3 hrs.

The lysates from the preceding were filtered at 5 hrs. and the filtrates titrated.

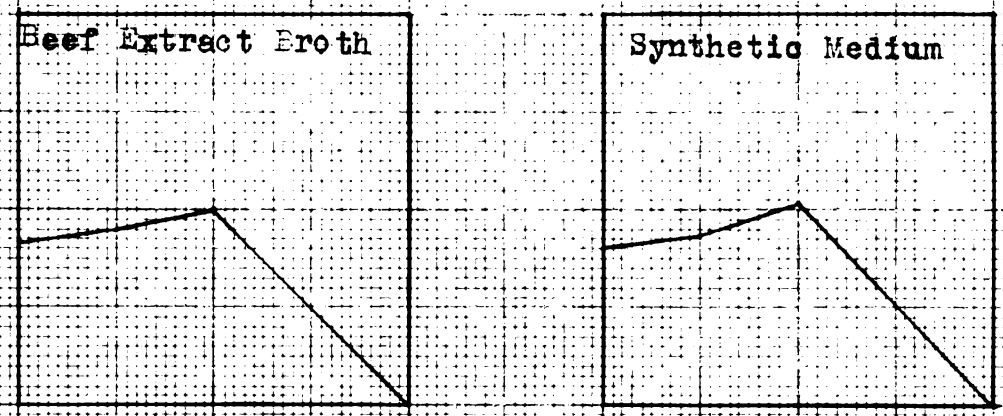
Titration Readings at 24 hours

	10^{2}	10^{-4}	10^{-6}	10^{-8}	10^{-10}	10^{-12}
E.coli						
With phage #M.K.13	++++	++++	++++	++++	++++	-
Beef Ext. Broth						
Synthetic	++++	++++	++++	++++	++++	-
With phage #M.K.16						
Beef Ext. Broth	++++	++++	++++	++++	++++	-
Synthetic	++++	++++	++++	++++	++++	-

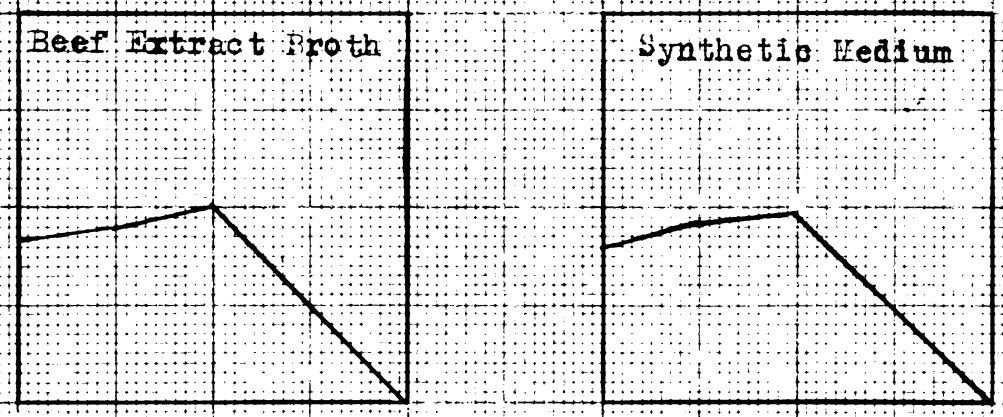
Ordinate = Logs. of approx. numbers of bacteria per cc.

Abscissa = Progress of lysis in hours.

E. coli with phage # M.K. 13



E. coli with phage # M.K. 16



The results of this group of experiments indicate quite clearly that so far as the quantity of phage produced in synthetic mediums to which the growth stimulating substances had been added is concerned, they are preferable for the preparation of this principle since their total nitrogen content is far below that of the ordinary extract broth with or without peptone. It remains to be determined, however, whether such mediums can be used successfully in the propagation of bacteriophage. This problem is investigated in Section B.

Section B. Transmission of Bacteriophage in Synthetic Medium.

The procedure employed in these experiments was similar to that used in Section B of Part I; that is, .5 cc of phage was added to a bacterial suspension to bring about lysis, the lysed bacterial suspension was passed through a Mandler filter and .5cc of this filtrate was added to a second bacterial suspension. After lysis had occurred the procedure was repeated and the series continued in this manner.

Beef extract broth and the synthetic medium broth with and without the growth stimulating substance were used. Staph. aureus, and Colon bacillus were the cultures employed.

There appears to be no occasion for considering these experiments separately since the procedures were essentially identiaal and the results are tabulated in Tables XVII to XXV inclusively. It is apparent from all of them that the serial passage of bacteriophage in the synthetic medium continued uninterrupted through varying numbers of contacts, and that the absence of of the growth stimulating substance resulted in the disappearance of the bacteriophage in all but one instance.

Table XVII.

Exp. (1) Culture: Staph. aureus # 1910

Phage: # 017873A (Staph.)

	Beef Extract Broth	Synthetic
1st contact	++++	++++
2nd contact	++++	++++
3rd contact	++++	++++
4th contact	++++	++++

(4th contact titrated)

Titration Readings at 48 hours

	10^{-2}	10^{-4}	10^{-6}	10^{-7}	10^{-8}	10^{-9}
Beef Extract Broth	++++	++++	++	-	-	-
Synthetic	++++	++++	+	-	-	-

Table XVIII

Exp. (2) Culture: Staph. aureus # 1963

Phage: # 017873A Staph.

	Beef Extract Broth (Lysis)	Synthetic (Lysis)
1st contact	++++	++++
2nd contact	++++	++++
3rd contact	++++	++++
4th contact	++++	++++
5th contact (titrated)	++++	++++
6th contact	++++	++++
7th contact	++++	++++
8th contact	++++	++++
9th contact	++++	++++
10th contact	++++	++++
11th contact	++++	++++
12th contact	++++	++++
13th contact	++++	++++
14th contact	++++	++++
15th contact (titrated)	++++	++++

Titration Readings at 48 hours.
(Filtrate from 5th contact)

	10^{-2}	10^{-4}	10^{-6}	10^{-7}	10^{-8}	10^{-9}	10^{-10}
Beef Ext.Broth	++++	++++	++++	+	-	-	-
Synthetic	++++	++++	++++	+	-	-	-

(Filtrate from 15th contact)

Beef Ext.Broth	++++	++++	++++	++++	++	-	-
Synthetic	++++	++++	++++	++++	++	-	-

Table XIX.

Exp. (3) Culture: Staph. aureus # 1961

Phage: # 017873A (Staph)

	Beef Extract Broth (Lysis)	Synthetic (Lysis)
1st contact	++++	++++
2nd contact	++++	++++
3rd contact	++++	++++
4th contact	++++	++++
5th contact	++++	++++
6th contact	++++	++++
7th contact	++++	++++
8th contact	++++	++++
9th contact	++++	++++
10th contact	++++	++++
11th contact	++++	++++
12th contact(titrated)	++++	++++
13th contact	++++	++++
14th contact	++++	++++
15th contact	++++	++++
18th contact	++++	++++
17th contact	++++	++++
18th contact(titrated)	++++	++++

Titration Readings at 48 hours

Filtrate from 12th contact

	10^{-2}	10^{-4}	10^{-6}	10^{-7}	10^{-8}	10^{-9}	10^{-10}
Beef Ext.Broth	++++	++++	-	-	-	-	-
Synthetic	++++	++++	-	-	-	-	-

Filtrate from 18th contact

Beef Ext. Broth	++++	++++	++++	++++	+	-	-
Synthetic	++++	++++	++++	++++	+	-	-

Table XX.

Exp. (4) Culture: Staph. aureus # 1963

Phage: # 90 (Staph.)

	Beef Extract Broth (Lysis)	Synthetic (Lysis)
1st contact	++++	++++
2nd contact	++++	++++
3rd contact	++++	++++
4th contact	++++	++++
5th contact(titrated)	++++	++++
6th contact	++++	++++
7th contact	++++	++++
8th contact	++++	++++
9th contact	++++	++++
10th contact	++++	++++
11th contact	++++	++++
12th contact	++++	++++
13th contact	++++	++++
14th contact	++++	++++
15th contact	++++	++++
16th contact	++++	++++
17th contact	++++	++++
18th contact	++++	++++
19th contact	++++	++++
20th contact(titrated)	++++	++++

Titration Readings at 48 hours

Titration of filtrate from 5th contact

	10^{-2}	10^{-4}	10^{-6}	10^{-7}	10^{-8}	10^{-9}
Beef Ext. Broth	++++	++	-	-	-	-
Synthetic	++++	++	-	-	-	-

Titration of filtrate from 20th contact

Beef Ext. Broth	++++	++++	-	-	-	-
Synthetic	++++	++++	-	-	-	-

Table XXI.

Experiments 5 and 6.

Note: In 5 and 6, synthetic media both with and without the activator were used as well as beef extract broth. Bacterial growth in the synthetic medium without the presence of activator was so slight that the presence or absence of lysis was hardly discernible by observation. Therefore, the presence of phage was tested for by adding 1 cc of the filtrate to a tube of beef extract broth containing 1 drop of culture suspension.

Exp.(5) Culture: Staph. aureus # 1963
Phage: #107873A (Staph.)

	Beef Extract Broth	Synthetic (with activator)	Synthetic (without activator)
	Lysis	Lysis	Lysis
1st contact	++++	++++	+
2nd contact	++++	++++	?
3rd contact	++++	++++	-

Exp.(6) Culture: Staph. aureus # 1961
Phage: E62B (Staph.)

	Beef Ext. Broth	Synthetic (with activator)	Synthetic (without activator)
	Lysis	Lysis	Lysis
1st contact	++++	++++	?
2nd contact	++++	++++	-
3rd contact	++++	++++	-

Table XXII

Exp.(7) Culture: E. coli # 7c
Phage: M.K. 13 (Coli phage)

	Beef Extract Broth (Lysis)	Synthetic (Lysis)
1st contact	++++	++++
2nd contact	++++	++++
3rd contact	++++	++++
4th contact	++++	++++
5th contact	++++	++++
6th contact	++++	++++
7th contact	++++	++++
8th contact	++++	++++
9th contact	++++	++++
10th contact(titrated)	++++	++++

Titration Readings at 24 hours of Filtrate from 10th contact

	10^{-2}	10^{-4}	10^{-6}	10^{-7}	10^{-8}	10^{-9}	10^{-10}
Beef Extract Broth	++++	++++	++++	++++	++++	++++	++++
Synthetic	++++	++++	++++	++++	++++	++++	++++

Table XXIII

Exp.(8) Culture: E. coli # 7c

Phage: M. K. 16 (coli)

	Beef Extract Broth (Lysis)	Synthetic (Lysis)
1st contact	++++	++++
2nd contact	++++	++++
3rd contact	++++	++++
4th contact	++++	++++
5th contact	++++	++++
6th contact(titrated)	++++	++++
7th contact	++++	++++
8th contact	++++	++++
9th contact	++++	++++
10th contact	++++	++++
11th contact	++++	++++
12th contact(titrated)	++++	++++
		++

Titration Readings at 24 Hours

Titration of filtrate from 6th contact:

	10^{-2}	10^{-4}	10^{-6}	10^{-8}	10^{-10}	10^{-12}	10^{-14}
Beef Extract Broth	++++	++++	++++	++++	++++	++++	-
Synthetic	++++	++++	++++	++++	++++	++++	-

Titration of filtrate from 12th contact:

Beef Extract Broth	++++	++++	++++	++++	++++	-	-
Synthetic	++++	++++	++++	++++	++++	++++	-

Table XXIV.

Exp(9) Culture: E. coli,#7c

Phage: M.K. 13 (Colon phage)

	Beef Extract Broth (Lysis)	Synthetic (Lysis)
1st contact	++++	++++
2nd contact	++++	++++
3rd contact	++++	++++
4th contact	++++	++++
5th contact	++++	++++
6th contact	++++	++++
7th contact	++++	++++
8th contact	++++	++++
9th contact	++++	++++
10th contact(titrated)	++++	++++
11th contact	++++	++++
12th contact	++++	++++
13th contact	++++	++++
14th contact	++++	++++
15th contact(titrated)	++++	++++

Titration Readings at 24 hours

Titration of filtrate from 10th contact

	10^{-2}	10^{-4}	10^{-6}	10^{-8}	10^{-10}	10^{-12}	10^{-14}
Beef.Ext.Broth	++++	++++	++++	++++	++++	-	-
Synthetic	++++	++++	++++	++++	++++	++	-

Titration of filtrate from 15th contact

Beef.Ext.Broth	++++	++++	++++	++++	++++	++++	-
Synthetic	++++	++++	++++	++++	++++	++	-

Table XXV.

Experiments 10 and 11.

Note: In 10 and 11, Synthetic media both with and without activator were used. Lysis was tested for as in 5 and 6; that is, by adding one cc of the filtrate to beef extract broth containing 1 drop of culture.

Exp.(10) Culture: E. coli 7c

Phage: M.K. 13 (Colon)

	Beef Ext. Broth (lysis)	Synthetic (with activator) (lysis)	Synthetic (without activator) (lysis)
1st contact	++++	++++	+
2nd contact	++++	++++	?
3rd contact	++++	++++	-

Exp.(11) Culture: E. coli 7c

Phage: M.K. 16 (Colon)

	Beef Ext. Broth (Lysis)	Synthetic (with activator) (Lysis)	Synthetic (without activator) (Lysis)
1st contact	++++	++++	Present
2nd contact	++++	++++	"
3rd contact	++++	++++	"
4th contact	++++	++++	"
5th contact	++++	++++	"
6th contact	++++	++++	"
7th contact	++++	++++	"
8th contact	++++	++++	"

Discussion

Contrary to expectations, the presence or absence of peptone in culture medium in concentrations up to 10% had relatively little effect upon the ultimate production of bacteriophage of moderately high titre. Nor was there any marked advantage in one peptone over another with the possible exception of Witte's which, especially in the higher concentrations, gave a low yield. It would appear, then, that in the preparation of bacteriophage for clinical use, little or no peptone need be used. In the presence of low concentrations of peptone, however, bacteriophage is apparently not successfully propagated.

There is, however, a very important consideration that intervenes here which considerably modifies any conclusions that may be drawn at the present time. Propagation of bacteriophage involves the serial transmission of the principle from culture to culture with intervening filtration. The filters customarily employed are made of infusorial earth, asbestos or collodion. Those most commonly used are the Berkfeld, Mandler, Chamberland, and Seitz. This introduces a conflicting factor in the transmission of the principle for any loss of potency is as

conceivably occasioned by absorption to the filter as by the action of any constituent of the medium. Thus, although Larkum and Semmes (12) reported that Seitz filters exerted no appreciable effect on the bacteriophage, the mediums they employed were constant in composition. In the work here reported, the Seitz filter proved unsuited for serial transmission of bacteriophage when low concentrations of peptone were employed, but no difficulty was encountered when the higher concentrations were used. Although no further attempt was made to investigate this phase of the problem, it appears entirely warranted to to assume that the loss of potency of bacteriophage on serial transmission was occasioned not so much by the effect of the peptone upon the principle itself as by the effect of peptone on the filtration of the product. Although this phase of the problem formed no part of the experiments planned, the observation could not be neglected and is, therefore, presented here.

From the standpoint of practical use of the observation made, it might be said that in as much as we are at present limited to certain types of filters in the

preparation of bacteriophage, care should be taken to provide conditions enabling these filters to perform effectively; that is, to allow the passage of bacteriophage. This is apparently assured in the presence of moderately high concentrations of peptone. In as much, however, as high concentrations of peptone are undesirable in the material to be adapted to clinical use, it is obvious that large lots of bacteriophage for therapeutic purposes must be made in a medium containing little or no peptone, and that serial passages of bacteriophage to provide the "starter" for each new lot of clinical material must be prepared and carried along in mediums containing higher concentrations of peptone (5%-10%).

Conclusions

The results of the comparative study of four brands of commercial peptone added to beef extract broth indicate that these peptones in concentrations up to 10% exert no marked effect on the quantity of bacteriophage produced. High concentrations of peptones, however, retard the progress of lysis.

The propagation of bacteriophage through serial transfers is materially affected by the presence of high concentrations of peptone. A more active phage

results when higher concentrations of peptone are incorporated in the medium.

Synthetic medium containing growth stimulating substance is a suitable medium for the production of bacteriophage giving a product equal in amount and potency to that produced in beef extract broth with 1.0% peptone. The use of synthetic medium without the growth stimulating substance is not satisfactory for the production of bacteriophage.

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