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I hereby recommend that the thesis prepared under my supervision by Irvin W. Gibby

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be accepted as fulfilling this part of the requirements for the degree of Doctor of Philosophy

Approved by:

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STUDIES ON THE NUTRITION OF BACTERIUM TULARENSE

A dissertation submitted to the
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by

Irvin W. Gibby

M. S. University of Cincinnati 1942

STUDIES ON THE NUTRITION OF BACTERIUM TULARENSE

From the time that Bacterium tularense was first isolated up to the present, classical descriptions of the organism have stated that growth occurred only on the surface of solid media. The media that have been described usually contained chemically complex materials such as blood or serum. Such media are obviously of limited value if precise chemical studies are to be conducted.

The purpose of my investigations has been to define as completely as possible liquid media capable of supporting growth of Bacterium tularense.

In a previous publication (1) liquid media suitable for growth of the organism were discussed. Chemically undefined substances were present in those media. Quantitative measurement of the growth promoting activity and refinement of these unknown materials have made necessary the development of special techniques, and have demonstrated much useful and interesting information about the characteristics of liquid cultures of the organism.

All important literature dealing with growth was summarized in my previous thesis (1). Since that time the following studies have been reported. Berkman (2) in 1942 reported the growth of several strains in a complex hydrolyzed gelatin-amino acid medium to which had been added thiamin or cocarboxylase. In these studies determination of growth was made visually by estimating turbidity on the usual one plus, two plus, three plus, four plus basis. I have shown this system to be inconclusive and unreliable (1). Berkman further stated that growth was light, that it appeared only after four days of incubation, and that heavy inocula were necessary. No subcultures were reported. Altogether, the evidence presented suggests that only very feeble growth could have occurred. It is very doubtful if continued subcultivation could have been obtained with the medium described.

In 1944 Steinhaus, et al., (3) reported a liquid medium which is essentially the usual solid medium without agar. This medium is undoubtedly useful in the work these authors are conducting but has little value as a chemically defined medium.

Section I

Materials and Methods: In performing previous growth experiments (1) the various media were dispensed into bulb tubes onto which side arms 8 cm. in length had been sealed. The bulbs were approximately 32 mm. in diameter but as much as ±5 mm. variation occurred. The turbidity of growing cultures was determined by tipping the medium from the bulb into the side arm and comparing visually with turbidity standards. This method was a great improvement over any previous one for obtaining approximate quantitative data. Nevertheless, such determinations of turbidity were only approximate at best, and it became highly desirable to eliminate the "personal factor." For this reason a photoelectric turbidity comparator was obtained.*

New pyrex bulb tubes were adapted to fit this machine by increasing the length of the side arm to 16 cm. Turbid suspensions contained in the side arms reflected light at a 90° angle into a photoelectric cell. This machine was described by Krebs, et al. (4).

As stated previously considerable variation had occurred in bulb diameters. Although no ideal ratio of surface to volume of media has been worked out it was

* I am very greatly indebted to Dr. Harold J. Kersten of the Department of Physics, University of Cincinnati for the construction and maintenance of this machine.

definitely known that a relatively large surface was very important. For this reason the diameter of the new bulbs was standardized at 40 mm. Variations greater than 1 mm. were not permitted. The side arms were constructed from selected pyrex tubing having an outside diameter of 10.5 mm. Variations greater than ±0.12 mm. were not permitted. The bulb tubes illustrated in Fig. 1 have an overall height of approximately 13 cm.

The readings obtained from the photoelectric turbidity comparator are in terms of microamperes (hereafter referred to as M. A.). In checking the uniformity of readings obtained a variation greater than ±2 M. A. for different tubes was not encountered when tested with any given suspension. Such variations decreased almost to the vanishing point and were regarded as negligible with turbid suspensions which read 50 M. A. or less. This has been the working range wherever the machine has been used in assay work.

The readings obtained from the photoelectric turbidity comparator increase with increasing turbidity. For all practical purposes, and within the limits of ordinary usage, M. A. readings are directly proportional to turbidity. This is not strictly true throughout the entire range of the machine but for readings between 0 and 50 presentation of graphic and numerical data in M. A. causes no important loss of accuracy.

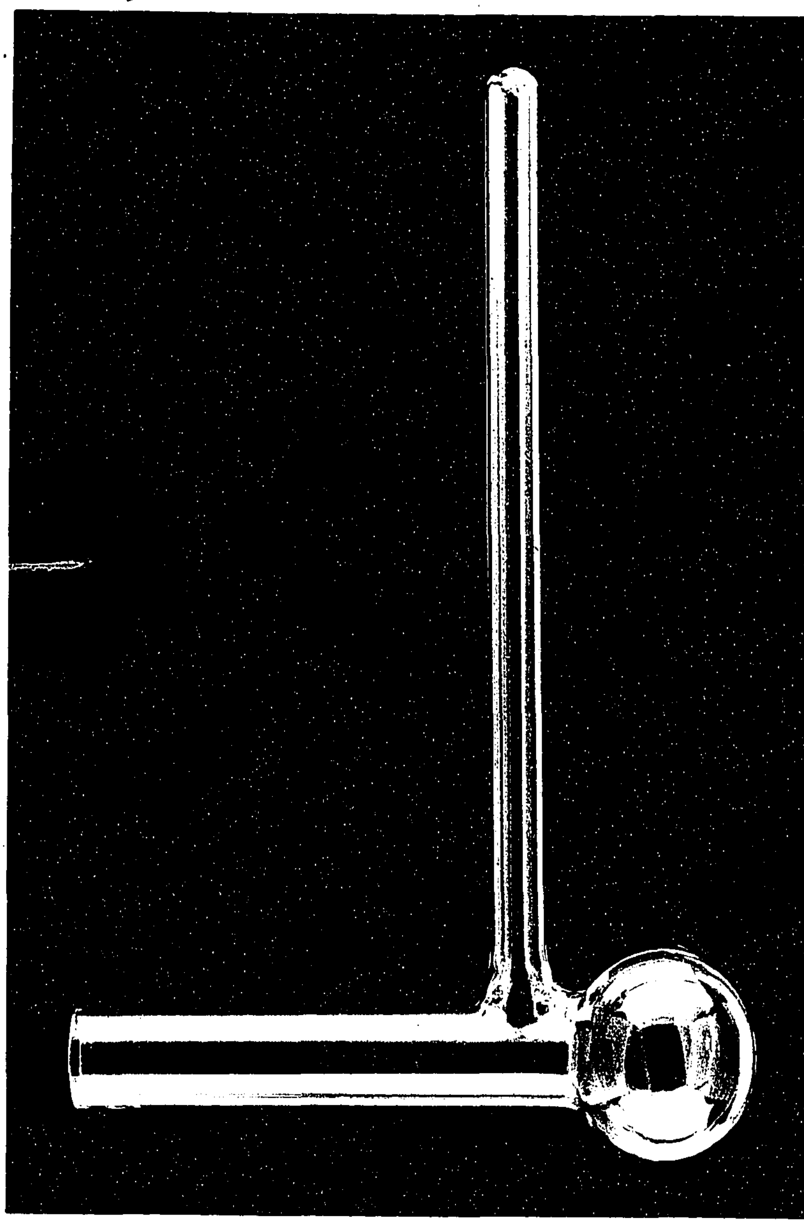


Fig. 1

In the work previously reported (1) certain essential materials and approximate optimal concentrations had been determined. The most simplified reliable medium defined at that time had the following composition:

Gelatin hydrolyzate	1.5- 2.0 per cent -- as gelatin
Blood cell extract	5.0-10.0 per cent -- as cells
Cystine	0.01 per cent
NaCl	1.5- 2.0 per cent total
Glycerine	0.5 per cent
$Na_2HPO_4 \cdot 12H_2O$	0.0625 per cent
KH_2PO_4	0.025 per cent
$MgSO_4$	0.0025 per cent

It had also been found that in this medium tryptophane, pimelic acid, nicotinic acid, pantothenic acid, biotin concentrate, and ferrous sulfate were without effect upon growth. This conclusion has been verified repeatedly.

Aside from the gelatin hydrolyzate the only substance which remained undefined was the blood cell extract. Accordingly, this material has received the emphasis of the present investigation. Blood cell extract was prepared in the following manner: One volume of packed unwashed human blood cells, from which all but a small percentage of the plasma had been removed, was diluted with 9 volumes of distilled water. The resultant fluid was brought to boiling with constant stirring. It was boiled for 5 to 10 minutes and, while still very hot, sufficient concentrated hydrochloric acid was added to lower the pH to 6.3. After cooling for 2 to 3 hours the material was placed in the refrigerator over night. By use of cheesecloth filters the coagulated hemoglobin was readily removed. The filtrate was light pink in color and was completely cleared of suspended material by pouring through the filter several times. The 1:10 filtrate was placed in a large distilling flask and concentrated under vacuum to one-fiftieth of its original volume. At this point the concentrate usually contained a great deal of precipitated material which was removed by filtration through fine paper. The filtrate was clear and tan-red in color.

Since most work was done with liquid preparations of blood cell extract, most measurements have been volumetric. Although solids content of much of the material is known, for the sake of convenience in preparing fractions, additions to basic media have been made according to the volume of packed cells represented by the extract or fraction. Thus by referring to the method of preparation it will be seen that the volume of the first crude filtrate can be assumed to represent one-tenth of its volume of blood cells. If this filtrate were concentrated fifty times, the concentrate would represent five times its own volume of blood cells, or 1 cc. of concentrate would equal 5 cc. of packed cells. Most stock solutions of blood cell extract have been concentrates made up in this fashion. Concentration of extracts was necessary so that they could be treated chemically; also appropriate dilutions could be readily made for use in media.

Concentrated extracts were originally stored in the refrigerator without sterilization. More recently they have been rapidly frozen and stored in a dry ice refrigerator.

The basic medium had the composition given above except that it was made up without blood cell extract. As all cultures were made up to a final volume of 5 cc. the stock medium was made up double strength -- for each tube 2.5 cc. were used.

In conducting an experiment the extract or fraction was diluted appropriately and added to basic medium in increasing quantities. After all additions had been made all media were adjusted to pH 6.9. The total volume for each tube was made up to 5 cc. with distilled water. The media were sterilized at 10 lbs. for 10 minutes.

Tubes were inoculated with 0.1 cc. of a suspension of bacteria which had been centrifuged from a 24 hour liquid culture and washed with saline.

M. A. readings and pH determinations were made at 24 hours, 48 hours, and 72 hours. In the early part of the work the pH values of cultures were determined by adding a loopful of culture to a drop of indicator on a spot plate. A platinum loop was used; the indicators were brom thymol blue, brom cresol purple, and brom cresol green. Although the general trend of such data was valid certain errors were inherent in this method. It will be shown that the use of unbuffered liquid media became necessary. With such media the pH of the mixture which resulted from adding a loopful of culture to a drop of indicator tended to be the pH of the indicator rather than the pH of the culture. Thus, cultures with pH values either greater or less than the pH of the indicator gave readings that were distorted toward the pH of the indicator. To avoid these errors as nearly as possible a new technique was worked out. Small pipettes were constructed from soft glass tubing with an inside diameter of 3 mm. A 200 mm. length of tubing was sealed at one end, and a hole approximately 5 mm. in diameter was blown in the side just at the sealed end of the tube. By immersing this portion of the pipette into any liquid a small sample could be withdrawn. The amount of sample could be varied at will by varying the angle at which the tube was held. These pipettes were sterilized in large test tubes, and at any desired time the pH of a culture could be determined by withdrawing approximately 0.05 cc. of medium.

By using a capillary dropper a small drop of a dilute solution of an appropriate sulfonphthalein dye was added through the side hole into the sample of medium. Mixing was effected by rocking the fluid up and down in the pipette. By comparing with standard buffers mixed with the same dye in the same way pH values could be determined which agreed with electrometric determinations to ±0.1 unit. The differences encountered in various cultures were of such magnitude that this degree of accuracy was sufficient.

Many preliminary experiments were conducted to determine optimal growth conditions. It has been known for several years that aeration greatly accelerates growth. Many different methods of aeration were tried in an attempt to utilize this stimulatory effect. The various schemes which have been tried can be briefly summarized as follows: Cultures were aerated slowly with coarse bubbles; rapidly with fine bubbles; air was blown on top of cultures so that no bubbling occurred; oxygen and oxygen-carbon dioxide mixtures were used instead of air; atmospheres of varying concentrations of oxygen were placed over cultures and the tubes were sealed, and cultures were shaken continuously with and without aeration. In practically all such experiments many cultures showed phenomenal growth in very short periods of time. No stimulation of growth in basic medium resulted from any aeration or shaking procedure.

However, these procedures were of no real value in assay work because the results as determined by the photoelectric turbidity comparator were very erratic. High readings were often obtained after 24 hours. Further incubation often resulted in still higher readings for some cultures and much lower readings for other cultures. Duplicate and triplicate tubes were practically never in agreement, differences being very great in some cases. Such results were of no statistical value whatever. Continuous shaking and aeration techniques were abandoned for assay work in which turbidity was measured by the photoelectric turbidity comparator.

Experiment 1

During the course of the investigation several lots of concentrated blood cell extract were made. These were tested for activity by combining aliquots with basic medium according to the method outlined. The quantities used, and the growth promoting effect of blood cell extract Lot No. 6, are shown in Table 1 and illustrated graphically in Fig. 2. This lot was one of the first highly potent extracts to be made. Even the smallest amount used had a pronounced growth promoting effect. Readings graphed against increasing concentrations showed increasing turbidity with a very regular curve up to 5 per cent. Beyond this amount the points were not quite so regular but the trend was still upward.

All cultures were adjusted to pH 6.9 prior to inoculation. Terminal pH values graphed against concentration gave a curve very similar to that for M. A. readings. Strongly acid end points were reached by the cultures containing a low concentration of extract, whereas increasing concentrations resulted in progressively higher pH values. Concentrations over 5 per cent resulted in approximately neutral pH values. No culture had a pH greater than 7.1. Daily pH determinations demonstrated clearly that those cultures that terminally were strongly acid had shown, day by day, a progressive drop in pH; the lower the concentration of

Table I

The effect of increasing concentrations of blood cell extract upon growth and pH

Per cent of blood cell extract	M. A. at 72 hours	pH at 72 hours
0	5	6.0
1	23	6.0
2	32	6.0
3	36	6.2
4	39	6.4
5	41	6.6
6	40	6.7
7	40	6.7
8	43	6.7
9	46	6.8
10	47	6.8
12.5	46	7.0
15	49	7.1
17.5	52	7.1
20	49	7.1

Determinations of pH made by the use of brom thymol blue, values less than 6.0 not obtainable by this method.

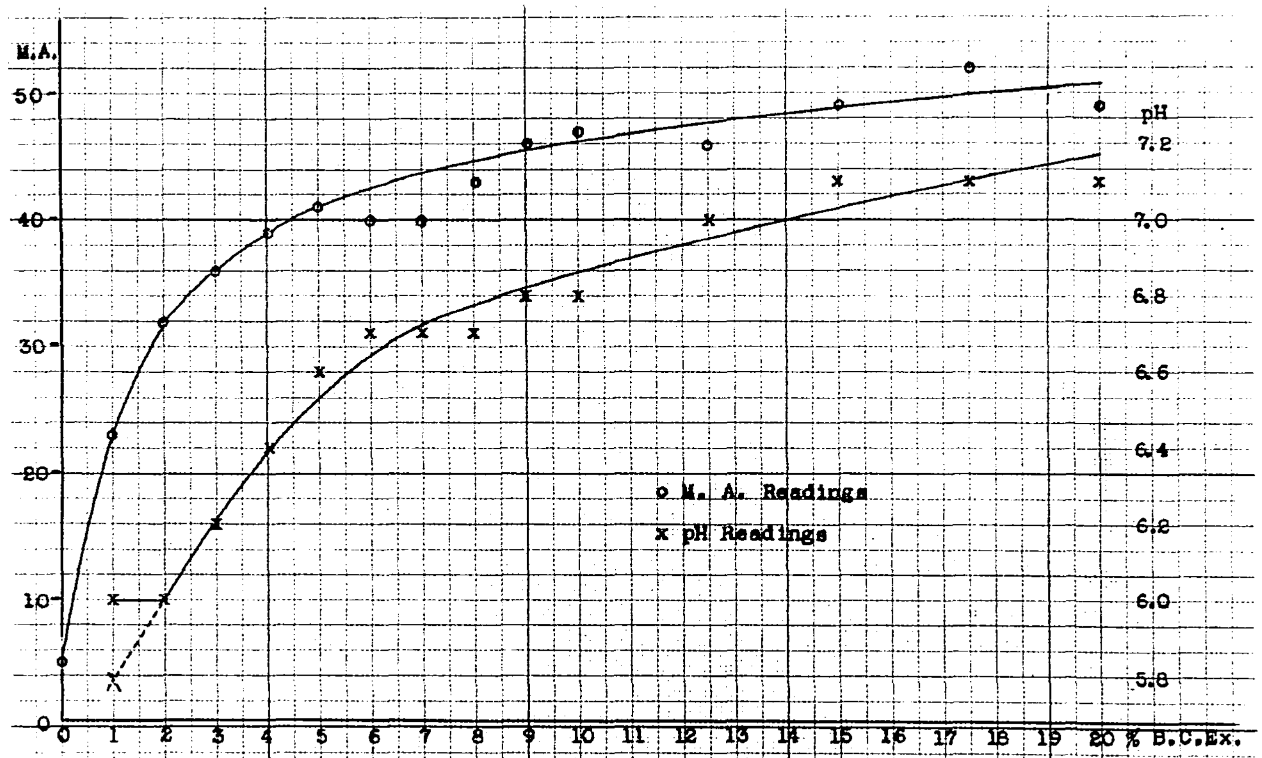

M.A.
50
40
30
20
10
0
pH
7.2
7.0
6.8
6.6
6.4
6.2
6.0
5.8
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 % B.C.Ex.
o M. A. Readings
x pH Readings

Fig. 2

extract, the more rapid was the pH drop. The initial reactions of all cultures were 6.9. The cultures with terminal pH values of 7.1 had become only slightly basic and without first becoming acid. Three cultures had terminal pH values of 6.7, two cultures had terminal pH values of 6.8 and one culture had a terminal pH value of 7.0. These cultures changed from their initial reactions only very slightly during the entire course of the experiment. Values of less than 6.0 are not recorded as these determinations were made using only brom thymol blue.

Experiment 2

It was known from experience that if a concentrated extract were heated to boiling considerable precipitation occurred. If such a heated solution were cleared by centrifugation and then autoclaved further precipitation resulted. No noticeable precipitation occurred when the diluted extract was combined in media and sterilized.

To determine the effect of such treatment upon activity of the concentrate the following experiment was conducted. A small amount of concentrated extract was adjusted to pH 6.9. The liquid was brought quickly to boiling and then rapidly cooled under running water. After separation of the precipitated material by strong centrifugation an aliquot of the liquid was taken for assay. The remainder was autoclaved at 10 lbs. for 10 minutes. After cooling the precipitate was removed as before. The two liquid specimens were separately combined with basic medium in increasing amounts. A control with untreated extract was also made. The results, shown in Table 2 and Fig. 3, demonstrated that whereas boiling had no effect, autoclaving seriously impaired the activity of the extract. Also, the pH stabilizing effect of the extract was largely destroyed by autoclaving.

The definite relationship of concentration of extract to pH drop which was described in the previous experiment

Table 2

The effect of boiling and autoclaving upon the growth promoting activity and pH stabilizing capacity of concentrated extract

Treatment of extract	Per cent of extract	pH at 24 hours	pH at 48 hours	pH at 72 hours	M. A. at 72 hours
Untreated	1	6.3	6.0	6.0*	21
	2	6.5	6.1	6.0	32
	3	6.6	6.3	6.0	36
	4	6.8	6.4	6.2	40
	5	6.8	6.6	6.4	45
Boiled	1	6.4	6.0	6.0*	22
	2	6.6	6.1	6.0	34
	3	6.7	6.3	6.1	37
	4	6.8	6.4	6.3	42
	5	6.9	6.6	6.4	46
Autoclaved	1	6.1	6.0*	6.0*	7
	2	6.1	6.0	6.0	11
	3	6.1	6.0	6.0	13
	4	6.2	6.0	6.0	15
	5	6.3	6.0	6.0	16

* pH values determined by use of brom thymol blue, lower values not obtainable by this method.

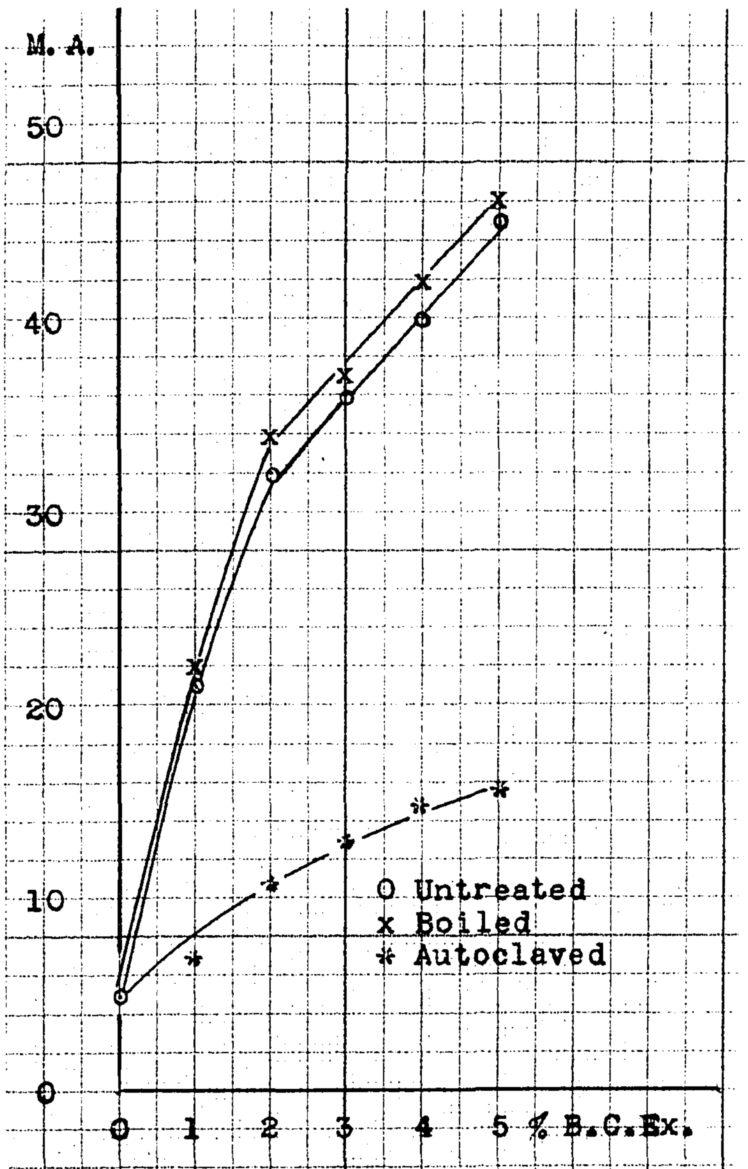

M. A.
50
40
30
20
10
0
O Untreated
x Boiled
* Autoclaved
0
1
2
3
4
5 % B.C.Ex.

Fig. 3

was again observed for the untreated and the boiled extracts. This relationship suggested that maintenance of neutrality might be due to a direct buffering action of the proteinaceous extract. On this basis the precipitation and removal of proteins from the autoclaved product may have been so great as to leave no effective buffering power. Thus limitation of growth may have been due to a rapidly increasing hydrogen ion concentration rather than to a loss of active material.

These data, and the similarity of the curve for M. A. readings and the curve of terminal pH values shown in Fig. 2, made necessary a consideration of the effect of pH upon growth.

Experiment 3

It is evident from the foregoing data that the quantity of phosphate salts present in the basic medium was insufficient to stabilize the pH of growing cultures. The total quantity of phosphate salts hitherto present in the basic medium was 0.0026 M. Accordingly, increasing concentrations were added in the ratio of 3 moles of Na_2HPO_4 to 1 mole of KH_2PO_4.

It was necessary to add the buffers as sterile solutions to avoid formation of a voluminous precipitate during autoclaving. Incubation at 37 °C. also caused the formation of precipitate if the total concentration exceeded 0.03 M.

Using total concentrations of 0.01 M., 0.02 M., and 0.03 M. in the ratio given, the effect of increased buffer was determined. For this purpose the boiled extract and the autoclaved extract discussed in Experiment 2 were used. If loss of proteinaceous buffering material were the important factor in the decreased activity of the autoclaved extract, then maintenance of neutrality by some other agent should restore this activity.

These two extracts were separately combined with basic medium in increasing concentrations, and the phosphate buffers were added according to the method outlined. The quantities used, and the pertinent results, are shown in Table 3 and Fig. 4.

The phosphate buffers were decidedly inhibitory. Both extracts showed greatest growth in the absence of buffer. In both cases better growth was obtained in the 0.01 M. cultures than in the 0.02 M. cultures, and these in turn showed more growth than the 0.03 M. cultures.

The pH was maintained at a higher level for a longer period of time in those cultures that contained buffers. Where 0.01 M. buffer was used with the autoclaved extract there was a substantial difference in pH to that of the same extract without buffer. At 48 hours the pH in the former was 6.7 throughout, whereas the pH in the latter was 6.0 or less. Growth in the buffer containing media was, however, substantially less. The lack of growth promoting ability of the autoclaved product could not be, therefore, entirely a matter of falling pH.

It had been determined that no precipitation occurred when phosphate buffers were added to casein hydrolyzate media. For this reason the above experiment was repeated in casein hydrolyzate basic media. The results were in complete agreement with those given here for gelatin hydrolyzate media.

Sodium carbonate was made up in stock solution, neutralized to pH 7.0, and sterilized by filtering through sintered glass. This solution was added aseptically to

Table 3

The effect of varying concentrations of phosphate buffers upon growth and pH

Per cent of boiled extract	Concentration of buffer	pH at 48 hours	pH at 72 hours	M. A. at 72 hours
1	0.00 M	6.0	6.0	20
2	"	6.2	6.0	37
3	"	6.4	6.0	40
4	"	6.6	6.1	41
5	"	6.7	6.3	42
1	0.01 M	6.7	6.5	13
2	"	6.7	6.4	23
3	"	6.7	6.4	30
4	"	6.7	6.4	33
5	"	6.7	6.4	36
1	0.02 M	6.9	6.6	8
2	"	6.9	6.5	16
3	"	6.9	6.5	22
4	"	6.9	6.5	26
5	"	6.9	6.5	28
1	0.03 M	7.0	7.0	5
2	"	7.0	6.8	15
3	"	7.0	6.7	19
4	"	7.0	6.6	27
5	"	7.0	6.8	27
Per cent of autoclaved extract	Concentration of buffer	pH at 48 hours	pH at 72 hours	M. A. at 72 hours
1	0.00 M	6.0	6.0	7
2	"	6.0	6.0	12
3	"	6.0	6.0	15
4	"	6.0	6.0	19
5	"	6.0	6.0	21
1	0.01 M	6.7	6.2	5
2	"	6.7	6.2	8
3	"	6.7	6.2	10
4	"	6.7	6.2	12
5	"	6.7	6.2	13
1	0.02 M	6.9	6.8	2
2	"	6.9	6.8	2
3	"	6.9	6.6	5
4	"	6.9	6.5	9
5	"	6.9	6.5	10
1	0.03 M	7.0	7.0	2
2	"	7.0	7.0	2
3	"	7.0	7.0	2
4	"	7.0	7.0	3
5	"	7.0	6.8	5

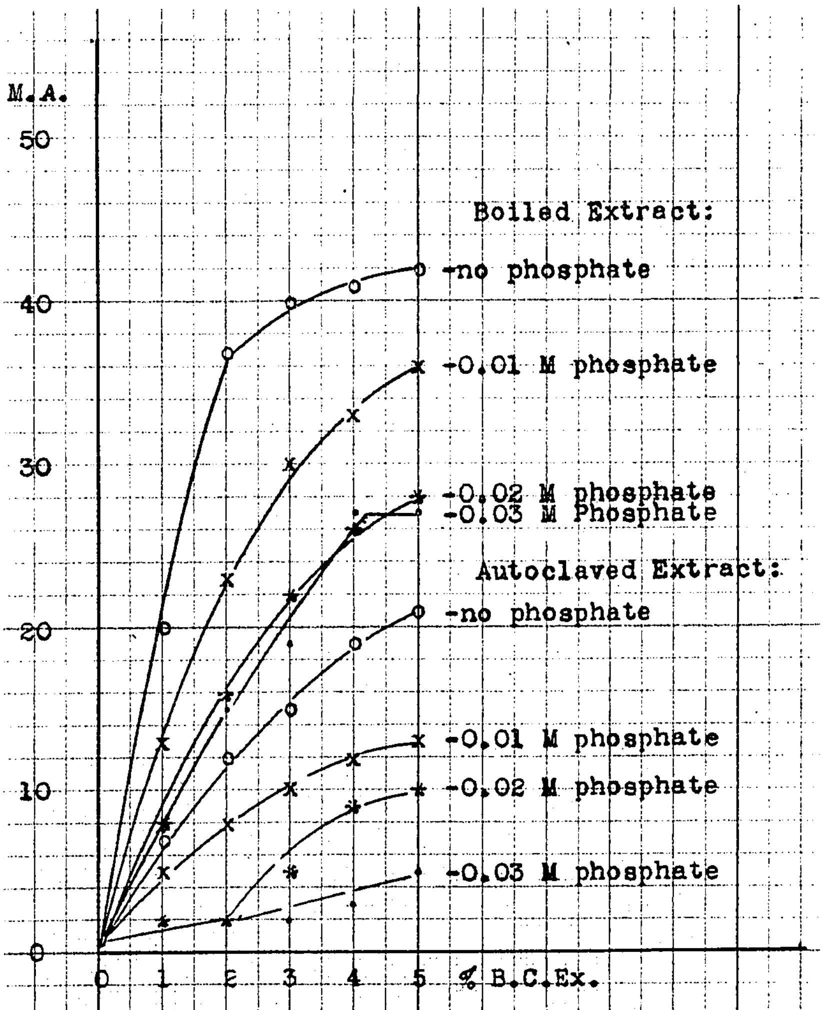

M.A.
50
40
30
20
10
0
Boiled Extract:
-no phosphate
-0.01 M phosphate
-0.02 M phosphate
-0.03 M Phosphate
Autoclaved Extract:
-no phosphate
-0.01 M phosphate
-0.02 M phosphate
-0.03 M phosphate
0
1
2
3
4
5
% B.C.Ex.

Fig. 4

sterile media containing the same extracts and made up in the same manner as given above. Essentially the same result was obtained. The best growth was obtained in media containing no carbonate. Growth was approximately inversely proportional to the concentration of carbonate.

Acetate buffers were also found to be similarly inhibitory. Sodium glycinate, although not inhibitory, had no stabilizing effect upon the pH. Increasing the concentration of gelatin or casein hydrolyzates in the basic medium also had no stabilizing effect on the pH.

Basic media used in all the experiments that follow did not contain phosphate salts.

Experiment 4

The data accumulated up to this time clearly showed that the initial pH could be maintained by using blood cell extract in sufficient quantity. Since none of the ordinarily used buffers was suitable for use with this organism an experiment was designed to determine the optimal pH for growth in the following manner.

A series of media was made up having pH values from 5.0 to 7.8 in steps of 0.4. Each of these media contained 10 per cent blood cell extract Lot No. 6 to stabilize the pH. The results obtained were wholly unexpected. The pH values of the 5.8, 6.2, and 6.6 cultures rose steadily to 6.9-7.0 in 48 hours. On the other hand the pH values of the 7.4 and 7.8 cultures dropped to 6.9-7.0 during the same period. All cultures with an initial pH between 5.8 and 7.0, inclusive, grew about equally well. Cultures with an initial pH of 7.4 or over grew more slowly, and the terminal M. A. readings were somewhat less than the 5.8-7.0 group. Growth in the 7.8 cultures was less than that of the 7.4 cultures. Cultures with a pH of 5.4 or below showed no growth. These data are presented in Table 4 and Fig. 5.

It is evident from the foregoing that the pH of growing cultures could be stabilized by a suitable quantity of blood cell extract only at neutrality. Growing cultures having initial pH values either below

Table 4

Growth and pH changes in cultures having various Initial pH values and an abundance of blood cell extract

Initial pH	pH at 24 hours	M. A. at 24 hours	pH at 48 hours	M. A. at 48 hours	pH at 72 hours	M. A. at 72 hours
5.0	4.8	1	4.8	2	4.8	2
5.0	4.8	1	4.8	2	4.8	2
5.4	5.3	1	5.3	2	5.4	2
5.4	5.3	1	5.3	2	5.4	2
5.8	5.9	10	6.9	27	7.0	37
5.8	5.9	10	6.9	28	7.0	37
6.2	6.2	10	7.0	26	6.8	36
6.2	6.4	10	7.0	27	7.0	38
6.6	6.9	8	6.9	27	7.0	38
6.6	6.9	8	6.9	27	7.0	38
7.0	7.0	8	6.9	26	6.8	40
7.0	7.0	8	6.9	26	6.8	39
7.4	7.4	5	6.9	18	6.8	33
7.4	7.4	5	6.9	19	6.8	37
7.8	7.8	3	6.9	10	6.9	25
7.8	7.8	3	6.9	10	6.9	24

Initial pH values were determined by the use of an electric potentiometer. Brom thymol blue, brom cresol purple, and brom cresol green were used for subsequent determinations.

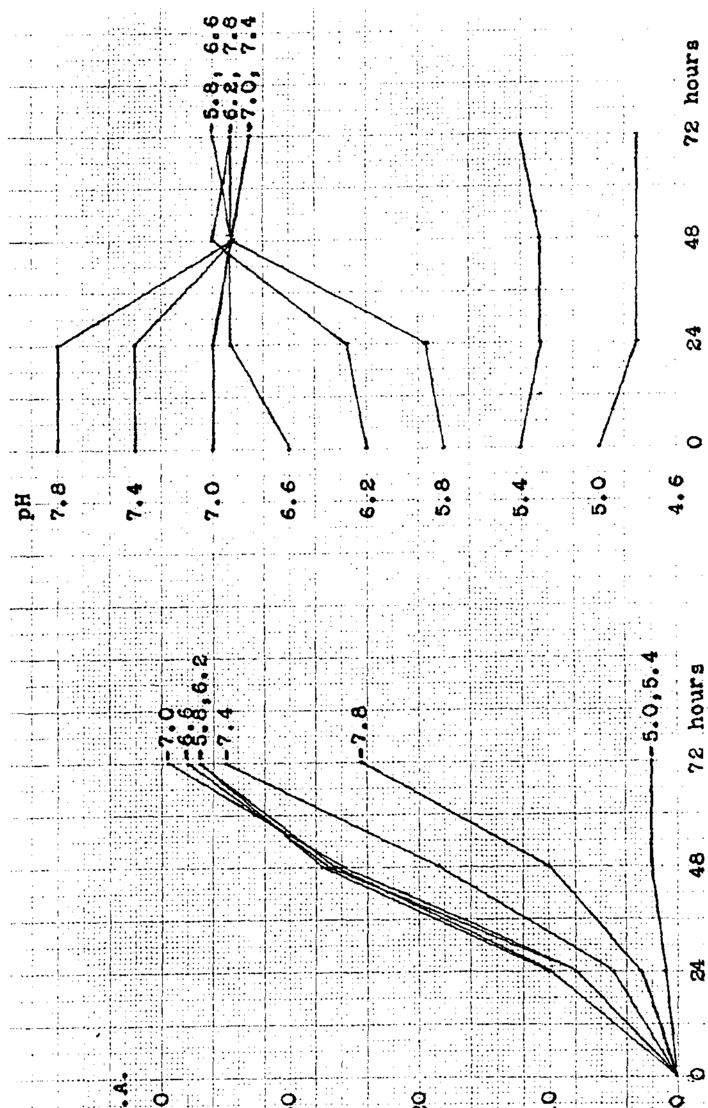

M.A.
40
30
20
10
0
24
48
72 hours
-7.0
-6.6
-5.8,6.2
-7.4
-7.8
-5.0,5.4
pH
7.8
7.4
7.0
6.6
6.2
5.8
5.4
5.0
4.6
-5.8, 6.6
-6.2, 7.8
-7.0, 7.4

Fig. 5

or above 7.0 rapidly became neutral. Neutralization of acid media can be accounted for only by the postulation of base production. The optimal pH cannot be stated from the above data. It is probable that almost any pH from 5.8 to 7.0 would be suitable. It is evident that values above 7.4 are not desirable.

Experiment 5

It was shown that acid media were rapidly neutralized by growing cultures containing an excess of blood cell extract. This constituted indisputable evidence of base production by the growing cultures. As it had been found repeatedly that cultures containing minimal quantities of extract became rapidly acid, it appeared probable that maintenance of neutrality in cultures that contained ample quantities of extract was due to base production rather than to direct buffer action of the proteins of blood cell extract, and that base production occured in an observable quantity only in the presence of an adequate supply of extract.

The following experiment was performed to obtain additional information on this matter.

A series of cultures containing quantities of extract from 1 to 5 per cent was set up in the usual manner. These were incubated for 72 hours. At the end of this time M. A. readings, pH values, and titratable acidity values were determined. It was found that the cultures containing 1 per cent extract had the lowest M. A. readings, the lowest pH values, and the highest titratable acidity values. The 2, 3, 4, and 5 per cent cultures showed, in this respective order, increasing M. A. readings, increasing pH values, and decreasing titratable acidity values. The parallelism previously noted between the curve of M. A. readings and the curve of pH values was again observed. Titratable acidity plotted against concentration gave a similar but inverted curve. Thus it is seen that the titratable acidity was roughly inversely proportional to pH, and directly proportional to hydrogen ion concentration.

The cultures which contained 5 per cent extract permitted a drop in pH from 6.9 to 6.3 in 72 hours. 0.25 cc. of 0.011 N NaOH was required to restore the pH to 6.9. The cultures that contained 1 per cent extract permitted a drop in pH from 6.9 to 5.1 during the same period. 2.0 cc. of 0.011 N NaOH were required to restore the pH to 6.9. If direct buffer action of the proteins of the extract were the only basis for resisting pH change, then regardless of the buffer system, the 5 per cent culture should have produced as much, if not more, titratable acid than the 1 per cent culture. From the figures given it is evident that this was not the case; the 1 per cent culture produced eight times as much titratable acid as the 5 per cent culture. These data are presented in Table 5 and Fig. 6.

Thus it became evident that the tendency of cultures containing ample extract to remain neutral was not due to direct buffer action of the proteins of the extract but rather to the production of alkaline material(s) during growth.

Table 5

The effect of various concentrations of blood cell extract upon growth, pH, and titratable acidity

Per cent extract	M. A. at 72 hours	pH at 72 hours	Titratable acidity* at 72 hours
1	21	5.1	2.00
2	31	5.5	1.50
3	34	5.8	0.75
4	39	6.1	0.50
5	40	6.3	0.25

* cc. of 0.011 N NaOH required to titrate to 6.9.

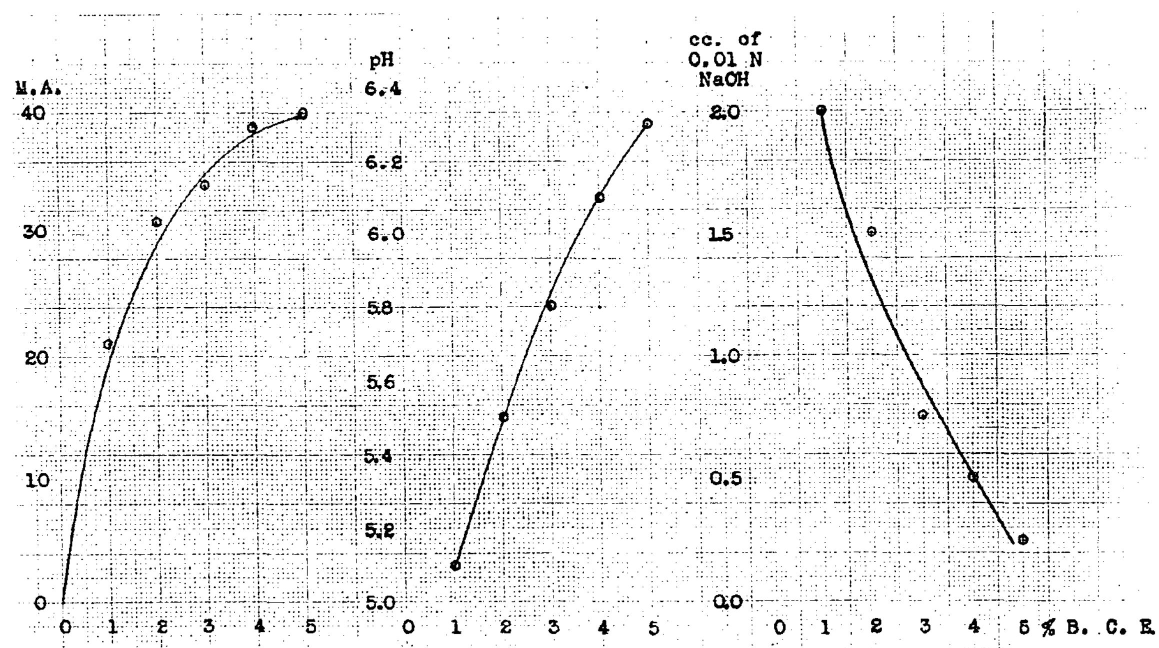

M.A.
40
30
20
10
0
0 1 2 3 4 5
pH
6.4
6.2
6.0
5.8
5.6
5.4
5.2
5.0
0 1 2 3 4 5
cc. of
0.01 N
NaOH
2.0
1.5
1.0
0.5
0.0
0 1 2 3 4 5 % B. C. E.

Fig. 6

Experiment 6

These experiments confirmed the production of alkaline material(s) by growing cultures. However, in one experiment it was shown that initially alkaline media readily became neutral. It appeared probable that carbohydrate metabolism with acid formation prevented highly basic cultures.

Cultures were set up which contained 5 per cent blood cell extract but no carbohydrate. Control cultures containing glycerol were made. Additional controls containing carbohydrate but no blood cell extract were set up. The bacteria used for inocula were washed three times with saline to remove as nearly as possible all carbohydrate. All media were adjusted to 6.9 prior to inoculation.

The pH of the carbohydrate deficient cultures rose to 7.8 in 48 hours. The pH of the media containing carbohydrate with or without extract dropped to 6.4 during the same period. Growth in the carbohydrate deficient medium was much less than that obtained in the complete medium but was significantly greater than that which occurred in the medium lacking in extract. These data are found in Table 6.

It was thus seen that in cultures capable of producing base,decidedly alkaline reactions were obtained in the absence of carbohydrate, and that to maintain the

Table 6

The effect of glycerol upon growth and pH

Media containing: B. C. Ex.	Glyc.	pH at 48 hours	M. A. at 72 hours
5 per cent	0 per cent	7.8	6
5	0	7.8	6
5	0.5	6.4	37
5	0.5	6.4	38
0	0.5	6.4	1
0	0.5	6.4	1

reaction near neutrality carbohydrate fermentation was necessary.

From the data so far presented it is evident that, given the proper circumstances, acid and base were produced simultaneously in growing cultures of Bacterium tularense. In the presence of a suitable quantity of extract essentially neutral reactions resulted. Results somewhat similar to this were obtained by Ayers and Rupp (5) for three numbered but unnamed cultures of the "alkali forming group". Neutral to slightly alkaline reactions were obtained in cultures which contained sodium citrate and dextrose. The presence of dextrose alone resulted in strongly acid reactions. Sodium citrate alone resulted in strongly basic reactions. Oxidation of the sodium citrate to alkaline carbonates was stated as the reason for its alkaline effects. Ishikawa (6) stated that B. ammoniagenes, B. aerogenes, B. morgani, B. proteus vulgaris, and Staphylococcus aureus decomposed urea in cultures. The presence of fermentable carbohydrate accelerated the production of ammonia from urea by these organisms and thus maintained alkaline reactions in the presence of fermentable carbohydrates. Ashworth (7) has described ammonia production from urea by the above mentioned organisms in the presence of mannite. Orcutt (8) stated in an abstract that "some members of the genus Bacillus carry on ammonification concurrent with acid production which tends to keep the pH near neutrality". He gave no data.

From the data presented it becomes clear why fermentation studies of Bacterium tularense have been difficult to appraise. It may be pointed out that either strongly acid or strongly basic terminal reactions are obtained readily from very feebly growing cultures, depending on the constituents of the media, whereas abundant growth may be obtained with no perceptible change in pH. Since no accurate method is available for determining quantity of growth on a slant, and since it is known that growth is readily supported on solid media without added carbohydrate, it becomes apparent that no criteria are known by which carbohydrate utilization can be determined when complex solid media are employed.

It has been demonstrated that the tendency for cultures to remain neutral is not due to direct buffer action of blood cell extract. It has further been demonstrated that the buffers ordinarily used are inhibitory. It is evident that the production of base is dependent upon some material(s) present in blood cell extract. Data presented in Experiment 2 demonstrated that the growth promoting activity of the extract was greatly decreased by a procedure which also diminished or destroyed the alkalinizing factor. It might well be that the alkalinizing factor is an intrinsic part of a complete medium and that metabolic base production is an inevitable end product in such media. Regarding this as a distinct possibility the work on fractionation of blood cell extract was resumed.

Section II

Although useful data had been obtained with methods given in the preceding section it soon became evident that uniform assay curves were not always obtainable. For reasons which were not apparent at that time turbidity readings graphed against concentrations of the various extracts frequently gave activity curves which were very irregular. Even with the same lot of extract uniform results were not always obtainable from one experiment to the next. The supply of one lot of extract was not inexhaustible, and other lots had to be prepared. Although the method of preparation which was outlined was followed exactly the activity curves obtained varied considerably from lot to lot. It is recognized that numerical data from experiments set up at different times are not strictly comparable but, to be useful, activity curves should show the same general shape. The following experiments are recorded to demonstrate the great variability that was encountered.

Experiments 7, 8, and 9

Of several lots of extract the pertinent data on three are presented as representative of the general situation. These extracts were made and assayed at different times. Hence, the data presented are not to be regarded as parts of a single experiment. An assay curve was given on extract Lot No. 6. For comparison a later trial with this lot is included. Assay curves for Lot Nos. 7 and 8 are also presented. These extracts were prepared according to the procedure given for No. 6. It should be pointed out that differences in behavior of an extract in a later assay could not be due to spoilage since these preparations were preserved either by freezing in the dry ice refrigerator or in the freezing unit of a mechanical refrigerator. Aliquots of each lot were combined with basic medium in amounts sufficient to give final concentrations as listed in Table 7. Turbidity readings were taken at 48 and 72 hours. pH values were determined at 72 hours. These data are recorded in Table 7 and Fig. 7.

According to the turbidity values maximum or nearly maximum activity was obtained at 2 per cent concentration of each extract. The curve for No. 6 is less regular than that shown in Fig. 2 for this extract. In the former determination considerable increase in

Table 7

Activity curves obtained with three different extracts as determined turbidimetrically

Per cent B. C. Ex.	Extract No. 6 M. A.	Extract No. 6 pH	Extract No. 7 M. A.	Extract No. 7 pH	Extract No. 8 M. A.	Extract No. 8 pH
0	2		2		1	
1	24	5.0	30	5.3	30	5.1
			30	5.3	31	5.3
2	32	5.4	32	6.7	35	6.7
			31	6.7	35	6.6
3	32	6.0	27	7.0	31	6.9
			26	7.0	29	7.0
4	33	6.2	21	7.2	30	7.2
			22	7.2	30	7.2
5	35	6.4	20	7.2	28	7.2
			20	7.2	30	7.2
7.5					28	7.2
					27	7.2
10					24	7.2
					24	7.2

Results obtained at the end of 72 hours' incubation

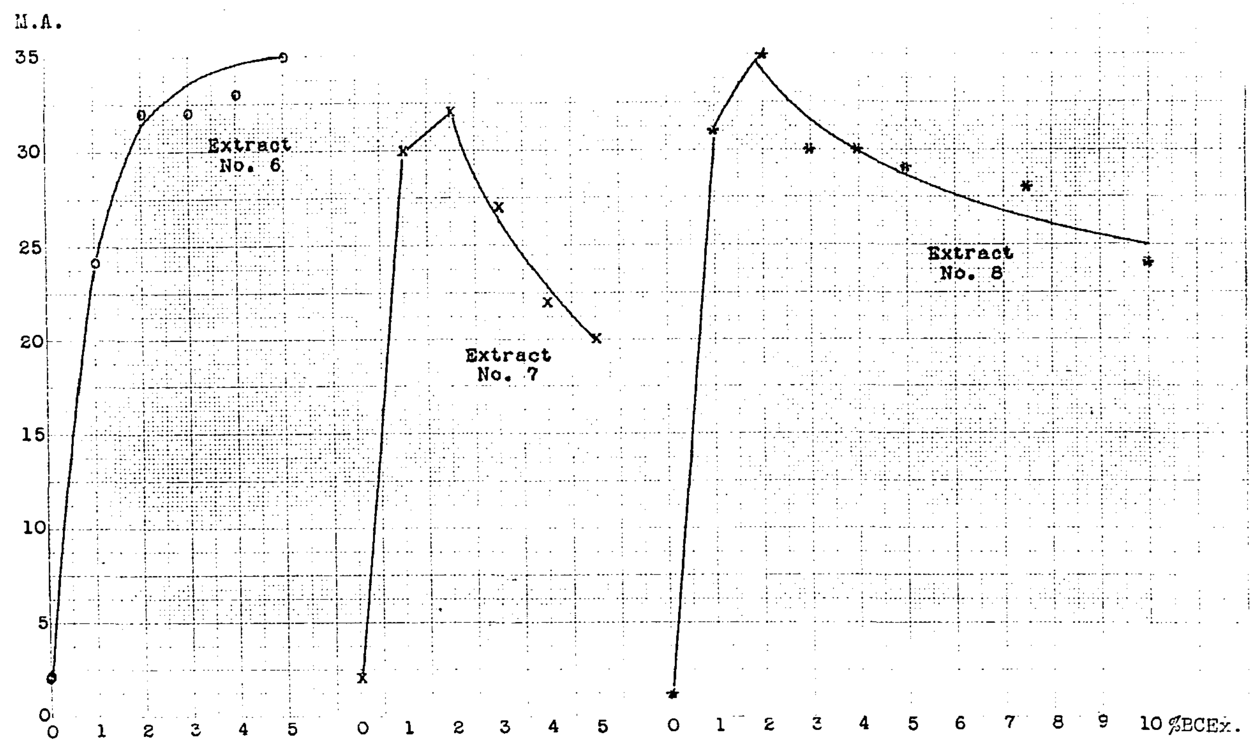

M.A.
35
30
25
20
15
10
5
0
Extract No. 6
Extract No. 7
Extract No. 8
0 1 2 3 4 5
0 1 2 3 4 5
0 1 2 3 4 5 6 7 8 9 10 %BCEx.

Fig. 7

turbidity accompanied increase in concentration in a uniform pattern up to 5 per cent, whereas in the latter determination nearly maximum activity was obtained at a concentration of 2 per cent, and further increase resulted in but very slight increase in turbidity.

For the cultures which contained extract No. 7 the readings showed maximum turbidity at 2 per cent. Increasing concentrations of the extract beyond this amount resulted in a progressive decline in turbidity. The activity curve of extract No. 8 was very similar to that for No. 7 except that in addition to the usual concentrations, media containing 7.5 and 10 per cent, respectively, of extract were made. The turbidity of the 7.5 per cent culture was less than that of the 5 per cent culture and the turbidity of the 10 per cent culture was less than that of the 7.5 per cent culture. Several other extracts were assayed and the results were similar to those already described.

The terminal pH values of the various cultures showed the usual trend for each extract. Low pH values were reached by the cultures containing small amounts. Cultures containing increasing concentrations showed increasing pH values. Extracts 6 and 7 were very similar in this respect. Cultures containing 5 per cent of these extracts reached a pH of 6.4.

Cultures containing 5 per cent of No. 8 showed pH values of 7.2. No. 8 was evidently a more alkalinizing extract than either No. 6 or No. 7.

With no other method of measurement available, and with no reason to doubt that turbidity values represented growth, it was necessary to explain the activity curves that were obtained. It was assumed that 2 per cent of extract was all that was necessary for maximum growth. Indeed, the curves that showed decreasing turbidity with increasing concentrations almost warranted the assumption that some material(s) acted as inhibitors when present in sufficient concentration. Therefore, the concentrations of extracts used in basic media for assay work were varied empirically to obtain more uniform activity curves. Instead of using 1 to 5 per cent, inclusive, the concentrations were changed to 0.5 to 2.5 per cent, inclusive, in intervals of 0.5 per cent. In this manner the problem of purification of blood cell extract was resumed.

Experiment 10

The effect of fractionation of blood cell extract with methanol was investigated as follows: To 1 volume of concentrated aqueous extract 9 volumes of methanol were added. After complete mixing the material was placed in the refrigerator over night. The mixture was then centrifuged and the supernatant was decanted. The precipitate was suspended in the original volume of water and filtered to remove insoluble material. The filtrate was light tan-yellow in color. It was designated fraction A.

The methanol supernatant was filtered, placed in a vacuum still, and carefully concentrated to dryness without the use of excessive heat. The residue was taken up in the original volume of water and filtered. A small amount of insoluble material was removed and the filtrate was tan-red in color. This filtrate was designated fraction B.

Since both fractions had been restored to the original volume they were tested individually and in combination in the same concentration as the original extract. The concentrations used, and the results obtained, are shown in Table 8 and Fig. 8.

It was found that both fractions were necessary to obtain the complete activity of the unfractionated extract. Fraction A was found to have almost no growth

Table 8

The effect of fractionation with methyl alcohol upon the growth promoting activity and pH stabilizing power of blood cell extract

Unfractionated extract	M. A. at 48 hours	pH at 72 hours
0.5 per cent	13	6.0
1.0	20	6.0
1.5	24	6.4
2.0	28	6.8
2.5	30	7.0
Fraction B		
0.5 per cent	14	6.0
1.0	14	6.1
1.5	15	6.8
2.0	17	7.0
2.5	19	7.1
Fraction A		
0.5 per cent	3	6.0
1.0	4	6.0
1.5	5	6.0
2.0	6	6.0
2.5	5	6.0
Fraction A plus Fraction B		
0.5 per cent	13	6.0
1.0	19	6.0
1.5	23	6.4
2.0	25	6.8
2.5	28	7.0

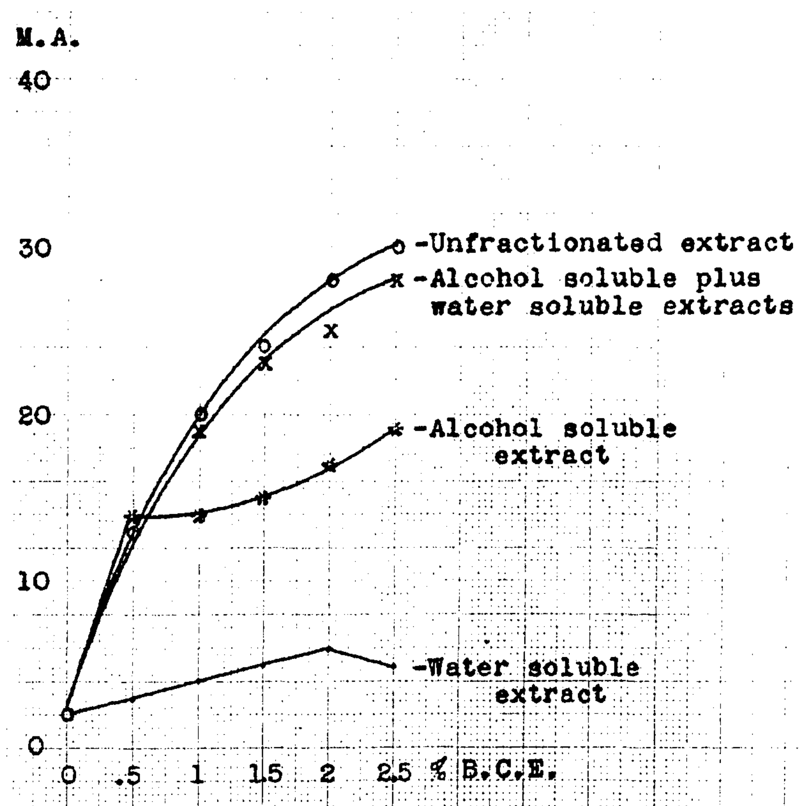

M.A.
40
30
20
10
0
0
.5
1
1.5
2
2.5
% B.C.E.
-Unfractionated extract
-Alcohol soluble plus water soluble extracts
-Alcohol soluble extract
-Water soluble extract

Fig. 8

promoting activity when used alone. Fraction B alone had about two-thirds of the growth promoting power of the unfractionated extract. From the activity curve of fraction B it is apparent that regardless of the quantity used the total activity of the unfractionated extract could not be obtained. When both fractions were used together growth was identical with that obtained with the whole extract. Fraction B contained all of the alkalinizing factor while fraction A had no stabilizing effect on pH. A total weight loss of 6.5 per cent was accomplished by the above procedure. The concentrated unfractionated extract contained 8.32 per cent solids, fraction B, 6.66 per cent solids, and fraction A, 1.12 per cent solids.

It was found that the active material of fraction B could be quantitatively precipitated from aqueous solution by acetone and dioxane. If dissolved in 90 per cent methanol, it could also be precipitated by ether and ethylene dichloride. Such precipitations were accompanied by no important weight loss, and no division of activity was accomplished. The precipitates were never crystalline but were viscous and gummy. Since no inert materials were lost by these procedures they are regarded as unimportant to this publication and are not reported in detail.

Many attempts have been made to crystallize active material from methanol and water solutions of fraction B.

The procedures used may be listed as follows: Concentrated solutions in methanol, and methanol solutions to which were added varying amounts of ether, acetone, dioxane or ethylene dichloride, were allowed to stand for many days at room temperature, in the refrigerator, and in the dry ice refrigerator. These procedures were varied by making such solutions acid or alkaline in varying degrees. Solutions of the fraction were treated with ammonium sulfate in varying concentrations. The active material was adsorbed chromatographically on various alumina adsorbents and on vegetable charcoal. It was recoverable from the alumina adsorbents but not from the charcoal. To date no active crystalline material has been obtained by any procedure.

Experiment 11

Since the active material of fraction B could not be crystallized by any of the methods employed it was subjected to hydrolysis. A sample of this fraction was mixed with sufficient concentrated hydrochloric acid to equal 5 per cent. The mixture was boiled in a reflux still for 12 hours. Most of the hydrochloric acid was then removed by repeated vacuum distillation. The residue was made up to the original volume in water and filtered. The hydrolyzed fraction was tested for activity in the presence and in the absence of fraction A. For comparison cultures containing unhydrolyzed fraction B were set up in the same manner. The combinations used, and the results obtained, are shown in Table 9 and Fig. 9.

For convenience in discussion the following symbols will be used:

B cultures -- cultures containing fraction B but not fraction A.

A-B cultures -- cultures containing both fraction A and fraction B.

HB cultures -- cultures containing hydrolyzed fraction B but not fraction A.

HB-A cultures -- cultures containing hydrolyzed fraction B and fraction A.

Table 9

Effect of hydrolysis of the alcohol soluble fraction upon growth and pH

Tube No.	Per cent of fraction		M. A. at 48 hours	M. A. at 72 hours	pH at 72 hours
1	0.5	B	14	13	6.0
2	1.0	"	14	17	6.1
3	1.5	"	15	17	6.8
4	2.0	"	17	18	7.0
5	2.5	"	19	18	7.1
6	0.5	A-B	15	16	6.0
7	1.0	"	18	20	6.0
8	1.5	"	23	25	6.4
9	2.0	"	25	30	6.4
10	2.5	"	27	30	6.9
11	0.5	HB	5	7	6.0
12	1.0	"	7	12	6.0
13	1.5	"	10	21	6.0
14	2.0	"	12	27	6.0
15	2.5	"	16	34	6.0
16	0.5	HB-A	6	8	6.0
17	1.0	"	8	17	6.0
18	1.5	"	10	26	6.0
19	2.0	"	15	30	6.0
20	2.5	"	20	31	6.0

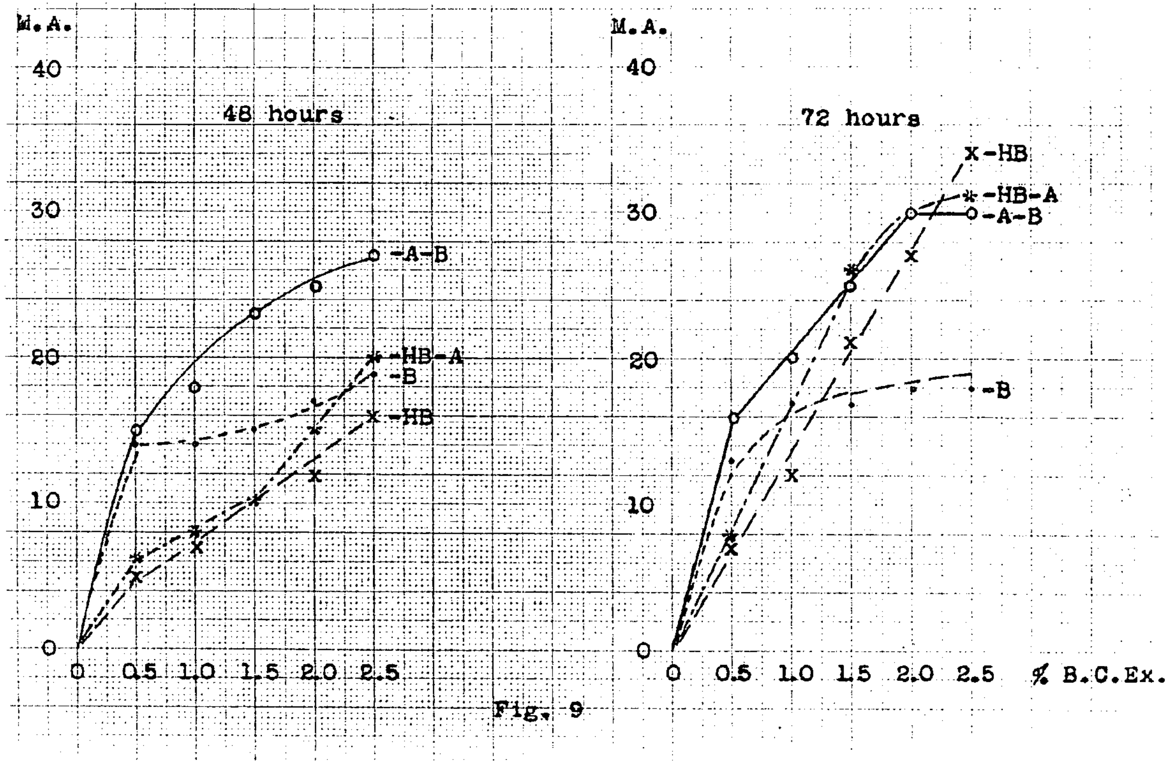

M.A.
40
30
20
10
0
48 hours
-A-B
-HB-A
-B
-HB
0 0.5 1.0 1.5 2.0 2.5
M.A.
40
30
20
10
0
72 hours
-HB
-HB-A
-A-B
-B
0 0.5 1.0 1.5 2.0 2.5 % B.C.Ex.

Fig. 9

The alkalinizing factor was either destroyed or very greatly diminished by hydrolysis. The pH values of the HB and the HB-A cultures were less than 6.0 in all concentrations at the end of 72 hours, whereas the pH values of the B or A-B cultures showed the typical picture of increasing pH values with increasing concentration.

After the first 48 hours the growth obtained in the HB and the HB-A cultures, as determined by M. A. readings, was much inferior to that obtained in the B or A-B cultures. This was especially true in the lower concentrations. At 72 hours the M. A. readings of the HB and HB-A cultures had greatly increased, whereas the readings for the B and A-B cultures had increased only slightly. In concentrations over 1 per cent the readings for the HB cultures had greatly surpassed those of the B cultures. Little difference existed in the readings of the HB, the HB-A, and the A-B cultures in the higher concentrations. At a concentration of 2.5 per cent of the respective fractions the readings of the HB cultures surpassed those of the A-B cultures. Since the HB fraction was derived from the B fraction, and since a combination of B and A fractions were shown to be necessary for maximum activity, the high M. A. readings of the HB cultures created a perplexing situation.

However, if cultures containing equivalent concentrations of the respective fractions were visually compared, the B and A-B cultures appeared much more turbid than the HB and HB-A cultures.

It became evident, therefore, that photoelectric measurement of turbidity with a reflecting instrument did not give sufficient information about cultures under certain conditions of growth.

Just prior to the completion of this experiment an alternative method of measuring growth, or perhaps more properly, of appraising media, had been devised. This method consisted of determining the actual packed volume of bacteria in any liquid medium. By weighing mercury the total volumes, and the volumes of the calibrated stems, were determined for several Van Allen hematocrit tubes. From these data it was possible to calculate a factor for each tube as follows: What per cent of the total volume is the volume of the stem? Let V_s equal the volume of the stem and V_t equal total volume, then

$$\frac{V_s \times 100}{V_t} = \text{stem volume per cent.}$$

Now since the stem is divided into 100 scale divisions,

$$\frac{V_s}{100} = \text{volume of 1 scale division of calibrated stem}$$

and

$$\frac{\frac{V_s}{100} \times 100}{V_t} = \text{volume per cent of 1 scale division of calibrated stem}$$

and

$$\frac{V_s}{V_t} = \text{factor.}$$

To measure the volume of cells in any suspension of organisms a tube was completely filled. The tube was sealed and centrifuged. After maximum packing was obtained the height of the column of packed cells was read off the calibrated stem. By multiplying with the appropriate factor for each tube, any reading could be converted directly to cell volume per cent. Owing to an objectionable feature in the design of commercially available Van Allen hematocrit tubes it was necessary to centrifuge first in a horizontal and then in an angle centrifuge. The speed of centrifugation was 4,000 r.p.m. and the time was 20 minutes for each machine. The horizontal centrifugation was necessary to get the bacteria into the stems and the angle centrifugation was necessary to pack the bacteria. The nature of the objectionable design, and the methods used in overcoming the necessity for two centrifugations, will be given in another place.

In Experiment 11, cell volume percentages were determined for tubes 8 and 9 from the A-B group, and tubes 18 and 19 from the HB-A group. Note that tubes 8 and 18 had M. A. readings of 25 and 26, respectively, and that tubes 9 and 19 each had an M. A. reading of 30. Cell volume percentages were found to be:

Tube No. 8	0.283 per cent	A-B culture
18	0.098	HB-A
9	0.296	A-B
19	0.110	HB-A

It is evident that with the same M. A. readings the A-B cultures had cell volume percentages approximately three times as great as those of the HB-A cultures. These data agreed with visual observations of turbidity on these cultures. When first devised, and while still in the experimental stage, the number of cell volume tubes and the number of spring sealing clips were very limited. For this reason cell volume percentages were not determined for all the cultures of this experiment.

The significance of this discrepancy between M. A. readings and cell volume became the basis for much speculation. A smaller cell volume might have been interpreted to mean fewer organisms or a smaller amount of bacterial protoplasm but no definite conclusions could be drawn. Samples of the various cultures were observed in dark field preparations by Foshay. He reported that no significant difference in numbers of organisms per field could be observed but that a predominance of large globular forms existed in the A-B cultures, whereas a predominance of small, compact, solid-appearing organisms existed in the HB-A cultures. Thus it could not be assumed that the smaller, denser organisms did not contain even more bacterial protein per organism than the larger less dense globular forms. On the other hand, a high turbidity reading must indicate either a large number of organisms or an increase in the reflective properties of a relatively smaller number of organisms. If a medium produced a large number of small compact organisms, could it be assumed to be a better medium than another which produced a relatively smaller number of "plump" organisms if the total reflective properties of the two cultures were essentially the same? In such a situation would a determination of actual numbers of organisms, either viable or non-viable, be of any assistance in appraising media?

Further, the alkalinizing factor was greatly diminished or destroyed by hydrolysis. High turbidity readings in acid cultures were one of the characteristics of the irregular results observed in Experiments 7, 8, and 9. The cultures containing hydrolyzed (albeit neutralized) blood cell extract became rapidly and strongly acid. These cultures showed what might be referred to, for lack of a better term, "abnormally" high turbidity values. If these turbidity values were "abnormal" then the question arose, Were the irregularities observed in Experiments 7, 8, and 9 due to this type of "abnormality"? To phrase the question differently, Do cultures which become acid give rise to a type of organism that is capable of reflecting more than the usual amount of light, thus resulting in "abnormally" high turbidity values? If so, then the whole matter of appraising media with turbidimetric methods would be brought into question. Obviously, this was a matter of primary importance. The resolution of these problems became the object of the experiments that follow.

Experiment 12

To open an investigation of the usefulness of turbidimetric methods for appraising media an experiment was designed to determine what, if any, differences existed between the activity curves obtained by turbidimetric measurements and the activity curves obtained by cell volume determinations in an ordinary assay of unhydrolyzed extract.

Blood cell extract Lot No. 9 was added to samples of basic medium in concentrations from 1 to 5 per cent. It was expected that cultures from these media would show an irregular turbidity curve as described in Experiments 7, 8, and 9. If the values obtained by cell volume determinations should result in a similar curve then the irregularities would have to be regarded as representing actual differences in amount of bacterial substance present. If the curves should be dissimilar then the choice of a method for appraising media could be regarded as an undecided matter, subject to further investigation.

The cultures were set up in 125 cc. Erlenmeyer flasks instead of the bulb tubes previously used. The total volume of each culture was 5 cc. The cultures were inoculated with 0.1 cc. of a suspension of organisms from a 24 hour liquid culture.

The organisms had been washed three times and resuspended to volume with saline.

At the end of 60 hours M. A. readings, cell volume percentages, and pH values were determined. The results are given in Table 10 and Fig. 10.

The alkalinizing effect of increasing amounts of blood cell extract was again apparent. The pH values of the 1 per cent cultures averaged 5.1. The pH values of the 2 per cent cultures were 6.9. All cultures containing more than 2 per cent of the extract had pH values of 7.2 or over.

The activity curves obtained by the two methods were strikingly dissimilar. High turbidity was obtained in the 1 per cent culture. Maximum turbidity was obtained in the 2 per cent culture. The 3, 4, and 5 per cent cultures showed less turbidity than the 2 per cent culture. The curve was irregular. On the other hand, the 1 per cent cultures showed an average cell volume per cent of 0.196. The 2 per cent cultures showed an average cell volume per cent of 0.382. The 3 per cent cultures showed an average cell volume of 0.46 and so on. Each successive increase in concentration of extract resulted in an increase in cell volume per cent.

Cell volume percentage values graphed against the logarithms of the concentrations of extract resulted in

Table 10

Comparison of results obtained on cultures measured: (1) turbidimetrically (2) by determination of packed cell volume

Flask No.	Per cent B. C. Ex.	Turbidity in M. A.	Cell volume per cent	Average	pH
1	1	43	0.198	0.196	5.0
2		43	0.194		5.2
3	2	48	0.384	0.382	6.9
4		50	0.379		6.9
5	3	40	0.456	0.460	7.2
6		44	0.466		7.3
7	4	39	0.538	0.547	7.3
8		40	0.556		7.4
9	5	43	0.607	0.600	7.4
10		42	0.593		7.4

Results obtained at the end of 60 hours' incubation

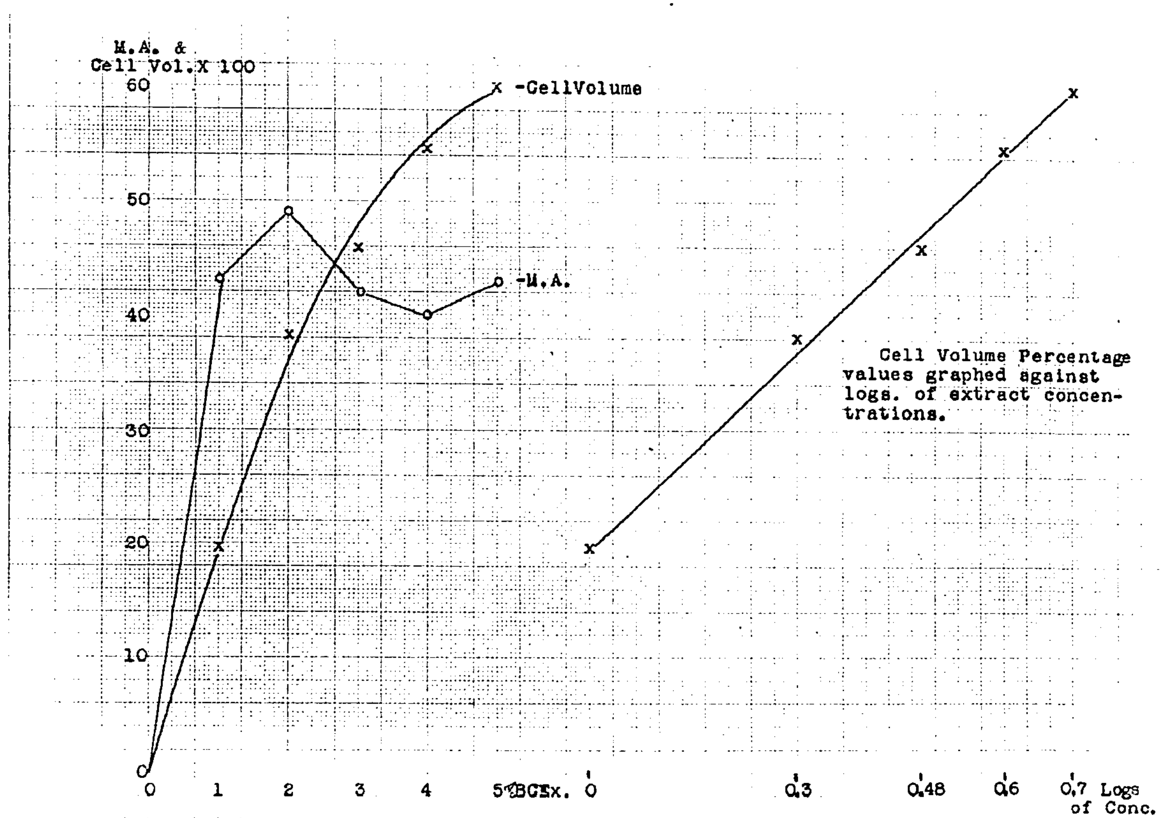

M.A. &
Cell Vol. X 100
60
50
40
30
20
10
0
-CellVolume
-M.A.
Cell Volume Percentage values graphed against logs. of extract concentrations.
0
1
2
3
4
0.3
0.48
0.6
0.7
Logs of Conc.

Fig. 10

a straight line. Within the limits of the experiment cell volume percentages increased in logarithmically decreasing increments as the concentration of the extract was increased.

It was concluded that turbidity measurements could not be regarded as necessarily representative of bacterial substance present in the cultures. The choice of a method of measurement was an open question and subject to further work, but from the standpoint of a quantitative interpretation it could reasonably be expected that cell volume determinations would have greater utility than turbidity readings.

Experiment 13

A discrepancy of major proportions had been shown to exist between turbidity readings and cell volume percentage values in various cultures. In this experiment the two methods were again used in the same manner as in Experiment 12. At the same time micro-Kjeldahl determinations of bacterial nitrogen were made as a standard of reference against which the two methods could be compared. The general procedure was a duplication of the previous experiment except that the final volume of each medium was 10 cc., and 250 cc. Erlenmeyer flasks were used as culture vessels.

At the end of 60 hours M. A. readings, cell volume percentages, and bacterial nitrogen determinations were made.

For the nitrogen determinations 5 cc. of each culture were placed in separate 15 cc. conical centrifuge tubes and sedimented in an angle centrifuge. The media were poured off and the bacteria were washed three times with saline. After the last sedimentation the packed bacteria were quantitatively transferred to micro-Kjeldahl flasks. Digestion, distillation, and titration of the specimens were according to the usual techniques.

The results are given in Table 11 and Fig. 11.

Table 11

Growth of cultures as determined by three different methods: turbidity, volume of cells, bacterial nitrogen

Flask No.	Per cent B. C. Ex.	Turbidity in M. A.	Cell volume per cent	Mgm. of nitrogen	Terminal pH
1	1	35	0.172	0.188	5.3
2		34	0.187	0.182	5.2
3	2	41	0.313	0.288	7.0
4		40	0.306	0.277	7.0
5	3	39	0.368	0.316	7.4
6		42	0.382	0.319	7.4
7	4	37	0.410	0.336	7.6
8		40	0.426	0.336	7.6
9	5	40	0.458	0.350	7.6
10		39	0.472	0.342	7.6

Results obtained at the end of 60 hours' incubation

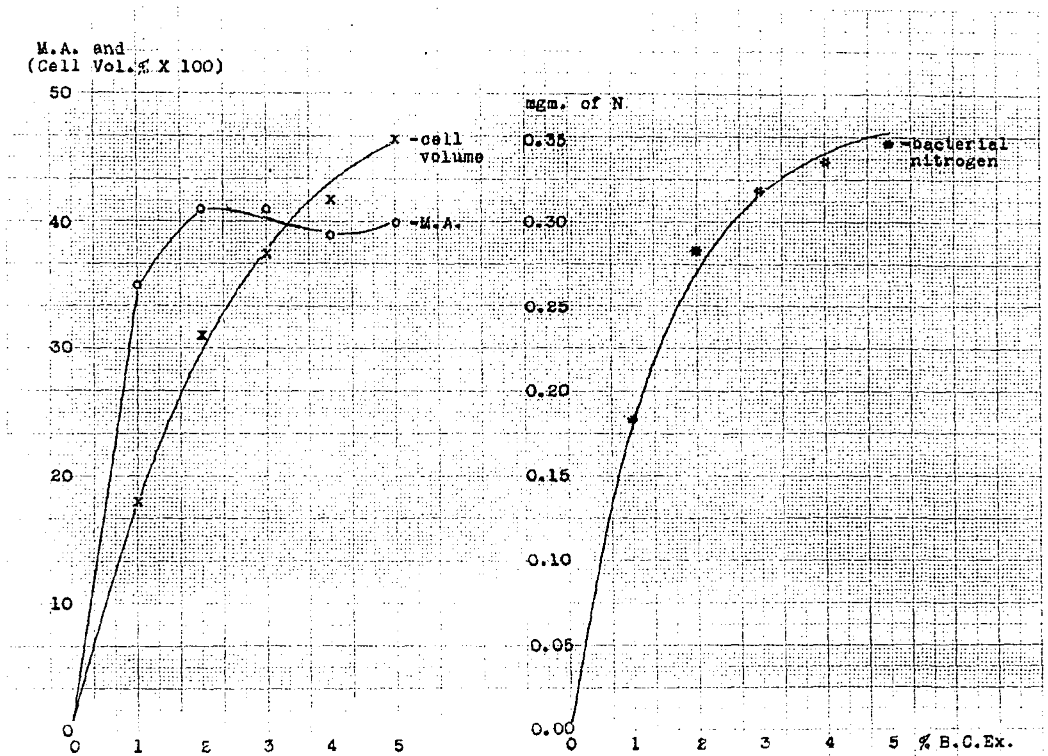

M.A. and
(Cell Vol.% X 100)
50
40
30
20
10
0
0
1
2
3
4
5
x -cell
volume
o -M.A.
mgm. of N
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
0
1
2
3
4
5
% B.C.Ex.
* -bacterial
nitrogen

Fig 11

The same discrepancy between turbidity readings and cell volume percentages was evident, and the general forms of the respective curves were the same in this experiment as they were in Experiment 12. Cell volume percentages and bacterial nitrogen values gave similar but not identical curves. Within the limits of the experiment increasing the quantity of blood cell extract increased bacterial nitrogen and cell volume percentages. The curves for bacterial nitrogen and cell volume percentages were uniform. Maximum turbidity was obtained in the 2 per cent culture. Further increase in extract did not increase the readings. The turbidity curve was irregular. Under the conditions of the experiment it was concluded that cell volume percentage values more nearly represented bacterial nitrogen values than did turbidity readings. Assuming that bacterial nitrogen was an accurate indicator of the quality of a medium, it followed that cell volume determinations were of greater value for this purpose than turbidity readings obtained with a reflecting turbidity comparator.

The alkalinizing effect of increasing amounts of blood cell extract was apparent in this experiment. The pH values of the 1 per cent cultures averaged 5.3. The pH values of the 2 per cent cultures were 7.0. All cultures containing more than 2 per cent of the extract had pH values of 7.4 or over.

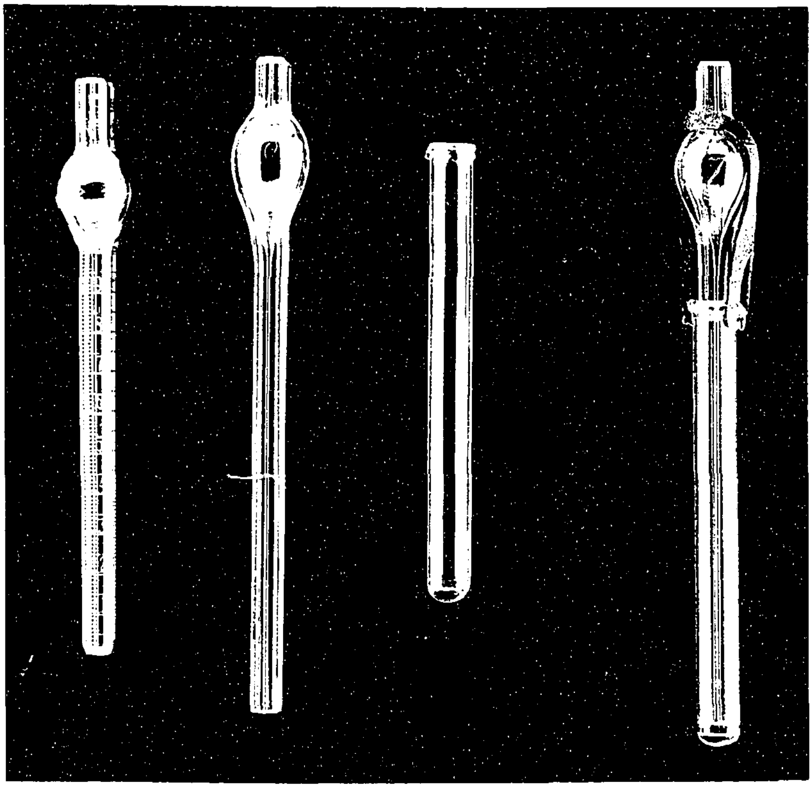
A range of pH values from strongly acid reactions to moderately and sometimes markedly basic reactions, associated with increasing concentrations of extract, has been a very constant finding. With cultures of an organism known to be highly pleomorphic the effect of widely varying hydrogen ion concentration upon the optical properties of the organisms cannot be assumed to be negligible. Although not proven in any degree it appeared reasonable to speculate that various forms with varying optical properties were produced by cultures growing at different reactions. In this manner the high turbidity values encountered in the 1 and 2 per cent cultures have been accounted for. In almost all cases the 1 and 2 per cent cultures showed slightly to strongly acid reactions. The lower turbidity values in cultures containing more than 2 per cent extract were almost always associated with relatively more basic reactions. Thus, the cultures containing higher hydrogen ion concentrations may tend to offset actual differences in growth by producing organisms which are more highly reflective than those organisms produced by more basic cultures.

It may be, however, that pH is only an associated phenomenon, and not directly responsible for the discrepancies observed. It appears probable that other unknown factors may influence the growth and the form of the organisms because the peculiarities in turbidity values, the "abnormal highs," are observed whether the pH range encountered is 5.2 to 6.4 or 5.0 to 7.6. Satisfactory explanation of the observed facts cannot be made at this time. Further work will be necessary.

As has been stated, commercially available Van Allen tubes were incorrectly designed for bacterial cell volume determinations. The lower portion of the bulb was not tapered gradually and evenly to the diameter of the stem, but the juncture was abrupt. It was necessary to use an angle centrifuge to obtain packing of the organisms. A tube with an untapered bulb, held at an angle, presented a curved pocket or ledge on which the organisms collected. To deliver the organisms into the stem required horizontal centrifugation. This procedure was time consuming and generally unsatisfactory.

For this reason cell volume tubes were constructed by the author. These tubes were hand blown from selected pyrex capillary tubing. They were constructed with gradual uniform tapers in the lower part of the bulbs. With these new tubes no horizontal centrifugation was necessary. The bacteria were delivered into the stems and were packed by a single centrifugation in the angle centrifuge (Sorvall -- type G). The tubes were numbered, and the total volume and the volume of the stem were determined for each tube. The volumes of the stems were determined with a micrometer burette (9). The volumes of the bulbs were determined by direct measurements with a microburette. From these data a factor was calculated for each tube as was described previously.

For the sake of completeness it is also mentioned that the use of commercially available spring clips was abandoned. Satisfactory sealing was not obtained in the majority of cases with such clips. A glass jacket to fit around the stem of the cell volume tube was devised. It was held in place by means of a rubber halter stretched over the bulb. A small rubber pad of the diameter of the lumen was placed in the bottom of the jacket to act as a seal and a cushion for the cell volume tube. With this device complete sealing was obtained in practically all cases. The glassware described is illustrated in Fig. 12.



Van Allen tube

Lamp blown tube

Glass jacket

Rubber halter

Assembled unit

Fig. 12

Experiment 14

It was stated that continuous shaking and aeration techniques produced cultures which grew very rapidly but that the turbidity readings obtainable were of no statistical value because of the great variability which was encountered. It had been determined that cell volume determination were more representative of bacterial protein than were turbidity readings in ordinary unshaken cultures. It appeared probable that this might also be true for shaken cultures.

To separate samples of basic medium increasing amounts of blood cell extract were added to give concentrations of 2, 4, 6, 8, and 10 per cent. These media were prepared in duplicate. They were inoculated in the usual way but were continuously shaken during incubation. At the end of 24 hours cell volume percentages and turbidity readings were determined. The results are presented in Table 12 and Fig. 13.

Maximum turbidity readings were obtained with the cultures containing 4 per cent extract. Further increase in concentration did not result in increased turbidity readings. The duplicates of the 6 per cent cultures and the duplicates of the 10 per cent cultures were not in agreement. Lack of agreement between duplicates has been found to be typical for turbidity

Table 12

Effect of shaking on growth of cultures as measured (1) turbidimetrically (2) by determination of packed cell volume

Flask No.	Per cent B. C. Ex.	Turbidity in M. A.	Cell volume per cent	Average cell volume
1	2	33	0.216	0.218
2		33	0.219	
3	4	107	0.410	0.401
4		105	0.392	
5	6	102	0.520	0.517
6		88	0.514	
7	8	99	0.578	0.576
8		103	0.573	
9	10	87	0.591	0.592
10		103	0.593	

Results determined at the end of 24 hours' incubation

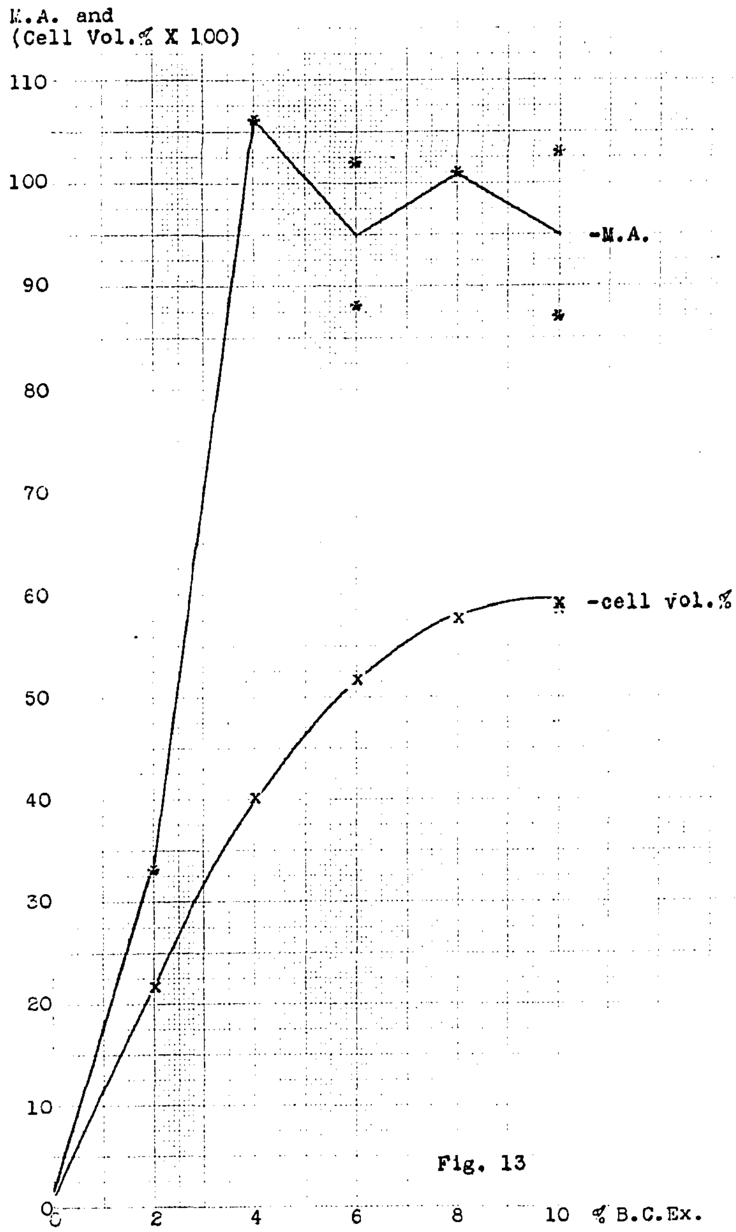

M.A. and
(Cell Vol.% X 100)
110
100
90
80
70
60
50
40
30
20
10
0
0
2
4
6
8
10
% B.C.Ex.
-M.A.
-cell vol.%

Fig. 13

readings on shaken cultures. The general irregularity of the curve is similar to that observed in unshaken cultures previously described.

Cell volume percentage values were found to increase as the concentration of extract was increased throughout the limits of the experiment. The curve obtained was uniform. Determinations on duplicate cultures showed close agreement. It was concluded that cultures could be shaken, and that statistically interpretable results could be obtained in 24 hours by the cell volume technique.

Experiment 15

It was determined previously that turbidimetric values obtained with a reflecting turbidity comparator could not be quantitatively interpreted in all cases. It became essential, therefore, to investigate a light transmission instrument. For this purpose an Evelyn colorimeter was used.

Cultures were set up with increasing concentrations of blood cell extract as previously described. In general, the construction of this experiment was a repetition of the former ones with the following important exceptions: (1) A new and more stimulatory blood cell extract solution was used, the concentration of which was measured in milligrams per cent rather than in percentage of packed cells. (2) Cultures were incubated for 24 hours only, and were shaken continuously. (3) The concentration of gelatin hydrolyzate in the basic medium was increased to 300 milligrams per cent of nitrogen. Cultures were set up in duplicate and inoculated with washed bacteria as before.

At the end of 24 hours readings were made with the Evelyn colorimeter, and with the reflecting turbidity comparator. Cell volume percentage values were also determined.

The color effect of uninoculated media was found to be negligible by testing a series of blanks having respective compositions identical to the media of the cultures.

The results, shown in Table 13 and Fig. 14, demonstrated that cell volume percentage values increased progressively as the concentration of blood cell extract was increased throughout the entire range of the experiment. From Fig. 15 it is seen that the curve formed by plotting these values is logarithmic.

With transmitted light the readings were inverse to turbidity values. Maximum light absorption occurred in the cultures that contained 100 milligrams per cent of blood cell extract. All cultures which had greater concentrations of blood cell extract did not show greater turbidity as measured by this method. While the readings were being taken it was suggested that the ideal or effective working range of the instrument had been exceeded. For this reason the above cultures were diluted 1:6 with blank media, and readings were taken on the diluted specimens. The curve obtained by plotting the readings of the diluted cultures was identical in form to that of the undiluted cultures. The use of various filters

Table 13

Growth of cultures as determined by volumes of cells and turbidity: (1) by reflected light (2) by transmitted light

Mgm. per cent B. C. Ex.	Cell volume (average)*	M. A. Series 1	M. A. Series 2	Colorimeter readings (average)
0	1.3	5	3	90
50	35	95	83	25
100	64.4	135	133	17
150	78.7	118	123	18
200	89	115	118	19
250	96	124	113	18
300	101	116	113	18

* These values equal cell volume percentages x 100

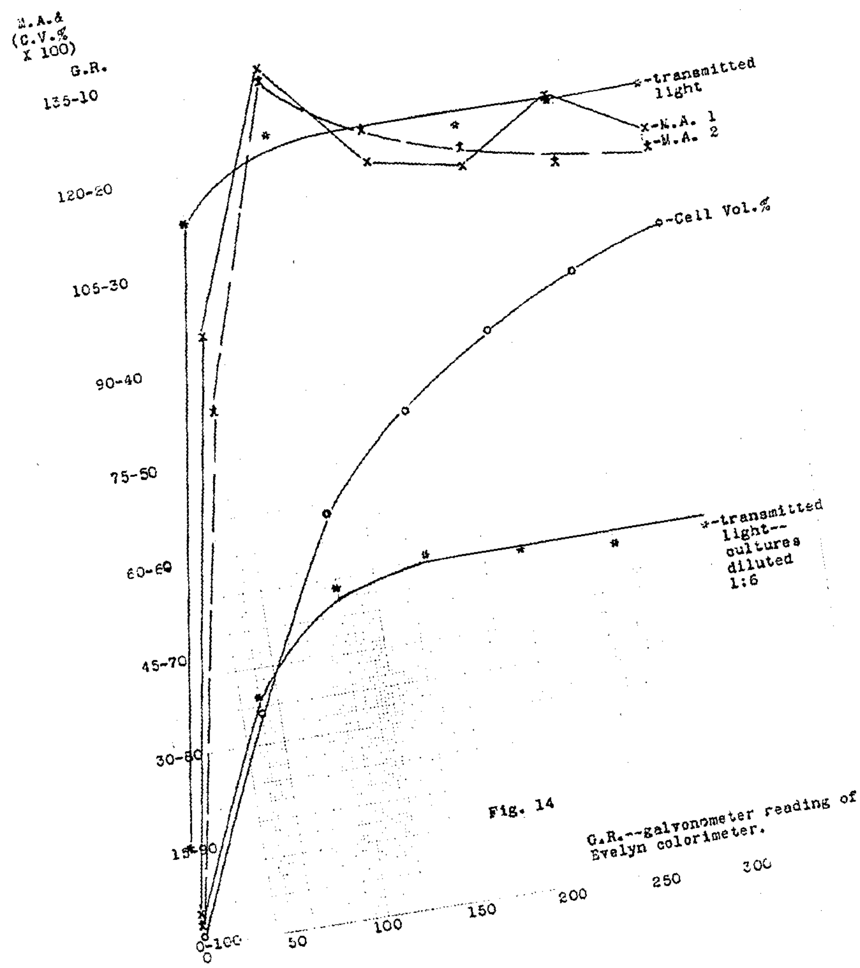
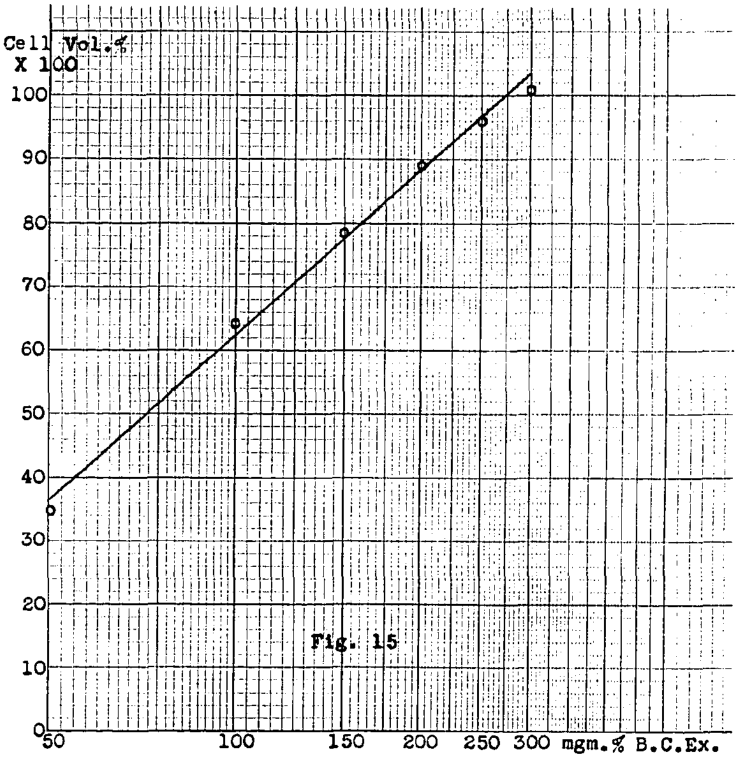


Fig. 14

G.R.--galvonometer reading of Evelyn colorimeter.


Cell Vol.%
X 100
100
90
80
70
60
50
40
30
20
10
0
50
100
150
200
250
300
mgm.% B.C.Ex.
Fig. 15

also made no difference in the general form of the curves obtained.

Readings on duplicate cultures were in close agreement if the results were determined either by the cell volume technique or by light transmission. For reflected light, however, readings on duplicate cultures sometimes differed by more than 10 units. For this reason the results obtained by this method were graphed separately for the two series. Maximum turbidity was obtained in the cultures containing 100 milligrams per cent blood cell extract. Cultures containing more than this amount actually showed less turbidity.

The differences between duplicate cultures, and the erratic nature of the curve beyond 100 milligrams per cent, are quite typical for shaken cultures when measured by the reflecting comparator.

Cell volume determinations were shown to parallel closely values for bacterial nitrogen. Wide discrepancies were shown to exist between cell volume percentage values and values obtained either by reflected or by transmitted light.

Under the experimental conditions given, photoelectric measurement of light, either reflected or transmitted, cannot be regarded as a strict function of the bacterial substance present in any particular suspension of *Bacterium tularense*.

Discussion

Bacterium tularense grows abundantly in liquid media that contain appropriate amounts of gelatin hydrolyzate, cystine, glycerol, sodium chloride, and blood cell extract. Although all of these ingredients are important for maximum growth, blood cell extract influences cultures in other important ways.

Growth may be limited by the paucity of an essential nutriment or by the accumulation of some metabolite which impedes multiplication. In current assay techniques complete removal of harmful waste products is not generally attempted but high concentrations of free acid or free alkali are usually avoided by the use of appropriate buffer systems. Critical pH levels inhibitory to growth are seldom approached.

Since no buffer system is known, which is not inhibitory for cultures of Bacterium tularense, extreme acidic and basic reactions are produced. Therefore, it is not possible to state whether growth is restricted by a lack of nutriments or by an incompatible concentration of hydrogen or hydroxyl ions.

The degree and the rapidity of pH changes are controlled by the concentration of extract; consequently, for each concentration the inhibitory effect is different. The amount of growth is not determined entirely by the nutriments available in a given medium; it is affected also by the different pH levels that are attained by the various cultures. Thus, the problem of assay assumes considerable complexity. In no other assay system with which the author is acquainted do the unknown factors influence cultures in this manner. The growth promoting properties and the alkalinizing effects are here mingled in one assay system.

A consideration of the acidic and basic reactions of cultures explains the difficulties that have been encountered in sugar fermentation studies of _Bacterium tularense_.

The lack of an acidic reaction or the presence of a basic reaction in a culture does not prove that sugar was not decomposed, because the reaction at any given time is the result of simultaneous production of acid and alkaline materials. Either process may be diminished or halted without preventing growth altogether. The terminal reaction represents only the difference in magnitude of the two processes.

The stimulatory effect of utilizable carbohydrate upon growth can be quantitatively measured in liquid cultures. No such measurement is possible in cultures prepared on agar slants in the usual way. Previous fermentation studies on Bacterium tularense have employed only acidic reactions to detect sugar fermentations.

Downs and Bond (10), discussing differences which had been noted between their own studies and those of Francis stated, "Our results agree closely with those of Francis except for the few strains which were negative in mannose and levulose. These negative strains were carefully checked but always failed to give fermentation although they grew well." These authors also stated, "An interesting aspect of these fermentation tests was the increase in alkalinity in all media in which the carbohydrate was not fermented. The color of the phenol red in these tubes showed a deep pink after the fourth day and reached a reaction of approximately pH 8.0 to 8.4 after the seventh day. The tubes showing fermentation reached a pH of 6.4 to 6.6 on the seventh to ninth day and then slowly decreased in acidity until at the seventeenth to the twenty-third day they also showed a reaction of pH 8.0 to 8.4."

Francis, (11) in discussing the fermentations of 60 strains reported, "In isolated instances the reaction swung to the alkaline side. * * * * Of 60 cultures studied, 53 fermented glycerol but 7 did not, 17 fermented levulose but 43 did not."

In both of these studies the lack of an acid reaction was regarded as evidence that sugar was not fermented although each report described alkaline reactions for some cultures and Downs and Bond described a shift from acid to alkaline reactions. The production of alkaline materials is clearly indicated by these descriptions, but the authors failed to notice the implications this holds. It is now clear that these techniques are not appropriate for the determination of carbohydrate utilization. Concomitant production of alkali may have neutralized considerable quantities of acid, thus invalidating the tests, insofar as negative results were interpreted to mean lack of carbohydrate fermentation.

Many investigators have shown that it is not permissible to regard an acid reaction as the only method by which sugar fermentation is detected. In a critical study of carbohydrate utilization, Wedum (12) demonstrated that many common bacteria utilized large quantities of carbohydrate without producing acidic reactions. Merrill (13) showed that members of the mycobacteria gave very basic reactions in cultures although several common sugars were utilized. Goldsworthy, Still and Dumares (14) described transient acid reactions that were followed rapidly by alkaline reactions for cultures of C. diphtheriae. Similar results for other organisms have been reported by many other investigators (15, 16, 17, 18, 19, 20, 21, 22).

Recognition of sugar fermentations is manifestly impossible in cultures which are capable of producing alkaline reactions even though sugar is being decomposed, if an acidic reaction is the sole criterion for sugar fermentation.

Many schemes have been employed for the appraisal of the quality of media and for the measurement of relative growth rate in microbiologic assays. Among those commonly described are: Viable cell counts, total counts, turbidity measurements, titratable acidity, oxygen uptake, terminal pH values, toxin production, dried weight of cells, packed cell volumes, and bacterial nitrogen values. No one method yields complete information about a culture. The method used is largely determined by the characteristics of the organism in question and the purpose of the experiment.

A culture of Bacterium tularense grown in the presence of a low concentration of extract reflects more light but has a lower bacterial nitrogen value than a culture grown in the presence of a high concentration of extract. It is unlikely that a larger number of organisms is responsible for the higher turbidity for the lower nitrogen values would imply the reverse. It may be however, that a larger number of very small organisms is present in the high turbidity suspension, and that each organism contains a relatively smaller quantity of protein. If such were the case, which medium would have the better quality; one which produced a large number of very tiny organisms with a low total protein or one that produced relatively fewer organisms but a higher total bacterial protein? In the present study it was assumed that a medium which for a given volume produced the highest bacterial nitrogen value in a given period of time was the best medium regardless of discordant results obtained by other methods.

An explanation for the disparity that exists between bacterial nitrogen values and turbidimetric data may be found in the extreme polymorphism of the organism.

The morphology of Bacterium tularense was described by Hesselbrock and Foshay (23) who demonstrated many forms that varied not only in size and shape but also in internal structure. The morphologic units of the organism "include globi and globules, flat and cylindrical bacillary forms, coccoid forms, delicate filaments and minimal reproductive units." Giant "cocci" as large as 3 microns in diameter and minute "cocci" as small as 0.3 microns in diameter were described. Filaments of various lengths were attached to many forms in a variety of ways: "fine filamentous structures united 3, 4, 5 or more than 20 bodies of either coccoid or bacillary shapes."

The internal structure of the various forms showed a great diversity of arrangement; "The chromatin-like particles within the coccoid forms were usually ring-shaped, discoidal, navicular, or crescentically obovoid, and almost always peripherally located. * * * * In larger globoid forms nuclear chromatin was often dispersed into peripheral granules. * * * * Occasionally filaments were seen within globules; some forked or branched, others unbranched and coiled within the cell walls."

It cannot be assumed that light is transmitted and reflected in a uniform manner by all of the bacterial forms described for <u>Bacterium tularense</u>. A large "coccus" would intercept more light than a minute "coccus". The density and the index of refraction of the internal "fluids", the size, shape, density, and distribution of granules and other internal structures all influence the amount of light intercepted, reflected, and transmitted. Each morphologic variant contributes a special optical effect. Since strongly acid cultures contained a predominance of small, dense, highly reflective organisms, and since M. A. readings on these cultures were disproportionately high, turbidimetric data were more dependent upon the optical properties of the variants than upon the concentration of bacterial substance.

The optical properties of bacteria were described by Mestre (24). He regarded bacteria in suspension as micro-lenses of extremely long focal length. "The distribution of flux incident on a single cell might consequently be expected to be characterized by an extraordinary anisotropy with almost all of the light scattered in a forward direction and only slightly deviated from its original direction."

This situation would probably hold true for suspensions of globi and coccoid forms of Bacterium tularense; the greater part of the light would be conducted forward and very little would be reflected and a low reading would be obtained on a reflecting instrument. Denser forms would reflect more light. Smaller forms would behave as lenses of much shorter focal length. This would increase the angle through which the light would be reflected and would result in much greater scattering of light. With such organisms a high reading would be expected on a reflecting instrument.

Since the optical properties of organisms grown in a low concentration of extract differ from those of organisms grown in a high concentration of extract, it is apparent that the quantity of some component of the extract affects the morphologic variation of the organism. It seems probable that the component responsible for this effect is the same substance that is responsible for variations in pH values of cultures.

Since low concentrations of alkalinizing factor permit a culture to become acid, and since a high concentration of alkalinizing factor causes a culture to become basic, it is probable that the effect upon the morphology is not due to the alkalinizing fraction directly but is due to the effect of various pH reactions upon growth. An acid environment would produce morphologic forms that are more highly reflective than those produced in a basic culture. Thus, turbidimetric data are not appropriate as a basis of comparison for cultures that contain different amounts of extract.

Gainey (25) in 1940 reported studies on Azotobacter and showed that turbidity, number of viable cells, and volume of packed cells could not be correlated under all circumstances. Under conditions which precluded cell division, turbidity steadily increased. The presence of gums and the tendency for the cultures to flocculate caused cell volume determinations to be inconsistent. Loofbourow and Dwyer (26) in 1938 showed that haemacytometer counts of yeast did not correlate well with turbidimetric measurements. In these studies turbidity was not a function of cell numbers alone.

Many varieties of photoelectric instruments have been designed for use with bacteria but in any photometric system turbidity or opacity are not proportional to concentration of bacterial substance unless form, size, and density of the individual organisms are uniform from one suspension to another. The extreme polymorphism of Bacterium tularense precludes the existence of a close quantitative relationship between the quantity of bacterial substance and the amount of light transmitted or reflected by suspensions under all conditions.

Growth of Bacterium tularense occurs only at the surface of solid media and is completely inhibited by anaerobiosis. In unshaken liquid media growth is very scanty if deep cultures are used. Shallow layers (5 mm. or less) of liquid media permit abundant growth but heavy yields are obtained only if cultures are aerated or shaken vigorously. These characteristics indicate that the organism requires a high concentration of free oxygen for rapid growth.

Preliminary work showed that blood cell extract was necessary for maximum oxygen uptake by suspensions of washed cells. This strongly indicates that some component or components of blood cell extract are vitally important in oxygen transfer mechanisms of the organism.

Since Menke (27) showed that the extract was without effect upon the dehydrogenation systems, it appears that the effect of the extract is exerted farther along in the oxidation-reduction processes, possibly operating in a manner similar to co-carboxylase in the liberation of carbon dioxide from carboxylic acids. The high oxygen demand of the organism would necessitate an efficient mechanism for dissipation of carbon dioxide.

The heat stability of the extract, and the fact that it is completely dialyzable, suggest that a similarity to the cytochromes is improbable. Furthermore, the extract has no absorption bands in visible light.

It is possible that the extract functions somewhat like Warburg's Atmungsferment which accepts hydrogen from cytochrome and is thereby reduced. Blood cell extract is a reducible substance. In the presence of sodium hydrosulfite the color of the extract is greatly bleached. This reaction is irreversible but some component of the extract may be denatured by the hydrosulfite.

The general properties of the extract suggest participation in the energy transfer systems of the organism:.

The extract is tan-yellow in color. It is reducible as described. The extract gives a faint biuret test, a faint xanthoproteic test and a positive test for sulfur. It contains about 5.4 per cent nitrogen on a basis of dried weight. A strong Molisch test and a negative Benedict's test indicate the presence of nonreducing carbohydrate. The presence of pentose is demonstrated by a strongly positive Bial's test. Phosphate is present in the extract. Mono- and Di-nucleotides and the carboxylases show similar properties. Ribose and phosphate are essential links in the nucleotides. Sulfur is present in co-carboxylase. Nitrogen compounds and substituted benzene derivatives are essential building blocks in these compounds. The nucleotides and carboxylases show oxidation-reduction properties. The exact role of blood cell extract is still undetermined but preliminary work indicates that its function is important to the energy transfer mechanisms of the organism.

Exclusive of the substances present in basic medium, blood cell extract supplies whatever accessory factors are necessary for maximum growth. A close approximation to complete media must have been attained because extremely small inocula are sufficient to initiate sustained growth, growth continues to be abundant upon repeated subcultivation and initial virulence is maintained.

The concentrated extract is easy to prepare and can be lyophilized and stored in the dry state without loss of activity. Thus preparation of media is greatly simplified.

The liquid media which have been defined present definite advantages over those previously available. They permit the introduction of quantitative measurements of growth promoting substances. Thus, an important beginning has been made toward the construction of a completely synthetic medium, and toward ultimate definition of obligate and accessory nutritional factors.

Quantitative chemical studies can now be performed upon the organism since the media permit large scale production methods that yield large quantities of bacterial cells. Commercial production of prophylactic vaccine now becomes a feasible undertaking and such vaccine would be free from agar and foreign proteins. Since all components of the media are dialyzable, it can readily be removed from the bacteria.

Studies of the physiology, of enzymatic and respiratory processes, of the organism will be greatly facilitated and it is possible that end products may be isolated and identified. In summary, many aspects of the chemistry of the organism can now be investigated with prospects for accurate and reproducible analyses far brighter than those offered by all previously known media.

Summary

A simplified liquid medium was devised which supported abundant growth of _Bacterium tularense_. The medium defined and the techniques described permit large scale production of the organism for chemical study or for the preparation of prophylactic vaccine.

Essential growth factors were found to be present in an aqueous extract of blood cells. As no growth is obtained in the absence of this extract, some of its components are obligate for the nutrition of _Bacterium tularense_ in the liquid medium described.

The concentrated blood cell extract is tan-yellow in color, is irreversibly reduced by sodium hydrosulfite, gives a faint biuret test, a faint xanthoproteic test and a positive test for sulfur. A strongly positive Bial's test indicates the presence of pentose. Benedict's test for reducing sugar is negative. The extract contains about 5.4 per cent nitrogen on a basis of dried weight. The extract was fractionated into two portions with methyl alcohol and both portions were found to be essential for maximal growth.

Some component of the extract governs the production of alkaline materials by growing cultures. Thus, in the presence of fermentable carbohydrate, acid and base are produced simultaneously and neutral or basic terminal reactions may occur. Therefore, absence of an acidic reaction does not signify inability to utilize carbohydrate.

The concentration of extract determines pH change in cultures during growth and, therefore, cultures that contain various concentrations of extract reach different pH reactions. Organisms grown under these conditions are morphologically dissimilar and so have different optical properties. Therefore, turbidimetric data do not vary proportionally as nitrogen values on such cultures.

A new method was devised for the measurement of bacterial growth rates whereby volume percentage of packed cells may be measured directly. These measurements paralleled closely the bacterial nitrogen values of any or all suspensions and hence were largely independent of the effects of morphologic variations.

BIBLIOGRAPHY

1. I. W. Gibby. Studies on the Cultural Requirements of Bacterium Tularense. Master's Thesis, University of Cincinnati Graduate School, Department of Bact. Cincinnati, Ohio (1942).
2. S. Berkman. Accessory Growth Factor Requirements of the Members of the Genus Pasteurella. Jour. Inf. Dis. 71:201 (1942).
3. E. A. Steinhaus, R. R. Parker and M. T. McKee. Cultivation of Pasteurella Tularensis in a Liquid Medium. Pub. Health Reps. 59:78 (1944).
4. R. P. Krebs, P. Perkins, A. A. Tytell and H. Kersten A Turbidity Comparator. Rev. Sci. Instruments 13:229 (1942).
5. S. H. Ayers and P. Rupp. Simultaneous Acid and Alkaline Bacterial Fermentations from Dextrose and the Salts of Organic Acids Respectively. Jour. Inf. Dis. 23:188 (1918).
6. M. Ishikawa. Influence of Carbohydrate on Bacterial Decomposition of Urea. Jour. Inf. Dis. 43:67 (1928).
7. P. G. Ashworth. The Production of Ammonia by Bacteria. Med. Jour. Australia 29 (5):125 (1942).
8. F. S. Orcutt. Acid Production from Carbohydrates by the Genus Bacillus as Influenced by the Lack of "Sparing Action" on Peptone. Jour. Bact. 45:15 (1943).

9. P. F. Scholander, G. A. Edwards, L. Irving. Improved Micrometer Burette. Jour. Biol. Chem. 148: 495 (1943).

10. C. M. Downs and G. C. Bond. Studies on the Cultural Characteristics of Pasturella tularensis. Jour. Bact. 30: 485 (1935).

11. E. Francis. Fermentation of Sugars by Bacterium tularense. Jour. Bact. 43: 343 (1942).

12. A. G. Wedum. Carbohydrate Utilization by Bacteria. Jour. Inf. Dis. 58: 234 (1936).

13. M. H. Merrill. Carbohydrate Metabolism of Mycobacterium. Jour. Bact. 20: 283 (1930).

14. N. E. Goldsworthy, J. L. Still and J. A. Dumares. Serum Enzymes and Fermentation Tests. Jour. Path and Bact. 46: 253 (1938).

15. H. J. Conn and G. J. Hucker. Use of Agar Slants in Detecting Fermentation. Jour. Bact. 5: 433 (1920).

16. George G. DeBord. Nitrogenous Metabolism in Bacterial Cultures. Jour. Bact. 8: 7 (1923).

17. E. B. Fred, W. H. Peterson and J. A. Anderson. Fermentation of Arabinose and Xylose. Jour. Bact. 8: 277 (1923).

18. J. H. Brown. A Study of Anaerobic Bacteria. Jour. Bact. 10: 513 (1925).

19. H. R. Stiles, W. H. Peterson and E. B. Fred. Sugar in Bacterial Cultures. Jour. Bact. 12: 427 (1926).

20. F. S. Jones, M. Orcutt and R. B. Little. Atypical Lactose Fermenting B. Coli. Jour. Bact. 23: 278 (1932).

21. H. E. Goresline. Studies of Agar-Digesting Bacteria. Jour. Bact. 26: 455 (1933).

22. J. Lemoyne Roberts. New Species of Genus Bacillus. Jour. Bact. 29: 229 (1935).

23. W. Hesselbrock and Lee Foshay. The Morphology of Bacterium tularense. Jour. Bact. 49: 209 (1945).

24. H. Mestre. Precision Photometer. Jour. Bact. 30:335 (1935).

25. P. L. Gainey. Measuring the Growth of Azotobacter. Jour. Bact. 48: 285 (1944).

26. J. R. Loofbourow and C. M. Dwyer. Relative Consistency of Weights and Counts in Determining Microorganism Populations by Photoelectric Nephelometers. Stud. Inst. Divi Thomae (Cincinnati) 2 (1): 129 (1938).

27. M. Menke. Metabolism of Bacterium tularense. Unpublished Results. Univ. of Cincinnati, Dept. Bact., Medical College, Cincinnati, Ohio. 1943.