BIOLOGY OF BACTERIUM LEPISEPTICUM.

I. Effects of Oxygen Tension and the Presence of Rabbit Blood on Growth, Dissociation, and Virulence.

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During the course of our studies of the rabbit respiratory infection, snuffles—pneumonia—septicemia,[1] we have isolated many strains of the microbic incitant, *Bacterium lepisepticum*. The cultures have been obtained from the nasal passages of normal and infected rabbits, from the lungs and heart's blood of animals dying of pneumonia, and from subcutaneous abscesses and middle ear infections. They differ one from another in minor respects but all possess the main characteristics of the Pasteurella, rabbit septicemia group.

A common type of the organism, described by Theobald Smith in 1887,[2] has been studied more recently by De Kruif.[3] His cultures, obtained from rabbits dying of spontaneous pneumonia, were designated as Microbe D and were characterized as follows: "Microbe D grows diffusely in serum and plain broth, forms rather opaque, fluorescing colonies on serum agar, and is highly virulent for rabbits."[3] Its acid agglutination optimum lies between pH 3.0 and 3.5.

We are familiar with De Kruif's cultures of Microbe D and have found the type to be widespread in the nasal passages of normal and infected rabbits in different localities and in the pleural fluid, lungs, and heart's blood of rabbits dying of spontaneous pneumonia.

A dissociation of this microbe with the appearance of another form, Type G, has been described by De Kruif (Table I). The

[1] Webster, L. T., *J. Exp. Med.*, 1924, xxxix, 837; xl, 109.

[2] Smith, T., *J. Comp. Med. and Surg.*, 1887, viii, 24.

[3] De Kruif, P. H., *J. Exp. Med.*, 1921, xxxiii, 773.

phenomenon was stated to occur in extract broth cultures, to be favored by high concentrations of peptone, and to be inhibited by beef infusion or undiluted serum. The variant, Microbe G, showed a granular growth in fluid media, formed a translucent, bluish non-fluorescent colony, was of low virulence, and floculated best in buffer solutions of pH 4.1 to 4.7.[4] De Kruif noted the occurrence of both Microbes D and G in the nasal passages of normal rabbits.[5]

This D to G transformation has occurred from time to time in many of our stock cultures and we have recovered Microbe G from the nasal passages of normal and infected rabbits. Besides confirming the work of De Kruif, the studies described in this paper permit a more complete understanding of this dissociation phenomenon and sudden drop in virulence.[6]

TABLE I.

Distinguishing Characteristics of Bacterium lepisepticum, Types D and G.

Type.	Virulence.	Colony.	Broth.	Acid agglutination.
				pH
D	+++	Fluorescent.	Diffuse.	3.0–3.5
G	±	Non-fluorescent.	Granular.	3.5–4.7

Growth of Microbe D in Meat Extract Broth, pH 7.4.

Experiment 1.—A culture of *Bacterium lepisepticum*, Type D, was inoculated into meat extract broth, pH 7.4, the medium used throughout these experiments, unless otherwise designated. After 17 hours incubation, broth dilutions, 10^{-1} to 10^{-9}, were made and plated on blood agar to determine the number of organisms per cc. in the original culture. ½ cc. from each dilution was then transferred to a test-tube containing 4½ cc. of broth. The tubes were incubated and examined for growth after 24, 48, and 72 hours.

In Table II the results of this and several succeeding experiments are shown. And it can be seen that under the conditions of Experiment 1, growth occurred only in tubes which had received an inocula-

[4] De Kruif, P. H., *J. Exp. Med.*, 1922, xxxv, 561.

[5] De Kruif, P. H., *J. Exp. Med.*, 1922, xxxvi, 309.

[6] A preliminary report of this work has recently appeared (Webster, L. T., *Proc. Sco. Exp. Biol. and Med.*, 1924–25, xxii, 139).

tion of 100,000 or more bacteria. The character of this growth was then studied more carefully by a serial counting method described in Experiment 2.

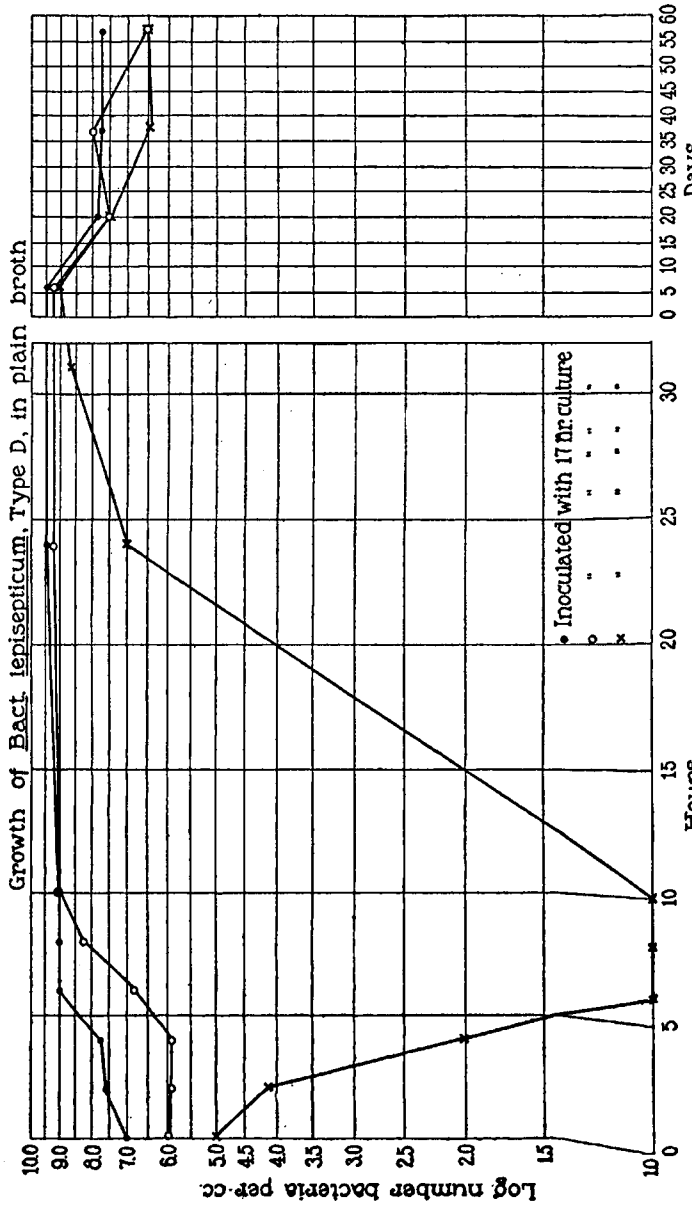
Experiment 2.—Three Erlenmeyer flasks of broth were inoculated with different quantities of a 17 hour broth culture of Microbe D and brought to a volume of 150 cc. Flask 1 received 10,000,000 per cc., Flask 2, 1,000,000, and Flask 3, 100,000. Immediately after inoculation and thereafter at hourly intervals, 1 cc. was removed from each flask and plated by the dilution method to estimate the number of living bacteria present.

TABLE II.

Growth Characteristics of Bacterium lepisepticum, Types D and G.

Experiment No.	Type.	Media.	No. of bacteria inoculated per tube.										D to G transformations.
			1,000,000,000	100,000,000	10,000,000	1,000,000	100,000	10,000	1,000	100	10	1	
1	D	Plain broth.	++	++	++	++	++	0	0	0	0	0	+++
3	G	" "	++	++	++	++	++	++	++	++	++	++	
5	D	" " + 0.6 per cent blood.	++	++	++	++	++	++	++	++	++	++	±
7	"	Plain broth + vaseline seal.	++	++	++	++	++	++	++	++	++	++	±
8	"	Plain broth + 0.00003 cc. blood.	++	++	++	++	++	++	++	++	++	++	±
9	"	Plain broth + 0.00003 cc. autoclaved blood.	++	++	++	++	++	++	++	++	++	++	±

The results of these counts are plotted in Text-fig. 1. The bacteria in Flask 1 increased in numbers immediately but slowly and reached a maximum of 1,000,000,000 per cc. in 6 hours. The number of organisms in Flask 2 remained stationary for 4 hours before a rise in the count was noted. And in Flask 3, the number per cc. fell rapidly to less than 10 and remained so for several hours, after which multiplication occurred and in 24 hours the maximum count of 1,000,000,000 was obtained. In other experiments still smaller inoculations resulted in a rapid drop to zero in the count with no subsequent rise.



TEXT-FIG. 1. Growth of *Bacterium lepisepticum*, Type D, in flasks of extract broth, pH 7.4.

When flasks were seeded with cultures in lag, the stationary phase continued for some hours but when sufficient numbers of bacteria in the period of logarithmic growth were inoculated, multiplication continued at the same rate until the maximum count of 1,000,000,000 per cc. was reached.

Thesé observations are quite similar to those of Chesney on the growth curve of pneumococcus.[7]

Growth of Microbe G in Meat Extract Broth, pH 7.4.

The behavior in broth of Microbe G, the so called type variant, was compared with that of the original strain, Type D, by tests similar to those described above; and it was found that a 16 hour culture seeded quantitatively into a series of nine broth tubes grew well in each instance. Inoculations of 1 or 2 organisms to 1,000,000,000 multiplied freely (Experiment 3, Table II). Furthermore, when frequent counts were made, it was found that a very few organisms, 18, 6, and 1½ hours old, when transferred to fresh media, began immediately to multiply without lag and continued to increase logarithmically until a maximum number of 1 billion per cc. was reached (Text-fig. 2).

Evidently then, meat extract broth under aerobic conditions is adequate for the optimum growth of Microbe G, the form of low virulence, but not for Type D, the highly virulent strain.

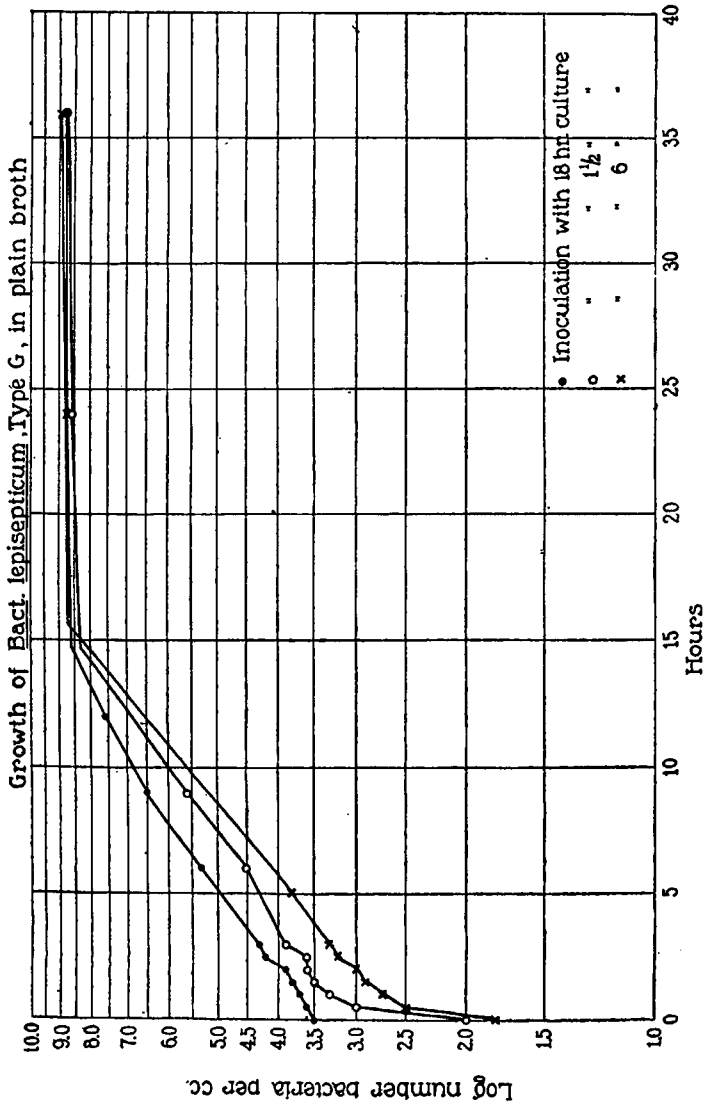
Growth of Microbes D and G in Extract Broth Plus Rabbit Blood.

Rabbit blood added to extract broth modified the growth of Microbe D in a striking manner.

Experiment 5.—Nine tubes containing 0.6 per cent citrated blood in 5 cc. of broth were inoculated with nine dilutions from 1:1 to 1:1,000,000,000, respectively, of a 16 hour bouillon culture of Microbe D. Certain dilutions were plated to determine the actual numbers of organisms present; and after 24 hours incubation, the number of organisms on the plates was counted and the tubes inspected.

A heavy growth occurred invariably in each tube (Table II, Experiment 5). The smallest number of organisms employed multiplied freely in this medium.

[7] Chesney, A. M., *J. Exp. Med.*, 1916, xxiv, 387.



TEXT-FIG. 2. Growth of *Bacterium lepisepticum*, Type G, in flasks of extract broth, pH 7.4.

A further analysis of this growth was carried out by means of serial counts as in Experiment 2.

Experiment 6.—An Erlenmeyer flask containing 150 cc. of broth plus 0.6 per cent rabbit blood was inoculated with 1 cc. of a 1:100,000 dilution of a 17 hour broth culture of Microbe D and incubated at 37°. At ½ or 1 hour intervals thereafter, 1 cc. was removed and counted by the dilution and plating method. After 1½ hours incubation, 20 cc. of culture was removed from Flask 1 and put into a second flask of similar blood broth. This new culture was then incubated and submitted to frequent counts. 4½ hours later, or 6 hours after inoculation, 1 cc. of a 1:100 dilution was again taken from Flask 1 and placed into a third flask of the same medium. Counts were begun on this fresh culture and were continued throughout the day on all three flasks.

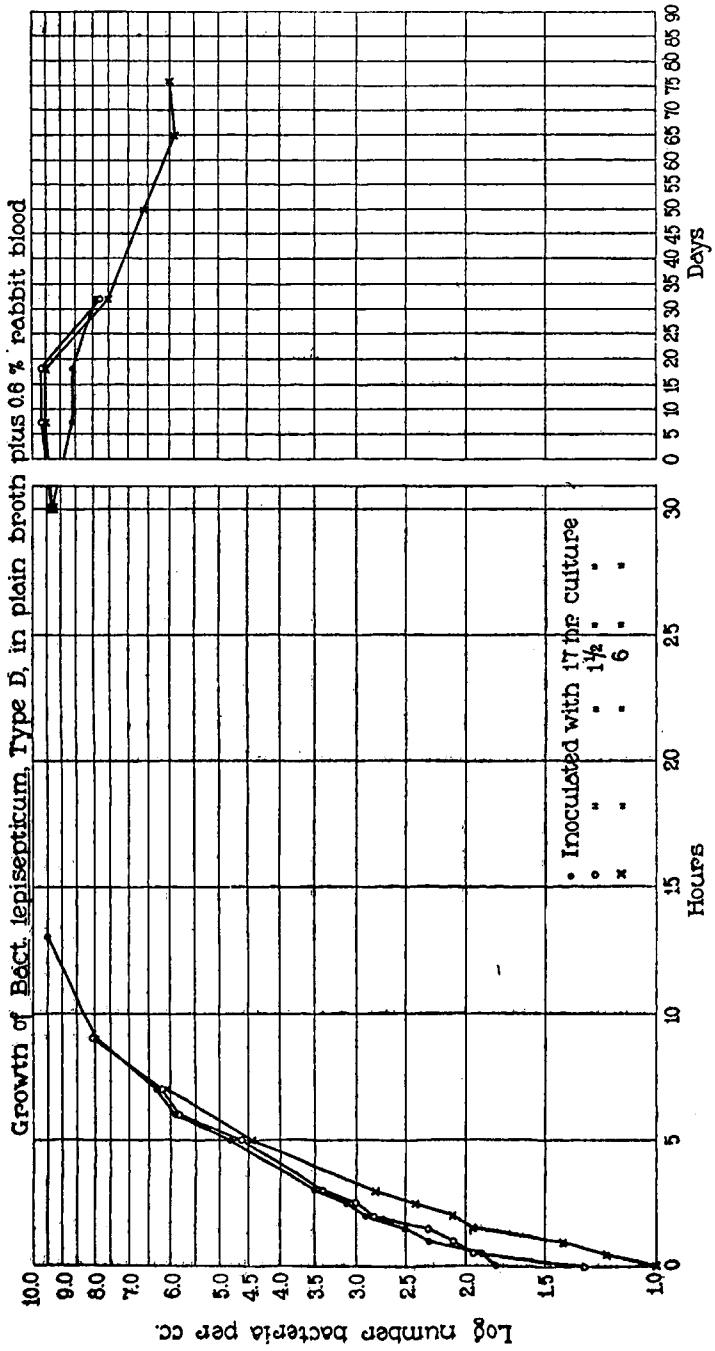
The growth of the bacteria in Flask 1 represents, therefore, the activity of a 17 hour culture of Microbe D in fresh blood broth media, that of Flask 2, the activity of a 1½ hour culture, and of Flask 3, a 6 hour culture under similar conditions. From Text-fig. 3, in which the counts are plotted, it may be seen that Flask 1 received an inoculation of 65 organisms, Flask 2, 21, and Flask 3, 6. In each instance a logarithmic increase in the number per cc. began immediately and continued for about 10 hours. When the count of 1,000,000,000 per cc. had been reached, no further increase was noted. For several weeks the counts remained about the same; then a slow and progressive drop occurred.

In brief, then, the addition of rabbit blood to extract broth furnishes optimum growth conditions for Type D by enabling the smallest number of organisms to multiply logarithmically without lag in a manner similar to the multiplication of Microbe G in plain broth.

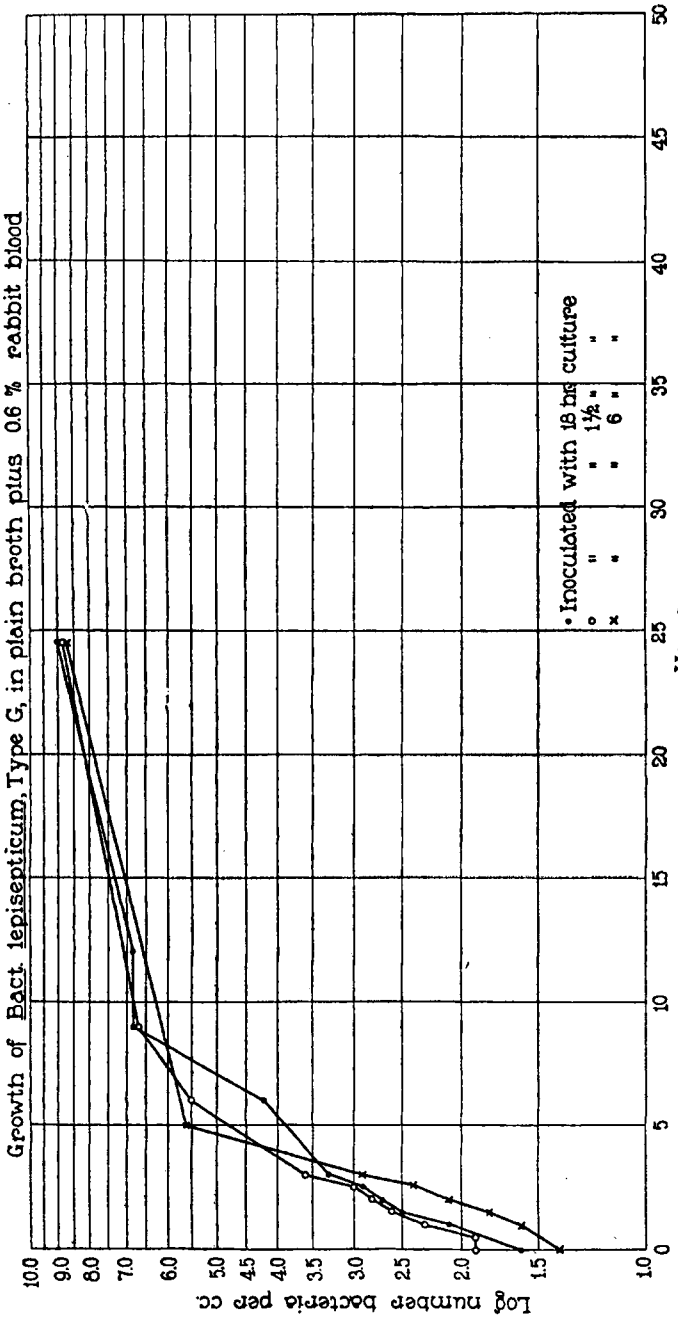
The behavior of Type G in blood broth did not differ essentially from that in plain broth (Text-fig. 4). Small inoculations were sufficient for growth, multiplication began immediately and continued logarithmically for 8 to 10 hours until a maximum number of about 500,000,000 was reached.

Dissociation of Bacterium D to Type G.

If broth tubes are inoculated with Type D and incubated for 48 hours or more as in Experiment 1, a scum or granular film appears at the surface of the liquid. When the tube is shaken, this material



TEXT-FIG. 3. Growth of *Bacterium lepisepticum*, Type D, in flasks of extract broth plus 0.6 per cent rabbit blood.



TEXT-FIG. 4. Growth of *Bacterium lepisepticum*, Type G, in flasks of extract broth plus 0.6 per cent rabbit blood.

settles and a new scum forms in a few hours. This scum proved to be made up of aggregations of Type G bacteria.

Apparently this change from Microbe D to G was occurring at the surface of the fluid where the oxygen tension was at atmospheric pressure and not in the depths of the media where methylene blue was rapidly decolorized. The following experiment was planned to test the influence of media at low oxygen tension on this dissociation phenomenon.

Experiment 7.—Tubes containing 5 cc. of meat extract broth were placed in boiling water for ½ hour, layered with vaseline at the surface of the fluid, and allowed to cool. Each tube was then inoculated with ½ cc. of a graded dilution from 1:1 to 1:1,000,000,000 of a 17 hour culture of Microbe D and incubated at 37°.

An abundant growth was present in all tubes 24 hours later (Table II, Experiment 7). And subsequently, for a 3 week period of observation, no D to G transformation occurred.

Later it was noticed that the D to G dissociation in broth tubes containing blood was strongly inhibited. So a conclusion was reached that a reduction in oxygen tension of extract broth or the addition of blood to this medium furnished conditions for the optimum growth of the virulent Microbe D and retarded the appearance of the variant G forms of low virulence.

Growth of Microbe D in Broth Plus Minute Amounts of Rabbit Blood.

We were impressed with the similarity of these results to those in certain experiments with pneumococcus published by Avery and his associates.[8] They believe that two substances are necessary for the optimum growth of pneumococci, a heat-labile vitamine-like factor, and a heat-stabile catalyst, probably of peroxidase nature. Both of these factors are present in blood and in fresh potato and when added to extract broth enable small numbers of bacteria to multiply freely without lag. They prepared an "artificial" peroxidase with gum arabic and ferrous sulfate which when combined with

[8] Avery, O. T., *et al.*, *J. Exp. Med.*, 1921–24, xxxiv–xl; *Proc. Soc. Exp. Biol. and Med.*, 1921, xviii–xix.

yeast extract and added to broth induced good growth of pneumococci. These experiments together with the researches of McLeod and Gordon[9] have given rise to the theory that certain bacteria which evolve peroxides require for active growth in aerobic media the presence of a peroxidase to split up these toxic substances.

It seemed to us quite possible that in our experiments blood was functioning in a similar manner. So tests were applied to determine the reactivity of the blood in high dilutions before and after autoclaving.

Experiment 8.—Tubes of broth plus 0.00003 cc. of rabbit blood were inoculated with a broth culture of Microbe D in dilutions increasing exponentially from 1 to 9.

Growth occurred promptly in every tube (Table II, Experiment 8), and for some weeks little or no D to G transformation occurred. The benzidine reaction in this medium was positive and further experiments showed that optimum growth without any dissociation was always obtained in broth to which sufficient blood had been added to give a positive benzidine test.

When flasks containing 150 cc. of broth plus 0.0003 per cent blood were inoculated with small numbers of 17, 6, and $1\frac{1}{2}$ hour cultures of Microbe D, precisely as in Experiment 6, and counted at short intervals thereafter, figures were obtained which showed in each case immediate logarithmic multiplication with a maximum number of 1,000,000,000 per cc. in 9 to 10 hours.

These results, plotted in Text-fig. 5, are very similar to those in Text-fig. 3 and show that very small amounts of blood, to the limit of the benzidine reaction, function just as well as large quantities in inducing optimum growth and permanence of type characteristics.

Experiment 9.—Autoclaved blood in similar small quantities, 0.0003 per cent, just sufficient to give a positive benzidine test, was added to test-tubes and to Erlenmeyer flasks. Microbe D was added to the tubes in various dilutions as in Experiment 8, and to flasks as in Experiment 6. Counts were made and the flasks and tubes were inspected after 1, 2, and 3 days and after 1, 2, and 3 weeks incubation.

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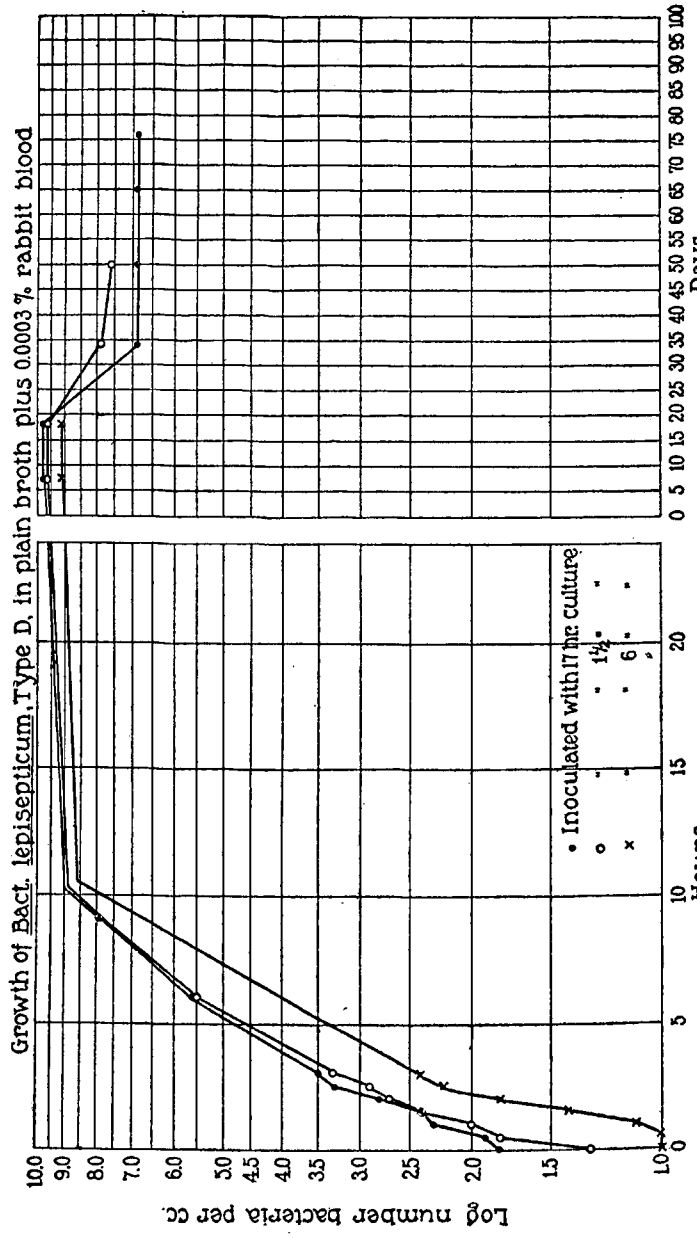
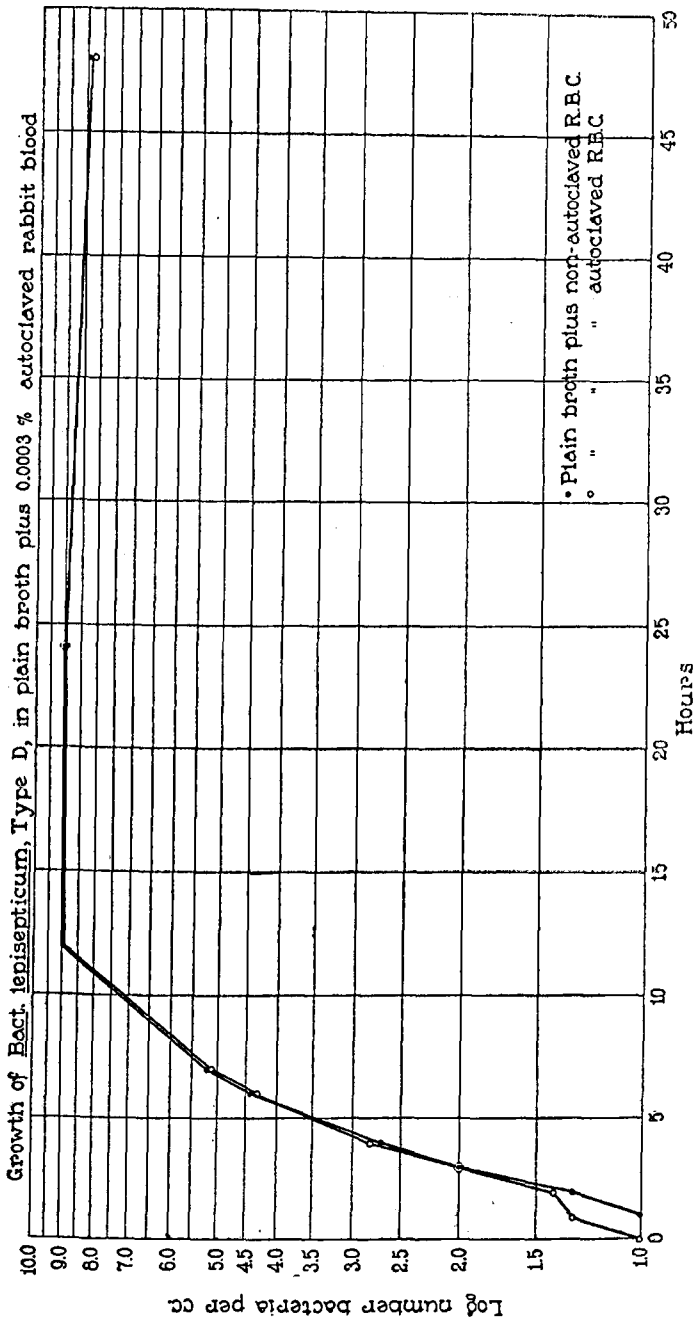
[9] McLeod, J. W., and Gordon, J., *J. Path. and Bact.*, 1922–23, xxv–xxvi.



TEXT-FIG. 5. Growth of *Bacterium lepisepticum*, Type D, in flasks of extract broth plus 0.0003 per cent rabbit blood.



TEXT-FIG. 6. Growth of *Bacterium lepisepticum*, Type D, in flasks of extract broth plus 0.0003 per cent autoclaved blood.

Good growth occurred in all tubes (Table II, Experiment 9) and the 17, 6, and $1\frac{1}{2}$ hour cultures in the flasks although inoculated in very small number, multiplied logarithmically (Text-fig. 6) and behaved similarly to the cultures in Experiment 6 (Text-fig. 3).

DISCUSSION.

These experiments show that virulent types of *Bacterium lepisepticum* require for optimum growth and maintenance of type purity and pathogenicity either a lowered oxygen tension in a general nutrient medium or, under atmospheric conditions, the presence of an accessory substance found in blood. This substance is active in high dilutions to the limit of the benzidine reaction, and is heat-stabile. Probably, therefore, it is not an ordinary food or of vitamine nature. Further experiments on this phase of the problem will be published shortly.

The apparent disagreement of De Kruif's experiments which showed that meat infusion and serum inhibited the D to G dissociation and that high concentrations of peptone with decreasing amounts of meat infusion favored the process is explainable by the presence of red cells or derivatives therefrom in the serum and meat infusion used in those experiments.

CONCLUSIONS.

1. *Bacterium lepisepticum*, Type D, inoculated in small amounts into meat extract broth, pH 7.4, under aerobic conditions failed to grow. Larger inoculations underwent a period of lag before the logarithmic growth phase began.

2. In this medium, dissociation of Microbe D to the variant G occurred readily.

3. A lowered oxygen tension in this broth resulted in growth of small numbers of Type D with no lag and great inhibition of the type dissociation process.

4. Similar optimum growth conditions were obtained by adding to extract broth, under aerobic conditions, autoclaved or unautoclaved rabbit blood in very small amounts, 0.0003 per cent, just sufficient to give a positive benzidine test.

5. The nature of this accessory substance is discussed.
6. The variant Type G grows equally well in plain or blood broth.

I wish to thank Mr. C. G. Burn for his assistance with the technical part of this work.