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ERRATA.

Page 15, Table 13, "Hyco" *should read* "Disinfectant B."
Page 230, line 2, "see Fig. 5" *should read* "see Fig. 3, Plate 5."
Page 231, line 12, "see also Plate 5" *should read* "see also Fig. 1, Plate 5."
Page 243, "Saccharose— *should read* "Saccharose+
B. communior" B. communior."
Page 244, line 11, *insert* "Three strains isolated by Melia from human feces."

Explanation of Plate 5 (p. 230).

Fig. 1 (at top).—Inclusion of oidiomycetes in uninuclear and multinuclear cells in liver (guinea-pig 53—see p. 231).

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A METHOD FOR THE BACTERIOLOGICAL STANDARDIZATION OF DISINFECTANTS.*†

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Part I.

INTRODUCTION.

There is no question but that great fraud is practiced in the exploitation of many of the so-called disinfectants that are offered for sale upon the market and are constantly being used by the credulous public. Although the germ theory of disease has long since become an established fact, the average layman either has only a vague conception of it or looks upon it with doubt and suspicion. This skepticism as to the nature of infectious diseases, and the ignorance as to the value of the agents and methods at our command for destroying infection, offer to those manufacturers of disinfectants who care to practice it a fruitful field for deception. Even physicians and health officers are frequently imposed upon by the manufacturers or vendors of so-called disinfectants or germicides.

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It is often too true that the deodorizing powers of these preparations are taken as an index of their germicidal efficiency which, in thus giving a false sense of security, may lead to disastrous results.

As an example, the sale of certain "chlorides" may be cited, the claims for the disinfecting power of which are extravagantly set forth on the label of the container but which, by laboratory examination, are shown to be almost devoid of disinfecting properties, even when used in undiluted strength and under the favorable conditions of a laboratory experiment. Other preparations are sold with the claim of a carbolic coefficient, which often is excessive and cannot be confirmed in other laboratories.

With the methods now customarily used in determining the value of a disinfectant in terms of its carbolic acid coefficient the results that may be obtained even by the same worker are misleading and subject to wide variations.

Therefore the necessity for a satisfactory method for the standardization of the large number of disinfectants offered for sale upon the market becomes evident, and particularly so to all those engaged in public health work.

The Rideal-Walker method[1] is now extensively used, but it is not without its faults. The Lancet method,[2] while not as simple or as easily performed as the Rideal-Walker method, seems to be the best one so far proposed.

Briefly stated, the carbolic acid coefficient in the Rideal-Walker method is arrived at by dividing the figure indicating the degree of dilution of the disinfectant that kills an organism in a given time by that expressing the degree of dilution of the carbolic acid that kills the same organism in the same time under exactly similar conditions. Leaving out details, the determination of the Rideal-Walker coefficient is substantially as follows:

Certain standard conditions are considered essential to the proper performance of the test. Phenol solutions of known strength are used; cultures are grown in a standard medium, transplants being made every 24 hours; the loops used for all inoculations are

[1] S. Rideal, and J. S. A. Walker, *Jour. Roy. San. Inst.*, London, 1903, 24, p. 424.

[2] "The Standardization of Disinfectants [unsigned]," *Lancet*, London, Vol. 177, Nos. 4498, 4499, and 4500.

of a standard size (about 4 mm. in diameter). Usually four dilutions of suitable strengths of the disinfectant to be used are made. Phenol controls of a suitable strength are also prepared. Five c.c. of each of these dilutions are placed in sterile test-tubes to which are added at intervals of one half-minute a 24-hour broth culture of *B. typhosus* in the proportion of 1 drop of culture to each cubic centimeter of disinfectant used (according to Partridge, one drop of culture equals about 0.1 c.c.).

At the end of two and a half minutes a loopful of each of the mixtures is inoculated into a test-tube containing 5 c.c. of standard broth, an interval of half a minute being thus allowed between taking the samples from the different dilutions. This is repeated at 5, $7\frac{1}{2}$, 10, $12\frac{1}{2}$, and 15 minutes. The broth tubes, after being incubated at 37° C. for 48 hours, are examined for growth.

The results of the examination are then noted, and if suitable comparative strengths of the disinfectant and carbolic acid have been selected the carbolic acid coefficient is determined as above stated.

The following table (Table 1) illustrates the manner of determining the carbolic acid coefficient of a disinfectant according to the Rideal-Walker method:

TABLE 1.

Date, May 18, 1910.

Name, "A."
Temperature of Medication, 20° C.
Culture Used, B. typhosus, 24-hr., extract broth, filtered.
Proportion of Culture and Disinfectant, 0.1 c.c.+5 c.c.
Organic Matter, None. *Kind*, None. *Amount*, None.
Subculture Media, Standard extract broth. *Reaction*, +1.5. *Quantity in Each Tube*, 10 c.c.

Sample	Dilution	Time Culture Exposed to Action of Disinfectant for Minutes:						Phenol Coefficient	Remarks
		$2\frac{1}{2}$	5	$7\frac{1}{2}$	10	$12\frac{1}{2}$	15		
Phenol.....	1:90	+	−	−	−				
	1:100	+	+	+	−	−	−		
Disinfectant "A"...	1:500	+	+	−	−	−	−		
	1:550	+	+	+	−	−	−		
	1:600	+	+	+	+	−	−		
								100)550 5.5 coefficient	

Early in our work it was realized that, on account of certain faults to be pointed out later, the Rideal-Walker method was not entirely satisfactory for determining the relative values of dis-

infectants in terms of pure phenol. When used under standard conditions as to temperature, organism, media, etc., *approximately* constant results may be obtained by different workers familiar with the technic; but a certain amount of dexterity which can only be obtained by practice is always necessary for carrying out the test.

The great objection to the method, however, and it is almost a vital one, is the latitude allowed in determining the coefficient. This point is strikingly brought out in the following tables (2, 3, and 4):

TABLE 2.

Date, May 18, 1910.

Name, "A."
Temperature of Medication, 20° C.
Culture Used, B. typhosus, 24-hr., extract broth, filtered.
Proportion of Culture and Disinfectant, 0.1 c.c.+5 c.c.
Organic Matter, None. *Kind*, None. *Amount*, None.
Subculture Media, Standard extract broth. *Reaction*, +1.5. *Quantity in Each Tube*, 10 c.c.

Sample	Dilution	Time Culture Exposed to Action of Disinfectant for Minutes:						Phenol Coefficient	Remarks
		2½	5	7½	10	12½	15		
Phenol.....	1:80	−	−	−					
	1:90	+	−	−	−				
Disinfectant "A"...	1:375	−	−	−					
	1:400	+	−	−	−				
	1:425	+	+	−	−	−	−		
								90)400	
								4.44 coefficient	

TABLE 3.

Date, May 18, 1910.

Name, "A."
Temperature of Medication, 20° C.
Culture Used, B. typhosus, 24-hr., extract broth, filtered.
Proportion of Culture and Disinfectant, 0.1 c.c.+5 c.c.
Organic Matter, None. *Kind*, None. *Amount*, None.
Subculture Media, Standard extract broth. *Reaction*, +1.5. *Quantity* in *Each Tube*, 10 c.c.

Sample	Dilution	Time Culture Exposed to Action of Disinfectant for Minutes:						Phenol Coefficient	Remarks
		2½	5	7½	10	12½	15		
Phenol.....	1:90	+	−	−	−				
	1:100	+	+	+	−	−	−		
Disinfectant "A"...	1:450	+	+	−	−	−	−		
	1:550	+	+	+	−	−	−		
	1:600	+	+	+	+	−	−		
								100)550	
								5.5 coefficient	

TABLE 4.

Name, "A." *Date*, May 18, 1910.
Temperature of Medication, 20° C.
Culture Used, B. typhosus, 24-hr., extract broth, filtered
Proportion of Culture and Disinfectant, 0.1 c.c.+5 c.c.
Organic Matter, None. *Kind*, None. *Amount*, None.
Subculture Media, Standard extract broth. *Reaction*, +1.5. *Quantity in Each Tube*, 10 c.c.

Sample	Dilution	Time Culture Exposed to Action of Disinfectant for Minutes:						Phenol Coefficient	Remarks
		2½	5	7½	10	12½	15		
Phenol.....	1:100	+	+	+	−	−	−		
	1:110	+	+	+	+	+	−		
Disinfectant "A"...	1:600	+	+	+	+	−	−		
	1:650	+	+	+	+	+	−		
	1:700	+	+	+	+	+	+		
								110)650	
								5.91 coefficient	

Tables 2, 3, and 4 give the determination of the carbolic coefficient for the same disinfectant under exactly similar conditions, the experiments being done on the same day.

In Table 2 the coefficient is 4.44; in Table 3 it is 5.5; in Table 4 it is 5.91. It will be seen, therefore, that according to the bias of the operator a coefficient may be obtained for the same disinfectant varying from 4.44 to 5.91, or a difference of 33.2 per cent—truly a wide variation.

With practice and by selecting for use certain strengths of the disinfectant and carbolic acid, the operator can regulate to a certain extent the time period at which the comparison will be made in determining the coefficient. Furthermore, if more than one strength of the carbolic acid control is used and the results show that more than one of the time periods will admit of comparison, the operator can arbitrarily select the one that will most advantageously suit the purpose of the experiment. Herein lie the principal objections to the Rideal-Walker method of determining the coefficient of disinfectants.

Other minor objections to the method that may be noted are lack of definiteness in the proportion of culture added to the disinfectant, a "drop" being a variable quantity; the latitude allowed in temperature, 18° to 20° C., being a rather wide variation (see results of our experiments at temperatures from 15° to 25° C.); the use of seeding tubes 5 inches in length by $\frac{5}{8}$ inch in diameter which, unless the tubes are handled, offer some difficulty in taking

plants therefrom, and if the tubes are removed for this purpose erroneous results may be obtained by shaking from the sides of the tube organisms that have not been fully exposed to the action of the disinfectant.

The coefficient, as determined by the Lancet Commission, is arrived at as follows: The figure representing the percentage strength of the weakest killing dilution of the phenol is divided by the figure representing the percentage strength of the weakest killing dilution of the unknown disinfectant, both at $2\frac{1}{2}$ and at 30 minutes, the mean resulting figure being taken as the true coefficient. An example of the determination of the carbolic acid coefficient by the Lancet method may be seen from the following table:

TABLE 5.*

DISINFECTANT X.

MINUTES	DILUTIONS							
	1–300	1–400	1–500	1–600	1–700	1–800	1–900	1–1000
	% 0.333	% 0.250	% 0.200	% 0.166	% 0.143	% 0.125	% 0.111	% 0.100
$2\frac{1}{2}$	o	o	+	+	+			
5		o	o	+	+	+		
$7\frac{1}{2}$		o	o	+	+	+		
10			o	+	+	+	+	
$12\frac{1}{2}$			o	+	+	+	+	
15				+	+	+	+	+
20				o	+	+	+	+
25				o	+	+	+	+
30				o	+	+	+	+

CARBOLIC ACID CONTROL.

MINUTES	PERCENTAGE DILUTIONS							
	1.10	1.00	0.917	0.846	0.786	0.733	0.687	0.647
$2\frac{1}{2}$	o	o	+	+				
5	o	o	+	+				
25					o	o	+	+
30					o	o	+	+

Room temperature 67° F.; + signifies growth; o signifies no growth; blank spaces signify not tested.

The coefficient is therefore:

$$\frac{\frac{1.00}{0.25}+\frac{0.733}{0.166}}{2}=\frac{4.0+4.4}{2}=4.2.$$

* Taken from the report of the Lancet Commission.

The important modifications of the Lancet method on the Rideal-Walker are in the increased number of dilutions employed, sometimes as many as twelve tubes being inoculated in each $2\frac{1}{2}$ minute interval; extension of the number of time intervals to 30 instead of 15 minutes; the use of *B. coli* and MacConkey's bile salt media for subcultures instead of *B. typhosus* and standard extract broth; the amount of the mixture of culture and disinfectant transferred to the subculture tubes; the method of determining the coefficient.

Other minor modifications are the use of special spoons for transplanting instead of loops; the use of "spoonfuls" of the culture for seeding instead of "drops"; a special apparatus for flaming the spoons; and the use of specimen pots instead of test-tubes to contain the mixture of disinfectant and culture.

The Lancet method, on the whole, seems to be a distinct advance over the Rideal-Walker; but nevertheless it appears to have certain rather serious faults.

The Lancet method requires the use of *B. coli* and MacConkey's bile salt media, and their use was proposed for the following claimed advantages over the *B. typhosus* and standard extract broth: (1) It is nonpathogenic; (2) a culture of very constant biological characters may be obtained by carrying on a culture every 24 hours in broth of the same standard composition; and (3) if MacConkey's bile salt media are used for subcultures the risk of misleading results from accidental contamination is practically eliminated.

These claimed advantages do not appear to us to be altogether well founded, for the following reasons: It is well known that there is a greater variability in the different strains of *B. coli communis* than in the various strains of *B. typhosus* and therefore there will be less variation in the organism used in the different laboratories if the latter be used than if *B. coli* is used. The use of a special medium which, by its color reactions, will prevent falsification of results does not greatly appeal to us; if the typhoid bacillus is used and an error is suspected it can be easily cleared up with antityphoid serum. Moreover, besides being comparatively expensive, bile salt media have a much greater restraining influence on

attenuated organisms of the typhoid-colon group, such as is the case after they have been exposed to the action of disinfectants, than is the case with extract broth, the reaction of which is +1.5.

Furthermore, with the Lancet method there seems to be no rule or standard as to the rate of decrease in strengths of the dilutions of disinfectants to be tested. It is evident, for instance, that different results will be obtained if one worker uses dilutions, say of 1:300, 1:350, 1:400, etc., and another uses 1:300, 1:325, 1:350, etc.

Aside from the use of the colon bacillus as a test organism, the special media for subcultures, and the lack of a standard scale for making dilutions, the Lancet method has but little to be objected to. The lengthening of the time for the performance of the test to 30 minutes does not seem to have any very special advantage. The special apparatus required, particularly the "spoons," interfere to some extent with the general employment of the method.

However, notwithstanding the objections noted, we believe the Lancet method to be a distinct improvement over any other that has been proposed up to this time; and that with it constant results can be obtained by different workers.

After doing considerable work with both the Rideal-Walker and the Lancet methods we came to the conclusion that, on account of the great variation in results that may be obtained with the former method, it was not a method to advise for the examination of disinfectants.

While consistent results can be obtained with the Lancet method, we believe, on account of the objections we have stated in the discussion of that method, that this method can be so modified as to greatly increase its usefulness. With this object in view we have taken up in detail each step and factor concerned in the successful carrying out of the test and propose the following method, to be described later, for the determination of the germicidal efficiency of disinfectants as compared with pure phenol.

In proposing this method we desire to make full acknowledgment of our use of the Rideal-Walker and the Lancet methods, especially the latter, as a basis for our work.

Part II.

PRINCIPAL FACTORS INVOLVED IN THE EXAMINATION OF DISINFECTANTS.

Lack of attention to the different factors concerned in the examination of disinfectants is responsible for a large percentage of the inconsistencies or discrepancies in results obtained by the same or by different workers when working with the same disinfectant. Unless strict attention is paid to the various influences involved it is useless to expect to find any method satisfactory.

In order the better to emphasize the effect of these influences upon the results obtained the various factors involved will be discussed under the appropriate headings.

TEST ORGANISM.

Unless different observers use the same species of organism there can be no possibility of uniformity in results. The coefficient obtained with different species may vary as much as 300 per cent. For this reason it is important that one species be selected for use as the test organism. It would be highly desirable if the same strain of this species could be used by all workers in the testing of disinfectants, as there is often a variation in the resistance of different strains of the same species. This objection does not apply as much to the typhoid organism as to the colon bacillus, and to some other bacteria.

We made a number of comparative tests with different strains of *B. typhosus* and *B. coli*, and found a very much greater difference in the resistance of different strains of the colon bacillus than of the typhoid bacillus.

It is most important that, before being used for a test, the organism be carried over on broth daily for at least one week. In all cases a 24-hour culture should be used, as there is a decided difference in the resistance of a 24-hour and a 48-hour culture, the latter being the more resistant.

In order to avoid clumps in the culture, the 24-hour broth culture should be well shaken and then filtered through sterile filter paper into a sterile test-tube. After this it should be placed

in the water bath in order that it may reach the standard temperature before being added to the disinfectant dilutions.

TEMPERATURE OF EXPERIMENT.

It is a well-known principle in the use of disinfectants that, within certain limits, the higher the temperature at which the disinfectant is used the greater are its germicidal properties. This increase in the germicidal properties of disinfectants through the influence of heat is not the same for all disinfectants; some, such as formaldehyde, are more strongly influenced than others. The following tables (6 to 9) show the influence of heat upon solutions of phenol and disinfectant "A." It will be seen that, at 15° C., phenol, in a dilution of 1:80, killed the typhoid organism in $2\frac{1}{2}$ minutes, while at a temperature of 30° C. the organism was killed in the same length of time by a dilution of 1:120.

On account of the great variation of temperature in the United States, especially during the summer, it becomes necessary that a standard temperature be adopted. We have adopted a temperature of 20° C. and have devised a simple water bath to be used for maintaining this temperature. This bath consists of a wooden box 20 inches deep, 21 inches long, and 21 inches wide. Inside this box a 14-quart agate-ware pail 10 inches deep is placed and sawdust is well packed around, sufficient being placed on the bottom of the box to bring the rim of the pail on a level with the top of the box.

A tightly fitting wooden cover is placed over the pail, so made that the edges project slightly over the rim. In the cover are a sufficient number of holes for the seeding tubes, a thermometer, and the tube containing the culture. About three inches below the rim of the pail a false bottom of wire gauze is placed; this is for the seeding tubes, etc., to rest on. Water is placed in the pail to within half an inch of the top.

When an experiment is to be made the temperature of the water in the pail is taken, and if above or below 20° C. it is brought to the desired temperature by the addition of either cold or hot water. It will be found that only very slight change takes place in the temperature of the bath in an hour and that it is an easy

matter to keep the temperature at the figure desired. It is of advantage, in regulating the temperature of the bath, to have a spigot in the bottom of the pail to draw off the water when so desired.

TABLE 6.

Date, August 11, 1910.

Name, Disinfectant "A" and phenol.
Temperature of Medication, 15° C.
Culture Used, B. typhosus, 24-hr., extract broth, filtered.
Proportion of Culture and Disinfectant, 0.1 c.c.+5 c.c.
Organic Matter, None. *Kind*, None. *Amount*, None.
Subculture Media, Extract broth. *Reaction*, +1.5. *Quantity*, 10 c.c

Sample	Dilution	Time Culture Exposed to Action of Disinfectant for Minutes:						Phenol Coefficient	Remarks
		2½	5	7½	10	12½	15		
Phenol.....	1:70	−	−	−					
	1:80	−	−	−	−				
	1:90	+	−	−	−	−	−		
	1:100	+	+	−	−	−	−		
	1:110	+	+	+	+	+	+		
	1:120	+	+	+	+	+	+		
Disinfectant "A"...	1:350	−	−	−					
	1:375	+	−	−	−	−			
	1:400	+	−	−	−	−	−		
	1:450	+	+	−	−	−	−		
	1:500	+	+	+	−	−	−		
	1:550	+	+	+	+	−	−		
	1:600	+	+	+	+	+	+		
	1:650	+	+	+	+	+	+		
	1:700	+	+	+	+	+	+		

TABLE 7.

Date, August 11, 1910.

Name, Disinfectant "A" and phenol.
Temperature of Medication, 20° C.
Culture Used, B. typhosus, 24-hr., extract broth, filtered.
Proportion of Culture and Disinfectant, 0.1 c.c.+5 c.c.
Organic Matter, None. *Kind*, None. *Amount*,
Subculture Media, Extract broth. *Reaction*, +1.5. *Quantity*, 10 c.c.

Sample	Dilution	Time Culture Exposed to Action of Disinfectant for Minutes:						Phenol Coefficient	Remarks
		2½	5	7½	10	12½	15		
Phenol.....	1:90	−	−	−					
	1:100	+	−	−	−	−	−		
	1:110	+	+	+	+	−	−		
	1:120	+	+	+	+	+	+		
Disinfectant "A"...	1:350	−	−	−					
	1:375	−	−	−	−				
	1:400	−	−	−	−	−			
	1:450	+	−	−	−	−	−		
	1:500	+	−	−	−	−	−		
	1:550	+	+	−	−	−	−		
	1:600	+	+	−	−	−	−		
	1:650	+	+	+	+	+	+		
	1:700	+	+	+	+	+	+		
	1:750	+	+	+	+	+	+		

TABLE 8.

Name, Disinfectant "A" and phenol. *Date*, August 11, 1910.
Temperature of Medication, 25° C.
Culture Used, B. typhosus, 24-hr., extract broth, filtered.
Proportion of Culture and Disinfectant, 0.1 c.c.+5 c.c.
Organic Matter, None. *Kind*, None. *Amount*,
Subculture Media, Extract broth. *Reaction*, +1.5. *Quantity*, 10 c.c.

Sample	Dilution	Time Culture Exposed to Action of Disinfectant for Minutes:						Phenol Coefficient	Remarks
		2½	5	7½	10	12½	15		
Phenol.....	1:100	−	−	−					
	1:110	−	−	−	−	−			
	1:120	+	−	−	−	−	−		
	1:130	+	+	+	−	−	−		
	1:140	+	+	+	+	+	−		
	1:150	+	+	+	+	+	+		
Disinfectant "A"...	1:450	−	−	−	−				
	1:500	−	−	−	−	−			
	1:550	−	−	−	−	−	−		
	1:600	−	−	−	−	−	−		
	1:650	+	+	−	−	−	−		
	1:700	+	+	+	−	−	−		
	1:750	+	+	+	−	−	−		
	1:800	+	+	+	+	+	+		

TABLE 9.

Name, Disinfectant "A" and phenol. *Date*, ————, 1910.
Temperature of Medication, 30° C.
Culture Used, B. typhosus, 24-hr., extract broth, filtered.
Proportion of Culture and Disinfectant, 0.1 c.c.+5 c.c.
Organic Matter, None. *Kind*, None. *Amount*,
Subculture Media, Extract broth. *Reaction*, +1.5. *Quantity*, 10 c.c.

Sample	Dilution	Time Culture Exposed to Action of Disinfectant for Minutes:						Phenol Coefficient	Remarks
		2½	5	7½	10	12½	15		
Phenol.....	1:110	−	−	−	−				
	1:120	−	−	−	−				
	1:130	+	−	−	−	−	−		
	1:140	+	+	−	−	−	−		
	1:150	+	+	+	−	−	−		
	1:500	−	−	−	−				
	1:550	−	−	−	−				
	1:600	−	−	−	−	−	−		
	1:650	−	−	−	−	−	−		
	1:700	−	−	−	−	−	−		
	1:750	+	−	−	−	−	−		
	1:800	+	+	+	−	−	−		
	1:850	+	+	+	+	+	+		
	1:900	+	+	+	+	+	+		

PROPORTION OF CULTURE TO DISINFECTANT.

As disinfection is the result of the chemical action of the disinfecting agent upon the test organism, mass action is an important factor in the testing of disinfectants. By this is meant that within certain limits the greater the number of bacteria added to the disinfectant dilution the stronger the dilution required to do the same work. For this reason it is important that the amount of

culture to be added to the dilution should be stated in definite quantities and not in "drops" or in "spoonfuls." We have adopted the practice of using 0.1 c.c. of a 24-hour broth culture. For measuring this we use a delivery pipette graduated in tenths.

The influence of different amounts of culture is shown in Tables 10 and 11.

TABLE 10.

Name, "B." *Date*, May 10, 1910.
Temperature of Medication, 20° C.
Culture Used, B. typhosus, 24-hr., broth culture, filtered.
Proportion of Culture and Disinfectant, 0.5 c.c.+5 c.c.
Organic Matter, None. *Kind*, None. *Amount*,
Subculture Media, Extract broth. *Reaction*, +1.5. *Quantity*, 10 c.c.

Sample	Dilution	Time Culture Exposed to Action of Disinfectant for Minutes:						Phenol Coefficient	Remarks
		2½	5	7½	10	12½	15		
Phenol.....	1:70	−	−					80)900	
	1:80	−	−	−	−	−			11.25
	1:90	+	−	−	−	−	−	100)1200	
	1:100	+	+	+	+	+	−		12.00
	1:110	+	+	+	+	+	+		2)23.25
	1:120	+	+	+	+	+	+		11.62
Disinfectant "B"...	1:800	−	−	−					
	1:900	−	−	−	−				
	1:1000	+	−	−	−	−	−		
	1:1100	+	+	+	−	−	−		
	1:1200	+	+	+	+	+	−		
	1:1300	+	+	+	+	+	+		
	1:1400	+	+	+	+	+	+		
	1:1500	+	+	+	+	+	+		

TABLE 11.

Name, "B." *Date*, May 16, 1910.
Temperature of Medication, 20° C.
Culture Used, B. typhosus, 24-hr., extract broth, filtered.
Proportion of Culture and Disinfectant, 0.1 c.c.+5 c.c.
Organic Matter, None. *Kind*, None. *Amount*,
Subculture Media, Extract broth. *Reaction*, +1.5. *Quantity*, 10 c.c.

Sample	Dilution	Time Culture Exposed to Action of Disinfectant for Minutes:						Phenol Coefficient	Remarks
		2½	5	7½	10	12½	15		
Phenol.....	1:80	−	−	−					
	1:90	+	−	−	−	−		80) 1300	
	1:100	+	+	−	−	−	−		16.25
	1:110	+	+	+	+	+	+	100) 1500	
	1:120	+	+	+	+	+	+		15.00
									2)31.25
Disinfectant "B"...	1:1100	−	−	−					15.62
	1:1200	−	−	−	−				
	1:1300	−	−	−	−	−	−		
	1:1400	+	−	−	−	−	−		
	1:1500	+	+	+	−	−	−		
	1:1600	+	+	+	+	+	+		

AMOUNT OF INOCULATION OF SUBCULTURE TUBES.

The amount of the mixture of culture and disinfectant transferred to the subculture tubes for observation as to the effect of the disinfectant upon the test organism has an important influence upon the result of the experiment. If in doing two experiments the subculture inoculations are made, one with loops and the other with spoons which carry over considerably more than the loops, it will be observed that a growth of the organism is often obtained after exposure to slightly stronger solutions of the disinfectant when the spoons are used than is the case with the loops. While this is not very marked, still the use of the spoon will give a slightly lower coefficient than the loop and it is therefore important that a standard-size loop be used for the inoculation of the subculture tubes, since the cost and the difficulty of procuring the spoons make them more or less impracticable. A large number of experiments were done both with loops and spoons, and Tables 12 and 13 show about the average difference in results:

TABLE 12.

Date, May 13, 1900.

Name, "B."
Temperature of Medication, 20° C.
Culture Used, B. typhosus, 24-hr., broth, filtered.
Proportion of Culture and Disinfectant, 0.1 c.c.+5 c.c.
Organic Matter, None. *Kind*, None. *Amount*,
Subculture Media, Standard broth. *Reaction*, +1.5. *Quantity*, 10 c.c.
Inoculations made with loops holding about 0.016 c.c.

Sample	Dilution	Time Culture Exposed to Action of Disinfectant for Minutes:						Phenol Coefficient	Remarks
		2½	5	7½	10	12½	15		
Phenol.....	1:80	−	−	−					
	1:90	+	−	−	−	−	−		
	1:100	+	+	+	+	−	−		
	1:110	+	+	+	+	+	+	80) 1300	
	1:120	+	+	+	+	+	+		16.25
Disinfectant "B"...	1:1100	−	−	−				100) 1600	
	1:1200	−	−	−					16.00
	1:1300	−	−	−	−				2)32.25
	1:1400	+	−	−	−	−	−		16.12
	1:1500	+	−	−	−	−	−		
	1:1600	+	+	+	−	−	−		
	1:1700	+	+	+	+	+	+		
	1:1800	+	+	+	+	+	+		

TABLE 13.

Date, May 11, 1910.

Name, "B."
Temperature of Medication, 20° C.
Culture Used, B. typhosus, 24-hr., broth, filtered.
Proportion of Culture and Disinfectant, 0.1 c.c.+5 c.c.
Organic Matter, None. *Kind*, None. *Amount*,
Subculture Media, Standard broth. *Reaction*, +1.5. *Quantity*, 10 c.c.
Inoculations made with spoon holding about 0.16 c.c.

Sample	Dilution	Time Culture Exposed to Action of Disinfectant for Minutes:						Phenol Coefficient	Remarks
		2½	5	7½	10	12½	15		
Phenol.....	1:80	−	−	−	−				
	1:90	+	−	−	−	−	−	80) 1200	
	1:100	+	+	−	−	−	−		15.00
	1:110	+	+	+	+	+	+	100) 1500	
	1:120	+	+	+	+	+	+		15.00
									2)30.00
Hyco.....	1:1100	−	−	−	−				15.00
	1:1200	−	−	−	−	−			
	1:1300	+	−	−	−	−	−		
	1:1400	+	+	−	−	−	−		
	1:1500	+	+	+	−	−	−		
	1.1600	+	+	+	+	+	+		
	1:1700	+	+	+	+	+	+		

MEDIA FOR SUBCULTURES.

There is probably no one factor, with the possible exception of temperature, that has more to do with irregularities in results than the media used for subcultures. Where the typhoid bacillus is used for the test organism, as in the Rideal-Walker method, and the method proposed by us, it is of paramount importance that the media have a reaction of just +1.5. A reaction greater or less than this exerts a decided inhibiting action upon the growth of the transplanted organism. This is an important point, for if the transplant is made from a test dilution which is just under the killing strength of the disinfectant, the inhibiting action of the media may be sufficient to prevent growth, thus giving a false result. In the hands of different workers a difference in the reaction of the media may result from the degree to which the color reaction in titration is carried. We always carry it to the point where the pink color is distinctly perceptible, but even then there seems to be at times a slight difference in various lots of our media.

The following experiments (Table 14) will show the marked effect of the difference in reaction of the subculture media, all the other conditions of the experiments being identical.

TABLE 14.

Date, August 4, 1910.

Name, Phenol.
Temperature of Medication, 20° C.
Culture Used, B. typhosus, 24-hr., in standard broth, filtered.
Proportion of Culture and Disinfectant, 0.1 c.c.+5 c.c.

Reaction of Media to Phenolphthalein	Dilution of Phenol	Time Culture Was Exposed to Action of Phenol, in Minutes:					
		2½	5	7½	10	12½	15
Neutral	1:100	−	−	−	−		
	1:110	−	−	−	−	−	
	1:120	−	−	−	−	−	−
	1:130	+	−	−	−	−	−
	1:140	+	+	+	−	−	−
	1:150	+	+	+	+	+	−
+0.5	1:100	−	−	−			
	1:110	−	−	−	−		
	1:120	+	−	−	−	−	−
	1:130	+	+	−	−	−	−
	1:140	+	+	+	+	−	−
	1:150	+	+	+	+	+	−
+1.0	1:100	−	−	−	−		
	1:110	+	−	−	−	−	−
	1:120	+	+	+	−	−	−
	1:130	+	+	+	+	+	+
	1:140	+	+	+	+	+	+
+1.5 (standard)	1:90	−	−	−			
	1:100	+	−	−	−	−	−
	1:110	+	+	+	−	−	−
	1:120	+	+	+	+	+	+
	1:130	+	+	+	+	+	+
+2.0	1:90	−	−				
	1:100	−	−	−	−	−	−
	1:110	+	−	−	−	−	−
	1:120	+	+	+	−	−	−
	1:130	+	+	+	+	+	+
	1:140	+	+	+	+	+	+

It is a noteworthy fact that the influence of the reaction of the subculture media upon the growth of the exposed organism was decidedly more pronounced after it had been exposed to phenol than to any of the other disinfectants tried.

It was found too that a more vigorous growth and a growth from stronger solutions were obtained when the exposed organisms were planted in meat broth than when they were planted in extract broth. It is therefore evident from the above that the reaction and character of the subculture media has an important bearing upon the results obtained in determining the phenol coefficient of disinfectants. However, as extract broth is more uniform in composition, more easily prepared, and cheaper than meat broth, we recommend that extract broth be always used, and when it is not, that the fact be so stated.

The amount of media in the tubes for subculture should be sufficient to prevent any antiseptic action, due to the transferred disinfectant. With substances such as chinosol and bichloride of mercury, this is often an important point.

It may be stated here that in our work with some disinfectants, particularly those containing coal-tar products, the disinfectant carried over in making the inoculations of the subcultures caused a distinct cloudiness in the media; but after 48 hours' incubation, this always cleared up so that there was no difficulty in making out the presence or absence of growth.

MacConkey's bile salt medium was given a limited trial with *B. coli communis*. We found that after exposing the *B. coli communis* to the action of a 1 per cent solution of carbolic acid and planting in MacConkey's medium and extract broth respectively every $2\frac{1}{2}$ minutes for 15 minutes, and incubating for 48 hours, there was a growth in all the tubes of the extract broth, but only in the $2\frac{1}{2}$ minutes' tube of MacConkey's medium. This condition or result was more marked with carbolic acid than with any other disinfectant tried.

When using the *B. typhosus* the possibility of contamination in the tubes of broth that show a growth at the end of 48 hours can be determined by the use of antityphoid serum.

ORGANIC MATTER.

We have said nothing as to the introduction of organic matter when making the tests, although we have made many experiments as to the influence of this factor in the examination of disinfectants. We have considered very carefully the advisability of the addition of some form of organic matter, but finally decided not to include it in the method proposed by us. After all, the comparative efficiency of a disinfectant can be made as well without as with organic matter, even though it may be claimed that in the practical use of the substances organic matter be present.

As there are certain disinfectants, such as bichloride of mercury, which are very adversely influenced by organic matter and others not at all—or favorably—affected, it does not seem to us wise to require the presence of organic matter, except where information

on this point is especially desired. It would be of value if the coefficient be determined both with and without the addition of organic matter. When organic matter is to be added we have preferred to use it in the form of a killed (by heat) 24-hour broth culture of the typhoid bacillus.

Part III.

HYGIENIC LABORATORY PHENOL COEFFICIENT.

Having discussed the necessity for a satisfactory method of standardizing disinfectants and the factors involved in the examination of disinfectants, we present below the method we have devised.

When this method is used for the standardization of disinfectants we recommend that it be referred to as the "Hygienic Laboratory Phenol Coefficient."

We prefer to use the word "phenol" instead of "carbolic acid" when speaking of the coefficient, especially since certain dealers advertise for sale carbolic acids which vary greatly in the proportion of phenol present.

MEDIA.

Standard extract broth is used, both for the culture to be tested and for the subcultures made after exposure to the disinfectant. The broth is made from Liebig's extract of beef and is in exact accordance with the standard methods adopted by the American Public Health Association for water analysis. Ten c.c. of the broth are put into each test-tube. This amount of broth has been found sufficient to avoid any antiseptic action of the disinfectant carried over. It is important that the reaction of the media is just $+1.5$.

ORGANISM.

For the test organism a 24-hour-old broth culture in extract broth of the *B. typhosus* (Hopkins) is used. Before beginning a test the culture should be carried over every 24 hours on at least three successive days. For carrying over the culture one loopful of a 4 mm. platinum loop is used.

Before being added to the disinfectant the culture is well shaken, filtered through sterile filter paper, and placed in the water

bath in order that it may reach a temperature of 20° C. before being added to the disinfectant.

TEMPERATURE.

A standard temperature of 20° C. has been adopted for all experiments. This temperature is obtained by the use of a specially devised water bath. The cultures and dilutions of the disinfectant are brought to this temperature before the beginning of the test.

PROPORTION OF CULTURE TO DISINFECTANT.

One-tenth c.c. of the culture is used, added to 5 c.c. of the disinfectant dilution. The amount of culture is measured with a pipette graduated in tenths of a cubic centimeter.

INOCULATION LOOPS.

For making the transfer of the culture after exposure to the disinfectant a platinum loop 4 mm. in diameter of 23 U.S. standard gauge wire is used. We have found it is of advantage to have at least 4, and preferably 6, loops. In order to save time in flaming the following method was devised:

A block about 3 inches wide, 10 inches high, and 12 inches long, containing four or six grooves, spaced two inches apart, is used. Into each of the grooves the platinum loop is laid so that the end of the loops extend about 5 inches beyond the side of the block. The first step in the operation is to sterilize each loop by flaming with a fan-tail Bunsen burner before beginning the experiment.

When ready to begin the operation the loop farthest from the operator is taken in the right hand and the inoculation made. It is then replaced in the groove with the right hand and the Bunsen burner (fan-tail) placed under it with the left hand. The next loop is then used, replaced in its groove, and the Bunsen burner placed under it with the left hand, the first loop having been heated to redness while the second loop was in use. This procedure is then continued until all the inoculations have been made. The time required in making the inoculations and in replacing the loop is short, it being found that 15 seconds is ample.

INCUBATION.

The subcultures are incubated 48 hours at 37° C., and the results then read off and tabulated.

DILUTIONS.

Capacity pipettes for the original dilutions are invariably used. For the phenol controls a standard dilution of pure phenol (Merck) is made and standardized by the U.S.P. method (Koppeschaar) to contain exactly 5 per cent of pure phenol by weight. From this stock solution the higher dilutions are made fresh each day for that day's test.

For the dilutions of the disinfectant a 5 per cent solution is made by adding 5 c.c. of the disinfectant to 95 c.c. of sterile distilled water. A standardized 5 c.c. capacity pipette is used for this and after filling the pipette all excess of the disinfectant on the outside of the pipette is wiped off with sterile gauze. The contents of the pipette are then delivered into a cylinder containing 95 c.c. of sterile distilled water and the pipette washed out as clean as possible by aspiration and blowing out the contents of the pipette into the cylinder. The contents of the cylinder are then thoroughly shaken and the dilutions up to 1:500 made from it, using delivery pipettes for measuring. For those disinfectants which do not readily form a 5 per cent solution we make a 1 per cent stock solution and from this make the dilutions greater than 1:100 in accordance with the second table of dilutions. If greater dilutions than 1:500 are to be made, a 1 per cent solution is made from the 5 per cent solution, and the higher dilutions made from this.

We have adopted the following scale for making dilutions:

For dilutions up to 1:70, increase or decrease by a difference of 5 (i.e., 5 parts of water).

From 1:70	to 1:160	by a difference of 10
From 1:160	to 1:200	by a difference of 20
From 1:200	to 1:400	by a difference of 25
From 1:400	to 1:900	by a difference of 50
From 1:900	to 1:1800	by a difference of 100
From 1:1800	to 1:3200	by a difference of 200

and so on if higher dilutions are necessary.

It is important that the cylinders used for making the dilutions be correctly graduated, as we have found disregard of this factor an important source of error. It is preferable to use standardized cylinders and pipettes, and we recommend that they be used whenever possible. They of course should be perfectly clean. For making the dilutions in accordance with the above scheme we have found the following tables of much service:

TABLE 15 (FOR DILUTIONS).

STOCK 5 PER CENT SOLUTION.

(5 c.c. disinfectant + 95 c.c. distilled water) = Solution A.

	c.c. of A	c.c. Dist. Water		c.c. of A	c.c. Dist. Water		c.c. of A	c.c. Dist. Water
1:20	= 20	+ 0	or	10	+ 0	or	4	+ 0
1:25	= 20	+ 5	or	10	+ 2½	or	4	+ 1
1:30	= 20	+ 10	or	10	+ 5	or	4	+ 2
1:35	= 20	+ 15	or	10	+ 7½	or	4	+ 3
1:40	= 20	+ 20	or	10	+ 10	or	4	+ 4
1:45	= 20	+ 25	or	10	+ 12½	or	4	+ 5
1:50	= 20	+ 30	or	10	+ 15	or	4	+ 6
1:55	= 20	+ 35	or	10	+ 17½	or	4	+ 7
1:60	= 20	+ 40	or	10	+ 20	or	4	+ 8
1:65	= 20	+ 45	or	10	+ 22½	or	4	+ 9
1:70	= 20	+ 50	or	10	+ 25	or	4	+ 10
1:70	= 20	+ 50	or	10	+ 25	or	4	+ 10
1:80	= 20	+ 60	or	10	+ 30	or	4	+ 12
1:90	= 20	+ 70	or	10	+ 35	or	4	+ 14
1:100	= 20	+ 80	or	10	+ 40	or	4	+ 16
1:110	= 20	+ 90	or	10	+ 45	or	4	+ 18
1:120	= 20	+ 100	or	10	+ 50	or	4	+ 20
1:130	= 20	+ 110	or	10	+ 55	or	4	+ 22
1:140	= 20	+ 120	or	10	+ 60	or	4	+ 24
1:150	= 20	+ 130	or	10	+ 65	or	4	+ 26
1:160	= 20	+ 140	or	10	+ 70	or	4	+ 28
1:160	= 20	+ 140	or	10	+ 70	or	4	+ 28
1:180	= 20	+ 160	or	10	+ 80	or	4	+ 32
1:200	= 20	+ 180	or	10	+ 90	or	4	+ 36
1:200	= 20	+ 180	or	4	+ 36	or	2	+ 18
1:225	= 20	+ 205	or	4	+ 41	or	2	+ 20½
1:250	= 20	+ 230	or	4	+ 46	or	2	+ 23
1:275	= 20	+ 255	or	4	+ 51	or	2	+ 25½
1:300	= 20	+ 280	or	4	+ 56	or	2	+ 28
1:325	= 20	+ 305	or	4	+ 61	or	2	+ 30½
1:350	= 20	+ 330	or	4	+ 66	or	2	+ 33
1:375	= 20	+ 355	or	4	+ 71	or	2	+ 35½
1:400	= 20	+ 380	or	4	+ 76	or	2	+ 38
1:450	= 20	+ 430	or	4	+ 86	or	2	+ 43
1:500	= 20	+ 480	or	4	+ 96	or	2	+ 48

TABLE 16 (FOR DILUTIONS).

STOCK 1 PER CENT SOLUTION.

(1 c.c. disinfectant + 99 c.c. distilled water) = Solution A.

	c.c. of A	c.c. Dist. Water		c.c. of A	c.c. Dist. Water		c.c. of A	c.c. Dist. Water
1:100	= 100	+ 0	or	10	+ 0			
1:110	= 100	+ 10	or	10	+ 1			
1:120	= 100	+ 20	or	10	+ 2			
1:130	= 100	+ 30	or	10	+ 3			
1:140	= 100	+ 40	or	10	+ 4			
1:150	= 100	+ 50	or	10	+ 5			
1:160	= 100	+ 60	or	10	+ 6			
1:160	= 100	+ 60	or	10	+ 6			
1:180	= 100	+ 80	or	10	+ 8			
1:200	= 100	+ 100	or	10	+ 10			
1:200	= 100	+ 100	or	10	+ 10	or	4	+ 4
1:225	= 100	+ 125	or	10	+ 12½	or	4	+ 5
1:250	= 100	+ 150	or	10	+ 15	or	4	+ 6
1:275	= 100	+ 175	or	10	+ 17½	or	4	+ 7
1:300	= 100	+ 200	or	10	+ 20	or	4	+ 8
1:325	= 100	+ 225	or	10	+ 22½	or	4	+ 9
1:350	= 100	+ 250	or	10	+ 25	or	4	+ 10
1:375	= 100	+ 275	or	10	+ 27½	or	4	+ 11
1:400	= 100	+ 300	or	10	+ 30	or	4	+ 12
1:400	= 10	+ 30	or	4	+ 12	or	2	+ 6
1:450	= 10	+ 35	or	4	+ 14	or	2	+ 7
1:500	= 10	+ 40	or	4	+ 16	or	2	+ 8
1:550	= 10	+ 45	or	4	+ 18	or	2	+ 9
1:600	= 10	+ 50	or	4	+ 20	or	2	+ 10
1:650	= 10	+ 55	or	4	+ 22	or	2	+ 11
1:700	= 10	+ 60	or	4	+ 24	or	2	+ 12
1:750	= 10	+ 65	or	4	+ 26	or	2	+ 13
1:800	= 10	+ 70	or	4	+ 28	or	2	+ 14
1:850	= 10	+ 75	or	4	+ 30	or	2	+ 15
1:900	= 10	+ 80	or	4	+ 32	or	2	+ 16
1:900	= 5	+ 40	or	4	+ 32	or	2	+ 16
1:1000	= 5	+ 45	or	4	+ 36	or	2	+ 18
1:1100	= 5	+ 50	or	4	+ 40	or	2	+ 20
1:1200	= 5	+ 55	or	4	+ 44	or	2	+ 22
1:1300	= 5	+ 60	or	4	+ 48	or	2	+ 24
1:1400	= 5	+ 65	or	4	+ 52	or	2	+ 26
1:1500	= 5	+ 70	or	4	+ 56	or	2	+ 28
1:1600	= 5	+ 75	or	4	+ 60	or	2	+ 30
1:1700	= 5	+ 80	or	4	+ 64	or	2	+ 32
1:1800	= 5	+ 85	or	4	+ 68	or	2	+ 34
1:1800	= 5	+ 85	or	4	+ 68	or	2	+ 34
1:2000	= 5	+ 95	or	4	+ 76	or	2	+ 38
1:2200	= 5	+ 105	or	4	+ 84	or	2	+ 42
1:2400	= 5	+ 115	or	4	+ 92	or	2	+ 46
1:2600	= 5	+ 125	or	4	+ 100	or	2	+ 50
1:2800	= 5	+ 135	or	4	+ 108	or	2	+ 54
1:3000	= 5	+ 145	or	4	+ 116	or	2	+ 58
1:3200	= 5	+ 155	or	4	+ 124	or	2	+ 62

SEEDING TUBES.

The seeding tubes are glass test-tubes 1 inch in diameter and about 3 inches long, with round bottoms. In order to measure the disinfectant into them they are placed in a suitable wooden stand to receive them. We found it convenient to use a wooden block containing six rows of 15 holes each for the disinfectant to be tested and a separate stand for the phenol controls. The tubes

are placed in the stand and each marked with the strength of dilution it is to contain. The rows of tubes running crosswise represent the same strength dilution, while the rows running lengthwise represent the different strengths to be used in the experiment.

Starting with the lowest dilution (i.e., the strongest), the cylinder is shaken, then 5 c.c. are measured into the tubes of the row to receive that strength, using a 5 c.c. delivery pipette. In order to economize glassware, the same pipette is used for measuring out the next dilution, first blowing out as much of the remaining liquid as possible, then drawing a pipetteful of the next dilution to be used and discarding that; then filling the pipette a second time, which is then emptied into the seeding tube.

The measuring out being completed, the tubes are placed in the water bath and allowed to stand a few minutes in order that the disinfectant solution may reach the standard temperature. We have not found it necessary to use cotton plugs in the seeding tubes. They are sterilized in paper-lined wire baskets, with the closed end of the tubes up.

SUBCULTURE TUBE-RACKS.

Wooden racks, with five rows of 14 holes each, are used for holding the subculture tubes, and as plants are made from each mixture of culture and disinfectant every $2\frac{1}{2}$ minutes up to 15 minutes six tubes are required for each dilution. Thus, in each rack we have ten rows of six tubes each with two empty cross rows of holes left, which are utilized by placing over in the next row each tube as it is planted. This makes it easy to keep run of the tubes that are planted. It is well also always to plant from the seeding tube in a certain hole in the water bath into a certain row of tubes in the rack. This, after a little practice, will help to avoid errors in planting.

METHOD OF CONDUCTING THE TEST.

If there are in one experiment more than 10 dilutions of the disinfectant, including the phenol controls, the stronger solutions of the disinfectant and phenol are tested first, as it will not be necessary to plant them after $7\frac{1}{2}$ minutes. The weaker solutions are

then immediately done and are planted every $2\frac{1}{2}$ minutes for 15 minutes.

For keeping the time a stop watch can be used, but an ordinary watch will serve the same purpose by simply starting on the $2\frac{1}{2}$ or 5 minute periods.

When everything is in readiness the culture is added to the disinfectant solutions with a sterile pipette in tenths of a cubic centimeter.

To add the culture, the seeding tube containing the disinfectant is removed from the water bath with the left hand and slanted at an angle of about 45°, and with the right hand the end of the pipette containing the culture is introduced and lightly touched against the side of the tube where the liquid has run away on account of the slanting. At the proper time the culture is allowed to run into the disinfectant solution, the pipette removed, the tube straightened up, gently shaken three times, and replaced in the water bath. The other tubes are done the same way in succession, and it will be found that 15 seconds is ample time for each tube. By adding the culture to the disinfectant with a pipette touched against the side of the seeding tube, accurate measurements can be made and each tube receive exactly the same amount of "seeding," which is not the case when the culture is added by the "drop."

If ten tubes are to be inoculated, only a few seconds will remain after inoculating the last tube before a plant from the first tube will have to be made.

The mixing tubes are not removed or disturbed in making the planting except to insert the loop or spoon into them, touch the bottom, withdraw, and then make the plant in broth. Every effort is made to insert and withdraw the loops and spoons in a uniform manner. The loops and spoons are bent to an angle of about 45° where they are joined on to the shank, and therefore are always filled with the mixture when withdrawn from the seeding tubes. After making the plants the loops or spoons are flamed as already described.

After an experiment is finished the date and any necessary details can be marked on one of the broth tubes and the rack placed in the incubator at 37° C. for 48 hours. At the end of this

time the results are recorded on a chart specially devised for the purpose.

DETERMINING THE COEFFICIENT.

After a large number of experiments we have concluded that the method employed by the Lancet Commission, with certain modifications, is the best one for determining the coefficient, i.e., the mean between the strength and time coefficients.

In performing the test, plants are made every $2\frac{1}{2}$ minutes up to and including 15 minutes. To determine the coefficient, the figure representing the degree of dilution of the weakest strength of the disinfectant that kills within $2\frac{1}{2}$ minutes is divided by the figure representing the degree of dilution of the weakest strength of the phenol control that kills within the same time. The same is done for the weakest strength that kills in 15 minutes. The mean of the two is the coefficient. The method of determining the coefficient will be seen in Table 17.

TABLE 17.

Name, "A." *Date*, May 18, 1910.
Temperature of Medication, 20° C.
Culture Used, B. typhosus, 24-hr., extract broth, filtered.
Proportion of Culture and Disinfectant, 0.1 c.c.+5 c.c.
Organic Matter, None. *Kind*, None. *Amount*, None.
Subculture Media, Standard extract broth. *Reaction*, +1.5. *Quantity in Each Tube*, 10 c.c.

Sample	Dilution	Time Culture Exposed to Action of Disinfectant for Minutes:						Phenol Coefficient	Remarks
		$2\frac{1}{2}$	5	$7\frac{1}{2}$	10	$12\frac{1}{2}$	15		
Phenol.....	1:80	−	−	−					
	1:90	+	−	−	−			80)375	
	1:100	+	+	+	−	−	−		4.69
	1:110	+	+	+	+	+	−	110)650	
									5.91
Disinfectant "A"...	1:350	−	−	−					2)10.60
	1:375	−	−	−					5.30=
	1:400	+	−	−	−				coefficient
	1:425	+	+	−	−	−	−		
	1:450	+	+	−	−	−	−		
	1:500	+	+	−	−	−	−		
	1:550	+	+	+	−	−	−		
	1:600	+	+	+	+	−	−		
	1:650	+	+	+	+	+	−		
	1:700	+	+	+	+	+	+		
	1:750	+	+	+	+	+	+		

TO DETERMINE THE COMPARATIVE COST PER UNIT OF EFFICIENCY.

When bids are solicited for supplying disinfectants they should be required to be made so as to show the comparative cost per

100 units of efficiency of the disinfectant as compared with 100 units of pure phenol. It is manifestly cheaper to purchase a disinfectant that sells for 60 cents a gallon than one that sells for 30 cents a gallon if the former has four times the efficiency of the latter.

The true cost of a disinfectant can be determined only by taking into consideration the phenol coefficient and the cost per gallon of the disinfectant.

The following table (18) is a good illustration of the value of a determination of the comparative cost per 100 units of disinfectant in terms of 100 units of pure phenol:

TABLE 18.

Disinfectant	Phenol Coefficient	Price per Gallon	Relative Cost per 100 Units of Efficiency as Compared with Pure Phenol
Car	2.12	$0.30	5.2
Chl	4.44	1.00	8.4
Phi	1.40	0.37	9.9
Cre	1.13	0.44	14.5
Nap	0.44	0.41	34.8
Zod	0.25	0.40	59.6
Pure phenol	1.00	2.67	100.0

It will be seen that the substance Chl has a higher coefficient than any of the others in the table, but its high cost per gallon results in its being placed second in cost per 100 units.

The cost per 100 units of efficiency as compared with pure phenol is obtained by first dividing the cost per gallon of the disinfectant by the cost per gallon of pure phenol; this gives the price ratio between the disinfectant and pure phenol; the cost ratio is then divided by the phenol coefficient, which gives us the cost per unit of efficiency as compared with pure phenol = 1. The cost per unit is then multiplied by 100 to give the cost per 100 units.

EXAMINATION OF COMMERCIAL DISINFECTANTS.

NOTE.—We are now engaged in a study of the various proprietary disinfectants found upon the market, using the method which we have described in this paper. We propose to determine, in this study, the phenol coefficient with and without organic matter and the comparative cost per unit efficiency of the disinfectants studied by us.

Our studies have gone far enough to show more plainly than we ever saw before the great fraud that is being practiced upon the American people, health officers and laymen alike, in the exploitation of some so-called "disinfectants."

THE APPLICATION OF CERTAIN LAWS OF PHYSICAL CHEMISTRY IN THE STANDARDIZATION OF DISINFECTANTS.*

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The idea of standardizing disinfectants was first seriously proposed by Rideal and Walker.[1] Previous to that time, despite the fact that the germicidal properties of a great many chemical substances had been thoroughly investigated, no scientific attempt had ever been made to establish a common basis of comparison. The results of any disinfection experiments are fundamentally influenced by such conditions as temperature, character of the organism employed, number of organisms in unit volume, and character of the medium. In the absence of complete data covering these points results are practically worthless, at least for purposes of comparison. Even with such data given it is still impossible, owing to the variable conditions obtaining in practice, to establish any relationship, or order of excellence, among the various disinfectants. At best we can only hope to establish such relationship under specified experimental conditions. Rideal and Walker proposed to establish phenol as a standard. This selection was made because phenol is a substance readily obtainable anywhere in a comparatively pure condition. The effect of phenol and that of any other disinfectant may be determined simultaneously using the same test organism. The germicidal power of the disinfectant under investigation is then expressed as the "carbolic-acid coefficient," this being the ratio of the concentrations of the two disinfectants which will kill all the test organisms in the same time. Rideal and Walker recognized also that the time required to disinfect was dependent upon the number of organisms initially present as well as upon the temperature, a fact previously pointed out by Krönig and Paul.[2] These variable factors were controlled as closely

* Received for publication July 18, 1910.

[1] *Jour. Roy. San. Insl.*, 1903, 24, p. 424.

[2] *Ztschr. f. Hyg.*, 1897, 25, p. 1.

as possible. To these possible variables, which it has been necessary for the present to fix arbitrarily, Chick[1] has added a third; namely, the time element. Instead of noting the time required for disinfection with varying concentrations of the disinfectant, she determines the concentration necessary to disinfect in a given time. She selects 30 minutes arbitrarily. In discussing the time element it is pointed out that in comparing the action of phenol and mercuric chloride upon *B. paratyphosus* at 20° C., the concentrations necessary to kill in 2.5 minutes have a ratio of 13.6 while those necessary to kill in 30 minutes have a ratio of 550. The selection of a constant time factor is therefore necessary if a constant carbolic acid coefficient is to be obtained. A coefficient thus obtained is truly indicative of the relative value of the disinfectant in question only at the concentration at which the test was made. At any other concentration a new coefficient would be obtained.

Chick therefore establishes three arbitrary conditions of experiment for the comparison of disinfectants. The temperature must be standard, preferably 20° C.; the initial number of bacteria must be the same in each comparison; and the time required to complete the disinfection must be fixt at 30 minutes, or some other constant value.

In thus fixing the variables entering the problem it is indeed possible to determine relative values or carbolic acid coefficients, under the restricted conditions of the comparison. But are not these variables precisely the ones which are not fixt in practice? Given the relative values of two disinfectants at 20° C., what relation exists at 0° or at 40°? Mercuric chloride will destroy germs in 30 minutes at a concentration of only two one-thousandths of that required by phenol for the same result. If results are desired in two or three minutes, however, the concentration must be about one-tenth of that of phenol. We may want a comparison under these conditions or under longer periods of time, four or twenty-four hours. In brief, by the establishment of arbitrary fixt conditions in our comparisons we deprive the results of their practical value. Such a course might still be permissible if the work

[1] *Jour. Hyg.*, 1908, 8, p. 92; also Chick and Martin, *ibid.*, p. 654.

involved in determining the complete relationships were very arduous or if the mathematical expression of these relationships were unwieldy or unusable. Such, however, is not the case. Not one constant, but three, one for each variable condition met with, are necessary to completely describe a disinfectant with reference to phenol and to one type of bacterium. This latter is the only variable entering the practical problem which must be arbitrarily fixt in the experiment. It seems at present to be quite necessary to determine the relative germicidal values of two disinfectants upon the actual kind of germ upon which they are to be employed and to qualify the final comparative results accordingly.

With this one fixt factor, however, and by the aid of certain simple principles of physical chemistry, it is possible to derive mathematical expressions involving all the other variables in the complete problem. These expressions are convenient to use and may be put in such form that the actual labor of making the tests is reduced to a minimum. A brief discussion of the principles involved will be necessary to their clear understanding. Unfortunately the calculus must be resorted to, but when possible the actual meaning of the differential expression is stated.

The particular law of physical chemistry which will be considered first is known as the law of velocity of reaction. It may be briefly stated as follows:

In any chemical reaction the amount of change in the reacting substances in unit time is directly proportional to some power of the concentrations of those substances. Just what factors determine the power in question need not be considered at present. It is sufficient to state that, in what are known as monomolecular reactions, it is the first power that is involved so that, in these cases, it may be stated that the velocity of the reaction or the amount of change in unit time is directly proportional to the concentration of the reacting substance.

It has been shown furthermore, by Madsen and Nyman,[1] and more conclusively by Chick, that the killing of bacteria by disinfectants simulates a monomolecular reaction in which the bacteria take the place of one of the reacting substances. The other reagent,

[1] *Ztschr. f. Hyg.*, 1907, 57, p. 388.

the disinfectant, is present in such excess that its concentration is not materially altered during the process. The effect of varying concentrations of the disinfectant will be discussed separately. For the present also the temperature will be considered constant. Under the assumed conditions, if b represent the number of bacteria present in unit volume after any time, t, has elapsed, we have the differential expression,

$$-\frac{db}{dt}=kb$$

where k is known as the velocity constant. This is merely a mathematical expression of the law cited above, and means that the change in the number of bacteria per unit time is proportional to the number present. To determine the total change in any elapsed time this is integrated to

$$\log \frac{B}{b}=kt$$

in which B is the number of bacteria initially present. The test of the applicability of this formula is the constancy of k under constant temperature conditions but with varying values of t, B, and b. The experiments previously cited have shown that k varies with the kind and concentration of the disinfectant, with the temperature, and with the bacterial species, but otherwise it is constant in each experiment. In other words, this constant is a definite measure of the value of the disinfectant. It indicates the rate of disinfection. The larger the value of k the more rapid the disinfection and the more efficient the disinfectant. It may be compared directly with the similar constant obtained with phenol. It will be noted particularly that k is independent of the initial number of bacteria present and furthermore that it does not involve complete disinfection. The point of practically complete disinfection is indefinite and its determination involves large percentage error. With such a formula as that given above, a point of 50 per cent reduction would serve the purpose much better, and a degree of accuracy is secured with a small number of tests which would necessitate many times that number under the other method.

The effect of varying the concentration of disinfectant must next be considered. Returning to the original equation and includ-

ing now the concentration of the disinfectant as well as of the bacteria, we obtain

$$-\frac{db}{dt}=KbC^n$$

in which C is the concentration of the disinfectant expressed in any convenient form, and n, an exponent indicating the order of the reaction. As previously pointed out, in a monomolecular reaction, n is unity, but for the disinfectant, n may have another value and must be considered. This constant, n, expresses mathematically what Chick has pointed out experimentally, that mercuric chloride may have a "carbolic-acid coefficient," of 13 at one concentration and 550 at another. If, therefore, n can be determined, it will be unnecessary to adopt a standard time of testing, and the properties of the disinfectant will be more fully determined. Integrating again

$$\log\frac{B}{b}=KC^nt$$

Hence, KC^n is the k of the previous expression. It varies with the concentration and may be determined as already indicated for two concentrations of disinfectants. Let the values be k and k', C and C' respectively. Then

$$n=\log\frac{k'}{k}\div\log\frac{C'}{C}^{1}$$

Having C and n, then it is possible to calculate K. This is the true velocity constant of the disinfectant, being independent of the concentration, and thus differing from k which is constant only at constant concentrations. The constant, n, may be called the concentration exponent. It defines the relative strengths of two different concentrations of the same disinfectant. The two constants, K and n, therefore describe the fundamental characteristics of a disinfectant at any given temperature. Before discussing them in detail, the relation of temperature may be considered. A second law of physical chemistry has been empirically established as follows:

As the temperature increases in arithmetical progression the

[1] Common logs may be used.

velocity of reaction increases in geometrical progression; or mathematically expressed

$$\frac{K'}{K} = \theta^{(T'-T)}$$

in which K' and K are the constants of the reaction at the temperatures T' and T respectively and θ is the temperature coefficient. It has been found in the case of most chemical reactions that the velocity increases from two to threefold for each rise of 10° C. Among the bacterial reactions, however, the increase is greater—varying, according to Chick, from two to fourfold in the case of metallic salts, to seven or eightfold for phenol and similar compounds. A third constant, the temperature coefficient, is therefore necessary to express this characteristic of a disinfectant. The agreement of the disinfecting reactions with the temperature law of chemical reactions has been satisfactorily demonstrated in many instances and may safely be assumed in all cases. Comparisons made at two temperatures 10° apart furnish a basis for the calculation of the temperature coefficient. With this coefficient determined, the value of the velocity constant at any temperature may be calculated from the following:

$$K_{T°} = K_{20°} \times \theta^{(T-20)}$$

These three constants, the velocity constant, the concentration exponent, and the temperature coefficient, define three essential characteristics of the disinfectant. The last two are independent of experimental conditions. The first, however, is determined also by the type of test organism employed. Therefore any standardization of a disinfectant must be referred to a particular organism. To further correct the result for the uncertain resistance of the particular culture used, the value of K may be divided by the value similarly obtained for phenol. This gives a coefficient independent of all variables except the nature of the test organism. If the temperature were not 20°, reduction can be made by the formula above.

The minimum amount of work required to determine the three constants of a disinfectant is much less than is called for by the present methods. The end point to be used will be a reduction of

from 50 to 75 per cent of the initial number, altho other points serve practically as well if not too near either end. The plate method can therefore be used and the organisms counted. Before adding the disinfectant, the number of bacteria in the initial water suspension is determined. To two tubes, disinfectant in two different concentrations is added. A third tube is treated like one of these and is kept at a temperature approximately 10° higher. After a convenient length of time, depending wholly upon the character of the organism and the strength of the disinfectant, the number of bacteria present in each tube is determined with the usual precautions as to neutralization of disinfectant and other details.

At the same time a single determination of K is made on phenol using the same bacterial culture. The value of n for phenol is found to be 6, and of θ, between 7 and 8. These apparently do not vary with the test organism. From the results from these four tubes, tested initially and finally, the ratio of the velocity constants of phenol and the unknown germicide, and the temperature coefficient and concentration exponent of the latter can be determined. Of course in a careful comparison one or two intermediate determinations of numbers would be made between the initial and final sets. Each additional determination allows of an independent determination of K, the results of which should then be averaged. As a matter of fact the mathematical and theoretical considerations are more accurate than the experimental work. Invariably when a large number of tests are made the average results are in conformity with the theory.

For the purpose of illustration an imaginary case has been worked out using the K, n, and θ values which have been calculated from a large number of experiments by Madsen and Nyman, Chick, and in the writer's laboratory. This illustration is given to show the mode of procedure, the amount of labor involved in the test, and the method of making the calculations.

To summarize, disinfectants differ in three independent ways and there are required three constants to indicate their relative efficiencies. These efficiencies are not determined by a comparison of one of the factors any more than the relative volumes of two pieces of wood are determined by a comparison of their lengths.

TABLE 1.

RESULTS OF TEST: ANTHRAX SPORES PER CUBIC CENTIMETER.

TIME (MINUTES)	PHENOL	MERCURIC CHLORIDE		
	5 per cent	1 per cent	0.5 per cent	
	20°	20°	20°	29°
0	125,000	125,000	125,000	125,000
2	.....	.....	.....	15,500
5	.....	7,900	.....	630
10	.....	545	9,250	4
15	.....	27	.....	.....
20	.....	.....	720	.....
30	.....	.....	60	.....
60	112,000	.....	.....	.....
120	101,000	.....	.....	.....
240	82,000	.....	.....	.....

CALCULATIONS.

Using the formula

$$\log \frac{B}{b} = KC^n t$$

expressing time in minutes, concentration in per cent, and using common logs, we obtain the following values of KC^n:

TABLE 2.

VALUES OF KC^n.

TIME (MINUTES)	PHENOL	MERCURIC CHLORIDE		
	5 per cent	1 per cent		0.5 per cent
	20°	20°	20°	29°
0	.....	....	....	....
2	.....	....	....	.453
5	.....	.240	....	.440
10	.....	.236	.113	.460
15	.....	.244	....	....
20	.....	....	.112	....
30	.....	....	.110	....
60	.00080	....	....	....
120	.00078	....	....	....
240	.00077	....	...	....
Average	.00078	.240	.112	.451

Having the value of $n=6$ already established for phenol, we obtain for this disinfectant

$$K_{20} = .000,000,05.$$
$$= .05 \times 10^{-6}$$

For the mercuric chloride we have at 20°:

$$KC^n = 0.240 \text{ at } C = 1$$
$$KC^n = 0.112 \text{ " } C = 0.5$$

Whence

$$n = \log \frac{.240}{.112} \div \log \frac{1}{0.5} \ldots\ldots n = 1.08$$

$$K_{20} = 0.24 \text{ at } C = 1 \ldots\ldots K_{20} = 0.24$$
$$0.24 \text{ “ } C = 0.5$$

at 29° and $C = 0.5$

$$KC^n = .451$$

Whence

$$K_{29^\circ} = 0.953$$

$$9 \log \theta = \log \frac{K_{29}}{K_{20}} = 0.600$$

$$\log \theta = 0.067 \ldots\ldots\ldots \theta = 1.17$$

Disinfectants vary in the rapidity of their action. Other things being equal, the disinfectant which accomplishes a certain destruction of bacteria in a given time is twice as efficient as one which accomplishes the same result in twice the time. This velocity factor we denote, in keeping with the nomenclature of physical chemistry, by K which we call the velocity constant. It indicates the velocity of the disinfection.

The second point of difference among disinfectants is the change in efficiency which they show with changing concentrations. Thus a 0.2 per cent mercuric chloride solution is twice as efficient (will do the work in half the time) as a 0.1 per cent solution. With phenol, however, in dilute solution, doubling the strength increases the efficiency or decreases the time 64 times. Conversely, halving the strength decreases the efficiency or increases the time 64 times. Other disinfectants have other ratios of increase with increasing strength. This factor we denote by the concentration exponent, n. This constant shows by what power of 2 the efficiency is increased if the concentration be doubled.

The third point of difference among disinfectants is their relation to temperature. Increasing the temperature 1° may increase the rapidity of disinfection by from 1.074 to 1.123 fold, or for 10° the change would be from 2 to 8 fold. The temperature coefficient, θ, shows how many fold the efficiency is increased by a rise of one degree in temperature.

Given these three constants of a disinfectant, results obtained at any one concentration, duration of time, and temperature can

be transformed to any other set of conditions by the following equations:

$$\log \frac{B}{b} = KC^n t$$

$$K_{T^\circ} = K_{20^\circ} \cdot \theta^{(T-20)}$$

B Initial number of bacteria present.
b Final " " " "
t Elapsed time.
C Concentration of disinfectant.
K Velocity constant calculated at temperature of the experiment.
K_{T° Same at any temperature T°.
K_{20° Same at any temperature 20°.
n Concentration exponent.
θ Temperature coefficient.

The point of view which is taken here also somewhat simplifies our conception of antiseptic as distinguished from germicidal properties. It must be admitted that with our ordinary methods of determining antiseptic properties there is no attempt made to determine whether there is or is not a true killing of the organism. The experiment is made with a very low concentration of disinfectant and proceeds for a long time. There is an apparent inhibition under these conditions, which may be really a true killing. It is evident from the form of our killing curve that a low rate of killing over a long time is just as efficient as a higher rate for a shorter period. There is no apparent reason thus far for the distinction between germicidal and antiseptic properties.

The real difficulty appears when two substances are compared in these respects. The fact, now well established, that one of the two may be the superior germicide and the other the superior antiseptic, leads at first thought to the belief that the two properties, germicidal and antiseptic, are unrelated and distinct. A brief consideration of the principles which have been laid down here will show that such a contention is at least unnecessary and probably unjustified.

Two disinfectants having different values of the concentration exponent, n, will have different relative efficiencies at different concentrations. Their efficiency-concentration curves may and in fact often do cross, so that at the point of crossing they are equally

efficient, while above that point the one, and below that point the other, is superior. It follows then that antiseptic action, or true disinfection at low concentrations for long times, will show a relation between two disinfectants quite different from that shown at higher concentrations and shorter times. A disinfectant with a high value of n loses efficiency rapidly with dilution and hence is found to be a poor antiseptic, while one with a smaller value of n loses its power less rapidly with dilution. It has already been shown that decreasing the concentration of phenol one-half decreases its efficiency 64 times, its concentration exponent being six; while mercuric chloride with an exponent of one suffers a loss of efficiency of only one-half by similar dilution.

It is hoped to present this matter more fully in a subsequent paper and to support it with experimental data. For the present it is sufficient to point out that there is no necessity for the distinction between germicidal and antiseptic properties, and that the latter are equally determined by the fundamental constants that are here proposed.

An interesting corollary may also be drawn from this discussion. The velocity constant measures the velocity of the reaction between the disinfectant and the germ. With a given germ it is a measure of the power of the disinfectant. With a given disinfectant it is likewise a measure of the resistance of the organism. Hitherto all attempts to express the relation of bacteria to disinfectants, including heat, have been arbitrary. Death points have been sought when no such points exist. The rate of dying, whether under the influence of heat, cold, or chemical poison, is unfailingly found to follow the logarithmic curve of the velocity law, if the temperature be constant. This curve never reaches a zero value altho approaching it indefinitely. When in practice the bacteria are all killed this fact is interpreted mathematically to mean that, in the volume tested, the number is less than one. The common observation that the larger the initial number the longer the time required to kill is a proof of this view. It requires a greater percentage reduction to reduce the number to less than one, the larger the initial numbers. Therefore these death points are merely accidental, depending in the first place wholly upon the technic adopted

and again upon the chance of the last few survivors living or dying, when for a considerable period of time these chances are about equal.

The velocity constant, K, defines the properties of the logarithmic curve, and hence can be made to define such fundamental properties of an organism as its resistance to heat. To measure this function we would not attempt to determine a temperature at which the organisms all die (the non-existent thermal death point) but rather the rate of dying at any fixt temperature. If then we add to this determination a determination of θ which shows how this rate of dying increases with rising temperature, we have fully defined the thermal properties of the organism. A similar line of reasoning would apply to the relation of the organism to any other germicidal agent.

UPON THE PRESENCE OF SUPPURATION IN THE TUBERCLES OF LEPROSY.*†

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Altho the majority of leprographers recognize that the cutaneous nodules in the tubercular type of leprosy may and do occasionally "break down" with the formation of a purulent or necrotic material, resulting in many cases in sinuses or ulcers, practically all observers are unanimous in stating that true pus formation or polymorphonuclear leukocytic invasion of the tubercles occurs only in the presence of a secondary bacterial invader such as the streptococcus or staphylococcus.

Sticker[1] remarks that there can be no suppuration without the presence of pyogenic cocci. Babes[2] considers the "breaking down" to be a true necrosis, and trophoneucrotic in origin. In a similar manner by all authors except Sugai,[3] the possibility of acute inflammatory processes on the leprous lesion is either neglected or denied.

This last-mentioned author reported in 1909 a series of cases, in which pus was found in the nodules, in one of which no organisms other than the leprosy bacillus were found. His methods of excluding the possibility of secondary invasion were somewhat incomplete in that cultivation was not attempted. He states, however, in his conclusions that the leprosy bacilli may cause suppuration without the presence of any pyogenic coccus.

That many or, perhaps, most of the abscess formations, suppurative arthritises, and other purulent processes occurring in leprous patients are due to the invasion of tissues whose resistance is lowered by one or other of the ordinary pyogenic bacteria is undoubtedly correct; that, however, such a condition is not a necessity the following case which came under the author's observation proves.

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[1] *Tropen-Krankheit* (Mense), Leipzig, 1905.

[2] *Nothnagel Encyclopaedie*, Wien ("Lepra").

[3] *Lepra* (Leipzig), 1909, 8, No. 3.

The patient, a young white male of 22 years, had suffered from leprosy during a period of between two and three years. During this time he had passed through several attacks of exacerbation of symptoms consisting of high fever, with swelling edema and reddening of the tubercles. Upon the occasion when seen by the author his temperature had been between 103° and 104° F. for three days. He was suffering from malaise anorexia and other evidences of a constitutional disturbance. The tubercles situated over the face and hands were much swollen, very red and tender: one swelling over the malar prominence on the left side had ruptured spontaneously and was discharging pus. A tubercle over the posterior surface of the left wrist was incised, resulting in the expulsion of about 25 c.c. of a yellowish-green purulent material.

This material was planted upon the ordinary and several special culture media, both aerobically and anaerobically, without the development of a single colony.

Smears were made and stained by Giemsa's method, carbol fuchsin with acid decolorization, and by Gram's. The cellular elements with the polychrome stain consisted almost exclusively of polymorphonuclear leukocytes, together with a small number of large vesicular nuclei, practically devoid of protoplasm. Leprosy bacilli were present in very large numbers. A few well-formed aggregations or globi were found, but for the most part the bacilli were lying singly or in small groups. A much larger percentage than usual appeared broken up in the form of granules. Certain numbers of pus cells contained a few bacilli, but in general the organisms were free. Absolutely no other bacteria could be demonstrated in any preparation.

In addition to the above case in which comparatively large pus collections were present, the routine examination of a number of leprosy nodules derived during life and at autopsy has shown that polymorphonuclear leukocytes may be found almost constantly in small numbers, although in no case have definite foci of such cells been seen.

The fact that acute exudative inflammatory changes should occur in leprosy is not strange when we consider the other granulomata, such as tuberculosis and glanders. Acute abscess formation

in tuberculosis is not frequent, but can hardly be said to be rare, and a moderate number of pus cells may be found in many lesions. As demonstrated by Duval,[1] working upon glanders in laboratory animals, all lesions from the acute to proliferative may be produced by varying the virulence of the organisms used.

An interesting observation, with reference to the clinical course of cases which develop the so-called lepra-fever, has been made by Dr. Ralph Hopkins of this city; namely, that immediately following the cessation of the attack the condition of the individual improves until both constitutionally and locally the patient is better than previous to the onset of acute symptoms. This condition of affairs occurred in the case described in this paper.

It is difficult to understand why the invasion of the lesions by acute inflammatory cells should take place in this sudden manner, but there would appear to be no doubt but that the action of these cells results in a destruction of many of the bacilli with consequent clinical improvement. The author considers that a routine examination of tubercles from cases of lepra-fever would in all probability show a comparatively large proportion of polymorphonuclears.

Since it does not seem reasonable that an alteration in virulence should suddenly take place simultaneously in the bacilli in lesions in different parts of the body, the cause of the alteration in the reaction to the irritant must be looked for in the individual suffering from the disease. In studying the local cellular changes in such cases, therefore, the opsonic index ought to be considered, for, since we know that the opsonin influences the phagocytic activity of the polymorphonuclear leukocytes, we should expect to find the index against *B. leprae* raised at the time during which the cells are more active. That this increased reaction is potent to injure the organisms, the degenerative condition morphologically of the organisms and the improvement of the disease clinically are evidence.

[1] Duval, *Jour. of Exp. Med.*, 1907, 9, No. 4.

THE SUSCEPTIBILITY TO PLAGUE OF THE WEASEL, THE CHIPMUNK, AND THE POCKET GOPHER.*

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The rodents forming the subject of this paper are difficult to secure alive, and consequently the evidence presented here of their susceptibility to plague infection is not as extensive as might be desired.

The weasel (*Putorius xanthogenys*).—But two of these rodents have lived long enough in captivity to make it feasible to use them for experimental purposes. This animal is of special interest in regard to its susceptibility to plague as it is a predatory rodent and is believed to be a natural enemy of rats and ground squirrels which as is well known are frequently found infected with plague in nature. Indeed, weasels and allied species are sometimes used as rat exterminators.

In the first experiment a weasel weighing 110 gms. was inoculated by the cutaneous method (vaccinated) with a 48-hour agar culture of *B. pestis* that had been isolated about 15 months before from the spleen of a naturally infected plague rat that had been trapped in San Francisco. The eleventh generation on agar was used. The weasel died about 48 hours after the inoculation. The only lesion was a slight reaction at the site of inoculation and an enormously enlarged spleen which was very dark in color. A pure culture of *B. pestis* was isolated from the liver of the animal. The control used was a guinea-pig weighing 350 gms. This animal died on the ninth day, and at autopsy presented the lesions of subacute plague. The ninth day is rather late for a guinea-pig to die of plague, and, in consequence, we may assume that the culture that proved fatal to the weasel in two days was not an especially virulent one.

In the second experiment the culture used was one isolated in San Francisco from a naturally infected plague rat, but subse-

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quently the culture had been passed through a long series of ground squirrels without subculture on artificial media. This passage through the ground squirrels seemed to have no appreciable influence upon the virulence of the culture. A 48-hour-old broth culture was injected subcutaneously, the dose being shown in the following table, which also shows the fate of the various animals inoculated.

TABLE 1.

Animal	Weight gms.	Dose of Culture	Days before Death	Lesions	Cultures
Weasel.....	265	2 c.c. Subcutaneously	3	Acute plague	B. pestis from spleen
Guinea-pig .	320	0.5 c.c. "	4	" "	None made
Guinea-pig .	380	Vaccinated	5	" "	" "
Guinea-pig .	305	Infected needle thrust under the skin	4	" "	Contaminated
White rat ..	85	0.5 c.c. subcutaneously	5	" "	B. pestis from liver
White rat ..	160	Infected needle thrust under the skin	Killed on 47th day	None	None made

The control guinea-pigs died in the usual time. One of the white rats used for control purposes succumbed to acute plague; the other was found to be healthy when killed. The dose of culture given to the weasel was so large that no very safe conclusion is to be drawn from the experiment.

A guinea-pig which was vaccinated with the spleen of the dead weasel died on the fourth day, showing that the bacillus had undergone no change in virulence by passage through the weasel.

The chipmunk (*Callospermophilus* [*Citellus*] *chrysodeirus*).[1]—This little rodent is usually called the Gilded Ground Squirrel. It is fairly closely related to *Citellus beecheyi* (California Ground Squirrel) which has been found naturally infected.

The specimen forming the subject of the first experiment was caught in a rat trap in San Francisco and was probably a pet that had escaped. The chipmunk was vaccinated with the bubo of a guinea-pig that had died on the sixth day after inoculation from a naturally infected ground squirrel. The chipmunk died on the fourth day and at autopsy presented the usual lesions of acute plague in squirrels. Smears from the bubo and from the spleen

[1] I am indebted to Professor Joseph Grinnell, of the Department of Mammalogy of the University of California, for the identification of this rodent.

showed an enormous number of pest-like bacilli. No cultures were made. Three wild rats (*Mus norvegicus*) were used as controls. Two of these died on the fourth day; the third, on the seventh day. All presented at autopsy the usual lesions of plague in rats. The controls demonstrated that the culture was quite virulent. The spleen of the dead chipmunk was used to vaccinate a guinea-pig, which died of plague on the sixth day, showing that the bacillus was virulent after passing through the chipmunk.

For the second experiment, we had three chipmunks that had been trapped in the vicinity of Lake Tahoe, California, a natural habitat of this rodent. In this experiment, the infecting agent was contained in the bubo of a guinea-pig, dead on the seventh day after inoculation, from a naturally infected ground squirrel. All of the animals shown in the following table were vaccinated with the same material.

TABLE 2.

Animal	Weight gms.	Days before Death	Lesions	Cultures
Chipmunk	230	3	Acute plague	B. pestis from liver
Chipmunk (*b*)	?	3	" "	" " " "
Chipmunk	?	3	" "	" " " "
Guinea-pig (control)	315	12	Chronic plague	None
Guinea-pig (control)	...	18	" "	"

It is a remarkable fact that the three chipmunks died on the same day (third). All presented the lesions of acute plague. That the control guinea-pigs died so long after inoculation (12 and 18 days) indicates that the bacilli contained in the bubo were but slightly virulent for guinea-pigs or that they were very few in number. That the guinea-pig dying on the eighteenth day harbored virulent plague bacilli is shown by the fact that pus from a bubo of this guinea-pig when used to inoculate another guinea-pig caused the death of the latter of acute plague in five days. A guinea-pig was vaccinated from the liver of one of the chipmunks (*b*). This guinea-pig died of acute plague on the fourth day.

California pocket gopher (*Thomomys bottae*).—In a previous paper[1] several experiments on the susceptibility of gophers to plague were reported. These experiments seemed to show that the gopher

[1] *Jour. Infect. Dis.*, 1909, 6, p. 283.

was relatively immune to infection with *B. pestis*. Since that report was made two additional experiments have been made, both of which tend to confirm the conclusion previously reached. In the first of the experiments reported here, a three-day broth culture of *B. pestis*, which had been isolated about two months before from the spleen of a naturally infected ground squirrel, was used as the infecting agent. The second generation on artificial media was used. As will be seen in the accompanying table an attempt was made to vary the dose as it was thought that possibly the immunity of gophers was such that it might be overcome by the use of a very large dose of culture. The results indicate that this is probably true. It is needless to state that all of the doses given were much larger than an animal would ever get under natural conditions. On account of the scarcity of gophers a more extensive variation in dose was impracticable.

TABLE 3.

Animal	Weight gms.	Dose of Culture	Days before Death	Lesions
Guinea-pig (control ...	780	0.1 c.c. subcutaneously	12	Subacute plague
Guinea-pig (control)...	563	0.1 c.c. "	7	" "
White rat (control)....	180	0.1 c.c. "	4	Acute plague
Gopher.....	200	0.1 c.c. intraperitoneally	Killed on 10th day, negative	None
Gopher.....	150	0.1 c.c. subcutaneously	Killed 10th day	Abscess at site (see below)
Gopher.....	135	0.1 c.c. "	5	Acute plague
Gopher.....	300	2.5 c.c. "	5	" "

The large doses were sufficient to kill the gophers on the fifth day. The smaller dose (identical with that given to the controls) had no influence on one of the gophers, and in the other merely gave rise to a local necrosis. A considerable quanity of the pus from the abscess in the latter case was injected beneath the skin of a guinea-pig and caused its death of acute plague on the sixth day. The two gophers that had not succumbed by the tenth day were chloroformed at that time, the object of killing the animals at such an early date being the detection of slight lesions that might have disappeared had the animals been kept longer. It is rather remarkable that the only gopher to escape unscathed in this experiment was the one in which the culture had been inoculated intraperi-

toneally. It should perhaps be stated here that, as the guinea-pigs died late, the culture was probably not a very virulent one.

In the second and last experiment a uniform dose of culture was administered to all of the animals in the series. The culture was one that had been obtained by aspirating the primary bubo of a child about 36 hours after the onset of the symptoms of plague. At the time of the present experiment, the culture had been carried upon artificial media for about seven months. The third generation was used. The results of the experiment are shown in the following table.

TABLE 4.

Animal	Weight gms.	Dose	Days before Death	Lesions
Guinea-pig	320	0.1 c.c. 48-hr. broth culture	5	Acute plague
Guinea-pig	360	" " " "	6	" "
White rat	100	" " " "	2	Early plague
Gopher	180	" " " "	3	Acute plague (B. pestis isolated from heart's blood and from liver)
Gopher	225	" " " "	Alive and well on 50th day	
Gopher	225	" " " "	" " " " " "	
Gopher	230	" " " "	" " " " " "	

The results in the case of the control animals indicate that the culture was fully virulent; nevertheless, but one of the gophers succumbed.

SUMMARY.

These experiments indicate that the weasel (*Putorius xanthogenys*) and the chipmunk (*Callospermophilus* [*Citellus*] *chrysodeirus*) are quite susceptible to plague infection.

As compared with control animals, the susceptibility of the gopher (*Thomomys bottae*) is rather slight. This agrees with the results of previous experiments.

THE EFFECT OF VACUUM DESICCATION ON THE VIRUS OF RABIES, WITH REMARKS ON A NEW METHOD.*†

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(From the Pathological Laboratory of the City Hospital and the Physiological Laboratory of St. Louis University.)

Shackell[1] has described an improved method of vacuum desiccation, the essential feature of which is that the material is kept solidly frozen during the process of drying. Animal tissues when dried by this method are preserved intact, show no shrinkage, are porous, and resist chemical changes and deterioration. In brief, the technique is as follows:

The material to be dried is placed in the bottom of a Scheibler's vacuum desiccating jar, in the upper part of which is a separate dish containing sulphuric acid. The temperature is reduced by placing the jar, half submerged, in a salt and ice mixture, and after thorough solidification of the material has resulted, a rapid vacuum is produced by a Geryk pump to less than 2 mm. of mercury. During the process of desiccation, the temperature in the lower half should be kept several degrees below 0° C. Unless the sulphuric acid be repeatedly shaken to prevent surface saturation with water, the time required for complete desiccation will be unduly prolonged.

Pasteur attenuated the virulence of rabic material by drying this in the presence of air over caustic potash. The time required for complete loss of virulence by this method depends upon the surrounding temperature. At 23° C. this point is reached in from six to eight days. Vansteenberghe[2] found that virulence may be maintained for several months if the brain be ground into a pulp, spread out in a *very thin layer*, and desiccated *in vacuo very rapidly*. Marie[3] repeated this experiment with partial success.

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[1] Shackell, *Am. Jour. Physiol.*, 1909, 24, p. 325.

[2] Vansteenberghe, *Compt. rend. Soc. Biol.*, 1903, 55, p. 1646.

[3] Marie, *L'étude expérimentale de la rage.*

We have found that, by using Shackell's method of desiccation, brains and cords may be desiccated *in toto* without destruction of virulence. The time required for the complete extraction of water is about 24 to 36 hours. A number of brains have been so treated and the infectivity of all has been preserved. After the completion of desiccation, these brains were placed in an ordinary desiccating jar over sulphuric acid and left continually exposed to light at the ordinary room temperature. One brain has remained infective for four months. The only precaution taken was to guard against moisture. Material thus dried is like chalk and is easily pulverized. It is, however, very hygroscopic and, after a few hours' exposure to the air, becomes leathery and rapidly loses its infectivity. Experiments are now being carried on to compare quantitatively the virulence of desiccated cord with that of fresh cords, after the method described by Harvey and McKendick.

Harvey and McKendick[1] have constructed a curve of infectivity which corresponds very closely to a curve representing the loss of weight of a cord when dried by the method of Pasteur. These writers state: "We may conclude that the small amount of water remaining in the cord from the ninth day onward is insufficient to keep the virus infective." It is the general belief that the attenuation of a rabic cord depends primarily upon its loss of water. Our work leads us to believe that it is the *method* of extracting the water which results in attenuation or destruction of virulence, and not the extraction of the water *per se*. To state it differently, slow desiccation attenuates and destroys the virus directly by reason of the concentration of salts and other substances which are in solution in the brain and cord. The action is therefore, in essence, a chemical one.

Desiccation of frozen material avoids any concentration of those intra- and extracellular salts or substances which are at the ordinary temperature in solution. With this method absolute dryness proceeds, cell by cell, from the surface. If this proposition be true, Vansteenberghe's and Marie's successes and failures are easy of explanation. These authors emphasize the fact that the cord must be spread in a very thin layer and the vacuum produced

[1] Harvey and McKendick, *The Theory and Practice of Anti-rabic Immunization* (Calcutta).

very rapidly. A vacuum rapidly produced will freeze a small quantity of water in a bell jar. The success of these workers, in our opinion, depended upon the freezing of their thinly spread material and its drying without concentration. Further support is given to our hypothesis by the fact that exposure of our thoroughly dried material to ordinary air destroys its virulence completely within a few hours. The absorbed atmospheric moisture is, in this case, sufficient to redissolve some of the salts and other soluble material in a most concentrated state and destroy by chemical means the inclosed virus.

ON THE ADMINISTRATION OF DIPHTHERIA TOXIN IN A COLLODION SAC.*

E. C. L. Miller.

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The therapeutic value of antidiphtheric serum is recognized by all who are conversant with the facts; as to how it acts and how its value can best be tested there is much less unanimity. The Germans, following Behring and Ehrlich, stand for the antitoxic unit as the full measure of therapeutic value, while the French, following Roux, contend that the antitoxic power of a serum is not a satisfactory measure of its therapeutic value. The reason why this question cannot be definitely settled is because diphtheria, as found in man, cannot be produced experimentally in animals. Hence the curative value of a serum can be tested only on human beings and these of course cannot be adequately controlled. On the other hand, the antitoxic value of a serum can be easily and accurately tested on guinea-pigs and this accounts for the universal use of the antitoxin unit. The question still remains: Does the antitoxic power of a serum adequately measure its curative power?

In the attempts which have been made to devise a satisfactory test for the therapeutic value of antidiphtheric serum the use of diphtheria cultures either on scarified mucous membranes, or by subcutaneous injection, has been abandoned because of irregular results, and recourse has usually been had to the subcutaneous injection of toxin followed after an interval by the injection of antitoxin. This method differs from the conditions found in man, chiefly as to the rapidity with which the toxin is absorbed. In man the toxin is absorbed from the false membrane at first slowly, later more rapidly, until some influence occurs which checks the production of toxin. When the toxin is injected subcutaneously it is absorbed rapidly and the system is suddenly overwhelmed with toxin. In casting about for some method of introducing toxin into animals more analogous to the manner of its absorp-

* Received for publication July 25, 1910.

tion from the false membrane in man, recourse was had to a collodion sac containing toxin, placed in the peritoneal cavity of a guinea-pig. It was hoped that the toxin would be absorbed slowly from the sac and produce death in a manner analogous to a case of diphtheria in man. After much preliminary work on the size and quality of sac and amount of toxin, a combination was found which would uniformly kill 400 gm. guinea-pigs in five, six, or seven days after the introduction of the sac.

Histological examination of the abdominal organs showed no lesions other than those usually found in diphtheria, hence we concluded that death was not due to any local action of the toxin but to the usual systemic action and that absorption from the sac

TABLE 1.

DETERMINATION OF L+ DOSE OF DIPHTHERIA TOXIN NO. 05656.
L+ DOSE TAKEN AS 0.27 C.C.

GUINEA-PIG	SERUM		TOXIN		REACTION TIME IN MINUTES	INJECTION	DEATH	INTERVAL IN DAYS
Weight	Name	Dose	B No.	Dose		Day	Day	
250	Ehrlich	1 unit	05656	0.25	20	9–16–09	9–24–09	8
"	"	"	"	.25	"	"	9–22–09	6
"	"	"	"	.26	"	"	9–22–09	6
"	"	"	"	.26	"	"	9–21–09	5
"	"	"	"	.27	"	9–21–09	9–25–09	4
"	"	"	"	.27	"	"	9–25–09	4
"	"	"	"	.27	"	"	9–24–09	3

corresponded quite well to absorption from the false membrane. In all cases careful bacteriological tests were made of the peritoneal fluid, the cultures being both aerobic and anaerobic and the media in all cases containing unheated animal proteid such as ascitic fluid, blood serum, liver extract, etc. In most cases the peritoneum was entirely sterile. The few cases in which infection occurred are not included.

The results here given are the outcome of this work with collodion sacs and altho the work is by no means finished, yet it seems of sufficient interest to report. It is the hope of the writer, who is unable to continue the work, that someone else may take it up for further investigation.

Preliminary Tests.—The M.L.D. and L+ dose of a test toxin were carefully worked out as shown in Tables 1 and 2, and a quan-

tity of this toxin was put up in small vials for use in this work. The antitoxic potency of two sera was determined; one being of relatively high potency (900 units per c.c.) and one of relatively low potency (200 units per c.c.) as shown in Table 3. Quantities of these sera were set aside.

Considerable time was consumed in determining the size and

TABLE 2.

DETERMINATION OF THE MINIMUM LETHAL DOSE OF DIPHTHERIA TOXIN No. 05656 M.L. DOSE TAKEN AS 0.004.

GUINEA-PIG	TOXIN		INJECTION	DEATH	INTERVAL IN DAYS	PERCENTAGE OF DEATHS
Weight	B No.	Dose	Day	Day		
250	05656	0.0032	9-28-09	.....	..	43
"	"	"	11-10-09	11-29-09	9	
"	"	"	12- 2-09	12- 6-09	4	
"	"	"	12- 7-09	.....	..	
"	"	"	12-15-09	.....	..	
"	"	"	12-28-09	1- 3-10	6	
"	"	"	1-15-10	1-21-10	6	
"	"	0.0034	10- 5-09	10-10-09	5	62½
"	"	"	10-11-09	10-18-09	7	
"	"	"	11-10-09	11-16-09	6	
"	"	"	12- 2-09	12- 6-09	4	
"	"	"	12- 7-09	.....	..	
"	"	"	12-15-09	12-22-09	7	
"	"	"	12-28-09	.....	..	
"	"	"	1-15-10	.....	..	
"	"	0.0036	10- 5-09	.....	..	71½
"	"	"	11-10-09	11-16-09	6	
"	"	"	12- 2-09	12- 6-09	4	
"	"	"	12- 7-09	12-11-09	4	
"	"	"	12-15-09	12-22-09	7	
"	"	"	12-28-09	1- 2-10	5	
"	"	"	1-15-10	.....	..	
"	"	0.0038	10- 5-09	10-10-09	5	80
"	"	"	10-11-09	10-18-09	7	
"	"	"	10-21-09	.....	..	
"	"	"	11-18-09	11-26-09	8	
"	"	"	11-10-09	11-13-09	3	
"	"	0.0040	10- 5-09	10-11-09	6	100
"	"	"	10-11-09	10-15-09	4	
"	"	"	10-21-09	10-28-09	7	
"	"	"	10-28-09	11- 1-09	4	
"	"	"	11- 4-09	11- 8-09	4	
"	"	"	11-10-09	11-16-09	6	
"	"	"	11-18-09	11-29-09	11	

quality of sac and the amount of toxin required to give the desired results. The ultimate results, only, of this phase of the work need be given. We found that a sac having a capacity of about 1.5 c.c. and containing 0.15 c.c. of toxin (about 37½ M.L. doses or 55 per cent of the L+ dose) would kill a 400 gm. guinea-pig in five, six, or seven days as shown in Table 4.

This secured a satisfactory interval and the dose (0.15 c.c.) has been adhered to since. It was early found that the collodion

sacs must be uniform in thickness and quality or the results would be irregular. After considerable experimenting a method of making them was developed, which has given satisfactory results and has since been adhered to.

The method is a modification of that given by Frost.[1] Nine parts of Schering's celloidin are added to 100 parts of a mixture of equal parts of absolute alcohol and ether in a bottle. The mixture is allowed to stand with occasional shaking for about 24 hours, then placed in a mechanical shaker and shaken until dissolved. After all air bubbles have disappeared, a portion is poured into a test-tube (size 12×100 mm.), the tube is rotated, and the excess poured out into another bottle, not back into the unused collodion. The tube is rotated in a horizontal position for about ½ a minute to distribute the adhering collodion uniformly, then it is placed in a vertical position with the open end down and allowed to drain for 70 seconds. The excess of collodion

TABLE 3.

POTENCY TEST OF TWO ANTIDIPHTHERIC SERUMS, NO. 013069 TESTING 900 UNITS PER C.C. AND NO. 013327 TESTING 200 UNITS PER C.C.

GUINEA-PIG	SERUM		TOXIN		REACTION TIME IN MINUTES	INJECTION		DEATH	INTERVAL IN DAYS	RELEASE
Weight	Name	Dose	R No.	Dose		Day	Hour	Day		
250	013069	0.00117	05656	0.27	20	9–24–09	4 P.M.			9–30–09
"	"	.00111	"	"	"	"	"			9–30–09
"	"	.00105	"	"	"	"	"	9–27–09	3	
"	013327	.0050	"	"	"	"	"			9–30–09
"	"	.0040	"	"	"	"	"	9–28–09	4	
"	"	.0033	"	"	"	"	"	9–27–09	3	

about the lip of the tube is now removed and the tube is placed in a vertical position with the mouth up for 2½ minutes. A capillary glass tube having an internal diameter of 1 mm. is now introduced into the test-tube nearly to the bottom, and air under ¾ of a pound pressure per square inch is allowed to blow through the capillary for three minutes. The tube is now filled with water and allowed to stand till the next morning, when the tube of collodion can easily be withdrawn from the glass tube. The collodion tubes are suspended in test-tubes of water by means of a thread and sterilized. After some practice, it is possible to make very perfect tubes by this method. When wanted for use a tube is withdrawn by means of the string from the test-tube in which it was sterilized and placed in a short sterile test-tube (about 12×25 mm.) held in the hand. The top can now be cut off where desired, the water withdrawn with a pipette, and the desired amount of toxin and water introduced. The tube is then closed by tying the string, and sealed with a drop of collodion. The sac thus prepared is introduced into the peritoneal cavity of a 400 gm. guinea-pig, strictly aseptic precautions being observed during the operation.

Curative Tests.—Having found a method by which guinea-pigs could be killed by the gradual absorption of diphtheria toxin

[1] *Laboratory Guide to Bacteriology*, p. 296. Madison, Wis., 1902.

from a collodion sac, antitoxin was administered to determine its value in saving the lives of those guinea-pigs into which such sacs had been inserted.

In all these tests the same amount of toxin was placed in the

TABLE 4.

CONTROL GUINEA-PIGS SHOWING TIME OF DEATH AFTER THE INTRODUCTION OF A COLLODION SAC CONTAINING $37\frac{1}{2}$ FATAL DOSES OF DIPHTHERIA TOXIN NO. 05656.

The average time of death of the 41 guinea-pigs in this series is almost exactly six days.

GUINEA-PIG	TOXIN IN SAC		INTRODUCTION OF SAC	DEATH	INTERVAL IN DAYS
Weight	R No.	Dose	Day	Day	
400	05656	0.15	9- 7-09	9-13-09	6
"	"	"	9-13-09	9-18-09	5
"	"	"	9-14-09	9-20-09	6
"	"	"	9-20-09	9-27-09	7
"	"	"	9-20-09	9-27-09	7
"	"	"	9-20-09	9-27-09	7
"	"	"	10- 1-09	10- 6-09	5
"	"	"	10- 6-09	10-12-09	6
"	"	"	10-12-09	10-18-09	6
"	"	"	10-19-09	10-24-09	5
"	"	"	10-18-09	11- 3-09	6
"	"	"	12-14-09	12-21-09	7
"	"	"	12-14-09	12-19-09	5
"	"	"	12-14-09	12-20-09	6
"	"	"	12-21-09	12-26-09	5
"	"	"	12-21-09	12-26-09	5
"	"	"	12-21-09	12-27-09	6
"	"	"	12-20-09	1- 5-10	7
"	"	"	12-20-09	1- 4-10	6
"	"	"	1-25-10	2- 1-10	7
"	"	"	1-25-10	1-31-10	6
"	"	"	1-25-10	1-31-10	6
"	"	"	2- 7-10	2-13-10	6
"	"	"	2-23-10	3- 2-10	7
"	"	"	2-23-10	3- 1-10	6
"	"	"	2-23-10	3- 1-10	6
"	"	"	3- 9-10	3-15-10	6
"	"	"	3- 9-10	3-15-10	6
"	"	"	3-23-10	3-29-10	6
"	"	"	4- 2-10	4- 7-10	5
"	"	"	4- 2-10	4- 7-10	5
"	"	"	4- 2-10	4- 8-10	6
"	"	"	4-11-10	4-16-10	5
"	"	"	4-11-10	4-16-10	5
"	"	"	4-11-10	4-17-10	6
"	"	"	4-11-10	4-17-10	6
"	"	"	4-13-10	4-20-10	7
"	"	"	4-29-10	5- 4-10	5
"	"	"	5- 7-10	5-12-10	5
"	"	"	6- 3-10	6-10-10	7
"	"	"	6-14-10	6-21-10	7

sacs, namely, $37\frac{1}{2}$ M.L.D. or as much as would be neutralized by 0.55 of a unit. In Table 5 the results are shown when the serum is administered in one dose by subcutaneous injection 24 hours after the introduction of the sac. It will be seen that the animals receiving antitoxin survive longer than the controls, the length

of time they survive being roughly proportional to the amount of antitoxin they receive up to a point beyond which they do not die. However, the results are very irregular, especially in relation to the amount of antitoxin necessary to really save the life of the guinea-pig. It seems absurd that it should require more than 80 units to save the life of the pig, when the entire amount of

TABLE 5.

SHOWING THE RESULTS OF INJECTING ONE DOSE OF ANTITOXIN VARYING FROM 1 TO 1,280 UNITS, 24 HOURS AFTER THE INTRODUCTION OF A SAC CONTAINING 37½ FATAL DOSES OF TOXIN.

GUINEA-PIG	SERUM		TOXIN IN SAC		INTERVAL IN HOURS	INTRODUCTION OF SAC	DEATH	INTERVAL IN DAYS	AV. INTERVAL
Weight	R No.	Dose	R No.	Dose		Day	Day		
400	013069	1 unit	05656	0.15	24	10- 6-09	10-12-09	6	
"	"	"	"	"	"	10-12-09	10-21-09	9	7.75 days
"	"	"	"	"	"	10-13-09	10-20-09	7	
"	"	"	"	"	"	10-28-09	11- 6-09	9	
"	"	2 units	"	"	"	10- 6-09	10-14-09	8	
"	"	"	"	"	"	10-12-09	10-22-09	10	10 days
"	"	"	"	"	"	10-13-09	10-22-09	9	
"	"	"	"	"	"	10-28-09	11-10-09	13	
"	"	5 units	"	"	"	10-12-09	10-25-09	13	
"	"	"	"	"	"	10-13-09	10-21-09	18	13.3 days
"	"	"	"	"	"	10-28-09	11-16-09	19	
"	"	10 units	"	"	"	9-28-09	10-13-09	15	14 days
"	"	"	"	"	"	10- 1-09	10-14-09	13	
"	"	20 units	"	"	"	10- 1-09	10-19-09	8	15.5 days
"	"	"	"	"	"	2- 3-10	2-16-10	13	
"	"	40 units	"	"	"	10- 1-09	10-14-09	13	19.5+ days
"	"	"	"	"	"	12-23-09	1-18-10	26	
"	"	"	"	"	"	2- 3-10	.....	..	
"	"	"	"	"	"	2-15-10	.....	..	
"	"	"	"	"	"	4-29-10	.....	..	
"	"	80 units	"	"	"	12-23-10	1- 9-10	17	
"	"	"	"	"	"	2- 3-10	2-23-10	20	
"	"	"	"	"	"	2-15-10	3- 3-10	16	
"	"	"	"	"	"	4-29-10	.....	..	
"	"	160 units	"	"	"	12-23-09	.....	..	
"	"	320 units	"	"	"	12-23-09	.....	..	
"	"	640 units	"	"	"	12-23-09	.....	..	
"	"	1280 units	"	"	"	12-23-09	.....	..	

toxin originally placed in the sac would be neutralized *in vitro* by one-half of a unit. The apparent explanation of the large amount of antitoxin required seemed to lie in the destruction of antitoxin by the tissues of the guinea-pig. Accordingly the method of administering the antitoxin was changed from one dose 24 hours after the introduction of the sac to four doses—one on the day of the operation and one on each of the three following days. The results are shown in Table 6.

It will be seen that when given in four doses on successive days following the introduction of the sac about as large doses are required as when given in a single dose; four doses of 20 units each being required to save the guinea-pig. In each case the animal survives as long as the antitoxin remains in its system. When the antitoxin is exhausted the toxin still diffusing from the sac kills the guinea-pig.

TABLE 6.

ANTITOXIN ADMINISTERED TO A SERIES OF FIVE GUINEA-PIGS IN FOUR SUCCESSIVE DOSES AT INTERVALS OF 24 HOURS FOLLOWING THE INTRODUCTION OF A COLLODION SAC.
Four doses of 20 units each were required to save the guinea-pig.

GUINEA-PIG	SERUM SUBCUTANEOUSLY		TOXIN IN SAC		INJECTION		DEATH	INTERVAL IN DAYS	RELEASE
Weight	R No.	Dose	R No.	Dose	Day	Hour	Day		
400	.....	.....	05656	0.15	2-16-10	3 P.M.			
"	013327	5 units	.....	....	2-16-10	5 P.M.			
"	"	"	.....	....	2-17-10				
"	"	"	.....	....	2-18-10				
"	"	"	.....	....	2-19-10		3-1-10	13	
400	.....	.....	05656	0.15	2-16-10	3 P.M.			
"	013327	10 units	.....	....	2-16-10	5 P.M.			
"	"	"	.....	....	2-17-10				
"	"	"	.....	....	2-18-10				
"	"	"	.....	....	2-19-10		3-6-10	18	
400	.....	.....	05656	0.15	2-16-10	3 P.M.			
"	013327	20 units	.....	....	2-16-10	5 P.M.			
"	"	"	.....	....	2-17-10				
"	"	"	.....	....	2-18-10				
"	"	"	.....	....	2-19-10				3-30-10
400	.....	.....	05656	0.15	2-16-10	3 P.M.			
"	013327	40 units	.....	....	2-16-10	5 P.M.			
"	"	"	.....	....	2-17-10				
"	"	"	.....	....	2-18-10				
"	"	"	.....	....	2-19-10				3-30-10
400	.....	.....	05656	0.15	2-16-10	3 P.M.			
"	013327	80 units	.....	....	2-16-10	5 P.M.			
"	"	"	.....	....	2-17-10				
"	"	"	.....	....	2-18-10				
"	"	"	.....	....	2-19-10				3-30-10

That the supply of antitoxin might continue as long as the supply of toxin, another series was started in which the serum was injected every third day, beginning with the day after the operation and continuing until the animal died or it was seen that there was no longer probability that it would die. Table 7 gives details and results. As will be seen, 1, 2, 4, and 8 units given every third day do not save the animal but prolong its life to 10, 13, 15, and 31 days respectively, while 16 and 32 units every third day

protect the animal completely. Another series was run in which the antitoxin was injected daily, beginning with the day of the operation and continuing till the animal died or the danger of death was past. Table 8 gives the details. In this case doses running from 0.1 to 2.0 units injected daily prolonged the life of the animal but failed to save it, while 4 units daily saved it. As a final step an attempt was made to administer the antitoxin as continuously as the toxin by allowing it also to diffuse from collodion sacs

TABLE 7.

ANTITOXIN ADMINISTERED EVERY THIRD DAY TO GUINEA-PIGS CARRYING SACS OF DIPHTHERIA TOXIN.

Sixteen units every third day saved the animal; eight units did not.

SERUM SUBCUTANEOUSLY		TOXIN IN SAC		INJECTION	DEATH	INTERVAL IN DAYS
℞ No.	Dose	℞ No.	Dose	Day	Day	
.....	.....	05656	0.15	2– 2–10		
013069	1 unit	.....	....	2– 3–10		
"	"	every third day up to		2– 9–10	2–12–10	10
.....	.....	05656	0.15	2– 2–10		
013069	2 units	.....	....	2– 3–10		
"	"	every third day up to		2–12–10	2–15–10	13
.....	.....	05656	0.15	2– 2–10		
013069	4 units	.....	....	2– 3–10		
"	"	every third day up to		2–15–10	2–17–10	15
.....	.....	05656	0.15	2– 2–10		
013067	8 units	.....	....	2– 3–10		
"	"	every third day up to		3– 2–10	3– 5–10	31
.....	.....	05656	0.15	2– 2–10		
013069	16 units	.....	....	2– 3–10		
"	"	every third day up to		3– 2–10	Released 3–30–10 in good condition.	
.....	.....	05656	0.15	2– 2–10		
013069	32 units	.....	....	2– 3–10		
"	"	every third day up to		3– 2–10	Released 3–30–10 in good condition.	

placed in the peritoneal cavity; both sacs being introduced at one operation. That there might be more surface for diffusion two sacs containing serum were used and the results are shown in Table 9. The results would indicate that there was some diffusion of antitoxin through the sacs, as those animals in which the sacs contained a large number of units did not die. However, attempts to obtain diffusion of antitoxin through similar sacs *in vitro* failed completely.

These curative tests emphasize the important part played by the destruction of antitoxin *in vivo* by showing clearly that a small

amount of toxin protected from destruction by being inclosed in a sac and slowly diffusing out into the peritoneal cavity is effective against relatively large doses of antitoxin injected into the subcutaneous tissue where they are exposed to rapid destruction.

They also emphasize the difference between this work with the sac and true diphtheria by showing that eight units every third day do not protect the guinea-pig from the continuous administration of small quantities of toxin. Eight units for a 400 gram guinea-pig correspond to 1,345 units for a man, and this quantity every third day would certainly protect a man from infection

TABLE 8.

AFTER THE INTRODUCTION OF THE SAC THE GUINEA-PIGS RECEIVED DAILY INJECTIONS OF ANTITOXIN.

Four units daily saved the pig, lesser amounts did not.

SERUM		TOXIN IN SAC		INJECTION	DEATH	INTERVAL IN DAYS
R No.	Dose	R No.	Dose	Day	Day	
013069	0.1 unit	05656	0.15	2–23–10		
Inj. daily	0.1 unit	up	to	3– 7–10	3– 8–10	13
013069	0.25 unit	05656	0.15	2–23–10		
Inj. daily	0.25 unit	up	to	3– 7–10	3– 8–10	13
013069	0.5 unit	05656	0.15	2–23–10		
Inj. daily	0.5 unit	up	to	3– 6–10	3– 7–10	12
013069	1.0 unit	05656	0.15	2–23–10		
Inj. daily	1.0 unit	up	to	3– 8–10	3– 9–10	14
013069	2 units	05656	0.15	2–23–10		
Inj. daily	2 units	up	to	3– 9–10	3–10–10	15
013069	4 units	05656	0.15	2–23–10		
Inj. daily	4 units	up	to	3–13–10	Released 3–30–10 in good condition.	

and probably cure most cases. Now the reason this quantity does not protect the guinea-pig is, in all probability, because the absorption of toxin is continuous irrespective of whether it be neutralized or not, while a corresponding quantity saves a man not alone because it neutralizes the toxin already absorbed but because it stops the supply of toxin. We know clinically that following an injection of serum the false membrane changes in appearance and comes off, but why it does so or what the power in the serum is which brings it about, we do not know. This work with sacs shows clearly that simple neutralization of the poison (antitoxic power) avails little if the source of toxin remains; that

an equally essential factor in cure is the stopping of the supply of toxin. We can test the neutralizing or antitoxic value of antidiphtheric sera accurately, but for this other equally important function—its action on the membrane—we have no test at present.

Prophylactic Tests.—The destruction of antitoxin *in vivo* plays so important a rôle that further tests were made to determine the rate of destruction from the standpoint of prophylaxis. The test was so arranged that a series of guinea-pigs all received the same dose of antitoxin, but on different days. After all had been injected with antitoxin they were all at one sitting injected with toxin to determine which were still protected by the serum. The injections of serum had been so arranged that the times intervening

TABLE 9.

BOTH TOXIN AND ANTITOXIN ADMINISTERED IN COLLODION SACS.

The guinea-pigs receiving large doses of antitoxin were not injured by the toxin.

GUINEA-PIG	SERUM IN TWO SACS		TOXIN IN SAC		INTRODUCTION OF SACS	DEATH	
Weight	R No.	Dose in Units	R No.	Dose	Day	Day	Hour
400	013069	2700	05656	0.15	4–21–10	Released 5–10–10 in good condition.	
"	"	540	"	"	"	Released 5–10–10 in good condition.	
"	"	108	"	"	"	4–29–10	

between the injection of serum and of toxin formed a regular series as 14, 12, 10, 8, 6, 4, 2 days.

Table 10 shows the results with two series, one receiving 10 units each and the other 5 units each. If we neglect the death of one pig after $7\frac{1}{2}$ days, the results show that the destruction of antitoxin is proportional to the amount of serum injected and the time. Those animals receiving 10 units were protected for four days and those receiving five units for two days.

The antitoxic power of blood serum seems to be intimately associated with certain of the globulins, and it is probable that the guinea-pig's power of destroying the antitoxin is limited to his power of destroying these foreign protein substances. Hence one would expect that the more protein the antitoxin was combined with the longer it would escape destruction.

An attempt was made to test this by using high potency serum (900 units per c.c.) in one series and low potency serum (250 units per c.c.) in the other. The results are given in Tables 10 and 11. Ten units of the high potency serum protected for 6 days and 5 units for 2 days. In both cases the low potency serum gave longer protection than the high potency serum, 6 and 2 as compared with 4 and 1.

An attempt was made to determine whether the admixture of normal horse serum or normal guinea-pig serum would prolong

TABLE 10.

To Show Length of Time Antitoxin Remains in the Guinea-Pigs, They Were Injected in Succession One or Two Days Apart with Antitoxin. At the End of the Series They All Received the Same Dose of Toxin.

The dose of toxin would be neutralized by 0.55 unit antitoxin.

Guinea-Pig	Serum		Toxin		Injection	Interval in Days	Death	Interval in Hours	Release
Weight	B No.	Dose	B No.	Dose	Day		Day		
250	013069	10 units	.....	....	2- 3-10	14	2-18-10	22	
"	"	"	.....	....	2- 5-10	12	"	"	
"	"	"	.....	....	2- 7-10	10	"	"	
"	"	"	.....	....	2- 9-10	8	"	26	
"	"	"	.....	....	2-11-10	6	2-20-10	72	
"	"	"	.....	....	2-13-10	4	.....	..	3-15-10
"	"	"	.....	....	2-15-10	2	.....	..	3-15-10
All the above inj. with			05656	0.15	2-17-10				
250	013069	5 units	.....	....	2- 9-10	8	2-18-10	22	
"	"	"	.....	....	2-11-10	6	"	26	
"	"	"	.....	....	2-12-10	5	2-19-10	36	
"	"	"	.....	....	2-13-10	4	2-20-10	72	
"	"	"	.....	....	2-14-10	3	"	72	
"	"	"	.....	....	2-15-10	2	2-25-10	7½ days	
"	"	"	.....	....	2-16-10	1	.....	..	3-15-10
All the above injected with			05656	0.15	2-17-10				

the time the antitoxin would protect the animal, but the difficulty in obtaining horse serum free of antitoxic power, and lack of time and opportunity to follow it up prevented getting definite results.

These prophylactic tests emphasize the fact that from the time of its introduction into the system antitoxin tends to be destroyed and eliminated; that the rapidity with which it is destroyed probably depends on the ability of the animal to dispose of the foreign protein with which it is associated and hence 10 units of low potency serum will remain undestroyed and protect the animal longer than 10 units of high potency serum.

An Unexplained Phenomenon.—In this work with diphtheria toxin in a collodion sac the question arose as to the amount of toxin still left in the sac after the death of the animal. To determine whether enough was left to kill another guinea-pig the sac was removed from the dead pig and transferred to the peritoneal cavity of a second guinea-pig. To our surprise the second guinea-pig died in a shorter time than the first. The test was repeated a number of times and usually the first animal died in about 5 to 7 days, the

TABLE 11.

TO SHOW LENGTH OF TIME LOW POTENCY SERA REMAIN IN THE GUINEA-PIG, COMPARE WITH TABLE 10.

The serum was injected subcutaneously, the toxin intraperitoneally.

GUINEA-PIG	SERUM		TOXIN		INJECTION	INTERVAL IN DAYS	DEATH	INTERVAL IN HOURS	RELEASE
Weight	Name and R No.	Dose	R No.	Dose	Day		Day		
250	013327	10 units	.....	....	2- 3-10	14	2-18-10	22	
"	"	"	.....	....	2- 5-10	12	"	"	
"	"	"	.....	....	2- 7-10	10	"	"	
"	"	"	.....	....	2- 9-10	8	"	"	
"	"	"	.....	....	2-11-10	6			3-15-10
"	"	"	.....	....	2-13-10	4			"
"	"	"	.....	....	2-15-10	2			"
All the above injected with			05656	0.15	2-17-10				
250	013327	5 units	.....	....	2- 9-10	8	2-18-10	26	
"	"	"	.....	....	2-11-10	6	"	"	
"	"	"	.....	....	2-12-10	5	2-19-10	36	
"	"	"	.....	....	2-13-10	4	2-20-10	72	
"	"	"	.....	....	2-14-10	3	2-20-10	72	
"	"	"	.....	....	2-15-10	2			3-15-10
"	"	"	.....	....	2-16-10	1			3-15-10
All the above injected with			05656	0.15	2-17-10				

second in 2 or 3 days, the third in 3 or 4 days, and the fourth in about the same time as the first. The experiments in detail are shown in Table 12.

It should perhaps be stated that in every case when a sac was removed bacteriologic tests were made of the peritoneal fluid on culture media containing unheated animal protein under both aerobic and anaerobic conditions. Usually the peritoneum was sterile. The few cases in which infection was found are not included.

In considering these results there seemed but two possible explanations: (*a*) that the sac became more permeable to the toxin or (*b*) that the toxin was in some way activated. To determine whether a sac would become more permeable we took a sac and filled

it with a 25 per cent solution of sodium chloride in water and suspended it in a flask containing 100 c.c. of distilled water. At intervals 1 c.c. of the water was titrated for salt with silver nitrate solution. Curve 1 shows the increase of salt content to approximate very closely to a regular curve; there is no indication of increased permeability to salt.

Again to test directly for an increased permeability of the sac to toxin we took a sac from an animal just dead and instead of transferring it to a second guinea-pig placed it in a test-tube containing

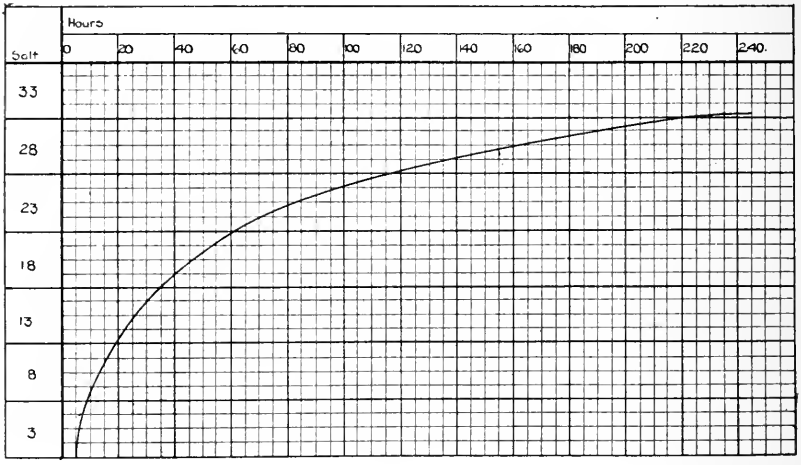

CHART 1.

·3 c.c. of sterile water and kept it in the incubator for five days. As a control we used a sac containing the usual dose of toxin (0.15 c.c.) but one which had never been in a guinea-pig and placed that in 3 c.c. of water in the incubator. Both sacs were kept under similar conditions for the same length of time and then the water from each was injected into a guinea-pig. Neither guinea-pig showed any toxic effect. Thinking the prolonged stay in the incubator might weaken the toxin, a second control was run at the same time to determine this point in this way: one, two, three, four, and five fatal doses of toxin (.005, .010, .015, .020, .025 c.c.) were diluted each to 3 c.c. with water and kept in the incubator with the sacs for the same length of time. Each amount was then injected into

a guinea-pig. All the guinea-pigs died: the one receiving one fatal dose in six days, the one receiving two fatal doses in three days, and all the rest in two days, thus showing that the toxin was not appreciably weakened by its stay in the incubator.

All attempts to obtain diffusion of toxin *in vitro* from collodion sacs have failed.

TABLE 12.

SHOWING THE TIME OF DEATH WHEN A SERIES OF GUINEA-PIGS IS KILLED WITH ONE COLLODION SAC. The second guinea-pig dies in a much shorter time than the first.

	GUINEA-PIG	TOXIN IN SAC	INTRODUCTION OF SAC	DEATH	INTERVAL IN DAYS
	Weight	R No. Dose	Day	Day	
Sac 1	400	05656 0.15	10– 1–09	10– 6–09	5
	"	Sac 1	10– 6–09	10– 8–09	2
	"	" "	10– 8–09	10–12–09	4
	"	" "	10–12–09	10–19–09	7
Sac 2	400	05656 0.15	10– 6–09	10–12–09	6
	"	Sac 2	10–12–09	10–14–09	2
	"	" "	10–14–09	10–18–09	4
Sac 3	400	05656 0.15	10–19–09	10–24–09	5
	"	Sac 3	10–25–09	10–28–09	3
Sac 4	400	05656 0.15	10–28–09	11– 3–09	6
	"	Sac 4	11– 3–09	11– 6–09	3
	"	" "	11– 6–09	11– 9–09	3
Sac 5	400	05656 0.15	11–18–09	11–24–09	6
	"	Sac 5	11–24–09	11–26–09	2
Sac 6	400	05656 0.15	12–21–09	12–26–09	5
	"	Sac 6	12–27–09	12–29–09	2
	"	" "	12–29–09	1– 2–10	4
Sac 7	400	05656 0.15	12–21–09	12–27–09	6
	"	Sac 7	12–27–09	12–30–09	3
Sac 8	400	05656 0.15	1–25–10	2– 1–10	7
	"	Sac 8	2– 1–10	2– 3–10	2
Sac 9	400	05656 0.15	1–25–10	1–30–10	5
	"	Sac 9	1–31–10	2– 2–10	2
Sac 10	400	05656 0.15	2– 7–10	2–15–10	8
	"	Sac 10	2–15–10	2–18–10	3

To determine how long after its introduction into the first pig the sac acquires the power of killing the second pig quickly, sacs were removed from the animals at varying intervals and placed in fresh pigs. The animals from which the sacs were to be removed were anesthetized, the wound opened, the sac removed carefully, and the wound again closed. In all cases they made good recoveries and seemed none the worse for the operations or the toxin. The sac removed 24 hours after its introduction killed a second pig in

four days, one 36 hours after in three days, and one 48 hours after in three days also. Here we have a sac remaining 48 hours in a pig and doing him so little damage that he could stand the toxin, two operations, and the trauma and shock incident to removal of the sac (the omentum tends to adhere to the sealed end of the sac so that it is difficult to remove the sac without trauma to the omentum); yet this same sac when transferred to a second pig kills it in 72 hours. The fact that these pigs die of toxin poisoning was in all cases controlled by the postmortem appearances, the absence of infection, and the uniformity of results.

Again we made a test to determine how much toxin was left in a sac after the first pig was dead. The entire contents of a sac from a guinea-pig that died in 6 days was divided into portions and injected into guinea-pigs in the following amounts: $\frac{1}{2}$, $\frac{1}{4}$, $\frac{1}{8}$, $\frac{1}{16}$, $\frac{1}{32}$ of the contents of the sac. All died except the one receiving the $\frac{1}{32}$ dose, and from the fact that the pig receiving the $\frac{1}{16}$ dose died in three days, it is probable that there were at least 20 M.L.D. left in the sac, or about half the amount put in ($37\frac{1}{2}$ to 40). Yet this sac with only half the original amount of toxin would kill a second guinea-pig in less than half the time required to kill the first pig.

SUMMARY.

In this work it has been shown:

1. That a guinea-pig may be killed by introducing a collodion sac containing diphtheria toxin into its peritoneal cavity; the slow absorption of toxin from the sac corresponding quite closely to the absorption of toxin from the false membrane in a case of diphtheria.

2. That to save the life of a guinea-pig by the administration of antitoxin after the introduction of a sac, requires an enormous dose or the long continued administration of moderate doses. The reason for this seems to be that the antitoxin administered subcutaneously, being a foreign protein, is subjected to rapid destruction by the tissues, while the toxin protected from such destruction by the sac continues to diffuse out and kills the animal after the protective power of the antitoxin has disappeared. The essential difference between these sac tests and the administration of antitoxin in diphtheria appears to be that in these tests the antitoxin has no

influence on the supply of toxin, while in diphtheria the administration of antitoxin is followed by destruction of the membrane, and consequently the supply of toxin ceases and the patient recovers. This power of antidiphtheric serum to destroy the membrane is probably quite as important as its antitoxic action, but unfortunately we have no means of measuring it.

3. That when used as a prophylactic, low potency sera protect for a longer time than those of high potency. Antitoxin protects until it is destroyed by the tissues. The more proteid it is combined with the longer will its destruction be delayed.

4. That diphtheria toxin in a collodion sac undergoes some change by which the sac altho containing less toxin than in the beginning is able to kill a second guinea-pig in half the time required to kill the first. This difference may be analogous to the short incubation period of tetanus toxin from the blood of an animal compared with the incubation period of tetanus toxin produced by the growth of the germs *in vitro*. It may also account for certain very rapidly fatal cases of diphtheria.

THE BIOLOGICAL REACTIONS OF THE VEGETABLE PROTEINS.*

I. ANAPHYLAXIS.

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SYNOPSIS.

1. INTRODUCTION.

The biological reactions of the vegetable proteins, with the exception of the phytotoxins, have received scant attention as contrasted with the great mass of investigations upon the proteins of animal origin. This is due in large part to the fact that nearly

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all the investigators in this field have been pathologists, bacteriologists, and clinicians, who were less familiar with vegetable proteins than with animal proteins. In studying these reactions the vegetable proteins offer distinct advantages over those of animal origin, since many of them can be isolated in a higher state of purity. On the other hand, the fact that but few of the vegetable proteins are soluble in water or physiological salt solution is a decided disadvantage in biochemical investigations, which in some cases is insurmountable.

Few references to vegetable proteins are to be found in the literature of immunology, with the exception of the numerous investigations of the phytotoxins, especially ricin and abrin. While these last investigations furnished the first evidence that the animal body would react to the presence of vegetable poisons by the production of antibodies, yet the reaction against the plant toxins with their typical toxin character is a special case, and need not be considered in connection with the general biological reactions of the ordinary plant proteins which are not toxins in the common use of the term. Against the phytotoxins we have the production of true antitoxins; against non-toxic vegetable proteins we may have the production of precipitins, agglutinins, opsonins, and also, under suitable conditions, the anaphylactic or hypersensitive state. There have been a few investigations of the precipitins produced by immunization with various impure extracts of plant tissues, and an even smaller number of experiments which demonstrate that vegetable proteins resemble animal proteins in being toxic to animals which have previously received a small sensitizing dose of the same protein. Concerning agglutinins and opsonins for vegetable substances we can find almost nothing in the literature.

2. HISTORICAL.

a) The Precipitin Reaction and Vegetable Proteins.

Kowarski[1] seems to have been the first to report (July 4, 1901) the observation of precipitins for vegetable proteins. He immunized rabbits with solutions of non-coagulable proteins from wheat flour, and obtained a precipitin for a saline extract of wheat flour. The immune serum also gave a distinct, but less heavy precipitate with extracts of the seeds of peas, rye, and barley, but not with an extract of the seeds of oats. From his observations he suspected that the specificity of vegetable proteins

may not be as marked as with animal proteins, but it is probable that the lack of specificity observed was due to the use of too concentrated solutions of the serum and protein.

At about the same time Jacoby[2] reported his observations on the precipitin reaction which occurs when anti-serum for ricin is added to a solution of ricin, but these experiments were not directed to ascertaining the biological reactions of a vegetable protein, as were the experiments of Kowarski. A similar demonstration of the possibility of producing specific precipitins for ricin was reported in the same year by Loewenstein.[4]

Albert Schütze[3] also described the production of an anti-serum for a soluble commercial preparation of vegetable protein ("Roborat") which reacted with this Roborat, but not with a solution of human muscle protein; further tests seem not to have been made. Later Schütze[5] reported some unsuccessful attempts to distinguish between different yeasts (top yeast, bottom yeast, grain yeast, potato yeast) by means of the precipitin reaction.

Ottolenghi[6] immunized with extracts of ergot of rye in physiological salt solution, and obtained a serum which gave a precipitate with aqueous extracts of ergot of rye, but did not react with watery extracts of rye or wheat flour.

The next investigation in this field that we can find recorded was by Bertarelli[7] who investigated beans, peas, lentils, and vetches. He used the non-coagulable proteins in aqueous solution as employed by Kowarski, and also saline extracts, or even fine suspensions of the pulverized seeds. Intravenous injections were found to be dangerous, especially with the bean extracts, possibly because of the agglutinating action of such seed extracts on the erythrocytes. Normal serum gave a precipitate with strong solutions of the proteins which Bertarelli used for immunizing, but only in concentrations of 1 in 10 to 1 in 50, varying with different animals and different proteins. Cautious subcutaneous immunization produced the most active sera, the maximum activity being reached only after continuing the immunizing treatment for six to seven weeks. Immune serum was obtained reacting against extracts of the seeds of *Phaseolus vulgaris* in dilutions as high as 1 in 300 to 1 in 3,000, and this serum reacted with other extracts of leguminous seeds only in concentrations of 1 in 60 to 1 in 100. The results with extracts of pea flour were quite similar. Immunizing against extracts of lentil flour he obtained anti-sera which reacted with this material in dilutions of 1 in 3,000 to 1 in 5,000, but with extracts of other leguminous seeds only in concentrations of 1 in 100 to 1 in 300. Likewise anti-serum against extracts of one sort of vetch or one variety of bean reacted with strong solutions of the proteins of the same genus. Therefore Bertarelli concludes that the precipitins which can be obtained by immunization with vegetable proteins are qualitatively but not quantitatively specific, and that to distinguish a protein by the precipitin reaction, dilutions of not less than 1 in 500 should be used. Attempts were made to differentiate the mushrooms by the precipitin reaction, but without success, for it was extremely difficult to obtain any precipitins whatever by using mushroom extracts and such active sera as were obtained did not react specifically.

Catastini,[10] on the other hand, claims to have obtained specific precipitins for aqueous extracts of various mushrooms, which exhibited quantitative differences with extracts from other than the homologous species. He states, however, that it is quite difficult to obtain precipitins with this material, which we may ascribe to the low content of protein in extracts of these fungi.

Magnus and Friedenthal[45] state that the press juice of *Agaricus campestris* contains but 0.1 per cent of protein, while truffle (*Tuber brumale*) press juice contains only

0.025 per cent, in contrast with press juice from yeast (*Saccharomyces cerevisiae*) which contains 2 per cent of protein (all estimated by the Esbach method). They immunized rabbits, one with each of these three plant extracts, and found that the anti-mushroom serum reacted only with the mushroom extract, the anti-yeast serum reacted well with the truffle extract but not with the mushroom extract, while the anti-truffle serum reacted slightly with the yeast but not at all with the press juice of *Agaricus campestris*. In subsequent papers they described their investigations of precipitin reactions with vegetable proteins, finding, in contradiction to Kowarski, that anti-sera for pea-meal extracts and wheat-flour extracts are mutually specific.[46] They also produced anti-sera for extracts of the seeds of *Ustilago jensenii*, *Mucor racemosus*, *Cocos nucifera*, *Sauromatum*, *Zea mays*, *Oryza sativa*, *Panicum italiacum*, *Triticum sativum*, *Avena sativa*, and *Pisum sativum*, all of which showed definite but limited specificity. Thus, anti-serum for maize flour did not react with extracts of rice flour but did react with extracts of the seeds of *Euchlaena mexicana;* wheat flour anti-serum reacted with extracts of rye and barley flour but not with extracts of oat flour; pea-meal anti-serum reacted with extracts of the seeds of vetch (*Vicia sativa*) but not with those of the seeds of the yellow lupine (*Lupinus luteus*). They consider that precipitins against extracts of vegetable substances are, if anything, more specific than precipitins against soluble animal substances. They also found[47] that precipitins resulting from immunization with any one part of a plant (rye) would react with extracts from all other parts of the same plant, e.g., seeds, leaves, and roots.

Gasis[8] in 1908 produced precipitins by immunizing with the non-coagulable proteins, obtained by boiling and filtering watery extracts of rye, rice, and various beans. He found these to show a high degree of specificity, contrary to Kowarski and Bertarelli, and states that if quantitative relations were taken into consideration closely related species could be distinguished; e.g., *Phaseolus vulgaris* could be distinguished from *Phaseolus multiflorus*. The proteins of rice seeds, however, seemed to be less specific than the proteins of the seeds of other cereals or of those of the seeds of leguminous plants.

Relander[9] published a preliminary report in the same year stating that he had found it possible to distinguish different varieties of grains and vetches by their precipitin reactions, but gave no details as to the materials or methods used. We have been unable to find any subsequent reports from this observer.

Magnus[48] properly criticizes the observations of Gasis on the ground that they were not sufficiently controlled, for many plant extracts, and especially those of the cereals, give precipitates with normal sera. He finds that the extract of rice flour produces especially heavy precipitates with normal serum, being followed closely by extracts of the flour of maize and oats, while extracts of rye, barley, and wheat flour produce practically no such precipitates. He states that in order to test such plant extracts as give precipitates it is necessary to first remove the precipitating elements by saturation with normal serum and filtration, then using the filtrate against the immune serum. Taking into account these and other sources of error, Magnus finds that the degree of immunization determines the range of reaction. If, for example, an animal is immunized but a short time with extracts of the seeds of one of the cereals, its serum may precipitate only the extract of the same species; a little later in the course of immunization, precipitates will appear for extracts of closely related species, and progressively a wider and wider list of cereals will react, until finally precipitates may be obtained with any and all *Gramineae*. Nevertheless, even with this extreme degree of immunity no reaction will be given by the serum with extracts derived from plants

not belonging to the *Gramineae*. As the order of appearance of reactions with heterologous extracts is always the same, it is possible in this way to secure a standard for estimating the relationship of plants by biochemical means.

Magnus[49] finds that the specificity of the precipitins resulting from immunization with physiological saline solutions of grain products is sufficiently marked to permit of detection of adulteration and mixing of such materials. In order to do this it is necessary to take into account quantitative as well as qualitative differences and in the case of closely related grains, differentiation can be secured only through the use of the saturation process. By this means Magnus was able to detect the presence of 3 per cent of wheat flour in rye flour, and $\frac{1}{2}$ of 1 per cent of "castor meal" (*Vicia faba*) in wheat flour.

Dunbar[50] was unable to obtain precipitins by immunizing with the pollen of rye, maize, solidago, and ambrosia, in which respect pollen differs from other plant structures. A complement-binding serum was obtained, however, which was peculiar in not reacting with other parts of the plant; as similar results were obtained with spermatozoa of fish and animals when contrasted with the flesh and serum of these animals, he considers that the sex cells are specifically distinct from all other elements of the organism, a view which is opposed by Magnus and Friedenthal.[51] Precipitins for extracts of rye leaves and grains were found by Dunbar to react more strongly with the homologous than with the heterologous extract.

Recently Wilenko[15] has studied the precipitation which occurs when extracts of various vegetable materials are added to sera or other protein solutions. This phenomenon was first observed by Kraus[16] who obtained a precipitate when ricin was added to sera of various origins, but only when the sera were from animals whose red corpuscles are agglutinated by ricin. Similar results have been obtained with abrin, and with physiological saline extracts of beans (*Phaseolus*), lentils (*Ervum*), peas (*Pisum*), and vetches (*Vicia*), by Landsteiner and Raubitschek,[17] and with *Datura stramonium* by v. Eisler and v. Portheim.[18] Wilenko[15] found that all normal sera gave precipitates with ricin,* abrin, and crotin, and with extracts of the seeds of oats and maize, bird sera reacting most strongly and carp serum the least. Solutions of hemoglobin, serum albumin, and egg albumin all gave precipitates with these same plant extracts, and even one plant extract may cause precipitation in another.† Excess of serum prevents the formation of a precipitate, while heating the plant extracts to 80° C. destroys their precipitating action. Solutions of ricin and extracts of maize absorb complement, when added to guinea-pig serum. These phenomena Wilenko ascribes to "normal precipitins" present in the plant extract, which he assumes, without advancing any proof of his contention, to be entirely analogous to the immune precipitins; neither does he prove that the reactions observed depend upon the proteins contained in his saline extracts of the seeds, rather than upon other extractive substances also present.

Raubitschek[21] immunized rabbits against saline extracts of lentils and beans, and found that the serum of these rabbits would inhibit the agglutination of red cor-

* Osborne, Mendel, and Harris, in their experiments with a highly purified ricin (*Am. Jour. of Physiol.*, 1905, 14, p. 259), found that the serum of a rabbit immunized with this ricin yielded a precipitate with the ricin, but the serum of a normal rabbit yielded *no* precipitate under the conditions of the experiment unpublished observation).

† In this connection we may point out that Hardy's experiments with serum globulin indicate that this protein has more pronounced acid than basic properties, whereas Osborne[54] found that edestin is more basic than acid, and it is known that when proteins of opposite reaction meet they commonly form precipitates.

puscles by such extracts, but would not inhibit agglutination by ricin; therefore it would seem probable that the natural phytagglutinins are each characterized by a specific antigen. He mentions, as a preliminary report, that these anti-sera have a specific inhibiting effect upon the development and growth of the seeds of the species furnishing the extracts used for immunizing. De Angelis[19] claims that specific precipitins can be obtained by immunizing with vegetable dyes.

b) ANAPHYLAXIS WITH VEGETABLE SUBSTANCES.

Little as is known concerning the behavior of the vegetable proteins as antigens in the precipitin reaction, their behavior in the anaphylaxis reaction has been even less considered. Rosenau and Anderson[11] observed in a brief series of experiments that guinea-pigs which had been sensitized with a watery extract of peas reacted violently (3 in 5 died) to a subsequent injection of the same material, establishing that vegetable extracts resemble animal and bacterial proteins in this respect. They performed no experiments to test the specificity of the reaction with pea extracts, nor with the extracts from yeasts and various bacilli which they found to sensitize guinea-pigs to the homologous extract. In a later paper they reported unsuccessful attempts to secure satisfactory reactions with edestin and excelsin dissolved in 10 per cent NaCl solution, using for the second or intoxicating dose 3 c.c. of a 1 per cent solution of edestin subcutaneously. Their failure in these last experiments we may ascribe to several different factors: first, the small quantity of relatively insoluble proteins used in the second dose; second, the subcutaneous injection, which is the least favorable route for producing the anaphylactic intoxication; third, precipitation of the protein because of diffusion of the salt which served as solvent for the protein, for Mendel and Rockwood[55] found that solutions of edestin in 0.5 per cent sodium carbonate, or of excelsin in 0.9 per cent sodium chloride, yield precipitates of unchanged protein when injected intraperitoneally; and fourth, the simultaneous injection of so much NaCl, for it has been found by Friedmann and Hartoch[13] that by injection of NaCl solution into either actively or passively immunized guinea-pigs the anaphylaxis reaction can be prevented; this inhibition of the reaction they ascribe to the fact that NaCl in sufficient quantities prevents the binding of complement.

Having observed that gelatin will not cause the anaphylactic reaction, Wells[14] suggested as an explanation for this failure the deficiency of aromatic radicals which is characteristic of gelatin, and for comparison investigated the behavior of zein and gliadin, zein being rich in tyrosine and phenylalanine, although devoid of tryptophane, while gliadin is poor in tyrosine and phenylalanine, although it contains tryptophane. Zein was found to actively intoxicate guinea-pigs sensitized with small quantities of zein, fatal reactions being frequently obtained. With gliadin, reactions were sometimes but not always obtained, and in only one of twenty experiments was a fatal intoxication observed.* Animals sensitized with zein did not react to gliadin, or conversely. These were the first, and until now the only recorded successful anaphylaxis experiments with pure proteins isolated from vegetable materials.

Raubitschek[21] sensitized rabbits with saline extracts of lentils and beans, and obtained severe, often fatal reactions, upon subsequent intravenous injections of the homologous extract; with the heterologous extract, reactions were also obtained, but usually less severe. The results secured by such intravenous injections are complicated by the strong hemagglutinating property of such extracts and hence the above experiments have relatively little significance.

* Later experiments with a more soluble preparation of gliadin have given more severe reactions.

Pozerski[41] obtained a form of anaphylaxis reaction by injecting guinea-pigs subcutaneously with papain in repeated small doses.* Uhlenhuth and Weidanz[52] injected into guinea-pigs raw linseed oil, commercial ricin, and cocoa butter, and later found them sensitive respectively to extracts of flax seeds, castor beans, and cocoa beans.

Karasawa[56] found that typical anaphylactic reactions are produced in guinea-pigs with saline extracts of ground rice, beans, and wheat; sensitization was by subcutaneous injection and the intoxicating dose was given intravenously. Passive sensitization was produced by injecting the serum of sensitized guinea-pigs or rabbits into normal guinea-pigs. One experiment showed that a dog reacted to rice extract with a sharp fall in blood pressure and decreased coagulability of the blood, these phenomena being characteristic of anaphylaxis in the dog. With these extracts a well-marked specificity was shown, guinea-pigs sensitized with rice extract not reacting to saline extracts of sago, rolled barley, wheat flour, beans, or maize; guinea-pigs sensitized with bean extracts not reacting to extracts of lentils, green peas, nut extracts, yellow peas, or "large beans." Only one experiment was performed with each of these substances, and the author failed to determine how much protein was present in the solutions used.

Schern[59] also records the use of the anaphylaxis reaction for the detection of the presence of small amounts of poisonous seeds mixed with food stuffs. He studied extracts of rye bran, peanut flour, and sesame-cake meal, which were contaminated or adulterated with castor-bean meal, cockle, charlock, and ergot.

c) AGGLUTININS AND OPSONINS.

The possibility of stimulating the formation of specific agglutinins for vegetable matter has been demonstrated by Cao[20], who found that by immunizing dogs with starch grains from different sources, it was possible to obtain sera agglutinating starch granules, the reaction showing definite specificity. On the other hand, Porges[43] was unable to increase the opsonic power of rabbit's serum for starch granules by repeated injections of starch.

d) CONCLUSIONS.

It is evident, from the above review of the literature, that the biological reactions of the vegetable proteins have been very inadequately considered, especially in regard to the behavior of isolated preparations freed as far as possible from other substances present in the crude extracts used in most recorded investigations. It is our purpose, in this and subsequent papers, to report investigations performed with vegetable proteins which have been purified as far as the means at present available will permit, and especially with proteins which have been studied as to their content of amino-acids. By using such material it is hoped to put the immunological reactions upon something more nearly approach-

* Wechselmann[22] considers as a form of anaphylaxis the hypersensitive condition which workers in satin wood (*Fagara flava*) acquire, resulting in erysipelatoid inflammation whenever the skin comes in contact with even a particle of the wood. The active agent seems, however, to be an alkaloid, and therefore this particular form of hypersensitivity is presumably not the same as hypersusceptibility to proteins.

ing a chemical basis than is possible by experiments with sera, egg white, and extracts of tissues and plants, all of which are of complex composition. It is probable that many of the vegetable proteins can be isolated in a purer condition than most of the animal proteins because they form a relatively large part of the reserve food material of seeds, in which they are associated chiefly with non-protein substances from which they can frequently be easily separated. These proteins consequently offer a better opportunity for a study of the biological relations of the proteins themselves than do the animal proteins, about the preparations of which usually much uncertainty exists as to their complete separation from other substances which may contribute, in part or in whole, to the results obtained by their use.

As a matter of convenience we have begun our investigations with a consideration of the anaphylaxis reaction as induced by vegetable proteins. Before describing our experiments it is necessary to discuss the degree of specificity exhibited in the anaphylaxis reaction as shown by previous investigations with proteins of animal origin.

3. SPECIFICITY OF THE ANAPHYLAXIS REACTION.

As yet the limits of specificity of the anaphylaxis reaction are not so well defined as with the precipitin and the complement fixation reactions. It is known that the precipitin reaction becomes less and less differential the more closely related are the sources of the proteins which are compared, and with those from very closely related species precipitins may show only quantitative differences. According to Bauer[23] the complement fixation reaction is much more specific than the precipitin reaction, and Brück[44] has claimed that he can distinguish different species of monkeys from one another by this means, and even that different secretions of body fluids of the same species can be thus distinguished. While this last statement failed of complete confirmation by Bauer, he found that the milk and blood of the same species can be differentiated by the complement fixation reaction.

In their first communication upon the subject of anaphylaxis, Rosenau and Anderson[24] reported a number of experiments made with the object of learning the degree of specificity of the reaction. They found in general that guinea-pigs sensitized with 1/250 c.c. of the serum of one species would in some instances show slight symptoms when 4 to 6 c.c. of the serum of some other species were given intraperitoneally, but as a rule these symptoms were not of any significance as compared with the violent reactions which occurred in the same animals when they were subsequently injected with the serum of the same species as had been used for the first injection. They therefore concluded that the reaction is specific if the degree of reaction is taken into account, that is, it is quantitatively specific. Recent work by these investigators

and many others has shown that the degree of specificity is perhaps even more marked than they had at first indicated, although the reports have not been entirely unanimous. One source of error has been pointed out by Uhlenhuth,[25] Pfeiffer,[26] and Thomsen;[27] namely, that some foreign sera are more or less toxic to normal guinea-pigs, and that unless carefully controlled such toxic effects may be interpreted as anaphylactic intoxication. So far as the specificity of the anaphylaxis reaction for foreign blood and serum is concerned, the results obtained by Thomsen[27] are most conclusive. Wishing to decide the possibility of medico-legal application of the anaphylaxis reaction for the determination of the nature of blood stains, he performed careful experiments in which the guinea-pigs were sensitized with small quantities of material obtained from dried blood drops, and found it possible to determine accurately the species of animal from which the drop of blood had been derived, by ascertaining with what sort of serum the sensitized animal would react. He found also, in agreement with the results of Rosenau and Anderson, that injection of a heterologous serum into a sensitized guinea-pig does not render it refractory to a subsequent dose of the homologous foreign serum unless the animals supplying the foreign sera are very closely related, e.g., sheep and goat. Neufeld[28] claims that human and monkey (*Affe*) blood cannot be distinguished by the anaphylaxis reaction, and it is well known that they react with the same precipitins. Trommsdorff[29] states that rat blood and mouse blood cannot be distinguished by this means, although by both the precipitin test and the complement fixation reaction they can be sharply differentiated, and more recently Pfeiffer and Mita[58] have claimed by using the fall of temperature as the index of reaction to distinguish rat blood from mouse blood.

On the other hand, a very high degree of specificity is claimed for the anaphylaxis reaction, at least in certain cases, by Thomsen,[30] who states that it is possible to differentiate the hemoglobin and the serum of the same blood by the anaphylaxis reaction, as guinea-pigs sensitized against foreign red corpuscles will not react with the serum of the same blood, or conversely.

With milk, interesting relations have been observed. Rosenau and Anderson[12] found that guinea-pigs sensitized with human milk or dog's milk did not react with cow's milk, but that when sheep's milk was used for sensitizing, a severe reaction might be obtained with cow's milk; this indicates that, as with serum, heterologous reactions are more likely to occur when the milk is from closely related species. Wells[14] found slight but uncertain reactions when serum and milk of the same species of animal were used against each other for the sensitizing and the intoxicating doses, but the number of experiments performed was too small to give significant results. The same criticism may also be applied to the observations of Uhlenhuth and Haendel[31] who claim that guinea-pigs sensitized with bovine serum will react with cow's milk, and conversely, which they contrast with the finding that precipitins for cow's milk react with bovine serum whereas precipitins for bovine serum react but feebly with cow's milk. Besredka[32] states that animals sensitized with cow's milk react also with goat's milk. Similarly Pfeiffer and Mita[42] obtained some slight reaction when goat's milk was injected into guinea-pigs that had been sensitized with cow's milk. Of particular significance is the observation of Rosenau and Anderson[33] that a guinea-pig sensitized with milk, egg white, and horse serum, either together or separately, will react with each of the three fluids given in series. The blood of such a triply sensitized animal when injected into other animals will likewise render them passively sensitive to all three.[12] The results of Gay and Southard,[34] indicating that any one of

these fluids may sensitize to the other two, are difficult of explanation in the light of many other observations to the contrary.

Uhlenhuth and Haendel[31] found that animals sensitized to chicken's serum will react to chicken's egg white, or yolk, or hemoglobin; if sensitized to chicken's hemoglobin they react to chicken's serum, and if sensitized to stroma of chicken's corpuscles they react to egg white or yolk. In all these heterologous reactions, however, there is not developed a refractory condition to the homologous protein, which indicates that there are probably separate as well as common sensitizing and intoxicating substances in even these closely related materials.

Rosenau and Anderson summarize their conclusion concerning the specificity of the anaphylaxis reaction as follows: "The anaphylactic reaction in the guinea-pig, therefore, seems to be specific in the sense that the precipitins are specific. That is, there is a group reaction in the proteins of allied species, but no reaction between the proteins of widely different species or between proteins of widely different origin." Subsequent developments have not materially modified the validity of this estimate.

Anaphylaxis with bacterial substance, which is perhaps more closely related to anaphylaxis with vegetable proteins, has also been found to exhibit a considerable degree of specificity. The pioneer demonstration by Rosenau and Anderson[11] that the anaphylactic reaction could be produced with extracts of bacterial cells has been repeatedly confirmed, and efforts made to determine the degree of specificity. While Delanoe[35] has claimed that there is no specificity whatever in bacterial anaphylaxis, the positive results of Holobut and Kraus seem to establish the existence of a marked degree of specificity both with active and passive sensitization. Holobut[36] found that specific reactions could be obtained with *B. typhosus*, *B. coli*, and cholera vibrios, and although interaction between colon and typhoid bacilli can be obtained this is to be looked upon as a group reaction, such as is obtained with agglutinins and precipitins. Kraus and Amiradzibi[37] also found that passive anaphylaxis was specific, and that the active poison of anaphylaxis could be produced only when the serum of sensitized animals was mixed with the homologous bacterial substance. Unfortunately, in all these experiments with bacteria the small number of experiments and the insufficiency of controls leaves the results in far from a satisfactory state.

As special cases of specific anaphylaxis should be mentioned the observations of Rosenau and Anderson upon anaphylaxis with placental substance, and the experiments of Kraus and others upon the specificity of anaphylaxis with crystalline lens extracts. The former authors[12,38] found that guinea-pigs could not be sensitized with blood from fetal guinea-pigs, whereas a certain degree of reaction could be obtained by sensitizing and reinjecting with extracts of guinea-pig placenta. With the protein of the crystalline lens peculiar relations as to specificity have been shown by Uhlenhuth with the precipitin reaction, which indicate that the precipitinogen of this protein is devoid of species specificity but that it has a specific relation to the crystalline lens. These ideas are based upon the well-corroborated observation that the precipitins produced by immunizing with lens substance will react with extract of crystalline lens from animals of most diverse species, but will not react with the serum or other proteins of even the homologous animal. Kraus[39] found a similar specificity when anaphylaxis experiments were performed with crystalline lens extract. Thus, animals sensitized with horse lens would react with extracts from the lens of the ox but would not react with horse serum; likewise, sensitization with ox serum did not make animals reactive to ox lens. Andrejew[40] has obtained similar results, but a critical con-

sideration of his protocols does not indicate that the reaction is as strikingly free from species specificity as his conclusions indicate; a very considerable percentage of the heterologous reactions were negative, while the homologous reactions were constant and violent. Pfeiffer and Mita[42] also found some evidence of the lack of species specificity of lens proteins, but they obtained much stronger reactions with the homologous than with the heterologous protein. They corroborate the lack of reaction to serum of animals sensitized with the homologous lens extract. These authors used as their measure of reaction the severe fall in temperature which takes place during the anaphylactic shock, and with this as an index rather than the symptoms of intoxication, they claim that the anaphylactic reaction is highly specific, distinguishing even between hemoglobin and serum of the same species. Uhlenhuth and Haendel[31] have made the remarkable observation that so highly specific is the lens protein that guinea-pigs can be sensitized to guinea-pig lens substance, and they even could sensitize a guinea-pig with the lens of one of its eyes and obtain a reaction by injecting later an extract of the lens of the other eye.

Taking the above-mentioned observations together it would seem that the anaphylaxis reaction, as it is usually observed, has much the same limits of specificity as the precipitin reaction and other immunity phenomena. That is, with some substances marked group reactions are shown, and with material from closely related species quantitative differences are not always constant; on the other hand, with some substances differences within a single species can be detected by the anaphylaxis reaction, e.g., serum and hemoglobin from the same blood, lens protein and serum protein, and also milk and serum from the same species. Taking the published results as they stand it would seem that the specificity of the anaphylaxis reaction is somewhat less sharply defined than the specificity shown by the precipitin reaction and the complement fixation reaction; however, it is possible that this is largely ascribable to the relatively coarse index of reaction furnished by the symptomatology of an intoxicated guinea-pig, as contrasted with the delicacy of observation possible with the precipitin and the complement fixation reactions. There are sources of confusion and error in the observation of symptoms, because of the possible intoxication by the foreign protein independent of anaphylaxis, differences in the rate of absorption, and sundry accidental injuries and intoxications that may complicate the results. Another ever-dangerous source of error lies in the infinitesimal dose that may cause sensitization, which, in the case of egg albumin, has been estimated as one-twenty-millionth of a gram.[14]

Such quantities of foreign proteins may be readily introduced unintentionally, and unless special mention is made of precautions taken to avoid such contamination one is justified in being skeptical of heterodox positive results. The hereditary transmission of sensitization adds a still further complication. It is probable that when all investigators appreciate fully these sources of difficulty, and when more accurate and more objective measures of anaphylactic shock are introduced, such as the fall of temperature, and perhaps other yet unknown objective standards, the anaphylaxis reaction will be found to be fully as specific as any other immunity reaction.

4. INFLUENCE OF DIET UPON ANAPHYLAXIS REACTIONS.

One factor which must come into consideration in studies of the biological reactions of the vegetable proteins is the influence of diet—a factor of less concern when animal proteins are injected into guinea-pigs or other graminivorous animals. It has been shown by several investigators that proteins fed to experimental animals are absorbed in greater or less amounts so little changed that they can be detected in the blood by the precipitin test, and are capable of acting as antigens, since animals so fed with unusual proteins develop precipitins for these proteins. Rosenau and Anderson also found that guinea-pigs fed upon horse serum and horse meat became sensitized to these proteins.

We have found that feeding of cow's milk, dried ox blood, and dried egg white daily for 3 to 8 weeks to guinea-pigs renders them sensitive to the corresponding proteins. This sensitization is specific, since animals fed upon ox blood and reacting to bovine serum did not react to dog serum, horse serum, human serum, or egg white, showing that during or before absorption the food protein that acted as anaphylactogen did not lose its specificity. We found that after two month's feeding the animals reacted less strongly than after one months' feeding, but we have not yet been able to carry on the feeding long enough to learn if complete immunity to an animal protein can be obtained in this way.

One set of experiments with zein, a protein obtained from Indian corn (*Zea mays*), gave results which indicate that there may

exist such an immune or refractory condition to a vegetable protein which is taken daily in the ordinary food of the guinea-pigs. It was noticed in a series of experiments performed one autumn with zein upon a certain lot of guinea-pigs that only slight or negative reactions could be obtained. During the previous winter and spring strong reactions had been obtained with the same preparation of zein with the guinea-pigs from the same breeder, and investigation showed that during the summer and autumn the breeder had been feeding his stock largely upon green corn, ears and all. This suggested that the daily ingestion of corn proteins had made the animals immune to zein, and experiments were performed to determine this. Young guinea-pigs, of approximately equal ages, just weaned, were obtained from another breeder who had never fed any form of corn product to his stock. To part of them moistened corn meal was given daily for six weeks as the chief food, while the other lot was fed upon oats, carrots, and hay. The animals then received each a sensitizing dose of zein, and three weeks later a second dose of the same protein; the animals which had been fed on corn meal showed no reaction whatever, while those that had not been thus fed all reacted to the zein. (These experiments are given in detail in Table 14, experiments 1–19.)

Experiments with oat proteins were also undertaken, because all our animals had been fed on oats since birth. Ground oats were extracted with 1 per cent KOH, and the filtrate neutralized with acetic acid, which threw down a considerable precipitate of proteins. These proteins, after being washed and dried, were dissolved in 0.1 per cent NaOH when used for the experiments. When injected into normal oat-fed guinea-pigs in 0.1 gm. doses this protein seemed to be somewhat toxic, for the animals after one hour or longer seemed slightly ill, inclined to lie down, and the coat became rough. The degree of intoxication varied much in different animals, some showing practically no symptoms. The effects were not very similar to the typical anaphylactic intoxication, in respect to the long latent period after injection, and the absence of cutaneous irritation and respiratory difficulty. Several guinea-pigs were then given doses of this oat protein varying from 0.001 to 0.1 gm. and after 15 days a second dose of 0.1

gm. was given. None showed a typical anaphylaxis reaction, although all became more or less ill after one hour or more as did the normal animals on receiving the first injection.

From these experiments it would seem that guinea-pigs may become sensitized to foreign animal proteins upon which they are fed, so that they react to the same protein. When raised upon corn and oats, however, they do not seem to become sensitized to the proteins derived from these grains, but rather to be immune, so that no satisfactory anaphylactic reaction can be obtained by two properly spaced injections. Whether this difference between the vegetable and the animal proteins depends upon the character of the proteins themselves, or upon the fact that the vegetable proteins have been taken in the food more abundantly and for a longer time than the animal proteins, thus favoring the establishment of a condition of immunity, is a matter that requires more investigation. From the results so far obtained, however, it seems probable that animals which have been rendered sensitive to a certain animal protein by feeding with this protein for a few days or weeks may become refractory to it when fed for a longer period. This suggests that it is the protracted and abundant feeding of vegetable proteins which accounts for the refractory condition observed in normal guinea-pigs when the anaphylaxis reaction is tried with vegetable proteins which are constituents of their customary food.

5. DESCRIPTION OF PREPARATIONS USED IN PRESENT WORK.

In the following pages we give some results of our investigations of the anaphylactic reaction obtained with preparations of several of the more extensively studied vegetable proteins. Since there is strong evidence that the anaphylactic reaction is in fact caused by proteins and not by some associated substance it is of fundamental importance to begin its study with the most carefully purified and chemically best defined preparations of the proteins that can be obtained.

Nearly all the published data respecting the anaphylaxis reaction relate to experiments with naturally occurring fluids which contain several protein substances, as well as many other substances

of known and unknown character. The only experiments as yet described with so-called pure preparations of proteins have been made with crystallized ovalbumin, ovomucoid,[60] crystallized edestin from the hemp seed, zein from maize, gliadin from wheat, and serum proteins separated from one another by fractional salting-out processes. Since these experiments plainly indicated the importance of greatly extending their range we have undertaken the work described in the following pages.

In regard to the chemical individuality of the preparations used in our experiments we have no conclusive evidence, for with the means at present available no preparations of proteins can be obtained which in this respect satisfy the requirements of the organic chemist. The preparations, however, represent those products which for the present we have to treat as chemical individuals.

That each of these preparations was completely separated from *all* the other proteins of these seeds is not certain. It is probable that those from the leguminous seeds (*Pisum*, *Vicia*, *Soja*) contained small quantities of some of the other proteins of the seed from which they originated, for the separation of these proteins presents peculiar difficulties which makes the production of absolutely pure preparations practically impossible. That these separations in the case of the preparations from the leguminous seeds were in fact imperfect is indicated by the results of our present experiments, as is later set forth in our discussion of the specificity of the reactions.

Most of the preparations of the proteins from the oil-seeds (*Ricinus*, *Cannabis*, *Cucurbita*, *Linum*, *Cocos*, *Bertholletia*) probably represent the best defined and, from a chemical standpoint, the purest protein preparations used in this work. Most of these were crystalline, and were obtained under conditions which our experience has shown readily yield products of a relatively high degree of purity.

The proteins from the cereals (wheat, rye, barley, maize) which were used for our anaphylaxis experiments were all obtained from solutions containing 75 per cent or more of ethyl alcohol. The method employed was such as to make it improbable that these

preparations contained more than minute traces, if any, of the other known protein constituents of the seed, and we have every reason to believe that these are among the best characterized of any proteins at present available for such studies.

The preparations from these three groups of seeds used in our present work represent not only biologically but also chemically different types of proteins. Those from the oil-seeds are globulins which dissolve readily in solutions of neutral salts and are in most cases precipitated by dialysis from such solutions in well-formed microscopic crystals. They are known to be characterized by a high content of basic nitrogen. With the exception of the globulins from the flax seed and cocoanut, which have not yet been examined in this respect, this basic nitrogen has been found to belong mostly to arginine. The proportions of the other amino-acids yielded by hydrolysis present no striking features. Differences have been recognized which leave no doubt that each of these proteins is chemically distinct, a conclusion now supported by the anaphylaxis reaction.

The proteins from the leguminous seeds which we used are likewise globulins but have never been obtained in crystals. They resemble one another in their solubility relations which are distinctly different from those of the proteins from the oil-seeds. These differences are difficult to describe but are readily appreciated by those who work with them. While the oil-seeds appear to contain but a single globulin, most of the leguminous seeds contain two or more. It is therefore difficult to make preparations from these seeds which offer the same assurance of purity as those from the oil-seeds. The proportion of the several amino-acids obtained by hydrolyzing these leguminous proteins is much the same for each, and very similar to that yielded by most animal proteins. Nevertheless, sufficient differences have been found between them to justify the assumption that each is a chemically distinct protein.

The cereal proteins used have been limited to those soluble in alcohol of 75 per cent or stronger. These are thus not only sharply differentiated from the other proteins used, but from all other known proteins of either animal or vegetable origin. They are

also characterized by yielding no lysine or glycocoll, very little arginine and histidine, and from 26 per cent (zein) to 42–43 per cent (hordein and gliadin) of glutaminic acid.

It is thus evident that the different proteins used in our experiments cover a wide range in properties, constitution, origin, and method of preparation and the results obtained with them should afford a sufficiently extensive review of the field under examination to serve as a substantial basis for further work.

In making most of these preparations clean seeds, apparently free from those of other species, were used, but we are not certain that a very small number of foreign seeds were not, in fact, present in the samples ground. We are also not certain that very slight contaminations with proteins from other seeds may not have occurred through imperfect washing of some of the bulky apparatus used in their preparation or from the dust of other preparations being blown about the laboratory in which large quantities of proteins of all sorts had been made and kept for several years.

Only the preparation of edestin, used in the experiments here described, came fully up to the requirements demanded by these considerations.

As will be seen from the following detailed description of the several preparations used, sufficient care was taken in making most of them to insure products of relatively high purity. We considered them to be suitable for a preliminary study of the anaphylaxis reaction, and this belief is supported by the results obtained with them, although in obtaining final data, especially in regard to the specificity of the reaction of the individual proteins and the relative anaphylactic power of each, preparations made with special precautions must be used. Those unfamiliar with the properties and character of these different vegetable proteins will find an extensive review of what is at present known of them in a paper recently published by one of us (O.) in *Ergebnisse der Physiol.*, 1910, 10, pp. 47–215. A detailed account of the methods used in preparing the different vegetable proteins has been given by the same author in Abderhalden's *Handbuch der biochemischen Arbeitsmethoden*, Berlin, 1909.

Some of the results which we have thus far obtained indicate

points to which special attention must be directed. The data here recorded must, therefore, be regarded as of a preliminary character from which only a general survey of the field has been obtained. It is our present intention to greatly extend the scope of this work and to confirm our results by many properly controlled experiments so that definite conclusions finally may be obtained.

Legumin, pea.—A large quantity of a preparation of legumin, which had already been freed from nearly all of the vicilin with which it was originally associated, was dissolved in 1 liter of water containing 76 gms. of $(NH_4)_2SO_4$. An equal volume of a saturated solution of this salt was then added gradually and then enough more crystals of $(NH_4)_2SO_4$ to make the solution $\frac{6}{10}$ saturated. The large precipitate which separated was filtered out and dissolved in 2 liters of water, the adherent sulphate being sufficient to bring nearly all of the protein into solution. An equal volume of a saturated solution of $(NH_4)_2SO_4$ was next added and then 250 gms. of crystals of this salt, thereby making the solution approximately $\frac{6}{10}$ saturated. The precipitate thus obtained was filtered out, freed from most of the adhering solution by pressing between filter papers, and redissolved by suspending in 4 liters of water and adding crystals of $(NH_4)_2SO_4$. Enough of this salt was then added to make the solution $\frac{7}{10}$ saturated. The legumin which was so precipitated was collected on a filter and when freed as far as possible from the solution it was dissolved in 10 per cent NaCl solution and filtered through a pulp filter. The solution was then dialyzed in running water for 4 days. The dialysis precipitate was washed first with water, then with dilute alcohol, digested with absolute alcohol, and dried over sulphuric acid. When this preparation was dissolved in NaCl solution it remained perfectly clear on boiling, thus indicating absence of vicilin. It also gave no Molisch reaction, from which we conclude that it contained no nucleic acid compounds or carbohydrates. This preparation can be considered to be as free from vicilin or other proteins of the pea as it is possible to make one with any reasonable amount of care.

Legumin, vetch.—The finely ground seeds were treated with three times their weight of 5 per cent NaCl solution, to which was previously added a quantity of cold saturated baryta solution just sufficient to neutralize to litmus the acid reaction of the meal. The extract was filtered perfectly clear and then dialyzed for 4 days until free from chlorides. The legumin which had separated on dialysis was then filtered out, redissolved in 5 per cent NaCl solution, and the solution which resulted filtered through paper pulp and again dialyzed for 4 days. The precipitate produced by dialysis was washed carefully by suspending in distilled water, all lumps being broken up by passing the suspension through fine bolting cloth. After sucking the legumin as dry as possible it was dehydrated by treating with absolute alcohol, digested with dry ether, and dried over sulphuric acid. Since legumin is the only protein that is precipitated by dialysis from extracts of the vetch the above process is sufficient to separate it from all but minute traces of legumelin and proteose which are also present in extracts of this seed.

Vicilin.—Finely ground pea meal was extracted with 10 per cent NaCl solution and the extract was filtered perfectly clear and dialyzed in running water until free from NaCl. The precipitate produced by dialysis was dissolved in $\frac{1}{10}$ saturated $(NH_4)_2SO_4$ and enough solid $(NH_4)_2SO_4$ added to the solution to make it $\frac{6.5}{10}$ saturated.

The precipitate was filtered out and the filtrate was saturated with $(NH_4)_2SO_4$. The precipitate which resulted was freed, as far as possible, from the solution, dissolved in 10 per cent NaCl solution, and, after filtering clear, the solution was dialyzed till nearly free from NaCl. The precipitate, which should contain any legumin still present, was filtered out and the solution treated with a current of CO_2. The precipitate thereby produced was dissolved in dilute NaCl solution and dialyzed until the globulin was precipitated. This was then washed with water and with alcohol and dried over H_2SO_4. The preparation thus obtained had the ultimate composition of vicilin but whether or not it was entirely free from any legumin is not certain. It was considered to be pure enough for preliminary experiments.

Vignin.—This preparation was made by extracting ground cow-peas with 5 per cent NaCl solution, filtering the extract perfectly clear and dialyzing for three days. The precipitate produced by dialysis was filtered out, suspended in a measured quantity of water, and dissolved by adding a weighed amount of NaCl. The solution was then filtered perfectly clear and the vignin precipitated by diluting with water until the solution contained 1 per cent of sodium chloride. The precipitate which resulted was allowed to settle, the supernatant solution was drawn off, and the precipitate sucked as dry as possible with a pump. After washing with alcohol and ether the preparation formed a pure white dusty powder. By this method of preparation the vignin was separated from the more soluble globulins, albumins, and proteose and from all of the other water-soluble constituents of the seed.

Glycinin.—The soy-beans were coarsely ground in the laboratory, freed from the greater part of the outer seed coats by a current of air, and the meal then extracted with petroleum benzine until most of the oil was removed. After freeing from adhering benzine by exposure to the air the coarse meal was ground fine and extracted with about three parts of 10 per cent NaCl solution. Owing to the very viscid character of the extract it was necessary to mix with the meal and solvent enough dry finely divided filter paper to make it possible to squeeze out the extract in a powerful screw press. The extract thus obtained was filtered perfectly clear and dialyzed for 4 days in running water. The globulin that separated was filtered out, dissolved again in NaCl solution, and again precipitated by dialysis. After repeating this process a third time the precipitated glycinin was washed thoroughly with water, absolute alcohol, and ether and then dried over H_2SO_4. By this repeated precipitation the glycinin was separated from the more soluble proteins of the seed and had the properties and ultimate composition of preparations formerly obtained by extensive fractionation from NaCl solution.

Castor-bean globulin.—Two preparations were used for our anaphylaxis experiments. That used for experiments 1–5 was obtained by removing the shells from carefully selected castor beans and extracting the nearly oil-free meal with 10 per cent NaCl solution. The extract was filtered perfectly clear and dialyzed for 60 hours in running water. The precipitate was dissolved in 10 per cent NaCl solution and the solution saturated with NaCl. The precipitate produced was filtered out; dissolved in a dilute solution of this salt, and the solution saturated with NaCl. The second precipitate so obtained was filtered out and again subjected to the same treatment. The third precipitate produced by saturating with NaCl was dissolved in dilute NaCl solution and the clear solution dialyzed. The globulin which separated was washed with water and alcohol, dehydrated with absolute alcohol, and dried over H_2SO_4.

The preparation used for experiments 6–39 was extracted from the oil-free meal of carefully selected castor beans from which the oil had been mostly extracted with

ether but from which the shells had not been removed. This meal was extracted with five times its weight of 10 per cent NaCl solution, the extract filtered perfectly clear and dialyzed for 4 days in running water. The precipitate which separated was washed with water, digested with absolute alcohol, and dried over H_2SO_4. Although this preparation was not further purified by reprecipitating, the results obtained with it agreed with those yielded by the more carefully purified preparation used for the first five experiments. The single precipitation by dialysis appears to have been sufficient to separate this globulin from the albumin (ricin) a small fraction of a milligram of which promptly kills a guinea-pig when subcutaneously injected.* From this it is probable that this crude preparation of the globulin was nearly if not wholly freed by a single precipitation by dialysis from the water-soluble proteins of the seed.

Edestin.—The hemp seeds used for making this preparation of edestin were carefully selected one by one, taking every precaution to avoid the presence of any other seeds. The seeds were then crushed under kerosene and freed from most of the oil by pressing with a hydraulic press and from the adherent kerosene by exposing to the air. The coarse meal was then ground and sifted in such a way, on sieves of suitable mesh, as to remove most of the outer coat of the seed. The meal was then extracted with a 10 per cent solution of NaCl. The extract was filtered perfectly clear and dialyzed for 4 days in running water. The crystalline precipitate thus obtained was washed with 50 per cent alcohol until the washings were free from chlorin, dehydrated by digesting with absolute alcohol, and then extracted with ether. The preparation was then dried in the air. In making this preparation apparatus which had never been used in the preparation of any vegetable proteins was used throughout, and the preparations may be regarded as entirely free from any substance others than those which it may have acquired from the hemp seeds themselves or from the water and reagents used in its preparation.

Squash-seed globulin.—Carefully selected fresh seeds of the squash were coarsely ground without removing the outer seed coats, and then extracted with benzine until most of the oil had been removed. By sifting the coarse meal on sieves of suitable mesh the greater part of the outer coats of the seeds was removed. The fine meal was then extracted with 10 per cent NaCl solution, the extract filtered perfectly clear and precipitated by dialysis. The crystalline precipitate was redissolved in 10 per cent NaCl solution, the solution filtered clear and reprecipitated by dialysis. The precipitate was washed with water, dehydrated with absolute alcohol, and dried over H_2SO_4.

Squash-seed globulan.—This preparation was obtained in a similar way to that employed in making the preparation of the squash-seed globulin and represents the part of the first dialysis precipitate which failed to dissolve in NaCl solution. It is, therefore, that part of the globulin of the squash seed which had been converted into the insoluble condition during the first dialysis.

Flax-seed globulin.—This was extracted from finely ground flax seed by a 10 per cent solution of $BaCl_2$. The extract was filtered perfectly clear and dialyzed in running water until the globulin was nearly all precipitated in octahedral crystals. The dialysis precipitate was washed with water, redissolved in NaCl solution and reprecipitated by dialysis. The crystalline separation was washed with water and alcohol and dried over H_2SO_4.

Cocoanut proteins.—The preparations of these proteins were made in the Sheffield Laboratory of Physiological Chemistry of Yale University and kindly presented to

* Osborne, Mendel, and Harris, *Am. Jour. of Physiol.*, 1905, 14, p. 278.

us by Professor Lafayette B. Mendel. The details of the method by which they were prepared are unknown by us.

Excelsin.—The oil-free meal of the Brazil nut was extracted with 3 per cent $(NH_4)_2SO_4$ solution heated to 50° and the perfectly clear extract dialyzed until the greater part of the dissolved excelsin was deposited as crystals. These were then washed thoroughly with dilute NaCl solution and then with dilute alcohol which was gradually increased in strength up to absolute alcohol. The preparation was then dried over H_2SO_4. The product thus obtained consisted wholly of hexagonal crystals and under the microscope appeared to be entirely free from any admixture with other substances.

Gliadin, wheat.—The preparation of gliadin used in these experiments was made from a high grade of wheat flour from which the gluten was obtained by washing with water. The gluten was coarsely divided by passing through a meat chopper and extracted with 70 per cent alcohol until practically all of the protein that was soluble therein had been removed. The extract was filtered perfectly clear and concentrated at a low temperature, care being taken to add from time to time a quantity of strong alcohol sufficient to prevent the gliadin separating from the solution. The concentrated extract was then precipitated by pouring into several volumes of water containing a little NaCl. The precipitate was then redissolved in 70 per cent alcohol and precipitated by pouring into a large volume of 95 per cent alcohol. The gliadin was then dehydrated with absolute alcohol, and digested twice with a large quantity of ether. After drying over H_2SO_4 it was ground to a fine white powder. During the preparation of this gliadin care was taken to avoid exposing its solutions to a temperature above 60° or 70°. This preparation was made with new apparatus and with special precaution to avoid its contamination with any other substances than those present in the seeds from which the flour was obtained or the reagents used in making it.

Gliadin, rye.—The rye flour used for this preparation was ground in the laboratory from a quantity of selected seeds which were presumably free from those of other species. Whether or not the seeds of wheat were entirely absent cannot be definitely stated because, owing to the similar appearance of these two grains, the exclusion of such a contamination cannot be assured without making a careful examination of each seed before grinding, which was not done in this case, for this preparation was not originally intended for anaphylaxis experiments. There is little doubt, however, that the seeds ground contained very few, if any, grains of wheat. The flour, from which most of the bran had been removed, was extracted with cold 70 per cent alcohol and the extract filtered perfectly clear and concentrated under diminished pressure from a water-bath at 70°, care being taken during the concentration to prevent separation of the gliadin by adding absolute alcohol as soon as the solution began to foam. The concentrated solution was cooled and then poured into a large volume of ice water, to which a very little NaCl was added in order to cause the gliadin to separate in a flocculent condition. The precipitate which settled after standing for some hours formed a coherent deposit from which the solution was poured off nearly completely. This deposit was then dissolved in a little warm alcohol and the cold concentrated solution again poured into much water as before. The gliadin was then dissolved in alcohol and poured into much absolute alcohol which gave a milky colloidal solution which immediately yielded a voluminous flocculent precipitate on adding a few drops of saturated NaCl solution. This precipitate soon settled to a coherent mass from which the alcohol was nearly completely poured off. This mass was then repeatedly digested with portions of ab-

solute alcohol until it could be rubbed down to a fine powder. This was then washed with ether and dried over H_2SO_4.

Hordein.—A high grade of very white barley flour was used in making this preparation. This was extracted with warm 70 per cent alcohol, the extract filtered perfectly clear and concentrated to a small volume under low pressure on a water bath. On cooling, nearly all of the hordein separated as a coherent mass which was washed with water, dissolved in warm 70 per cent alcohol, and its clear solution poured into several volumes of ice water. The hordein was then again dissolved in a small quantity of 70 per cent alcohol and precipitated by pouring into much absolute alcohol. After digesting the hordein with absolute alcohol and ether it was dried over H_2SO_4 and obtained as a snow-white powder.

Zein.—This preparation was made from finely ground yellow maize meal by extracting with warm 92 per cent alcohol. The extract was filtered perfectly clear, concentrated under diminished pressure, and precipitated by pouring into a large volume of cold water containing a very little NaCl. The precipitate was then dissolved in warm 92 per cent alcohol and again precipitated as before by pouring into water. After redissolving in a small quantity of warm 92 per cent alcohol the clear solution was poured into a large volume of absolute alcohol and the precipitate digested with absolute alcohol and ether and then dried over H_2SO_4.

6. METHOD OF CONDUCTING EXPERIMENTS AND DESCRIBING RESULTS.

The experiments were performed with healthy young guinea-pigs, nearly all from one stock which was known to give strong anaphylaxis reactions. (There are some strains of guinea-pigs, as reported by Vasconcellos[53], and as we also have observed, which will not give satisfactory reactions.) In most instances the weight was about 300 gms. at the time of the first injection, some being a little smaller but none as heavy as 500 gms. The animals were fed, unless otherwise specified, exclusively upon oats, carrots, and hay. The vegetable proteins were generally dissolved in 0.2 per cent NaOH and the solution diluted with an equal volume of water before injection, making a 0.1 per cent NaOH solution, which ordinarily caused no symptoms of itself, although occasionally certain protein solutions caused an immediate transient irritation, the animals jumping about madly for a moment, but quieting down in a minute or two so that the anaphylaxis reaction was not obscured. All injections were made into the peritoneal cavity, with a blunt needle to avoid perforation of the viscera. Because of the tendency of vegetable proteins to cause agglutination of erythrocytes, and because our proteins were in alkaline solution, no intravascular injections were made.

To avoid contamination of the injected proteins with foreign proteins special precautions were adopted, necessary because of the extremely small dose of protein which may sensitize an animal. Only all-glass syringes were used, and after each set of injections they were thoroughly washed and then left standing over night in a cleaning fluid consisting of concentrated sulphuric acid and potassium dichromate. All scale pans, mortars, and other glass utensils with which the proteins came in contact were cleaned in the same manner. The needles were washed first with water and then with alcohol, and were kept constantly in absolute alcohol.

In recording the effects produced after the usual incubation period by the second or intoxicating dose, certain terms are used with the following constant significance.

1. "Doubtful" symptoms. The animal scratches itself a few times and perhaps seems a little uneasy or a trifle ill; the temperature remains normal or falls not more than 1° to 1.5° C. Such results may be occasionally observed after injection of foreign proteins into non-sensitized animals, and are not regarded as of any significance.

2. "Slight" symptoms. The animal does not become seriously ill, but scratches itself vigorously, is either very restless or very somnolent or both alternately, hair roughened, eyes usually lachrymate and partly closed. The temperature usually falls 1° to 1.5° C. Such a reaction is believed to indicate usually, if not always, a real sensitization and a true anaphylactic intoxication, but it may possibly sometimes, but rarely, occur from foreign proteins without sensitization.

3. "Moderate" symptoms. More seriously ill than under 2. Usually the hair becomes very rough, with respiratory distress and marked lachrymation, fall of temperature of 1.5°–3.5° C.; frequently the animal lies down and sometimes there is violent coughing.

4. "Severe" symptoms. Same as under 3 but so much more severe as to threaten to cause death, but followed by complete recovery in a few hours. In these animals the temperature usually falls 3° to 4°, or even more.

The results of our experiments are given in the following tables:

7. TABLES OF RESULTS OBTAINED.

TABLE 1.

CASTOR-BEAN GLOBULIN (*Ricinus communis*).

Sensitizing Dose	Days' Interval	Second Injection	Symptoms	Subsequent Injections and Remarks
Castor-bean Globulin		Castor-bean Globulin		
1. 0.0 (Control)...	..	0.1	none	
2. 0.0 (Control)...	..	0.1	doubtful	
3. 0.0 (Control)...	..	0.1	none	
4. 0.002.....	18	0.1	died in 1 hr.	
5. 0.0002.....	18	0.1	" " 6 hrs.	
6. 0.002.....	21	0.1	moderate	
7. 0.0002.....	21	0.1	died in 1 hr.	
		Flaxseed Globulin		
8. 0.002.....	18	0.1	slight	died 24 hrs. later
9. 0.0002.....	18	0.1	"	36 " "
10. 0.0001.....	18	0.1	doubtful	48 hrs. later reacted slightly to castor-bean globulin
11. 0.00002.....	18	0.1	"	48 hrs. later reacted severely to castor-bean globulin.
12. 0.002.....	27	0.1	slight	4 days later reacted severely to castor-bean globulin
13. 0.001.....	27	0.1	none	4 days later reacted fatally to castor-bean globulin
14. 0.0002.....	27	0.1	died in 1 hr.	
15. 0.002.....	27	0.1	slight	4 days later reacted severely to castor-bean globulin
16. 0.001.....	27	0.1	"	4 days later reacted fatally to castor-bean globulin
17. 0.0002.....	27	0.1	died in 35 min.	
		Squash-seed Globulin		
18. 0.002.....	21	0.1	doubtful	3 days later reacted moderately to castor-bean globulin
19. 0.001.....	21	0.1	"	3 days later reacted fatally to castor-bean globulin
20. 0.0002.....	21	0.1	"	3 days later reacted moderately to castor-bean globulin
21. 0.0001.....	21	0.1	"	3 days later reacted fatally to castor-bean globulin
		Edestin		
22. 0.007.....	17	0.1	none	
23. 0.002.....	17	0.1	doubtful	48 hrs. later reacted severely to castor-bean globulin
24. 0.001.....	17	0.1	none	
25. 0.0002.....	17	0.1	"	
		Excelsin		
26. 0.01.....	17	0.1	none	
27. 0.001.....	17	0.1	"	
28. 0.0002.....	17	0.1	"	48 hrs. later reacted severely to castor-bean globulin
		Castor-bean Globulin		
Zein				
29. 0.001.....	20	0.1	none	
Squash-seed Globulin				
30. 0.01.....	22	0.1	"	
31. 0.002.....	22	0.1	"	24 hrs. later reacted fatally to squash-seed globulin
32. 0.0002.....	22	0.1	"	24 hrs. later reacted fatally to squash-seed globulin
Edestin				
33. 0.01.....	22	0.1	"	
34. 0.002.....	22	0.1	"	24 hrs. later reacted fatally to edestin
35. 0.001.....	22	0.1	"	
36. 0.0002.....	22	0.1	severe	24 hrs. later reacted severely to edestin
Excelsin				
37. 0.002.....	21	0.1	none	
38. 0.001.....	21	0.1	"	
39. 0.0002.....	21	0.1	"	48 hrs. later reacted fatally to excelsin

TABLE 1.—*Continued.*

Sensitizing Dose	Days' Interval	Second Injection	Symptoms	Subsequent Injections and Remarks
Flaxseed Globulin		Castor-bean Globulin		
40. 0.01	20	0.1	slight	48 hrs. later reacted moderately to flax-seed globulin
41. 0.01	20	0.1	"	48 hrs. later reacted moderately to flax-seed globulin
42. 0.002	20	0.1	"	48 hrs. later reacted moderately to flax-seed globulin
43. 0.0002	20	0.1	"	48 hrs. later reacted moderately to flax-seed globulin

TABLE 2.

FLAX-SEED GLOBULIN (*Linum usitatissimum*).

Sensitizing Dose	Days' Interval	Second Injection	Symptoms	Subsequent Injections and Remarks
		Flax-seed Globulin		
1. 0.0 (Control)	..	0.1	none	Temperature fell 1.1° C.
2. 0.0 (Control)	..	0.1	"	" " 1.6° C.
Castor-bean Globulin				
3. 0.002	27	0.1	slight	4 days later reacted severely to castor-bean globulin
4. 0.001	27	0.1	none	4 days later reacted fatally to castor-bean globulin
5. 0.0002	27	0.1	died in 1 hr.	
6. 0.002	27	0.1	slight	4 days later reacted severely to castor-bean globulin
7. 0.001	27	0.1	doubtful	4 days later reacted fatally to castor-bean globulin
8. 0.002	18	0.1	slight	died 24 hrs. later
9. 0.0002	18	0.1	"	" 36 " "
10. 0.0001	18	0.1	doubtful	48 hrs. later reacted slightly to castor-bean globulin
11. 0.00002	18	0.1	"	48 hrs. later reacted severely to castor-bean globulin
12. 0.0002	27	0.1	died in 35 min.	
Squash-seed Globulin				
13. 0.008	20	0.1	none	3 days later reacted fatally to squash-seed globulin
14. 0.001	20	0.1	"	
15. 0.0001	20	0.1	"	3 days later reacted fatally to squash-seed globulin
Flax-seed Globulin		Squash-seed Globulin		
16. 0.002	20	0.1	none	24 hrs. reacted severely to flax-seed globulin
17. 0.001	20	0.1	slight	24 hrs. reacted severely to flax-seed globulin
18. 0.001	20	0.1	doubtful	24 hrs. reacted fatally to flax-seed globulin
19. 0.0002	20	0.1	none	24 hrs. reacted fatally to flax-seed globulin
		Castor-bean Globulin		
20. 0.01	20	0.1	slight	24 hrs. reacted moderately to flax-seed globulin
21. 0.01	20	0.1	"	24 hrs. reacted moderately to flax-seed globulin
22. 0.002	20	0.1	"	24 hrs. reacted moderately to flax-seed globulin
23. 0.0002	20	0.1	moderate	24 hrs. reacted moderately to flax-seed globulin

TABLE 3.

SQUASH-SEED GLOBULIN (*Cucurbita maxima*).

Sensitizing Dose	Days' Interval	Second Injection	Symptoms	Subsequent Injections and Remarks
Squash-seed Globulin		Squash-seed Globulin		
1. 0.0 (Control) ...	..	0.1	doubtful	
2. 0.0 (Control) ...	..	0.1	"	
3. 0.0 (Control) ...	..	0.1	"	
4. 0.01	23	0.1	died in 75 min.	
5. 0.008	23	0.1	" " 45 "	
6. 0.001	23	0.1	slight	
7. 0.001	23	0.1	died in 50 min.	
8. 0.0002	22	0.05	" " 45 "	
		Excelsin		
9. 0.01	20	0.1	moderate	3 days later reacted fatally to squash-seed globulin
10. 0.002	20	0.1	died in 2 hrs.	
11. 0.001	20	0.1	moderate	3 days later reacted slightly to squash-seed globulin
12. 0.01	21	0.1	"	24 hrs. later reacted slightly to squash-seed globulin
13. 0.002	21	0.1	"	
14. 0.001	21	0.1	"	
15. 0.0002	21	0.1	"	
16. 0.01	18	0.1	doubtful	48 hrs. later reacted moderately to squash-seed globulin
17. 0.002	18	0.1	"	48 hrs. later reacted fatally to squash-seed globulin
18. 0.001	18	0.1	doubtful	48 hrs. later reacted fatally to squash-seed globulin
19. 0.0002	18	0.1	"	48 hrs. later reacted fatally to squash-seed globulin
		Flax-seed Globulin		
20. 0.008	20	0.1	none	3 days later reacted fatally to squash-seed globulin
21. 0.001	20	0.1	"	
22. 0.0001	20	0.1	"	3 days later reacted fatally to squash-seed globulin
		Edestin		
23. 0.01	21	0.1	slight	
24. 0.002	21	0.1	"	
25. 0.001	21	0.1	"	
26. 0.0002	21	0.1	"	24 hrs. later reacted fatally to squash-seed globulin
27. 0.01	18	0.1	none	48 hrs. later reacted fatally to squash-seed globulin
28. 0.002	18	0.1	"	48 hrs. later reacted fatally to squash-seed globulin
29. 0.001	18	0.1	"	48 hrs. later reacted fatally to squash-seed globulin
30. 0.0002	18	0.1	"	48 hrs. later reacted fatally to squash-seed globulin
		Castor-bean Globulin		
31. 0.01	22	0.1	none	
32. 0.002	22	0.1	"	24 hrs. later reacted fatally to squash-seed globulin
33. 0.0002	22	0.1	"	24 hrs. later reacted fatally to squash-seed globulin
Excelsin		Squash-seed Globulin		
34. 0.01	21	0.1	none	6 days later reacted fatally to excelsin
35. 0.001	21	0.1	doubtful	6 days later reacted fatally to excelsin
36. 0.0002	21	0.1	none	6 days later reacted severely to excelsin

TABLE 3.—*Continued.*

Sensitizing Dose	Days' Interval	Second Injection	Symptoms	Subsequent Injections and Remarks
		Squash-seed Globulin		
Castor-bean Globulin				
37. 0.002.....	21	0.1	doubtful	3 days later reacted moderately to castor-bean globulin
38. 0.001.....	21	0.1	"	3 days later reacted fatally to castor-bean globulin
39. 0.0002.....	21	0.1	"	3 days later reacted moderately to castor-bean globulin
40. 0.0001.....	21	0.1	"	3 days later reacted fatally to castor-bean globulin
Edestin				
41. 0.01.....	21	0.1	doubtful	24 hrs. later reacted slightly to edestin
42. 0.002.....	21	0.1	none	24 hrs. later reacted moderately to edestin
43. 0.001.....	21	0.1	"	24 hrs. later reacted doubtfully to edestin
44. 0.0002.....	21	0.1	"	24 hrs. later reacted moderately to edestin
Flax-seed Globulin				
45. 0.002.....	20	0.1	none	24 hrs. reacted severely to flax-seed globulin
46. 0.001.....	20	0.1	slight	24 hrs. reacted severely to flax-seed globulin
47. 0.001.....	20	0.1	doubtful	24 hrs. reacted fatally to flax-seed globulin
48. 0.0002.....	20	0.1	none	24 hrs. reacted fatally to flax-seed globulin
Squash-seed Globulan				The following six experiments with globulan
49. 0.02.....	15	0.125	slight	
50. 0.002.....	15	0.125	severe	
51. 0.0002.....	15	0.125	died in 90 min.	
52. 0.1.....	100	0.125	slight	
53. young of 52, 6 weeks old.....		0.1	"	Inheritance test
54. Young of 52, 6 weeks old.....		0.1	slight	Inheritance test
		Squash-seed Globulin		
Squash-seed Globulin				
55. 0.1.....	17	0.1	severe	Died after 6 hours
56. 0.2.....	17	0.1	"	" " " "
57. 0.3.....	17	0.1	"	Recovered
58. 0.00005.....	18	0.1	died in 30 min.	
59. 0.000001.....	18	0.1	" " " "	
60. 0.0000005.....	18	0.1	moderate	Temp. fell 3.3°
61. 0.0000005.....	18	0.1	died in 60 min.	
62. 0.0000001.....	18	0.1	none	No fall in temp.
63. 0.0000001.....	18	0.1	doubtful	" " " "
64. 0.00000001.....	18	0.1	none	
65. 0.00000001.....	18	0.1	none	
66. 0.0005.....	21	0.020	moderate	Died during night
67. 0.0005.....	21	0.010	severe	Died after 6 hours
68. 0.0005.....	21	0.005	moderate	Temp fell 2.7°
69. 0.0005.....	21	0.005	"	" " 2.7°
70. 0.0005.....	21	0.004	"	" " 3.3°
71. 0.0005.....	21	0.003	slight	" " 1.6°
72. 0.0005.....	21	0.001	doubtful	" " 1.6°
73. 0.0005.....	21	0.001	slight	" " 2.7°
74. 0.0005.....	21	0.0005	none	No fall in temp.
75. 0.0005.....	21	0.0001	"	" " " "
76. 0.0005.....	6	0.1	"	" " " "
77. 0.0005.....	7	0.1	"	" " " "
78. 0.0005.....	8	0.1	moderate	Temp. fell 3.3°
79. 0.0005.....	9	0.1	died in 60 min.	

TABLE 4.

EDESTIN (*Cannabis sativa*).

Sensitizing Dose	Days' Interval	Second Injection	Symptoms	Subsequent Injections and Remarks
Edestin		Edestin		
1. 0.0 (Control) ...	..	0.1	none	No fall in temperature
2. 0.1.....	18	0.25	doubtful	Animal used before with animal proteins
3. 0.02.....	18	0.15	moderate	Animal used before with animal proteins
4. 0.002.....	18	0.15	"	Animal used before with animal proteins
5. 0.002.....	18	0.25	severe	Animal used before with animal proteins
6. 0.0002.....	18	0.15	slight	Animal used before with animal proteins
7. 0.002.....	21	0.1	moderate	Animal used before with animal proteins
8. 0.01.....	19	0.1	"	24 hrs. later reacted negatively to edestin
9. 0.00005.....	19	0.1	slight	
10. 0.00001.....	19	0.1	moderate	
11. 0.000005.....	19	0.1	"	
12. 0.000001.....	19	0.1	slight	
13. 0.0000005.....	20	0.1	moderate	
14. 0.0000001.....	20	0.1	"	
15. 0.0005.....	19	0.1	died in 2 hrs.	
16. 0.0005.....	19	0.04	severe	
17. 0.0005.....	18	0.02....	"	Temp. fell 5°. 48 hrs. later no reaction to edestin
18. 0.0005.....	17	0.01	moderate	Temp. fell 2.5°
19. 0.0005.....	17	0.005	slight	Temp. fell 2.6°. 48 hrs. later moderate reaction to edestin
20. 0.0005.....	18	0.002	"	Temp. fell 1.6°. 48 hrs. later no reaction to edestin
21. 0.0005.....	19	0.001	doubtful	48 hrs. later no reaction to edestin
		Castor-bean Globulin		
22. 0.01.....	22	0.1	none	
23. 0.002.....	22	0.1	"	24 hrs. later reacted fatally to edestin
24. 0.001.....	22	0.1	"	
25. 0.0002.....	22	0.1	severe	24 hrs. later reacted severely to edestin
		Excelsin		
26. 0.01.....	21	0.1	slight	24 hrs. later reacted negatively to edestin
27. 0.002.....	21	0.1	moderate	24 hrs. later reacted moderately to edestin
28. 0.001.....	21	0.1	"	24 hrs. later reacted fatally to edestin
29. 0.0002.....	21	0.1	"	24 hrs. later reacted moderately to edestin
30. 0.01.....	18	0.1	none	48 hrs. later reacted doubtfully to edestin
31. 0.002.....	18	0.1	"	48 hrs. later reacted doubtfully to edestin
32. 0.001.....	18	0.1	"	48 hrs. later reacted doubtfully to edestin
33. 0.0002.....	18	0.1	"	48 hrs. later reacted doubtfully to edestin
		Squash-seed Globulin		
34. 0.01.....	21	0.1	doubtful	24 hrs. later reacted slightly to edestin
35. 0.002.....	21	0.1	none	24 hrs. later reacted moderately to edestin
36. 0.001.....	21	0.1	"	24 hrs. reacted doubtfully to edestin
37. 0.0002.....	21	0.1	"	24 hrs. later reacted moderately to edestin

TABLE 4.—*Continued.*

Sensitizing Dose	Days' Interval	Second Injection	Symptoms	Subsequent Injections and Remarks
Castor-bean Globulin		Edestin		
38. 0.007	17	0.1	none	
39. 0.002	17	0.1	doubtful	48 hrs. later reacted severely to castor-bean globulin
40. 0.001	17	0.1	none	
41. 0.0002	17	0.1	"	
Excelsin				
42. 0.01	21	0.1	doubtful	
43. 0.002	21	0.1	"	48 hrs. later reacted severely to excelsin
44. 0.001	21	0.1	"	
45. 0.0002	21	0.1	"	
Squash-seed Globulin				
46. 0.01	21	0.1	slight	
47. 0.002	21	0.1	"	
48. 0.001	21	0.1	"	
49. 0.0002	21	0.1	"	24 hrs. later reacted fatally to squash-seed globulin
50. 0.01	18	0.1	none	48 hrs. later reacted fatally to squash-seed globulin
51. 0.002	18	0.1	"	48 hrs. later reacted fatally to squash-seed globulin
52. 0.001	18	0.1	"	48 hrs. later reacted fatally to squash-seed globulin
53. 0.0002	18	0.1	"	48 hrs. later reacted fatally to squash-seed globulin

TABLE 5.

EXCELSIN (*Bertholletia excelsa*).

Sensitizing Dose	Days' Interval	Second Injection	Symptoms	Subsequent Injections and Remarks
Excelsin		Excelsin		
1. 0.0 (Control)	..	0.1	slight	Temperature fell 1.6°
2. 0.0 (Control)	..	0.1	doubtful	No fall in temperature
3. 0.0 (Control)	..	0.1	none	" " " "
4. 0.0 (Control)	..	0.1	"	Temperature fell 1°
5. 0.0 (Control)	..	0.1	"	" " " 1.5°
6. 0.02	17	0.125	moderate	Animal previously used with animal proteins
7. 0.01	17	0.125	died in 40 min.	Animal previously used with animal proteins
8. 0.002	17	0.125	" " 120 "	Animal previously used with animal proteins
9. 0.0002	17	0.125	" " 90 "	Animal previously used with animal proteins
10. 0.002	19	0.1	" " 40 "	
11. 0.001	19	0.1	" " 45 "	
12. 0.0002	19	0.1	" " 30 "	
		Vignin		
13. 0.1	17	0.12	none	24 hrs. later reacted severely to excelsin
14. 0.02	17	0.1	"	24 hrs. later reacted severely to excelsin
		Glycinin		
15. 0.002	16	0.1	doubtful	3 days later reacted fatally to excelsin
16. 0.001	16	0.1	none	3 days later reacted fatally to excelsin
17. 0.0002	16	0.1	"	3 days later reacted fatally to excelsin

TABLE 5.—*Continued.*

Sensitizing Dose	Days' Interval	Second Injection	Symptoms	Subsequent Injections and Remarks
Excelsin		Squash-seed Globulin		
18. 0.01	21	0.1	none	6 days later reacted fatally to excelsin
19. 0.001	21	0.1	doubtful	6 days later reacted fatally to excelsin
20. 0.0002	21	0.1	none	6 days later reacted severely to excelsin
		Edestin		
21. 0.01	21	0.1	doubtful	
22. 0.002	21	0.1	"	48 hrs. later reacted severely to excelsin
23. 0.001	21	0.1	"	
24. 0.0002	21	0.1	"	
		Castor-bean Globulin		
25. 0.002	21	0.1	none	
26. 0.001	21	0.1	"	
27. 0.0002	21	0.1	"	48 hrs. later reacted fatally to excelsin
Glycinin		Excelsin		
28. 0.01	22	0.1	"	48 hrs. later reacted severely to glycinin
29. 0.002	22	0.1	"	48 hrs. later reacted severely to glycinin
30. 0.001	16	0.1	doubtful	3 days later reacted severely to glycinin
31. 0.0002	16	0.1	none	3 days later reacted severely to glycinin
Squash-seed Globulin				
32. 0.01	20	0.1	moderate	3 days later reacted fatally to squash-seed globulin
33. 0.002	20	0.1	died in 2 hrs.	
34. 0.001	20	0.1	moderate	3 days later reacted slightly to squash-seed globulin
35. 0.01	21	0.1	"	24 hrs. later reacted fatally to squash-seed globulin
36. 0.002	21	0.1	"	
37. 0.001	21	0.1	"	
38. 0.0002	21	0.1	"	
39. 0.01	18	0.1	doubtful	48 hrs. later reacted moderately to squash-seed globulin
40. 0.002	18	0.1	"	48 hrs. later reacted fatally to squash-seed globulin
41. 0.001	18	0.1	"	48 hrs. later reacted fatally to squash-seed globulin
42. 0.0002	18	0.1	"	48 hrs. later reacted fatally to squash-seed globulin
Castor-bean Globulin				
43. 0.01	17	0.1	none	
44. 0.001	17	0.1	"	
45. 0.0002	17	0.1	"	48 hrs. later reacted severely to castor-bean globulin
Edestin				
46. 0.01	21	0.1	doubtful	24 hrs. later reacted negatively to edestin
47. 0.002	21	0.1	moderate	24 hrs. later reacted moderately to edestin
48. 0.001	21	0.1	"	24 hrs. later reacted fatally to edestin
49. 0.0002	21	0.1	"	24 hrs. later reacted moderately to edestin
50. 0.01	18	0.1	none	48 hrs. later reacted doubtfully to edestin
51. 0.002	18	0.1	"	48 hrs. later reacted doubtfully to edestin
52. 0.001	18	0.1	"	48 hrs. later reacted doubtfully to edestin
53. 0.0002	18	0.1	"	48 hrs. later reacted doubtfully to edestin

TABLE 6.

COCOANUT PROTEINS (*Cocos nucifera*).

	Sensitizing Dose	Days' Interval	Second Injection	Symptoms
1. 0.1	Cocoanut Protein F	19	0.15 Cocoanut Protein F	severe
2. 0.002	" " "	19	0.15 " " "	"
3. 0.01	" " "	19	0.15 " " "	"
4. 0.01	" " "	17	0.15 " " "	moderate
5. 0.01	" " "	17	0.16 " Globulin B	slight
6. 0.025	" Globulin B	24	0.16 " Protein F	moderate
7. 0.005	" " "	17	0.1 " Globulin B	severe
8. 0.005	" " "	17	0.1 " " "	"

TABLE 7.

LEGUMIN, PEA (*Pisum sativum*).

Sensitizing Dose	Days' Interval	Second Injection	Symptoms	Subsequent Injections and Remarks
Pea Legumin		Pea Legumin		
1. 0.0 (Control)	..	0.1	none	
2. 0.0 (Control)	..	0.1	"	No fall in temperature
3. 0.02	22	0.2	moderate	Had previously been used with animal protein
4. 0.002	22	0.2	"	Had previously been used with animal protein
5. 0.0002	22	0.2	slight	Had previously been used with animal protein
6. 0.00002	22	0.2	doubtful	Had previously been used with animal protein
7. 0.05	14	0.125	moderate	
8. 0.02	14	0.125	"	3 days later no reaction to pea legumin
9. 0.01	17	0.125	"	
10. 0.01	14	0.125	"	
		Vetch Legumin		
11. 0.01	14	0.125	doubtful	3 days later reacted moderately to pea legumin
12. 0.01	19	0.1	severe	
13. 0.002	19	0.1	died in 40 min.	
14. 0.001	19	0.1	" " 45 "	
15. 0.0005	19	0.1	" " 60 "	
16. 0.01	18	0.1	moderate	48 hrs. later reacted doubtfully to pea legumin
17. 0.002	18	0.1	"	48 hrs. later reacted slightly to pea legumin
18. 0.001	18	0.1	moderate	48 hrs. later reacted negatively to pea legumin
19. 0.0002	18	0.1	doubtful	died 36 hours later
		Vicilin		
20. 0.01	20	0.1	died in 20 min.	
21. 0.002	20	0.1	" " 35 "	
22. 0.001	20	0.1	" " 40 "	
23. 0.0002	20	0.1	" " 90 "	
		Vignin		
24. 0.01	20	0.1	doubtful	4 days later reacted severely to pea legumin
25. 0.002	20	0.1	"	4 days later reacted fatally to pea legumin
26. 0.001	20	0.1	"	4 days later reacted severely to pea legumin
27. 0.0002	20	0.1	"	4 days later reacted fatally to pea legumin
28. 0.01	22	0.1	"	24 hrs. later reacted fatally to pea legumin
29. 0.002	22	0.1	"	
30. 0.001	22	0.1	"	
31. 0.0002	22	0.1	"	24 hrs. later reacted severely to pea legumin

TABLE 7.—*Continued.*

	Sensitizing Dose	Days' Interval	Second Injection	Symptoms	Subsequent Injections and Remarks
	Vetch Legumin		Pea Legumin		
32.	0.02.....	17	0.125	slight	24 hrs. later reacted moderately to vetch legumin
33.	0.01.....	14	0.1	moderate	3 days later reacted slightly to vetch legumin
34.	0.01.....	18	0.1	slight	24 hrs. later reacted slightly to vetch legumin
35.	0.002.....	18	0.1	moderate	24 hrs. later reacted slightly to vetch legumin
36.	0.001.....	18	0.1	slight	24 hrs. later reacted severely to vetch legumin
37.	0.0002.....	18	0.1	moderate	24 hrs. later reacted fatally to vetch legumin
38.	0.01.....	18	0.1	doubtful	48 hrs. later reacted doubtfully to vetch legumin
39.	0.002.....	18	0.1	"	48 hours later reacted doubtfully to vetch legumin
40.	0.001.....	18	0.1	"	48 hrs. later reacted doubtfully to vetch legumin
41.	0.0002.....	18	0.1	"	48 hours later reacted doubtfully to vetch legumin
	Vicilin				
42.	0.01.....	20	0.1	severe	
43.	0.002.....	20	0.1	died in 6 hrs.	
44.	0.001.....	20	0.1	" " 50 min.	
45.	0.0002.....	20	0.1	" " 6 hrs.	
	Vignin				
46.	0.01.....	22	0.1	doubtful	48 hrs. later reacted severely to vignin
47.	0.002.....	22	0.1	"	
48.	0.001.....	22	0.1	"	
49.	0.0002.....	22	0.1	"	48 hrs. later reacted fatally to vignin

TABLE 8.

LEGUMIN, VETCH (*Vicia sativa*).

	Sensitizing Dose	Days' Interval	Second Injection	Symptoms	Subsequent Injections and Remarks
	Vetch Legumin		Vetch Legumin		
1.	0 0 (Control) ...	..	0.1	none	
2.	0.0 (Control) ...	..	0.1	"	
3.	0.0 (Control) ...	..	0.1	doubtful	No fall in temperature
4.	0.0 (Control) ...	..	0.1	"	" " " "
5.	0.1.....	20	0.125	moderate	Had previously been used with animal proteins
6.	0.02.....	20	0.125	"	Had previously been used with animal proteins
7.	0.0002.....	20	0.125	died in 165 min.	Had previously been used with animal proteins
8.	0.05.....	20	0.125	moderate	Had previously been used with animal proteins
9.	0.01.....	14	0.125	severe	
10.	0.01.....	14	0.125	died in 150 min.	
			Glycinin		
11.	0.002.....	20	0.125	none	3 hrs. later reacted moderately with vetch legumin
12.	0.01.....	18	0.1	"	24 hrs. later reacted moderately with vetch legumin
13.	0.002.....	18	0.1	"	24 hrs. later reacted negatively with vetch legumin
14.	0.001.....	18	0.1	"	24 hrs. later reacted moderately with vetch legumin
15.	0.0002.....	18	0.1	"	24 hrs. later reacted negatively with vetch legumin

TABLE 8.—*Continued.*

Sensitizing Dose	Days' Interval	Second Injection	Symptoms	Subsequent Injections and Remarks
Vetch Legumin		Pea Legumin		
16. 0.01	14	0.1	moderate	3 days later reacted slightly with vetch legumin
17. 0.01	18	0.1	slight	24 hrs. later reacted slightly with vetch legumin
18. 0.002	18	0.1	moderate	24 hrs. later reacted slightly with vetch legumin
19. 0.001	18	0.1	slight	24 hrs. later reacted severely with vetch legumin
20. 0.0002	18	0.1	moderate	24 hrs. later reacted fatally with vetch legumin
21. 0.01	18	0.1	doubtful	48 hrs. later reacted doubtfully with vetch legumin
22. 0.002	18	0.1	"	48 hrs. later reacted doubtfully with vetch legumin
23. 0.001	18	0.1	"	48 hrs. later reacted doubtfully with vetch legumin
24. 0.0002	18	0.1	"	48 hrs. later reacted doubtfully with vetch legumin
		Vicilin, Pea		
25. 0.01	19	0.1	died in 45 min.	
26. 0.002	19	0.1	" " 55 "	
27. 0.001	19	0.1	" " 9 hrs.	
28. 0.0005	19	0.1	severe	24 hrs. later reacted severely with vetch legumin
29. 0.002	17	0.1	moderate	48 hrs. later reacted severely to vetch legumin
30. 0.0004	17	0.1	"	48 hrs. later reacted moderately to vetch legumin
31. 0.0004	17	0.1	"	48 hrs. later reacted severely to vetch legumin
32. 0.00005	17	0.1	"	48 hrs. later reacted severely to vetch legumin
33. 0.00005	17	0.1	"	48 hrs. later reacted severely to vetch legumin
		Vignin		
34. 0.01	21	0.1	none	3 days later reacted moderately with vetch legumin
35. 0.002	21	0.1	slight	3 days later reacted moderately with vetch legumin
36. 0.001	21	0.1	severe	3 days later reacted moderately with vetch legumin
37. 0.0002	21	0.1	"	3 days later reacted severely with vetch legumin
38. 0.01	18	0.1	none	48 hrs. later reacted doubtfully with vetch legumin
39. 0.002	18	0.1	moderate	48 hrs. later reacted doubtfully with vetch legumin
40. 0.001	18	0.1	none	48 hrs. later reacted moderately with vetch legumin
41. 0.0002	18	0.1	"	48 hrs. later reacted moderately with vetch legumin
		Vetch Legumin		
42. 0.005	16	0.1	severe	24 hrs. later reacted severely to vicilin
43. 0.005	16	0.1	"	24 hrs. later reacted severely to vicilin
44. 0.002	16	0.1	slight	3 days later reacted slightly to vicilin
45. 0.001	16	0.1	very severe	3 days later reacted slightly to vicilin
46. 0.0005	16	0.1	" "	3 days later reacted slightly to vicilin
47. 0.01	18	0.1	slight	48 hrs. later reacted moderately to vicilin
48. 0.002	18	0.1	none	48 hrs. later reacted fatally to vicilin
49. 0.001	18	0.1	"	48 hrs. later reacted slightly to vicilin
50. 0.0002	18	0.1	doubtful	48 hrs. later reacted moderately to vicilin

TABLE 8.—*Continued.*

Sensitizing Dose	Days' Interval	Second Injection	Symptoms	Subsequent Injections and Remarks
Pea Legumin		Vetch Legumin		
51. 0.01	19	0.1	severe	
52. 0.002	19	0.1	died in 40 min.	
53. 0.001	19	0.1	" " 45 "	
54. 0.0005	19	0.1	" " 60 "	
55. 0.01	18	0.1	moderate	48 hrs. later reacted doubtfully to pea legumin
56. 0.002	18	0.1	"	48 hrs. later reacted slightly to pea legumin
57. 0.001	18	0.1	"	48 hrs. later reacted negatively to pea legumin
58. 0.0002	18	0.1	doubtful	died 36 hrs. later
Vignin				
59. 0.002	20	0.1	severe	24 hrs. later reacted fatally to vignin
60. 0.0002	20	0.1	died in 4 hrs.	
61. 0.01	18	0.1	" " 2 "	
62. 0.002	18	0.1	none	48 hrs. later reacted fatally to vignin
63. 0.001	18	0.1	"	48 hrs. later reacted fatally to vignin
64. 0.0002	18	0.1	doubtful	48 hrs. later reacted fatally to vignin

TABLE 9.

VICILIN (*Pisum sativum*).

Sensitizing Dose	Days' Interval	Second Injection	Symptoms	Subsequent Injections and Remarks
Vicilin		Vicilin		
1. 0.0 (Control)	..	0.1	slight	
2. 0.0 (Control)	..	0.1	none	
3. 0.0 (Control)	..	0.1	slight	Temperature fell 1°
4. 0.0 (Control)	..	0.1	"	" " 1.5°
5. 0.05	18	0.16	severe	Had been used before with animal proteins
6. 0.01	18	0.16	slight	Had been used before with animal proteins
7. 0.001	18	0.16	"	Had been used before with animal proteins
8. 0.0001	18	0.20	moderate	Had been used before with animal proteins
9. 0.005	16	0.125	died in 65 min.	Fresh animals
10. 0.005	16	0.125	" " 95 "	" "
		Vetch Legumin		
11. 0.005	16	0.1	severe	24 hrs. later reacted severely with vicilin
12. 0.005	16	0.1	"	24 hrs. later reacted severely with vicilin
13. 0.002	16	0.1	slight	3 days later reacted slightly with vicilin
14. 0.001	16	0.1	very severe	3 days later reacted slightly with vicilin
15. 0.0005	16	0.1	" "	3 days later reacted slightly with vicilin
16. 0.01	18	0.1	slight	48 hrs. later reacted moderately with vicilin
17. 0.002	18	0.1	none	48 hrs. later reacted fatally with vicilin
18. 0.001	18	0.1	"	48 hrs. later reacted slightly with vicilin
19. 0.0002	18	0.1	doubtful	48 hrs. later reacted moderately to vicilin

TABLE 9.—*Continued.*

	Sensitizing Dose	Days' Interval	Second Injection	Symptoms	Subsequent Injections and Remarks
	Vicilin		Pea Legumin		
20.	0.01	20	0.1	severe	
21.	0.002	20	0.1	died after 6 hrs.	
22.	0.001	20	0.1	" in 50 min.	
23.	0.0002	20	0.1	" after 6 hrs.	
	Vetch Legumin		Vicilin		
24.	0.01	19	0.1	died in 45 min.	
25.	0.002	19	0.1	" " 55 "	
26.	0.001	19	0.1	" " 9 hrs.	
27.	0.0005	19	0.1	severe	24 hrs. later reacted severely with vetch legumin
28.	0.002	17	0.1	moderate	48 hrs. later reacted severely to vetch legumin
29.	0.0004	17	0.1	"	48 hrs. later reacted moderately to vetch legumin
30.	0.0004	17	0.1	"	48 hrs. later reacted severely to vetch legumin
31.	0.00005	17	0.1	"	48 hrs. later reacted severely to vetch legumin
32.	0.00005	17	0.1	"	48 hrs. later reacted severely to vetch legumin
	Pea Legumin				
33.	0.01	20	0.1	died in 20 min.	
34.	0.002	20	0.1	" " 35 "	
35.	0.001	20	0.1	" " 40 "	
36.	0.0002	20	0.1	" " 90 "	
	Glycinin				
37.	0.01	21	0.08	doubtful	
38.	0.002	21	0.08	"	24 hrs. later reacted severely with glycinin
39.	0.001	21	0.08	"	
40.	0.0002	21	0.08	moderate	24 hrs. later reacted fatally with glycinin

TABLE 10.

VIGNIN (*Vigna sinensis*).

	Sensitizing Dose	Days' Interval	Second Injection	Symptoms	Subsequent Injections and Remarks
	Vignin		Vignin		
1.	0.0 (Control)	..	0.1	doubtful	
2.	0.0 (Control)	..	0.1	none	
3.	0.01	24	0.1	severe	
4.	0.002	20	0.1	died in 60 min.	
5.	0.001	20	0.1	" " 30 "	
6.	0.0002	20	0.1	" " 50 "	
			Vetch Legumin		
7.	0.002	20	0.1	severe	24 hrs. later reacted fatally to vignin
8.	0.0002	20	0.1	died in 4 hrs.	
9.	0.01	18	0.1	" " 2 "	
10.	0.002	18	0.1	none	48 hrs. later reacted fatally to vignin
11.	0.001	18	0.1	"	48 hrs. later reacted fatally to vignin
12.	0.0002	18	0.1	doubtful	48 hrs. later reacted fatally to vignin
			Pea Legumin		
13.	0.01	22	0.1	"	48 hrs. later reacted severely to vignin
14.	0.002	22	0.1	"	
15.	0.001	22	0.1	"	
16.	0.0002	22	0.1	"	48 hrs. later reacted fatally to vignin

TABLE 10.—*Continued.*

Sensitizing Dose	Days' Interval	Second Injection	Symptoms	Subsequent Injections and Remarks
Vignin		Glycinin	doubtful	24 hrs. later reacted fatally to vignin
17. 0.01	20	0.1		
18. 0.002	20	0.1	none	
19. 0.001	20	0.1	"	
20. 0.0002	20	0.1	"	24 hrs. later reacted fatally to vignin
21. 0.01	24	0.1	doubtful	48 hrs. later reacted fatally to vignin
Excelsin		Vignin		
22. 0.1	17	0.1	none	24 hrs. later reacted severely to excelsin
23. 0.02	17	0.1	"	24 hrs. later reacted severely to excelsin
Glycinin				
24. 0.01	20	0.1	slight	
25. 0.002	20	0.1	severe	24 hrs. later reacted severely to glycinin
26. 0.001	20	0.1	"	24 hrs. later reacted severely to glycinin
27. 0.0002	20	0.1	severe	
28. 0.01	18	0.1	moderate	72 hrs. later reacted slightly to glycinin
29. 0.002	18	0.1	slight	72 hrs. later reacted slightly to glycinin
30. 0.001	18	0.1	none	72 hrs. later reacted slightly to glycinin
31. 0.0002	18	0.1	moderate	72 hrs. later reacted slightly to glycinin
Hordein				
32. 0.02	20	0.1	none	
Vetch Legumin				
33. 0.01	21	0.1	"	3 days later reacted moderately to vetch legumin
34. 0.002	21	0.1	slight	3 days later reacted moderately to vetch legumin
35. 0.001	21	0.1	severe	3 days later reacted moderately to vetch legumin
36. 0.0002	21	0.1	severe	3 days later reacted severely to vetch legumin
37. 0.01	18	0.1	none	48 hrs. later reacted doubtfully to vetch legumin
38. 0.002	18	0.1	moderate	48 hrs. later reacted doubtfully to vetch legumin
39. 0.001	18	0.1	none	48 hrs. later reacted moderately to vetch legumin
40. 0.0002	18	0.1	"	48 hrs. later reacted moderately to vetch legumin
Pea Legumin				
41. 0.01	20	0.1	doubtful	4 days later reacted severely to pea legumin
42. 0.002	20	0.1	"	4 days later reacted fatally to pea legumin
43. 0.001	20	0.1	"	4 days later reacted severely to pea legumin
44. 0.0002	20	0.1	"	4 days later reacted fatally to pea legumin
45. 0.01	22	0.1	"	24 hrs. later reacted fatally to pea legumin
46. 0.002	22	0.1	"	
47. 0.001	22	0.1	"	
48. 0.0002	22	0.1	"	24 hrs. later reacted severely to pea legumin
Hordein				
49. 0.006	19	0.1	doubtful	24 hrs. later reacted severely to hordein
50. 0.002	19	0.1	none	24 hrs. later reacted slightly to hordein
51. 0.001	19	0.1	"	24 hrs. later reacted severely to hordein

TABLE 11.

GLYCININ (*Soja hispida*).

	Sensitizing Dose	Days' Interval	Second Injection	Symptoms	Subsequent Injections and Remarks
	Glycinin		Glycinin		
1.	0.1	16	0.125	slight	Previously used with animal proteins
2.	0.02	16	0.125	"	Previously used with animal proteins
3.	0.002	16	0.125	severe	Previously used with animal proteins
4.	0.005	22	0.15	moderate	Previously used with animal proteins
5.	0.0005	22	0.15	slight	Previously used with animal proteins
6.	0.0005	22	0.15	"	Previously used with animal proteins
7.	0.01	17	0.1	severe	Normal animal
8.	0.002	17	0.1	"	" "
9.	0.001	17	0.1	died in 3 hrs.	" "
10.	0.0002	17	0.1	" " 7 "	" "
11.	0.001	16	0.1	severe	3 days later reacted negatively to glycinin
			Excelsin		
12.	0.001	16	0.1	doubtful	3 days later reacted severely to glycinin
13.	0.0002	16	0.1	none	3 days later reacted severely to glycinin
14.	0.01	22	0.1	"	2 days later reacted severely to glycinin
15.	0.002	22	0.1	"	2 days later reacted severely to glycinin
			Vignin		
16.	0.01	20	0.1	slight	
17.	0.002	20	0.1	severe	24 hrs. later reacted severely to glycinin
18.	0.001	20	0.1	"	24 hrs. later reacted severely to glycinin
19.	0.0002	20	0.1	"	
20.	0.01	18	0.1	moderate	72 hrs. later reacted slightly to glycinin
21.	0.002	18	0.1	slight	72 hrs. later reacted slightly to glycinin
22.	0.001	18	0.1	none	72 hrs. later reacted slighty to glycinin
23.	0.0002	18	0.1	moderate	72 hrs. later reacted slightly to glycinin
			Vicilin		
24.	0.01	21	0.08	doubtful	
25.	0.002	21	0.08	"	24 hrs. later reacted severely to glycinin
26.	0.001	21	0.08	"	
27.	0.0002	21	0.08	moderate	24 hrs. later reacted fatally to glycinin
	Vetch Legumin		Glycinin		
28.	0.01	18	0.1	none	24 hrs. later reacted moderately to vetch legumin
29.	0.002	18	0.1	"	24 hrs. later reacted negatively to vetch legumin
30.	0.002	20	0.125	"	3 hrs. later reacted moderately to vetch legumin
31.	0.001	18	0.1	"	24 hrs. later reacted negatively to vetch legumin
32.	0.0002	18	0.1	"	24 hrs. later reacted moderately to vetch legumin
	Excelsin				
33.	0.002	16	0.1	doubtful	3 days later reacted fatally to excelsin
34.	0.001	16	0.1	none	3 days later reacted fatally to excelsin
35.	0.0002	16	0.1	"	3 days later reacted fatally to excelsin

TABLE 11.—*Continued.*

Sensitizing Dose	Days' Interval	Second Injection	Symptoms	Subsequent Injections and Remarks
Vignin		Glycinin		
36. 0.01	20	0.1	doubtful	24 hrs. later reacted fatally to vignin
37. 0.002	20	0.1	none	
38. 0.001	20	0.1	"	
39. 0.0002	20	0.1	"	24 hrs. later reacted fatally to vignin

TABLE 12.

GLIADIN, WHEAT (*Triticum vulgare*).

Sensitizing Dose	Days' Interval	Second Injection	Symptoms	Subsequent Injections and Remarks
Gliadin (wheat)		Gliadin (wheat)		
1. 0.0 (Control)	..	0.1	none	
2. 0.005	22	0.1	moderate	
3. 0.005	22	0.1	died in 1 hr.	
4. 0.005	25	0.1	severe	
5. 0.005	25	0.1	died in 1 hr.	
6. 0.1	24	0.1	" " 1 "	
		Gliadin (rye) (*Secale cereale*)		
7. 0.01	24	0.1	died in 20 min.	
8. 0.002	24	0.1	" " 10 "	
9. 0.001	24	0.1	severe	3 days later reacted moderately to wheat gliadin
10. 0.0002	24	0.1	severe	3 days later reacted moderately to wheat gliadin
11. 0.001	18	0.1	died in 10 min.	
12. 0.0002	18	0.1	severe	48 hrs. later reacted slightly to wheat gliadin
13. 0.0001	18	0.1	died in 12 min.	
14. 0.00002	18	0.1	moderate	48 hrs. later reacted negatively to wheat gliadin
Gliadin (rye)		Gliadin (wheat)		
15. 0.01	24	0.1	severe	3 days later reacted negatively to rye gliadin
16. 0.002	24	0.1	severe	3 days later reacted slightly to rye gliadin
17. 0.0002	24	0.1	none	3 days later reacted negatively to rye gliadin
18. 0.001	18	0.1	severe	died 24 hrs. later
19. 0.0002	18	0.1	died in 80 min.	
20. 0.0001	18	0.1	" " 80 "	
21. 0.00002	18	0.1	severe	died 36 hrs. later
Gliadin (wheat)		Zein		
22. 0.02	26	0.25	none	
23. 0.08	26	0.25	"	
24. 0.005	22	0.1	doubtful	3 days later reacted severely to wheat gliadin
25. 0.005	22	0.1	"	3 days later reacted fatally to wheat gliadin
26. 0.002	17	0.1	none	48 hrs. later reacted moderately to wheat gliadin
27. 0.001	17	0.1	"	48 hrs. later reacted moderately to wheat gliadin
28. 0.0002	17	0.1	"	48 hrs. later reacted fatally to wheat gliadin
Zein		Gliadin (wheat)		
29. 0.005	22	0.1	doubtful	
30. 0.005	22	0.1	slight	
31. 0.01	20	0.1	none	24 hrs. later reacted slightly to zein
32. 0.002	17	0.1	"	48 " " " severely " "
33. 0.001	17	0.1	"	48 " " " " " "
34. 0.0002	17	0.1	"	48 " " " " " "

TABLE 13.

HORDEIN (*Hordeum vulgare*).

Sensitizing Dose	Days' Interval	Second Injection	Symptoms	Subsequent Injections and Remarks
Hordein		Hordein		
1. 0.0 (Control) ...	..	0.1	none	No fall in temperature
2. 0.0 (Control) ...	..	0.1	"	
3. 0.02	23	0.1	slight	
4. 0.01	23	0.1	died in 2 hrs.	
5. 0.002	23	0.1	severe	
6. 0.006	19	0.1	"	
7. 0.002	19	0.1	"	
8. 0.001	19	0.1	slight	
		Zein		
9. 0.02	22	0.1	none	24 hrs. later reacted slightly to hordein
10. 0.01	22	0.1	"	24 hrs. later reacted fatally to hordein
11. 0.002	22	0.1	"	24 hrs. later reacted severely to hordein
		Vignin		
12. 0.006	19	0.1	doubtful	24 hrs. later reacted severely to hordein
13. 0.002	19	0.1	none	24 hrs. later reacted slightly to hordein
14. 0.001	19	0.1	"	24 hrs. later reacted severely to hordein
Zein		Hordein		
15. 0.01	15	0.1	doubtful	3 days later reacted slightly to zein
16. 0.002	15	0.1	none	3 " " " " " "
17. 0.001	15	0.1	"	3 " " " " " "
18. 0.0002	15	0.1	"	3 " " " " " "

TABLE 14.

ZEIN (*Zea mays*).

Sensitizing Dose	Days' Interval	Second Injection	Symptoms	Subsequent Injections and Remarks
Zein		Zein		
1. 0.02	17	0.20	died in 1 hr.	Animal not raised on corn diet
2. 0.004	17	0.25	" " 40 min.	" " " " " "
3. 0.002	18	0.20	" " 12 hrs.	" " " " " "
4. 0.0004	17	0.20	severe	" " " " " "
5. 0.0002	18	0.20	"	" " " " " "
6. 0.01	25	0.20	died in 6 hrs.	" " " " " "
7. 0.01	25	0.20	" " 6 "	" " " " " "
8. 0.005	25	0.15	severe	" " " " " "
9. 0.02	23	0.1	none	" raised on corn diet
10. 0.01	23	0.1	"	" " " " "
11. 0.002	23	0.1	"	" " " " "
12. 0.01	17	0.1	severe	" " " bread and milk
13. 0.01	17	0.1	slight	" " " " " "
14. 0.01	18	0.1	none	" " " corn meal
15. 0.002	18	0.1	"	" " " " "
16. 0.001	18	0.1	"	" " " " "
17. 0.0003	18	0.1	"	" " " " "
18. 0.005	18	0.1	moderate	" " " oats and carrots
19. 0.001	18	0.1	severe	" " " " " "
		Gliadin (wheat)		All animals below raised on diet free from corn
20. 0.01	20	0.1	none	24 hrs. later reacted slightly to zein
21. 0.002	17	0.1	"	48 " " " severely " "
22. 0.001 ...	17	0.1	"	48 " " " " " "
23. 0.0002	17	0.1	"	48 " " " " " "
		Hordein		
24. 0.01	15	0.1	doubtful	3 days " " slightly " "
25. 0.002	15	0.1	none	3 " " " " " "

TABLE 14.—*Continued.*

Sensitizing Dose	Days' Interval	Second Injection	Symptoms	Subsequent Injections and Remarks
Zein		Hordein		
26. 0.001.....	15	0.1	none	3 days later reacted slightly to zein
27. 0.0002.....	15	0.1	"	3 " " " " " "
		Castor-bean Globulin		
28. 0.01.....	20	0.1	none	
Hordein		Zein		
29. 0.02.....	22	0.1	"	24 hrs. later reacted slightly to hordein
30. 0.01.....	22	0.1	"	24 hrs. later reacted fatally to hordein
31. 0.002.....	22	0.1	"	24 hrs. later reacted severely to hordein
Gliadin (wheat)				
32. 0.002.....	17	0.1	"	48 hrs. later reacted moderately to gliadin
33. 0.001.....	17	0.1	"	48 hrs. later reacted moderately to gliadin
34. 0.0002.....	17	0.1	"	24 hrs. later reacted fatally to gliadin
35. 0.02.....	26	0.25	"	
36. 0.08.....	26	0.25	"	
37. 0.005.....	22	0.1	doubtful	3 days later reacted severely to gliadin
38. 0.005.....	22	0.1	"	3 days later reacted fatally to gliadin

8. COMPARISON OF ANAPHYLAXIS REACTIONS OBTAINED WITH VEGETABLE AND ANIMAL PROTEINS.

a) MINIMUM SENSITIZING AND INTOXICATING DOSES.

As can be seen from these tables the vegetable proteins have been found to be suitable for anaphylaxis work, giving reactions when used in amounts comparable to those required for producing reactions with animal proteins. In general, however, it may be said that the reactions are somewhat less severe and less constant in degree than those usually observed by the commonly used animal proteins, such as egg albumin and various sera.

The only quantitative experiments reported with purified animal proteins[14] gave as the minimum fatally sensitizing dose of crystallized egg albumin one-thousandth of a milligram (0.000,001 gm.) while five one-hundred-thousandths of a milligram (0.000,000,05 gm.) sensitized animals so that they gave some reaction on a subsequent injection of egg albumin. Fatal intoxication of sensitized 300 gm. guinea-pigs was usually produced by five-tenths milligram (0.0005 gm.) of egg albumin given intraperitoneally. For comparison a series of experiments were per-

formed with edestin from hemp seed, the results of which are given in Table 4, experiments 1–21.

These experiments show that the sensitizing power of edestin is not noticeably less than that of crystallized egg albumin, as one ten-thousandth of a milligram (0.000,000,1 gm.), the smallest sensitizing dose tried, rendered the guinea-pig sensitive to edestin. The intoxicating power of edestin is, however, much less than that of egg albumin, serum proteins, etc., for the minimum intoxicating dose of this preparation of edestin was two to five milligrams (0.002 to 0.005 gm.), while severe reactions were not obtained with less than 20 to 40 milligrams. These quantities are large when compared with the half-milligram (0.0005 gm.) dose of egg albumin which produced fatal intoxication in sensitized guinea-pigs.

Since edestin appeared to be the least toxic, anaphylactically, of all the vegetable proteins that we have tried, with the possible exception of glycinin, we determined also the minimum sensitizing and intoxicating doses of squash-seed globulin, which seemed the most active vegetable protein used by us. The results of these experiments are given in Table 3, experiments 58 to 75. It will be seen that a sensitizing dose of one two-thousandth of a milligram (0.000,000,5 gm.) sensitized guinea-pigs fatally, while one ten-thousandth of a milligram (0.000,000,1 gm.) did not render the animals sensitive; these doses are not essentially different from those found as the minimum active doses of egg albumin and edestin, indicating that the degree of activity of different proteins in the anaphylaxis reaction bears no relation to the minimum sensitizing dose. To a certain extent the same is true for the minimum intoxicating dose, as is shown by a comparison of the results obtained with edestin and with squash-seed globulin. Although the customary intoxicating dose of 0.1 gm. usually causes death in sensitized pigs when the protein is squash-seed globulin, and the same dose of edestin usually causes only moderate and rarely severe symptoms, yet it was found that the minimum intoxicating dose was practically the same with each. It required ten to twenty milligrams of squash-seed globulin to cause severe reactions, 3 to 5 milligrams causing but slight symptoms, and amounts below that producing

doubtful or no reactions in sensitized pigs. The following may be proposed as a possible explanation of this unexpected result: Edestin seems to owe much of its inefficiency in the anaphylaxis reaction to its slight solubility in the body fluids, it having been shown that alkaline solutions of edestin are precipitated in the peritoneal cavity; therefore, no matter what the amount of injected edestin may be, the peritoneal fluid cannot dissolve and carry quickly to the blood more than a small quantity of this protein, not enough ordinarily to cause severe intoxication, and no more whether 40 or 200 mg. are injected. As the minimum intoxicating doses of edestin and squash-seed globulin are practically the same, the greater toxicity of the latter probably does not depend upon a difference of composition but upon its greater solubility in the body fluids, so that it is possible for a fatal does to be absorbed from the peritoneum into the blood.

b) RELATIVE ACTIVITY OF DIFFERENT VEGETABLE PROTEINS.

Of the several vegetable proteins used in these experiments, distinctly the most toxic to sensitized guinea-pigs was, as mentioned above, squash-seed globulin. This protein almost always caused fatal reactions, which were characterized by a rapid course and violent symptoms. Vignin, excelsin, and the globulin from castor bean were almost as toxic as squash-seed globulin to sensitized animals, although on the whole the reactions were not usually quite as sharp and rapid as with squash-seed globulin. Zein, vicilin, and gliadin frequently caused fatal and usually marked reactions, but these were less severe than the reactions with the proteins above named, while the legumins, hordein, and glycinin, caused fatal reactions only exceptionally. As before mentioned, edestin is distinctly inferior to the other proteins in producing anaphylactic intoxication. The following table gives some suggestion of the toxicity exhibited by the various proteins, but is not an exact indication, for in many of the experiments the intoxicating dose was given as a control to sensitized guinea-pigs that had failed to react to an injection of a heterologous protein 24 to 72 hours previously, and in such animals the reaction is com-

monly less severe and always less constant in degree than in animals which have not had the heterologous protein.

Numbers							Percentages				
Fatal	Severe	Moderate	Slight	Doubtful or Negative	Total		Fatal	Severe	Moderate	Slight	Doubtful or Negative
7	5	3	1	0	16	Castor-bean globulin.....	43	30	19	6	0
18	0	0	1	0	19	Squash-seed globulin.....	95	0	0	5	0
2	3	5	3	2	15	Edestin.....	13	20	33	20	13
11	4	1	0	0	16	Excelsin.....	69	25	6	0	0
0	5	2	1	0	8	Cocoanut proteins.....	0	63	25	12	0
3	3	4	0	0	10	Legumin, pea.....	30	30	40	0	0
2	2	11	0	4	19	Legumin, vetch.....	11	11	56	0	22
3	0	2	2	0	7	Vicilin.....	44	0	28	28	0
11	4	0	0	0	15	Vignin.....	72	28	0	0	0
3	8	0	2	0	13	Glycinin.....	23	62	0	15	0
7	4	5	0	0	16	Gliadin.....	44	25	31	0	0
2	6	0	4	0	12	Hordein.....	17	50	0	34	0
5	5	1	1	0	12	Zein.....	42	42	8	8	0

The reason or reasons for the differences in anaphylactic activity shown by these vegetable proteins we have not yet determined. One factor that may be of importance is the solubility of the several proteins in the body fluids, for we have found that there is usually more or less precipitated matter in the abdominal cavities of guinea-pigs which have received injections of proteins dissolved in 0.1 per cent NaOH. The bearing of this precipitation upon the slight toxicity of edestin has already been discussed on pp. 71 and 107.

c) SYMPTOMATOLOGY OF REACTIONS WITH VEGETABLE PROTEINS.

The observed symptoms of anaphylactic intoxication with vegetable proteins were not essentially different from those produced by animal proteins, but there were distinct quantitative differences. With the vegetable proteins the period which elapsed between the injection into a sensitized animal of the second or intoxicating dose and the appearance of symptoms was usually longer. Commonly distinct reactions did not appear in less than 20 to 30 minutes, and in fatal cases death usually occurred after 45 to 60 minutes, whereas with egg white and sera symptoms usually appeared within 15 minutes after intraperitoneal injection, frequently in 5 to 10 minutes, and death often occurred a few minutes later. As a rule the symptoms produced with the

vegetable proteins were less violent, the stormy convulsive attacks so common with serum and egg albumin intoxication being seldom observed; also severe paralysis was not often produced. Sneezing, coughing, and scratching were less marked in most cases. Roughening of the hair and lachrymation were usually pronounced, and there was the typical sharp fall in temperature, but even in the fatal cases the onset and course of the symptoms were seldom violent, and the animals died more quietly. In exceptional cases, however, severe and violent symptoms with a rapidly fatal termination were produced by the vegetable proteins. In most of the fatal cases autopsy did not reveal the distended emphysematous lungs, and hemorrhages were less extensive although commonly present; that is, the conditions more closely approximated those seen in cases of serum intoxication when death has occurred slowly, without violent symptoms. The abdominal cavity usually contained considerable fluid, and masses of what seemed to be coagulated or precipitated protein. How much of this insoluble material represented precipitated vegetable proteins and how much was lymph proteins we have not investigated.

The period of incubation after injection of the sensitizing dose before the animal became sensitive was about the same as with the animal proteins, although strong reactions were not usually obtained before the fourteenth day, and for the best results it was better to wait twenty days. In a series of experiments (Table 3, Nos. 76–79) performed with squash-seed globulin for the purpose of securing definite data upon the minimum incubation period, it was found that with intraperitoneal injections no symptoms could be obtained in less than 8 days; a parallel series with egg albumin gave exactly the same result.

It has been observed with plant proteins, even more strikingly than with animal proteins, that there is commonly a less active sensitization by large doses than by small doses. If our records are gone over it will be noticed that as a usual thing animals which received a sensitizing dose of 0.01 gm. protein reacted less violently to the second dose of the same protein than animals that had received but 0.001 gm. or 0.0002 gm., as shown for example by experiments 34–36, Table 3, squash-seed globulin, and espe-

cially by experiments 55–57, in which large sensitizing doses were purposely given. Possibly this phenomenon depends upon the slow absorption of the relatively insoluble vegetable proteins, thus favoring the development of a partial immunity or defective sensitization such as is observed frequently when large amounts of serum are given for the sensitizing dose.

9. Specificity of Vegetable Proteins as Shown by the Anaphylaxis Reaction.

a) General Considerations.

The vegetable proteins examined show distinct characteristics as regards specificity. In most cases sensitization with one protein rendered the animal sensitive only to the same protein but not to proteins from other more or less closely related plants. Thus castor-bean globulin (see Table 1) did not render guinea-pigs sensitive to the globulin from the squash seed, hemp seed, or Brazil nut. Occasionally atypical results were obtained, especially in the case of flax-seed globulin (experiments 8 to 17, Table 2). Of ten animals sensitized with castor-bean globulin and injected later with flax-seed globulin two died promptly with typical symptoms of anaphylaxis, two died after 24 to 36 hours, two showed slight symptoms, while the other four showed little or no reaction.

In Table 1 it is also shown that edestin, excelsin, and squash-seed globulin as well as zein did not sensitize guinea-pigs to castor-bean globulin. Here too one atypical reaction is observed (No. 36, Table 1). It is evident from these and similar irregular results that it is unsafe to draw conclusions from any small number of experiments, as some writers have incautiously done.

The reason for this peculiar irregularity of reaction is not clear and no such marked irregularity was obtained with other vegetable proteins. It certainly is not due to the presence of minute quantities of contaminating flax-seed globulin in our preparation of castor-bean globulin, for otherwise reactions should have been observed with those animals which had received larger sensitizing doses than some of those which did react. As most of our preparations were not originally made for use in anaphylaxis experiments no special precautions had been taken in their handling to

avoid contamination by minute traces of other proteins. To detect or avoid possible errors from this source, therefore, the sensitizing dose was purposely varied in each series from 0.01 to 0.0002 gm. and the second injection was made with but 0.1 gm. of protein, which would not contain an intoxicating dose of any admixed protein unless the impurity amounted to 1 to 10 per cent, and our extensive experience indicates that there has been no undetected error because of such impurities in the material.

As is the case with animal proteins, we have found that injection of a heterologous vegetable protein into a sensitized animal does not ordinarily render it refractory to a subsequent injection of the homologous protein as does a non-fatal reaction to the homologous protein, a point of importance in considering the specificity of the reactions. It is generally found, however, that the reaction is somewhat less severe when the homologous protein is injected 24 to 72 hours after a heterologous protein, and less often fatal than is usual with simple anaphylaxis experiments. This is probably at least partly due to incomplete absorption from the peritoneum, in consequence of the inflammatory reaction and fibrinous deposits in lymph spaces and on the peritoneal surfaces that follow the injection of the vegetable proteins. At least this factor is too evident to permit one to conclude that there has been any immunological process which reduces the reactivity of the animal. It was also noticed that animals that have been used for anaphylaxis experiments are not suitable for subsequent anaphylaxis experiments, even with very dissimilar proteins. For reasons of economy guinea-pigs that had been used in experiments with animal proteins were sometimes used shortly afterward in orientation experiments with vegetable proteins, where only gross phenomena were sought. A few only of these experiments are recorded in the accompanying tables, chiefly for comparison, and it will be seen that although reactions were obtained they were usually less severe than when fresh guinea-pigs were used (see Tables 4, 5, 7, 8, 9, 11). How much of this decrease in reactivity depends upon the factors mentioned above, and how much upon the existence and participation of non-specific anaphylactogens we have not attempted to determine.

b) SUMMARY OF RESULTS WITH COMMENTS.

The outcome of the individual experiments which are given in the tables is evident upon inspection of the data there given, but there are some features which require discussion.

Table 1. *Castor-bean globulin.*—That toxic symptoms should result from the injection of the globulin extracted from the castor bean might be expected from what we know of the great toxicity of ricin and the commonly assumed absorption of one protein by another when precipitated from solutions containing each of them. That our preparation of the castor-bean globulin did not contain a sufficient quantity of ricin to cause toxic symptoms is shown by the control experiments 1–3 and also by experiments 29–39, in only one of which were any symptoms of a toxic character observed. Our experiments with the castor-bean globulin were especially directed to a study of its anaphylactic relations to the globulins of the squash seed, flax seed, and hemp seed (edestin), because these four globulins are so nearly alike in crystalline form, solubility, physical properties, ultimate composition, and proportion of their decomposition products, that only a most critical comparison has revealed differences between them sufficient to justify the conclusion that they are distinct substances. It is therefore of interest to note that the only evidence of an anaphylaxis reaction observed between these four proteins is shown by the irregular and inconstant reactions produced in guinea-pigs sensitized by castor-bean globulin, and reinjected with flax-seed globulin, or conversely. It is possible that this difference in behavior between the flax-seed globulin and the others is caused by some difference in its structure which, if discovered, might shed much light on the nature of the anaphylaxis reaction.

Table 2. *Flax-seed globulin.*—Absolute specificity exists between this protein and the closely related globulin of squash seed, but castor-bean globulin and flax-seed globulin show a slight degree of interaction. Animals sensitized with flax-seed globulin react slightly but definitely to castor-bean globulin and are rendered partially refractory to the flax-seed globulin; but when the reverse reaction is tried and flax-seed globulin is injected into animals sensitized with castor-bean globulin, remarkably irregular reactions are

obtained, four of ten dying, five giving slight, and one no symptoms. Such irregular results are baffling, and many more experiments must be performed before any attempt can be made to interpret them.

Table 3. *Squash-seed globulin.*—Although animals sensitized with squash-seed globulin did not react to the three similar globulins from the castor bean, flax seed, or hemp seed (edestin), there was marked evidence of a reaction with excelsin, though in most cases no protection was imparted thereby. On the other hand, guinea-pigs sensitized with excelsin did not react to subsequent injections of squash-seed globulin. Excelsin differs both in crystalline form and ultimate composition to such a degree from the four globulins above mentioned, that it has long been recognized as a distinctly different protein. The structural differences between excelsin and these other globulins may however not be so great as these facts indicate, for the analyses of the products of hydrolysis of squash-seed globulin and of excelsin have given very similar results. In view of the wide difference, biologically, in the origin of these two proteins, their anaphylactic and chemical relations deserve further careful study.

Table 4. *Edestin.*—Edestin showed the least capacity to cause satisfactory anaphylactic reactions of any of the proteins tested. Compared with squash-seed globulin the difference in this respect was marked, although these two proteins appear to be, so far as known, chemically very similar to one another. On account of the relatively slight toxicity to guinea-pigs sensitized with edestin itself, and its tendency to cause slight atypical symptoms when injected into normal animals, our results were not as decisive as those obtained with the other proteins, especially when the refractory or "anti-anaphylactic" condition of the guinea-pigs was tested. Between the three similar globulins—mentioned in discussing Table 1—and edestin no reaction was obtained, but as was the case with animals sensitized with squash-seed globulin, there was also some reaction when excelsin was injected into guinea-pigs sensitized with edestin.

Table 5. *Excelsin.*—Although excelsin rendered guinea-pigs highly sensitive to a subsequent injection of excelsin, no evidence

of any reaction was obtained when such animals were treated with any of the other proteins tested, except edestin, which caused symptoms of a doubtful character. It is to be noted that while squash-seed globulin rendered guinea-pigs slightly sensitive to excelsin, this latter did not make them sensitive to squash-seed globulin. These last atypical results were obtained with one strain of guinea-pigs and not with another, and probably represent more a natural susceptibility to excelsin than a true sensitization; normal control guinea-pigs occasionally showing more or less atypical reactions when given 0.1 gm. doses of excelsin.

Table 6. *Cocoanut proteins.*—The preparations used in these experiments do not represent products of sufficiently definite character to require special comment.

Tables 7, 8, 9, 10, and 11. *Legumin, pea; Legumin, vetch; Vicilin; Vignin; Glycinin.*—The results given in these tables require discussion together, as they depend largely on the nature of the preparations used, as well as on the nature of the proteins contained in the seeds, peas, vetches, cow-peas, and soy-beans from which they were obtained. Legumin is the chief protein constituent of the first two seeds, preparations from each of which are so nearly alike in all respects that a most extensive and rigid comparison has as yet revealed no positive difference between them.* It is not surprising therefore to find that guinea-pigs sensitized with pea legumin showed marked anaphylaxis reactions when subsequently injected with vetch legumin. Conversely when sensitized with vetch legumin reactions were obtained with pea legumin, which, however, were usually much less severe, although approximately as strong as those obtained by sensitizing and intoxicating with pea legumin itself.

Besides legumin, the pea contains another protein of slightly different properties and composition to which the name vicilin has been given. Vicilin, which has not been found in the seeds of the vetch, is so difficult to separate from legumin that it is probable that no preparations of either one of these proteins from the pea have ever been made which did not contain enough of the other to cause the extremely delicate anaphylaxis reaction. The

* Cf. Osborne, "Die Pflanzenproteine," *Ergebnisse der Physiol.*, 1910, 10, p. 119.

preparations of pea legumin and vicilin used for our experiments were made by extensive fractional precipitation with $(NH_4)_2SO_4$ and represent products which, according to our past experience, should be very pure, especially in respect to the separation of the one protein from the other. It is, therefore, striking to find that four animals sensitized with pea legumin promptly died when treated with vicilin and that of four sensitized with vicilin and treated with pea legumin three died, though less quickly, and one became severely ill. That the reaction between vicilin and pea legumin, when tested either way, was so much more severe than between pea legumin and pea legumin (in our experiments no fatal reactions were obtained with the latter combination) suggests that the extensive fractional precipitation with $(NH_4)_2SO_4$ has contributed to the result in some way that deserves further careful study. A similar result has been reported by Gay and Adler[57] with preparations of serum proteins made by fractional precipitation with $(NH_4)_2SO_4$, and one of us (W.) has obtained results with the proteins of egg white which suggest that in natural protein mixtures there may be present substances which decrease the anaphylactic activity of some of the proteins.

When vicilin was used for sensitizing, a distinct reaction was obtained when vetch legumin was subsequently injected, but much less severe than when pea legumin was used. On the other hand, when vetch legumin was used for sensitizing and pea vicilin for intoxicating, the results were fatal in three cases, severe in one, and moderate in four, with no effective protection conferred against the vetch legumin. When vicilin was used for sensitizing, a distinct and often severe, but in no case fatal, reaction was obtained.

The preparation of vetch legumin was made by repeated precipitation from NaCl solutions and was not subjected to fractional precipitation with $(NH_4)_2SO_4$. It is difficult to find a satisfactory explanation of the results obtained with these three proteins unless the fractional precipitation with $(NH_4)_2SO_4$ has involved some factor which in some way concerns the anaphylaxis reaction. It is our intention to investigate this matter further, for it would seem as if we had here a clue which will lead to a better understanding of this important reaction.

Vignin, the principal protein of the cow-pea, closely resembles legumin in properties, composition, and relative proportion of the decomposition products which it yields on hydrolysis. The slight differences between these proteins are, however, sufficient to characterize them as chemically distinct substances. The preparation of vignin used in these experiments was made by repeated precipitation from NaCl solutions and was not subjected to fractionation with $(NH_4)_2SO_4$.

Unlike legumin, from the pea or vetch, vignin gave a very strong anaphylaxis reaction. Animals sensitized with vignin reacted in several cases with vetch legumin, but only doubtfully with pea legumin. Animals sensitized with vetch legumin reacted to vignin in some cases, in others not at all, and acquired only partial protection. Those sensitized with pea legumin gave, at the most, only doubtful reactions and showed no protection, the second injection of pea legumin reacting more strongly even than in those experiments where the second dose of pea legumin followed the first without the intervening dose of vignin. It is possible that the fractionation of the pea legumin with $(NH_4)_2SO_4$ had an influence in this case, but further experiments are necessary before definite conclusions can be drawn. Experiments with vicilin and vignin, which have not been tried, should also be made. Guinea-pigs sensitized with vignin did not react with glycinin, which is a protein of very similar properties and composition, obtained from the soy-bean. On the other hand, those sensitized with glycinin in most cases reacted to a considerable extent with vignin, but without pronounced protection.

The seeds of the pea, vetch, cow-pea, and soy-bean all contain small amount of an albumin-like protein (legumelin) which, so far as is now known, may be identical in each of these seeds. Sufficient data are not, however, available to indicate the real relation of the preparations of this protein from the different seeds. It is possible that the interreactions of legumin, vicilin, vignin, and vicilin may be connected with slight contaminations of our preparations with traces of legumelin, and that the difference in behavior of the preparations of legumin from the pea and vetch may be due to a more complete separation from legumelin by the

fractional precipitation with $(NH_4)_2SO_4$ employed in making the pea legumin.

Between proteins of markedly different structure neither vignin nor glycinin showed any reaction, e.g., excelsin+vignin, or hordein+vignin; glycinin+excelsin, or excelsin+glycinin.

Table 12. *Gliadin, wheat.*—The anaphylactic relations of gliadin from the wheat and that from the rye kernel offer a special interest, for preparations of these two proteins from each of these seeds are so nearly alike that the only marked difference yet noted is a slight difference in the specific rotation, observed in but a single case, and, therefore, requiring confirmation before it can be accepted as final.

It is interesting to note that our experiments show that sensitizing with preparations from one seed yield as severe symptoms on afterward injecting those from the other seed as when the same preparation was used for each injection. Owing to the similar appearance of rye and wheat and their frequent cultivation in neighboring fields it is possible that the flour from which our preparations were obtained was not entirely free from seeds of the other species. In order to exclude the effect of such contamination the amount of the sensitizing dose was varied widely without giving results which indicated that any contamination existed which was sufficient to affect the results; furthermore, the efficient protection exhibited by animals which had recovered from intoxication with the heterologous gliadins is conclusive proof that we are here dealing with a sensitization with one protein, and not with a mixture of two distinct proteins from wheat and rye respectively.

It is also interesting to find that between gliadin and the somewhat similar alcohol-soluble zein from maize, no reaction occurs.

Table 13. *Hordein.*—Hordein, the alcohol-soluble protein of barley, is similar to gliadin in solubility and the proportion of its products of hydrolysis. The anaphylactic relations of these two proteins have not yet been determined. Like gliadin, hordein gave no reaction with the alcohol-soluble zein nor with the very dissimilar vignin.

Table 14. *Zein.*—The most important fact shown by the results given in this table is the effect of diet on the anaphylaxis reaction. With guinea-pigs which had never been fed with corn a promptly fatal result was obtained in most cases and severe results, with but two exceptions, in the others, whereas those animals that were raised on corn diet gave no reaction whatever. It may be noticed that the toxic dose in the first eight cases was twice that given in all the other experiments here cited, but numerous control experiments show that this large dose of zein does not itself cause noticeable intoxication (see expts. 35 and 36).

c) CLASSIFICATION OF VEGETABLE PROTEINS ACCORDING TO THEIR SPECIFICITY.

On the basis of these experiments we may, provisionally, classify the vegetable proteins investigated according to their biological reactions, as follows. (The protein first named was used for sensitizing, and the second for intoxicating.)

A. SPECIFIC.

Castor-bean globulin+edestin
Squash-seed globulin+flax-seed globulin
" " " +castor-bean globulin
Flax-seed globulin+squash-seed globulin
Edestin+castor-bean globulin
" +squash-seed globulin
Excelsin+vignin
" +glycinin
" +squash-seed globulin
" +castor-bean globulin
Legumin, vetch+glycinin, apparently some protection.
Vignin+legumin, pea
" +glycinin
" +hordein
Glycinin+excelsin
" +vicilin
Gliadin+zein
Zein+gliadin
" +hordein, apparently some protection.
" +castor-bean globulin
Hordein+zein
" +vignin

B. NON-SPECIFIC.

Legumin, pea+legumin, vetch, 3 results fatal, 1 severe, 3 moderate, and 2 doubtful; protection distinct.
" " +vicilin, pea, all results fatal; protection not ascertained.

Legumin, vetch+legumin, pea,	1 result moderate, 1 slight, and 4 doubtful; distinct protection.
" " +vicilin, pea,	3 results fatal, 1 severe, 4 moderate; no protection.
Vicilin+legumin, vetch,	4 results severe, 1 slight, 1 doubtful, 2 none; no pronounced protection.
" +legumin, pea,	3 results fatal, 1 severe; protection not determined.
Vignin+legumin, vetch,	2 results fatal, 1 severe, 1 doubtful, 2 none; no protection.
Gliadin, wheat+gliadin, rye,	4 results fatal, 3 severe, 1 moderate; some protection.
" rye+gliadin, wheat,	2 results fatal, 4 severe, 1 none; distinct protection.

c. DOUBTFUL.

Castor-bean globulin+flax-seed globulin,	2 results fatal, 5 slight, 2 doubtful, 1 none; no protection.
" " " +squash-seed globulin,	4 results doubtful; no protection.
Squash-seed globulin+excelsin,	1 result fatal, 6 moderate, 4 doubtful; no protection.
" " " +edestin,	4 results slight, 4 none; no protection.
Flax-seed globulin+castor-bean globulin,	3 slight, 1 moderate; distinct protection.
Excelsin+edestin,	4 results doubtful; no protection.
Edestin+excelsin,	3 results moderate, 1 slight, 4 none; doubtful protection.
Legumin, pea+vignin,	8 results doubtful; no protection.
" vetch+vignin,	2 results severe, 1 moderate, 1 slight, 4 none; no pronounced protection.
Glycinin+vignin,	3 results severe, 2 moderate, 2 slight, 1 none; no protection.
" +vicilin,	1 result moderate, 3 doubtful; no protection.

d) CONCLUSIONS.

The only positive reactions between proteins from different seeds occurred between the legumins from the pea and vetch, vicilin from the pea and legumin from the vetch, the gliadins from wheat and rye, and vignin and vetch legumin. The reaction between the two latter proteins was rendered doubtful by some of the experiments in which doubtful or negative reactions were obtained and by the absence of any protection. Preparations of legumin from the pea and vetch are very similar, if not identical, as are also those of gliadin from wheat and rye; while legumin and vignin, though much alike both in their properties and the proportion of their decomposition products, are evidently not identical. The reactions between the legumins and vicilin are probably due to an incomplete separation of these two proteins from the other proteins of the seeds.

All of the doubtful reactions were obtained between proteins having very similar properties and yielding similar proportions of decomposition products. However, in several cases the reactions were wholly specific between proteins which were even more nearly alike according to our present knowledge.

It would seem from such data as we have thus far obtained that the chemical constitution of the protein was more concerned in the anaphylaxis reaction than the biological relations of the seeds from which they originate, but more evidence is required before this conclusion can be definitely accepted.

The results obtained with the preparations of legumin from the pea and those from the vetch, and also of vicilin, make it important to determine the effect of extensive fractionation with $(NH_4)_2SO_4$ on the outcome of anaphylaxis experiments.

10. SUMMARY.

Up to the present time nearly all the studies of the biological reactions of proteins have been made with mixtures of proteins such as exist in the fluids which occur naturally in the animal body, or extracts containing soluble constituents of animal or plant tissues. In order to determine the chemical relations of the biological reactions it is fundamentally important to use preparations of proteins of the highest possible purity, and especially those whose composition is known, as far as our analytic measures permit.

Since technical difficulties at present render an exact isolation of animal proteins an almost impossible task, it would seem best to undertake the study of the chemical principles and processes of the biological reactions with preparations of vegetable proteins, many of which can be isolated in a relatively pure condition. This paper gives the results of a preliminary survey of the field.

A review of the literature shows that very few investigations have been made of the biological reactions of vegetable material, and that most of these have been made with substances of indefinite character. There is already sufficient evidence to indicate that the specificity of the anaphylaxis reaction corresponds to that of the other biological reactions; and that this

reaction involves, in all probability, essentially the same sort of reaction to a foreign protein antigen as do the precipitin and complement fixation reactions. Because a large amount of data can be obtained in a comparatively short time, we chose the anaphylaxis reaction for our first orienting experiments with preparations of some of the vegetable proteins, whose chemical composition has already been investigated by analytic methods.

Experiments have been performed with globulin from the castor bean (*Ricinus communis*), flax seed (*Linum usitatissimum*), and squash seed (*Cucurbita maxima*); with edestin from hemp seed (*Cannabis sativa*), excelsin from the Brazil nut (*Bertholletia excelsa*); with proteins from the cocoanut (*Cocos nucifera*), legumin and vicilin from peas (*Pisum sativum*), legumin from the vetch (*Vicia sativa*), vignin from the cow-pea (*Vigna sinensis*), glycinin from soy-bean (*Soja hispida*), gliadin from wheat flour (*Triticum vulgare*) and rye flour (*Secale cereale*), hordein from barley flour (*Hordeum vulgare*), and zein from maize (*Zea mays*).

It has been found that all these proteins cause typical anaphylaxis reactions in sensitized animals, with all features essentially the same as when serum and other animal materials containing soluble proteins are used. The minimum doses which produce sensitization and the time of incubation are about the same as with animal proteins, but as a rule the symptoms are of somewhat slower onset and less stormy course than are those obtained with foreign sera, and the minimum intoxicating doses are larger. There are also considerable differences in the toxicity of the several vegetable proteins to sensitized animals, but the reasons for these differences have not yet been investigated. The most toxic proteins, as measured by the frequency of severe and fatal reactions, were the globulin of the squash seed, vignin, excelsin, and castor-bean globulin, which usually caused death when given in 0.1 gm. doses to properly sensitized animals. Edestin caused the least severe reactions of any of the proteins, while hordein and glycinin seldom caused fatal reactions; nevertheless the minimum sensitizing and intoxicating doses of edestin and squash-seed globulin are essentially the same.

The influence of the food of the guinea-pigs upon the anaphy-

laxis reaction is of particular importance in experiments with vegetable proteins, since the natural food of guinea-pigs is vegetable. Experiments showed that continuous feeding with a vegetable protein rendered guinea-pigs immune to this protein, so that they could not be sensitized to it. Although brief feeding with animal proteins (cow's milk, foreign sera, egg albumin) renders the animal sensitive to the corresponding animal protein, probably sufficiently protracted feeding with animal proteins will likewise confer immunity. The sensitization through feeding is specific for the protein fed, showing that during the processes preceding and including absorption of the food protein, no change takes place which robs it entirely of its biological specificity.

Marked specificity of reaction was shown, within certain limits, by the vegetable proteins employed. The close similarity, if not identity, of the legumins of the pea and vetch was shown by the interreaction of these proteins, and the close relation to vicilin from the pea was also indicated. The near relation or probable identity of the gliadins from wheat and rye was also shown. In some instances doubtful results were obtained, for example with some guinea-pigs, castor-bean globulin and flax-seed globulin interacted strongly, while with others similarly treated no reactions were obtained. The significance of the results of experiments upon specificity, with particular reference to the relation of chemical composition to the specificity of the biological reactions, is discussed.

The results obtained indicate the importance of future investigations along several lines which are now being followed as rapidy and thoroughly as possible.

REFERENCES.

1. KOWARSKI. *Deut. med. Wchnschr.*, 1901, 27, p. 442.
2. JACOBY. *Hofmeister's Beitr.*, 1901, 1, p. 51.
3. SCHÜTZE. *Ztschr. f. Hyg.*, 1901, 38, p. 493.
4. LOEWENSTEIN. *Prag. med. Wchnschr.*, 1901, 26, p. 374.
5. SCHÜTZE. *Deut. med. Wchnschr.*, 1902, 28, p. 804.
6. OTTOLENGHI. *Abst. in Biochem. Centralbl.*, 1903, 1, p. 646.
7. BERTARELLI. *Centralbl. f. Bakt.* (2. Abt.), 1904, 11, pp. 8 and 45.
8. GASIS. *Berl. klin. Wchnschr.*, 1908, 45, p. 358.

9. RELANDER. *Centralbl. f. Bakt.* (2. Abt.), 1908, 20, p. 518.
10. CATASTINI. *Abst. in Centralbl. f. Bakt.* (Ref.), 1907, 40, p. 83.
11. ROSENAU AND ANDERSON. *Jour. Med. Res.*, 1907, 16, p. 381.
12. ———. *Bull. No. 45, Hygienic Lab.*, 1908.
13. FRIEDMANN AND HARTOCH. *Ztschr. f. Immunität* (Orig.), 1909, 3, p. 581.
14. WELLS. *Jour. Infect. Dis.*, 1908, 5, p. 449.
15. WILENKO. *Ztschr. f. Immunität* (Orig.), 1910, 5, p. 91.
16. KRAUS. *Wien. klin. Wchnschr.*, 1901, 14, p. 693.
17. LANDSTEINER AND RAUBITSCHEK. *Centralbl. f. Bakt.* (Orig.), 1908, 45, p. 660.
18. v. EISLER AND v. PORTHEIM. *Ztschr. f. Immunität* (Orig.), 1909, 1, p. 297.
19. DE ANGELIS. *Abst. in Centralbl. f. Bakt.* (Ref.), 1909, 44, p. 530.
20. CAO. *Ann. d'Igiene Sper.*, 1904, 14, p. 83.
21. RAUBITSCHEK. *Wien. klin. Wchnschr.*, 1909, 22, p. 1752.
22. WECHSELMANN. *Deut. med. Wchnschr.*, 1909, 35, p. 1389.
23. BAUER. *Ztschr. exp. Path. u. Ther.*, 1910, 7, p. 417.
24. ROSENAU AND ANDERSON. *Bull. No. 29, Hygienic Lab.*, April, 1906.
25. UHLENHUTH. *Ztschr. f. Hyg. u. Infkr.*, 1897, 26, p. 384.
26. PFEIFFER. *Ztschr. f. Hyg. u. Infkr.*, 1905, 51, p. 183.
27. THOMSEN. *Ztschr. f. Immunität* (Orig.), 1909, 1, p. 741.
28. NEUFELD—quoted by TROMMSDORFF.
29. TROMMSDORFF. *Arb. a. d. kais. Ges.-Amte*, 1909, 32, p. 560.
30. THOMSEN. *Ztschr. f. Immunität* (Orig.), 1909, 3, p. 539.
31. UHLENHUTH AND HAENDEL. *Ztschr. f. Immunität* (Orig.), 1910, 4, p. 761.
32. BESREDKA. *Ann. Inst. Pasteur*, 1909, 23, p. 166.
33. ROSENAU AND ANDERSON. *Jour. Infect. Dis.*, 1908, 4, p. 552.
34. GAY AND SOUTHARD. *Jour. Med. Res.*, 1908, 19, p. 5.
35. DELANOE. *Compt. rend. Acad. Sci.*, 1909, 148, p. 1539.
36. HOLOBUT. *Ztschr. f. Immunität* (Orig.), 1909, 3, p. 639.
37. KRAUS AND AMIRADZIBI. *Ztschr. f. Immunität* (Orig.), 1910, 4, p. 607.
38. ROSENAU AND ANDERSON. *Jour. Med. Res.*, 1908, 19, p. 37.
39. KRAUS, DOERR, AND SOHMS. *Wien. klin. Wchnschr.*, 1908, 21, p. 1084.
40. ANDREJEW. *Arb. a. d. kais. Ges.-Amte*, 1909, 30, p. 450.
41. POZERSKI. *Compt. rend. Soc. Biol.*, 1908, 64, p. 631.
42. PFEIFFER AND MITA. *Ztschr. f. Immunität* (Orig.), 1909, 4, p. 410.
43. PORGES. *Ztschr. f. Immunität* (Orig.), 1909, 2, p. 4.
44. BRÜCK. *Berl. klin. Wchnschr.*, 1907, 44, p. 793.
45. MAGNUS AND FRIEDENTHAL. *Ber. deut. Bot. Gesell.*, 1906, 24, p. 601.
46. ———. *Ibid.*, 1907, 25, p. 242.
47. ———. *Ibid.*, 1907, 25, p. 337.
48. MAGNUS. *Ber. deut. Bot. Gesell.*, 1908, 26a, p. 532.
49. ———. *Landwirtsch. Jahrb.*, 1909, 38 (Erg. Bd. 5), p. 206.
50. DUNBAR. *Ztschr. f. Immunität* (Orig.), 1910, 4, p. 740.
51. MAGNUS AND FRIEDENTHAL. *Ztschr. f. Immunität* (Orig.), 1910, 5, p. 505.
52. UHLENHUTH AND WEIDANZ. *Praktische Anleitung f. Ausführung der biologischen Eiweissdifferenzierungsverfahrens*, 1909.
53. VASCONCELLOS—quoted by ROSENAU AND ANDERSON, *Arch. Int. Med.*, 1909, 3, p. 533.

54. OSBORNE. *Zeit. physiol. chem.*, 1901, 33, p. 240; *Jour. Amer. Chem. Soc.*, 1902, 24, p. 39.
55. MENDEL AND ROCKWOOD. *Amer. Jour. Physiol.*, 1904, 12, p. 336.
56. KARASAWA. *Ztschr. f. Immunität* (Orig.), 1910, 5, p. 509.
57. GAY AND ADLER. *Jour. Med. Res.*, 1908, 18, p. 433.
58. PFEIFFER AND MITA. *Ztschr. f. Immunität* (Orig.), 1910, 6, p. 727.
59. SCHERN. *Arch. f. wiss. u. prakt. Tierheilk.*, 1910, 36, p. 590.
60. WELLS. *Jour. Infect. Dis.*, 1909, 6, p. 506.

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THE TRANSMISSION OF TRYPANOSOMA LEWISI BY RAT FLEAS (CERATOPHYLLUS SP. AND PULEX SP.), WITH SHORT DESCRIPTIONS OF THREE NEW HERPETOMONADS.* †

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After several years of doubt and argument, rigid experiment and accurate observation have brought to definite knowledge the fact that certain invertebrates serve as true intermediary hosts for blood trypanosomes. By the last clause, I do not mean to convey the idea that the trypanosome necessarily passes through a sexual cycle within the invertebrate host. This much has been proved, that it establishes itself in the invertebrate in such a manner as to make possible for an indefinite period its introduction into its vertebrate host by the bite of the intermediate host. In other words, the fact of a cyclical method of transmission has been established. This does not annul the fact that the invertebrate may in addition act as a mere mechanical carrier.

Recent experiments on the tsetse fly and the rat flea have been

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so carefully carried on and with so many positive results, that there can be no doubt that these insects are more than mere mechanical carriers of trypanosomes. In the *Bulletin of the Sleeping Sickness Bureau* (1910), experiments on the tsetse fly are recorded which show beyond doubt that trypanosomes remain infective in the fly for a long time. Minchin and Thomson (1910) have admirably shown by experiment the same to be true of the rat trypanosome in the flea. Those who have read their report must have been impressed with the large majority of positive results obtained under the most rigid control of factors—results which can have no other meaning than that the rat flea acts as an intermediary host for *T. lewisi*. Nuttall (1908) clearly showed that rat fleas and rat lice are capable of transmitting *T. lewisi;* his experiments, however, were not such as would prove whether fleas and lice act as intermediary hosts or merely as mechanical carriers.

Whatever mistakes have been made by confusing trypanosomes with insect flagellates, it is now certain from experiment that the trypanosome does not always degenerate when taken into the stomach of the insect, but on the contrary often remains there for an indefinite period in such condition as to cause infection in vertebrate hosts.

Adding to these experiments the observations of developmental changes which take place in trypanosomes ingested by insect hosts, the evidence in favor of the cyclical method of transmission becomes overwhelmingly convincing. Among the observers who have brought forward strong evidence to this effect are Schaudinn (1904), Prowazek (1905), Minchin (1908), Breinl and Hindle (1909), and Gonder (1910). In addition to their observations, I will show in this paper that *T. lewisi* undergoes a cycle of development in the rat fleas (*Ceratophyllus* sp. and *Pulex* sp.)

Patton has been wisely conservative about accepting the evidence brought forward to show that insects act as true hosts for the transmission of trypanosomes. In various papers, for example, Patton and Strickland (1908) and Patton (1909*a*), he and Strickland have contended that forms described by various investigators as stages in the life history of trypanosomes were really stages of insect flagellates belonging to the genera *Crithidia* and *Herpetomonas*.

Such confusion has undoubtedly occurred on account of ignorance of the life histories and wide distribution of insect flagellates. Their work on these genera has been valuable in throwing doubt on much of the former research, in that it shows the absolute necessity of knowing what natural flagellates are present in the intermediate form which is being investigated as the host or carrier of trypanosomes. Patton's works (1907, 1908, 1909*b*, 1910*a*) and Miss Porter's papers (1909, 1910*a*, 1910*b*) on *Crithidia* and *Herpetomonas* have given such complete knowledge of their life histories that it is now possible to undertake upon sure footing the investigation of the changes which trypanosomes undergo in their intermediate hosts. Patton's criticisms deserve the highest praise for the part they have served in showing the imperative care that must be exercised against drawing unwarranted conclusions.

A complete discussion, in this connection, of the literature giving evidence for and against a cyclical development of trypanosomes within certain invertebrates would make entirely too great a volume. Many excellent works have, therefore, been purposely omitted from this discussion.

In a paper on the rat trypanosome (1907) I described meagerly a few stages of a flagellate from the intestine and rectum of a rat flea, supposing them to be developmental stages of *T. lewisi*. Patton (1909*b*, p. 354) holds that I have described a typical *Herpetomonas*. In a later paper (1909) I accepted Patton's suggestion tentatively. In the present work I will show that Patton was probably correct, a true *Herpetomonas* being present.

The rats of Lincoln, Neb., harbor two fleas (*Ceratophyllus* sp. and *Pulex* sp.).[1] They both contain herpetomonad forms which are so similar that I regard them as one and the same species. To these flagellates I assign the name *Herpetomonas pattoni*, in recognition of Patton's extensive work on insect flagellates. The cycle passed through by the trypanosome is exactly the same in both fleas. Therefore, a separate description of the cycles of the *Her-*

[1] An identification of the species of these fleas was impossible owing to the uncertain knowledge concerning the group. Professor C. F. Baker to whom the fleas were sent informed me that the former is nearest to *C. lucifer* (Roths.), but not that. This form was formerly identified for me as *C. fasciatus*. (See my paper, 1909, p. 1.) The second flea, he informs me, is possibly *P. brasiliensis* (Baker), which may be the same as *P. cheopis* (Roths.) and *P. pallidus*.

petomonas and the trypanosome in the respective fleas will not be necessary.[1]

The flagellates found in the fleas are either pure *Herpetomonas*, pure *T. lewisi*, or a mixture of the two. The following table shows the conditions as they were found to exist.

	No Flagellates	*Herpetomonas* and *Trypanosoma*	*Herpetomonas*	Developing Trypanosomes	Trypanosomes Not Developing
			Ceratophyllus.		
From infected rats...	5	2	1	7	3
From uninfected rats.	23	2	7	2	..
			Pulex.		
From infected rats...	7	3	8	23	..
From uninfected rats.	17	3	25	9	1
			TOTAL PERCENTAGES.		
From infected rats...	20.3	8.5	15.2	51	5.1
From uninfected rats.	45	5.6	36	12.3	1.1

It should be observed that a much higher percentage of fleas taken from uninfected rats than of fleas from infected rats have *no* flagellates; that the percentage of fleas harboring *Herpetomonas* alone is likewise higher from uninfected rats than from infected ones; that, on the other hand, the percentage of fleas possessing trypanosomes alone is much lower from uninfected than from infected rats; and, finally, that the percentage of those harboring a mixed infection is about the same in both cases. These results raise certain questions. Why did 20.3 per cent of the fleas taken from infected rats have *no* flagellates at all? Why did 12.3 per cent of the fleas taken from uninfected rats contain trypanosomes? These questions are satisfactorily answered by the fact that uninfected and infected rats associate intimately, especially when a half-dozen of each kind are caught together in the same trap, as was sometimes the case. Fleas may easily crawl from uninfected to infected rats, and vice versa. Thus we would expect to find

[1] The flea, *Pulex* sp., harbors a gregarine. The only forms observed were the late stages of cyst formation. They are found in the lumen of the stomach, as many as 24 in number. About 40 per cent of the fleas were found to be infected. Spores are formed within the cysts in great numbers. Within the spores eight spindle-shaped sporozoites develop. The reason why no other stages were seen lies in the probable fact that the earlier stages develop in fleas younger than any examined. Ross (1909) states that the early stages of the gregarine of the dog flea were found only in very young fleas.

about such conditions as are represented by the given percentages. There are two other factors which will help to explain these percentages. It is possible that some of the rats called uninfected were really infected, but so lightly that the parasites were overlooked; and, secondly, that some might have recently harbored trypanosomes, but had recovered at the time of examination. The fact that Strickland (1909) found, out of 45 fleas taken from 12 rats infected with trypanosomes, only two harboring *T. lewisi*, may be partially explained by the possibility that many of them had recently crawled from uninfected rats on to infected ones, but had not yet bitten the latter. There must, however, have been other reasons, which I will mention later.

DEVELOPMENT OF T. LEWISI IN THE FLEA.

The digestive tract of fleas was carefully dissected out in sodium citrate or normal salt solution and different regions of it examined separately. Permanent preparations were made by teasing the portion containing flagellates in a small drop of sodium citrate or normal salt solution and then inverting the mixture over the mouth of a bottle containing 2 per cent osmic acid for about ten seconds. After drying, the preparations were treated with absolute alcohol and stained with Giemsa's stain or iron hematoxylin. The drying may have destroyed the finer cytological structure, but, comparing with Breinl and Hindle's results from wet methods used on *T. lewisi* in the louse, no structures important in a study on the life cycle have been destroyed.

The trypanosome is taken into the stomach of the flea, where it remains but a short time. Even before the blood has completely lost its structure under the influence of digestion, it is oftentimes impossible to find any trypanosomes. They, however, do not die off. They migrate posteriad into the intestine, where important changes take place. This fact may explain Strickland's failure to find trypanosomes. If he was not careful to tease out the tiny intestine and rectum, he would in most cases have missed the trypanosomes, although they might have been present. In the anterior end of the intestine just posterior to the malpighian tubes are found the most typical, unchanged trypanosomes.

Generally a few are present which conform in every detail to the structure of *T. lewisi* in the blood of the rat (Pl. 1, Figs. 1 and 2). These undergo a series of modifications and divisions in the intestine. The first change to be seen is a diminution in the size which progresses until the parasite becomes quite small, measuring about 10 μ long by 2 μ broad (Pl. 1, Figs. 3–6). As the flagellate becomes smaller the nucleus moves toward the posterior end (Pl. 1, Figs. 4–6). The portion of the body posterior to the blepharoplast becomes absorbed so that the blepharoplast comes to lie clear at the posterior end even against the wall, where it becomes large and rounded and stains densely with Giemsa's stain (Pl. 1, Figs. 5–7). The trypanosome now resembles very closely *T. vespertilionis* in the blood of the bat, described by Gonder (1910). In these forms I have sometimes seen the flagellum ending in a small granule near the blepharoplast. Occasionally these forms degenerate. In such cases a little cytoplasm and a flagellum attached to the blepharoplast are all that remains (Pl. 1, Fig. 8). In most forms which do not degenerate the blepharoplast now moves forward and may be found just posterior to, at the side of, or anterior to, the nucleus. As this takes place the undulating membrane, which has thus far been very prominent, may disappear (Pl. 1, Figs. 9–12). A karyosome is present in the nucleus. Such forms as are represented by Fig. 12 are exactly like adult *Crithidia*. Similar crithidia-like forms have been represented by Gonder as stages in the development of *T. vespertilionis* in the digestive tract of the bat mite, *Leiognathus arcuatus*, and by Minchin (1908) as stages in the development of *T. grayi*. Minchin and Thomson (1910) state that the rat trypanosome changes into a crithidia-like form in the flea.

Division may take place in these forms. The process is similar to division in *Crithidia* (Pl. 1, Figs. 13, 14). Trypanosomes in this stage are often seen attached by their flagellar ends to the intestinal wall, and in this respect simulate *Crithidia* very closely.

Another explanation for Strickland's failure to find trypanosomes in many fleas, even though taken from infected rats, may be found in the fact that the trypanosome changes into a true crithidia-like form. Strickland (1909) states that he discovered *Crithidia* in the

intestine of fleas. His words are: "In 8 fleas (*Ctenophthalmus agyrtes*), from 5 different rats infected with *T. lewisi*, I found *Crithidia ctenophthalmi*." It should be noted that these fleas all came from rats infected with trypanosomes, which fact strongly suggests that his *Crithidia* were really transformed trypanosomes. Patton and Strickland (1908, p. 334) give several figures of *C. ctenophthalmi*, the first four of which are almost identical with my Figs. 7, 9, 10, 11, and 12, which I believe represent true trypanosomes. I have never found any true *Crithidia* present.

The other individuals of the type represented by Figs. 5, 6, and 7, which do not change into the *Crithidia* type, curl upon themselves to form an oval rounded mass. The anterior end with its projecting flagellum is lapped back against the posterior end containing the blepharoplast (Pl. 1, Figs. 15, 16). The contiguous edges fuse and the oblong nucleus which was in the middle of the body now lies opposite the blepharoplast in the larger end of the mass. The flagellum encircles the body. Breinl and Hindle (1909, Figs. 34–36) described a similar form, but were unable to determine whether it developed by the coiling up of a normal trypanosome or in some other manner. I never observed any further change in these forms other than is shown in Fig. 17. It is probable that the flagellum is lost and a rounded cyst results, something like Figs. 18 and 19, the latter being similar to a cyst described by Prowazek (1905, Pl. 3, Fig. 52).

Development of the crithidia-like forms may proceed along two separate lines which come to the same end. Like true *Crithidia*, individuals may agglutinate by the anterior ends, forming rosettes, or they may form cysts directly without agglutination. In the first case the anterior ends gradually become thickened and more rigid, the free flagellum and membrane having been lost (Pl. 1, Figs. 20, 21). In the latter figure the form designated "*a*" is the most completely encysted. It is marked by a strong, apparently stiff, ridge beginning near the posterior margin and passing diagonally across the anterior half of the parasite. The form represented by "*a*" in Fig. 20 resembles very closely herpetomonad stages which are described later (Pl. 3, Figs. 50, 51). The rosettes may become much larger than those figured. They show a marked

resemblance to rosettes from the rat louse, figured by Prowazek (1905, Pl. 3, Figs. 53, 54). The bodies of these forms are not rigid like those of *Crithidia* and *Herpetomonas* rosettes, from which they can be distinguished by their soft flexible appearance. In one flea a rosette of trypanosomes, some with their flagella centrally directed, others with them peripherally located, was found in the stomach.

The direct change of crithidia-like forms into solitary cysts is more common. Unlike the case of *Crithidia* and *Herpetomonas*, solitary cysts are almost exclusively found. In fact, this character is so striking that one can be pretty sure whether he is dealing with the trypanosome, without further observation. The anterior end of a crithidia-like form becomes thickened and compressed toward the posterior end, at the same time often showing a peculiar plastic twist (Pl. 1, Figs. 22–24). Thus the whole anterior end, including membrane, if present, and flagellum, becomes plastic and rolled together. The flagellum often remains in the anterior end as an irregular mass, staining slightly with Giemsa's stain (Pl. 2, Fig. 25). On the edge of the body generally opposite the flagellum and blepharoplast there soon appears a heavy ridge which is somewhat curved and raised so that the cyst becomes more or less triangular in cross-section (Pl. 2, Figs. 26, 27). In many cases another lighter line crossing the heavier one at the anterior end may appear (Pl. 2, Figs. 25 and 28). When seen from the side these cysts somewhat resembling kernels of buckwheat have quite a different appearance. They look like asymmetrical bowls with rounded bottoms, the heavy raised edge being the bottom of the bowl, as can be seen in profile when they roll over (Pl. 2, Figs. 29, 30). These forms measure about 5 μ long by 2 μ wide at the anterior end. The posterior end is always acute. During the formation of these cysts longitudinal division is frequent (Pl. 2, Fig. 31). But, different from the case of *Herpetomonas* and *Crithidia*, when division is complete the two daughter cells separate so that solitary cysts result. Division is a factor in the reduction of size.

The further changes were hard to determine. It is probable, however, that some at least of this type round off to form oval

or round cysts (Pl. 1, Figs. 18, 19). In one flea infected with trypanosomes only, a mass of round forms was found, each containing a single chromatin element (Pl. 2, Fig. 32). The lines of separation between the individual bodies were very faint, and in some places not recognizable.

What becomes of these cysts I am unable to say. Many of them must be passed out with the feces, and may, therefore, be taken up by other fleas or by rats. Cysts ingested by rats might pass through the membrane of the digestive tract and enter the blood stream.

A sexual process in trypanosomes has been described by various authors and discredited by others. No one has yet seen an unquestionable case of conjugation of male and female. Yet figures very suggestive of such a process have been published. Before much change in the size or form of *T. lewisi* has taken place in the rat flea, it is possible that conjugation occurs. In one instance a stage very suggestive of such a process was found (Pl. 2, Fig. 33). The specimen was very well stained, and has been accurately represented. It is possible that the posterior end of one flagellate merely underlies the other. However, there are two significant features which should be noted. The posterior end of the larger female (?), unlike all other adult forms found in the flea, is a large rounded mass twisted half-way round. It contains a large round blepharoplast which is apparently connected with a similar blepharoplast in the smaller male (?) form, by two delicate threads. I believe the anterior end of the smaller individual has no connection with the larger form, but merely underlies its edge. This specimen is very similar to Prowazek's conjugating forms in the case of the rat louse. (See his Pl. 3, Fig. 38, 1905.) No other signs of a sexual development like that described by him could be found.

Of great importance is the question regarding the migration of the trypanosome to the region of the flea's proboscis, whence it may be introduced into fresh rats by the bite of the flea. Minchin and Thomson (1910) have experimentally shown that the trypanosome establishes itself in the flea, where it remains for an indefinite period of time capable of being transmitted to rats by the flea's

bite. They further state, as I have also shown, that the trypanosome establishes itself in the flea by the "multiplication of crithidia-like forms in the rectum." This multiplication begins in the anterior part of the intestine and passes backward to the rectum.

Search was made for trypanosomes passing through the wall of the intestine and rectum. No sign of such a migration was ever observed. Yet Miss Porter (1910*b*) actually saw *Crithidia melophagia* pass through the intestinal wall of the sheep-tick. It is therefore probable that the trypanosome, after it has become a *Crithidia* in structure, passes through the wall and makes its way to the region of the proboscis. I found in a section of the viscera two crithidia-like forms just outside the intestinal wall.

The heads, including the probosces, of several fleas were ground up in salt solution. After smearing and staining, forms as shown in Fig. 34 were sometimes found. These bodies having but one chromatin mass are quite similar to the small oval forms found by Miss Porter in the puparia of the sheep-tick (see her Figs. 79 and 85). They might be forms homologous with the "latent bodies" described by Salvin-Moore, Breinl and Hindle (1908) as a stage in the development of *T. gambiense* and *T. lewisi* in the blood of the rat. If these bodies, which I found in the heads of fleas, are real trypanosomes, doubtless they would, upon being introduced into a fresh rat, like the "latent bodies," develop into typical trypanosomes.

I believe that it is very improbable that the trypanosome makes its way forward from the intestine to the proboscis through the lumen of the digestive tract, having found no evidence of such a path. More extended observations and careful technic will be necessary to reveal the facts of the case.

HERPETOMONAS OF THE RAT FLEAS (CERATOPHYLLUS SP. AND PULEX SP.).

About one-third of all fleas examined were found to be infected with a true *Herpetomonas*. Unlike the transitional forms between the trypanosome and crithidia-like forms, there were no intermediate forms to be found between the trypanosome and the *Herpetomonas*. There can be no doubt, as my figures plainly indicate, that the flea harbors a true *Herpetomonas*.

According to Patton, *Herpetomonas* has three stages in its life history: preflagellate, flagellate, and postflagellate. Working from this point of view, he and Miss Porter have been successful in determining the complete life cycles of several insect flagellates. The preflagellate stage is passed in the anterior portion of the alimentary tract, the flagellates as they develop passing backward and encysting in the rectum. For example, in *H. lygaei*, the preflagellate stage is found in the crop, the flagellate stage farther back, and the postflagellate in the rectum. In the case of the fleas I have made a careful examination of the whole digestive tract back to the malpighian tubes without finding any stage of the herpetomonad cycle, except in two cases where it was probable that forms from the intestine or rectum were accidentally introduced in the preparations during the dissections. These negative results lead me to believe that all three phases of life cycle are confined to the intestine and rectum, i.e., the portion of the digestive tract posterior to the malpighian tubes. In specimens where only a few parasites were found, forms evidently belonging to the preflagellate stage were present.

Preflagellate stage.—The infection probably begins in the anterior part of the intestine with round or oval forms which are ingested cysts only slightly modified. This form enlarges, its cytoplasm becoming more granular (Pl. 2, Fig. 35). At the same time the nucleus enlarges, while its chromatin becomes loose. A flagellum may originate directly (Pl. 2, Figs. 36, 37), or division take place, flagella forming later (Pl. 2, Figs. 38, 39). If division proceeds without a separation of the daughter cells, rosettes are produced (Pl. 2, Fig. 40). The individuals gradually elongate and develop long flagella. Some are more rapid in this process, the result being rosettes made up of young oval forms and typical adults (Pl. 2, Figs. 41, 42). All the younger forms are characterized by a granular cytoplasm and large loose nuclei.

Flagellate stage.—This stage is passed principally in the intestine, although adult flagellates are occasionally seen in the rectum. The adult flagellate (Pl. 2, Figs. 43, 44) is a typical *Herpetomonas* with the blepharoplast near the anterior end and the nucleus about the middle of the body. Like other herpetomonad forms, the

flagellum is inserted in the anterior end, no undulating membrane being present. Its path to the blepharoplast is a clear area as in other herpetomonads. The body may be long and slender (Pl. 2, Fig. 43), more or less spindle-shaped with a pointed posterior end (Pl. 3, Fig. 45), or oval with blunt posterior end (Pl. 3, Fig. 46). The elongated slender forms often show a twisting of the body, as is common in other insect flagellates (Pl. 2, Figs. 41 and 44). In some of the small individuals the posterior end is sometimes notched (Pl. 3, Fig. 47). The nucleus, round or oval, contains a central karyosome. The blepharoplast is rod-shaped, and generally lies transversely in the anterior end of the body. It may, however, pass back near the nucleus as in *Crithidia*. These forms measure, flagellum included, about 20 μ long by 1.5 μ to 3 μ thick. The size, however, varies greatly with the shape of the body. The flagellum may be longer or shorter than the body.

In fresh preparations the adult *Herpetomonas* is easily distinguishable from the adult trypanosome. The former has a rigid body and moves by means of the flagellum alone, while the latter has a very soft, flexible body which exhibits writhing, wormlike movements.

Breinl and Hindle (1909) have described herpetomonas-like forms as stages in the trypanosome cycle within the louse. Some of their figures are more crithidia-like than herpetomonas-like. Others are distinctly herpetomonas-like. They recognized the possibility of a mixed infection of *Herpetomonas* and *Trypanosoma*, but came to no definite conclusion. However, their herpetomonas-like figures, and the fact that fleas harbor a true *Herpetomonas*, strongly suggest that the louse likewise may be infected with a true *Herpetomonas*.

Postflagellate stage.—The cyst formation is very similar to that described for *H. lygaei* by Patton (1908). The adult forms may attach to the walls of the intestine and rectum and undergo encystment or remain free in the tract, where they form large rosettes similar to those described by me in an earlier paper (1907; see Figs. 13 and 14). Encystment is marked by loss of flagellum and a reduction in size due to repeated divisions without much growth (Pl. 3, Figs. 48, 49). The individuals composing rosettes and lining the walls of the tract become rigid and spindle-shaped with

sharp posterior ends. In the earlier stages they also develop thin, clear, winglike sides, while a central area running longitudinally remains thick and dense (Pl. 3, Figs. 50, 51). This area stains much more intensely than the sides. Occasionally, however, a portion of this area where the path of the flagellum lies is marked by a clear area (Pl. 3, Figs. 51, 52). If the forms tend to separate when division takes place so that large rosettes are not formed, the parasites are found either singly, in couplets, triplets, or quadruplets (Pl. 3, Figs. 53–56). In groups of twos, threes, or fours, the central anterior ends become very broad, so that triangular forms result. These forms later round off to produce oval or round cysts (Figs. 53, 54). Other quite common types of encysting forms are represented by additional Figs. 57 and 58. In those represented by the former figure, the path of the flagellum is marked by a clear area, on either side of which is a dark, dense band. In some cases the blepharoplast is evidently extruded (Pl. 3, Figs. 59, 60). It is not at all uncommon to find small round forms with only one chromatin body. In a few instances forms were seen with very heavy walls, staining red with Giemsa's stain (Pl. 3, Fig. 61). The final result of encystment appears normally to be a small round form containing blepharoplast and nucleus, and measuring about 2 μ to 3 μ in diameter (Pl. 3, Fig. 53). The whole intestine and rectum are sometimes packed full of such forms almost exclusively.

No observations were made to determine how this flagellate is transmitted from flea to flea. Miss Porter (1910*b*) has shown by careful observations that *C. melophagia* may be transmitted from one flea to another by the ingestion of cysts from the feces or through the egg from parent to offspring. Since the sheep-tick, a blood-sucking insect, is accustomed to ingesting feces, it is probable that the flea also becomes contaminated in the same manner.

TWO NEW HERPETOMONADS FROM FLIES (CALLIPHORA COLORADENSIS [HOUGH] AND SARCOPHAGA SARRACENIAE [RILEY]).

I wish in this connection to give only short preliminary descriptions of some of the stages of these flagellates. To the flagellate of the first fly I assign the name *Herpetomonas calliphorae*, and to that of the second, *Herpetomonas lineata*.

Herpetomonas calliphorae.—The adults of this flagellate occur in the stomach, or mid-gut, of the fly. They measure, flagellum included, about 30 μ in length by 2 μ in diameter. The body is long and slender, with tapering posterior and anterior ends (Pl. 3, Fig. 62). The nucleus is elongated and lies posterior to the center of the body. It contains many chromatin granules. The cytoplasm is finely granular and contains chromatoid granules. The blepharoplast is generally oval or round, especially preceding division. Longitudinal division is preceded by a thickening of the body. The blepharoplast enlarges, becomes rounded, and divides. The flagella apparently split (Pl. 3, Figs. 63, 64).

Encystment occurs in the ordinary manner. Forms approaching encystment become shortened and thickened (Pl. 3, Fig. 65). Their size is reduced by division (Pl. 3, Fig. 66). The flagellum is lost and small triangular forms with sharp posterior ends are produced (Pl. 4, Fig. 67). Oftentimes these develop a strong darkly staining ridge along one side (Pl. 4, Fig. 68). In this respect they slightly resemble a similar stage in the trypanosome cycle (Pl. 2, Fig. 27). But in the trypanosome the blepharoplast tends to remain nearer the posterior end. In the *Herpetomonas* a clear area marking the path of the flagellum is generally visible. Encystment finally results in the formation of round or oval forms measuring 2 μ to 3 μ in diameter.

The most interesting feature about this *Herpetomonas* is the presence of individuals of the trypanosome type. In them a large round blepharoplast lies against the wall in the rounded posterior end, while the flagellum passes forward along the body and projects a short distance beyond the attenuated anterior end. The body gently tapers with soft curves from the posterior to the anterior end (Pl. 4, Figs. 69–72). The side of the body along which the flagellum runs is often thin and gives the appearance of an undulating membrane (Figs. 70 and 72). These forms measure about 15 μ to 20 μ long. I was unable to find any transitional stages between them and the ordinary herpetomonad type. They resemble very closely one stage of *Rhynchomonas luciliae* figured by Patton (1910; see Fig. 6). But in his form the flagellum never projects beyond the body. Forms similar to his other figures

were not found. Nevertheless, these forms may be *Rhynchomonas* and have no connection with the *Herpetomonas* of the fly.

Herpetomonas lineata.—The forms that I studied were obtained from the digestive tract posterior to the malpighian tubes. The adults are characterized by their extremely long and threadlike tails. The anterior end forms an oval blunt head which tapers posteriorly into a long thread. This thread may be no thicker than a large flagellum, but differs from it in that it is a cytoplasmic projection and is more rigid. It is dragged behind and is capable of slight wavy motions. It may be almost straight and as smooth as a flagellum, or granular and angular (Pl. 4, Fig. 73). In the living condition it is generally perfectly straight or slightly wavy. These forms strongly resemble the attenuated adults of *H. muscae domesticae* and *H. sarcophagae* figured by Prowazek (1905). He gives the length of the former as 30 μ to 50 μ, and says that the latter is very similar. In *H. lineata* these adults reach the astonishing length of 385 μ without flagellum, which is always absent in the longest forms. Many measure over 200 μ. The head generally measures about 2 μ to 3 μ in diameter. The blepharoplast and nucleus are always very plain in the head region. The former is generally rounded, except during division, when it becomes rod-shaped. These extremely long forms are often found in process of division (Pl. 4, Fig. 74). The line of separation begins at the anterior end and evidently passes even out to the tip of the threadlike tail. One extremely large form was found dividing (Pl. 4, Fig. 75). I believe the tail of this one had been broken off. Such cases were common.

These long forms tend strongly toward agglutination. Their flagella become rolled up and contracted in a round mass at the anterior end of the body. This mass stains a deep red with Giemsa's stain (Pl. 4, Figs. 73 and 76). As many as fifty have been seen agglutinated by their anterior ends in perfectly symmetrical rosettes.

From these long forms with short flagella, or none at all, there are gradations all the way down to short spindle-shaped forms with long flagella (Pl. 4, Figs. 77, 78). Intermediate with respect to size is a form which resembles the trypanosome and *Crithidia*.

The blepharoplast carrying the flagellum with it moves clear behind the nucleus, which becomes very long and narrow (Pl. 4, Fig. 79). The body of the flagellate is always very slender and sickle-shaped, the posterior end giving the appearance of a sharp beak. A complete series of gradations from the type illustrated by Fig. 77 to this type is to be found. In Fig. 80 an intermediate stage is represented. This form resembles a *Crithidia*. The flagella of all these forms are always longer than the body. They may all be found agglutinated in rosettes with the long-tailed forms.

Here we have a case in which a true herpetomonad form passes over into the likeness of a trypanosome. The only difference is the lack of an undulating membrane, which, however, is absent in some stages of the trypanosome cycle. Were these forms extremely rare, they would have very little significance. On the contrary, they are as numerous as the other types.

DISCUSSION.

It is important to notice the great similarity between forms represented by Figs. 69 to 72, and the stage in the life cycle of *T. lewisi* represented by Figs. 5 to 7. If the former are herpetomonad forms, then the commonest distinctions, such as position of blepharoplast and presence of membrane, between the two genera break down. Transitions between the two genera are complete. On the other hand, if the former belong to the genus *Rynchomonas*, then the distinctions between this genus and *Trypanosoma* disappear. It might be urged that the forms represented by Figs. 5 to 7 do not belong to the trypanosome cycle. But they must be trypanosomes, because they never occur alone, being always associated with the other stages of the trypanosome cycle, and are connected with them by minute transitions. Someone might suggest that the forms from the fly are trypanosomes mixed in with the herpetomonad forms. This is not probable, because it is hard to conceive how a non-biting fly could become contaminated with trypanosomes.

In this investigation some important connections between the blood trypanosomes and insect flagellates have been brought out.

The evidence is ample that trypanosomes change over into true crithidia-like forms when taken up by insects acting as hosts. Some, it is true, will claim that we are confusing the two genera, and that these crithidia-like forms are natural flagellates of the insect. However, the gradations from a true trypanosome into a true *Crithidia* even in the same rosette are so perfect that it cannot be denied that these crithidia-like forms represent a stage in the trypanosome cycle (Pl. 1, Figs. 20, 21). Moreover, the crithidia-like forms are never found except in association with the typical trypanosome stages like Figs. 2 and 5. Furthermore, the trypanosome, in contrast to its behavior in the blood of the vertebrate, agglutinates in the insect host by its *anterior* end as is the case with *Crithidia*.

Thus we have forms making a perfect transition from the genus *Trypanosoma* to the genus *Crithidia*, thereby breaking down the distinctions between the two genera. The natural *Crithidia* of insects, such as *C. melophagia*, are not known to be connected with any blood trypanosome.[1] But the changing of a blood trypanosome over into a true *Crithidia*, when taken up by an insect host, signifies that the natural *Crithidia* of insects are the more primitive, and that the trypanosomes are merely insect *Crithidia* which have been successfully introduced into the blood stream of vertebrates. Likewise the Leishman-Donovan bodies represent natural herpetomonad forms which have been successfully introduced into vertebrate hosts. It has been shown that these bodies when sucked up by their invertebrate host return in their life cycle to the true herpetomonad type.

The facts given in this paper show that a very close connection exists between the four genera, *Herpetomonas*, *Rynchomonas*, *Crithidia*, and *Trypanosoma*. I do not believe it is wise to reduce them to a single genus, although distinctions between them do break down at various points. There are still great differences between them when their whole life cycles are considered.

[1] Since this article was sent to press, a paper by Woodcock (1910) on Avian Haemoprotozoa has been published. On p. 713 he states that he has discovered a true trypanosome in the blood of a sheep which was harboring sheep-ticks infected with *C. melophagia*. He says: "There can be little or no doubt that the *Crithidia melophagia* is simply a developmental phase of this sheep-trypanosome in its alternate, insectan host." While there is considerable evidence favoring his conclusion, it seems to me that there is still a possibility that *C. melophagia* is a true insect flagellate which has never been successfully introduced into the sheep's blood, and that the trypanosome which he discovered is an entirely distinct form.

It gives me pleasure to acknowledge my indebtedness to Professor Robert H. Wolcott, head of the Department of Zoölogy of the University of Nebraska, through whom the facilities of that laboratory were placed at my disposal for the carrying out of this investigation. I wish further to thank him for the personal interest he has shown and for the suggestions he has offered in the preparation of the manuscript. To Professor Myron H. Swenk of the Department of Entomology and to Professor C. F. Baker of Pomona College, I am indebted for the identification of the fleas. I am also under obligations to Mr. B. W. Coquillet of the United States National Museum, for his service in identifying the flies.

NOTE.

Since this article was sent to the publisher I have received a paper from Swellengrebel and Strickland (1910) on the rat trypanosome. In general the flagellates figured by them from rat fleas are identical with my forms. There are, however, two notable differences; they have not figured any typical adult herpetomonads nor any cysts like those shown in Plate I, Figs. 22–24, and Plate II, Figs. 25–30. A comparison of our papers will reveal the fact that the forms which I have considered to be true herpetomonads are considered by them to be stages in the trypanosome development. On account of this fact their arrangement of the stages of development is quite different from mine. They state that 4 per cent of their control fleas, which were fed upon an uninfected rat, developed flagellates. Thus, their experiment does not preclude the possibility of an infection with a natural herpetomonad. I am still of the opinion that such a form was harbored by the fleas which I investigated. Further experiment is needed to decide this question.

LITERATURE CITED.

BREINL, A., AND HINDLE, E. 1909. "Observations on the Life History of *Trypanosoma lewisi* in the Rat Louse (*Haemotopinus spinulosis*)," *Ann. Trop. Med. and Parasit.*, 3, pp. 553–64.

BRUCE, D. B., HAMERTON, A. E., BATEMAN, H. R., AND MACKIE, F. P. 1910. "Trypanosomes and Tsetse Flies," *Bull. Sleeping Sickness Bureau*, 2, pp. 155–63.

GONDER, R. 1910. "*Trypanosoma vespertilionis* (Battaglia)," *Centralbl. f. Bakteriol.*, 53, pp. 293–302.

MINCHIN, E. A. 1908. "Investigations on the Development of Trypanosomes in Tsetse Flies and Other Diptera," *Quart. Jour. Mic. Sci.*, 52, pp. 159–260.

MINCHIN, E. A., AND THOMSON, J. D. 1910. "The Transmission of *Trypanosoma lewisi* by the Rat-Flea (*Ceratophyllus fasciatus*)" (Preliminary Communication), *Proc. Roy. Soc.*, 82, pp. 273–85.

NUTTALL, G. H. F. 1908. "The Transmission of *Trypanosoma lewisi* by Fleas and Lice," *Parasitology*, 1, pp. 296–301.

PATTON, W. S. 1907. "Insect Flagellates," *Annual Report . . . . King Inst. Prevent. Med.*,[1] Guindy, Madras, pp. 10–12.

———. 1908. "*Herpetomonas lygaei*," *Arch. f. Protistk.*, 13, pp. 1–18.

———. 1909*a*. "A Critical Review of Our Present Knowledge of the Haemoflagellates and Allied Forms," *Parasitology*, 2, pp. 91–143.

———. 1909*b*. "The Life Cycle of a Species of *Crithidia* Parasitic in the Intestinal Tracts of *Tabanus hilarius* and *Tabanus* sp.," *Arch. f. Protistk.*, 15, pp. 333–62.

———. 1910*a*. "Experimental Infection of the Madras Bazaar Fly, *Musca nebula* Forb., with *Herpetomonas muscae domesticae* (Burnett)," *Bull. Soc. Path. Exotique*, 3, pp. 264–73.

———. 1910*b*. "*Rynchomonas luciliae*, nov. gen., nov. spec. A New Flagellate Parasitic in the Malpighian Tubes of *Lucilia serenissina* (Walk.)," *ibid.*, 3, pp. 300–303.

PATTON, W. S., AND STRICKLAND, C. 1908. "A Critical Review of the Relation of Blood-sucking Invertebrates to the Life Cycles of the Trypanosomes of Vertebrates, with a Note on the Occurrence of a Species of *Crithidia, C. ctenophthalmi*, in the Alimentary Tract of *Ctenophthalmus agyrtes* (Heller)," *Parasitology*, 1, pp. 322–46.

PORTER, ANNIE. 1909. "The Life-Cycle of *Herpetomonas jaculum* (Léger), Parasitic in the Alimentary Tract of *Nepa cinerea*," *Parasitology*, 2, pp. 367–91.

———. 1910*a*. "The Morphology and Life-History of *Crithidia gerridis*, as Found in the British Water-Bug, *Gerris paludum*," *ibid.*, 2, pp. 348–66.

———. 1910*b*. "The Structure and Life-History of *Crithidia melophagia* (Flu.), an Endo-Parasite of the Sheep-Ked, *Melophagus ovinus*," *Quart. Jour. Micr. Sci.*, 55, pp. 189–224.

PROWAZEK, S. 1904. "Die Entwicklung von *Herpetomonas*, einen mit den Trypanosomen verwandten Flagellaten" (Vorläufige Mitteilung), *Arb. a. d. kais. Gesundheitsamte*, 20, pp. 440–52.

———. 1905. "Studien über Säugetier-Trypanosomen," *ibid.*, 22, pp. 351–95.

ROSS, E. H. 1909. "A Gregarine Parasitic in the Dog-Flea, *Ctenocephalus serraticeps*," *Ann. Trop. Med. and Parasit.*, 2, pp. 359–63.

SALVIN-MOORE, J. E., BREINL, A., AND HINDLE, E. 1908. "The Life History of *Trypanosoma lewisi*," *Ann. Trop. Med. and Parasit.*, 2, pp. 197–220.

SCHAUDINN, F. 1904. "Generations- und Wirtswechsel bei *Trypanosoma* und *Spirochaete*" (Vorl. Mitteil.). *Arb. a. d. kais. Gesundheitsamte*, 20, pp. 387–439.

STRICKLAND, C. 1909. "On the Supposed Development of *Trypanosoma lewisi* in Lice and Fleas, and the Occurrence of *Crithidia ctenophthalmi* in Fleas," *Parasitology*, 2, pp. 81–90.

SWELLENGREBEL, N. H., AND STRICKLAND, C. 1910. "The Development of *Trypanosoma lewisi* outside the Vertebrate Host," *Parasitology*, 3, p. 360.

SWINGLE, L. D. 1907. "Some Studies on *Trypanosoma lewisi*," *Trans. Amer. Micr. Soc.*, 27, pp. 111–21.

———. 1909. "A Study on the Life History of a Flagellate (*Crithidia melophagi*, n. sp.) in the Alimentary Tract of the Sheep-Tick (*Melophagus ovinus*)," *Jour. Infect. Dis.*, 6, pp. 98–121.

WOODCOCK, H. M. 1910. "Studies on Avian Haemoprotozoa. I, On Certain Parasites of the Chaffinch (*Fringilla coelebs*) and the Redpoll (*Linota rufescens*)," *Quart. Jour. Mic. Sci.*, 55, p. 641.

[1] Not seen by me.

EXPLANATION OF PLATES.

PLATE 1.

All figures are magnified 2,866 diameters.

FIG. 1.—Adult *Trypanosoma lewisi* from the blood of rat.

FIG. 2.—Adult *T. lewisi* from the intestine of the rat flea.

FIGS. 3–7.—Trypanosomes from intestines of fleas showing the successive changes undergone. The blepharoplast and nuclei move together, while the posterior end contracts so that the blepharoplast comes to lie against the posterior wall.

FIG. 8.—Degenerating trypanosome from the intestine of a flea.

FIGS. 9–12.—Crithidia-like trypanosomes from the intestines of fleas. In Fig. 9 the undulating membrane is pronounced; in Fig. 10 it has almost disappeared; while in Figs. 11 and 12 it is entirely absent, the true crithidia form being assumed.

FIG. 13.—Trypanosome from the intestine of the flea, in process of division. The cytoplasm is not yet divided.

FIG. 14.—A later stage in division of the trypanosome in the intestine of the flea. The cytoplasm is more than half divided.

FIG. 15.—Trypanosome from intestine of flea. The body is bent back upon itself.

FIG. 16.—A later stage than is shown in Fig. 15. The contiguous edges have fused so that an oval mass bordered by the flagellum results.

FIG. 17.—The next stage in the rounding-off process of the trypanosome.

FIGS. 18 and 19.—Probably represent the final result of the rounding-off process. In the latter figure the blepharoplast has disappeared.

FIGS. 20 and 21.—Agglutinated trypanosomes from the intestine of the flea. Note the close resemblance to *Crithidia* rosettes.

FIGS. 22–24.—Three successive stages in the formation of solitary cysts from such crithidia-like forms as represented by Fig. 10. In Fig. 24 a diagonal ridge is shown. From intestine and rectum of fleas.

PLATE 2.

All figures, except 40 and 42, are magnified 2,866 times.

FIGS. 25–28.—Other types of solitary cysts formed from trypanosomes. The broad ends are anterior. In Figs. 25 and 28 two ridges crossing each other at the anterior end are shown. In Figs. 25–27 note the blepharoplast pressing the wall out to form a spine.

FIGS. 29 and 30.—Side views of solitary cysts similar to those represented by Figs. 25–28. The upper ends are anterior.

FIG. 31.—Division of trypanosome preceding the formation of cysts. From the intestine of flea.

FIG. 32.—Probably a mass of trypanosome cysts. The lines of separation between the individuals are barely visible.

FIG. 33.—Possibly male and female trypanosomes in process of conjugation. From intestine of flea.

FIG. 34.—A form found in crushed head of flea infected with trypanosomes.

FIG. 35.—Young stage of *Herpetomonas pattoni*, the flagellum not having yet formed. From intestine of flea.

FIGS. 36 and 37.—Little later stages of *H. pattoni* in which flagella have developed. From intestine of flea.

FIGS. 38 and 39.—Showing division of young *H. pattoni* and the formation of flagella. From intestine of flea.

FIG. 40.—Rosette of young herpetomonads formed by the division of such a form as Fig. 38. ×2,400.

FIGS. 41 and 42.—Rosettes of herpetomonads, the flagellated forms of which represent later stages than are shown in FIG. 40. Fig. 42 is magnified 2,400 diameters.

FIGS. 43 and 44.—Typical adult *H. pattoni*. In the latter figure note the twist in the posterior end of the body. From intestine of flea.

PLATE 3.

All figures except 62, 63, and 64 are magnified 2,866 diameters.

FIGS. 45 and 46.—Other adult *H. pattoni* from intestine of flea. In Fig. 45 the anterior end is blunt, while the posterior end is pointed. In Fig. 46 the opposite is true.

FIG. 47.—Adult herpetomonad with the posterior end notched.

FIGS. 48 and 49.—Division forms preceding encystment. In Fig. 48 the flagella have disappeared.

FIGS. 50–52.—Herpetomonad forms preparing for encystment. Note the thin winglike sides and a dense longitudinal band. In Fig. 51 a free flagellum still persists on one of the individuals. In Fig. 52 the path of the flagellum in one individual is marked by a light area. From intestine of flea.

FIGS. 53–56.—Encysted herpetomonad forms, which are respectively single, double, triple, and quadruple. In Fig. 55 note the broad anterior ends centrally located. From rectum of fleas.

FIGS. 57 and 58.—Common types of encysted *H. pattoni* from rectum and intestine of rat flea. In the former figure note the broad anterior and sharp posterior end. The path of the flagellum is marked by a clear area bounded on either side by a dense band.

FIG. 59.—Herpetomonad cyst from which the blepharoplast is being extruded. From rectum of flea.

FIG. 60.—Herpetomonad cyst from which the blepharoplast has probably been extruded. From rectum of flea.

FIG. 61.—Herpetomonad cyst with thick wall. Nucleus and blepharoplast both present. From rectum of flea.

FIG. 62.—Adult *Herpetomonas calliphorae* from the stomach of the fly, *Calliphora coloradensis*. ×2,000.

FIG. 63.—Adult *H. calliphorae* with flagellum splitting at its base. ×2,000.

FIG. 64.—Adult *H. calliphorae* with dividing blepharoplast and flagellum splitting in the middle. ×2,000.

FIG. 65.—*H. calliphorae* with broad posterior end.

FIG. 66.—*H. calliphorae* in which the blepharoplast has moved clear to the posterior end and the size of the body has been reduced.

PLATE 4.

FIGS. 67 and 68.—Cysts of *H. calliphorae* from the intestine of the fly. Note the broad anterior ends. In the former figure the blepharoplast is posterior to the nucleus. In the latter figure there is a longitudinal curved ridge. ×2,866.

FIGS. 69–72.—Trypanosome-like forms from the digestive tract of the same fly as contained the above *Herpetomonas*. Note that the blepharoplast lies against the posterior wall and that an undulating membrane is somewhat distinct. The flagellum projects beyond the anterior end of the body. ×2,866.

FIG. 73.—Adult forms of *H. lineata* agglutinated by their blunt anterior ends. Note the extremely long threadlike posterior ends. The upper individual contains many granules in the threadlike projection. From intestine of fly, *Sarcophaga sarraceniae*. ×700.

FIG. 74.—Division of adult *H. lineata* from intestine of fly. ×1,200.

FIG. 75.—Very large form of *H. lineata* just beginning division. The posterior end is probably broken off. ×2,000.

FIG. 76.—Adult *H. lineata*. Note the long pointed posterior end, and the round body at the anterior end, which is the contracted flagellum. ×1,200.

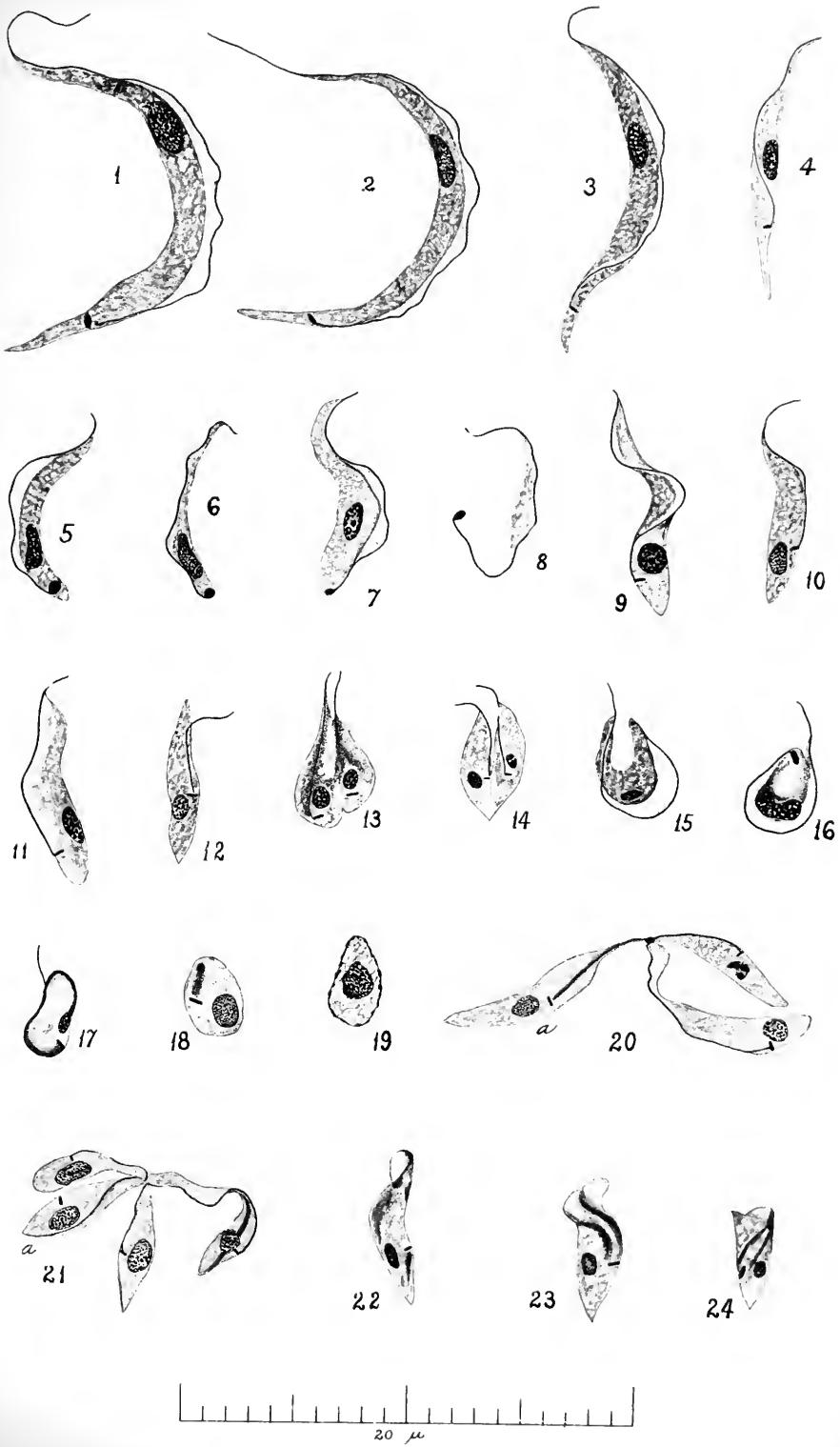
FIG. 77.—Typical adult form of *H. lineata* with free flagellum. ×1,200.

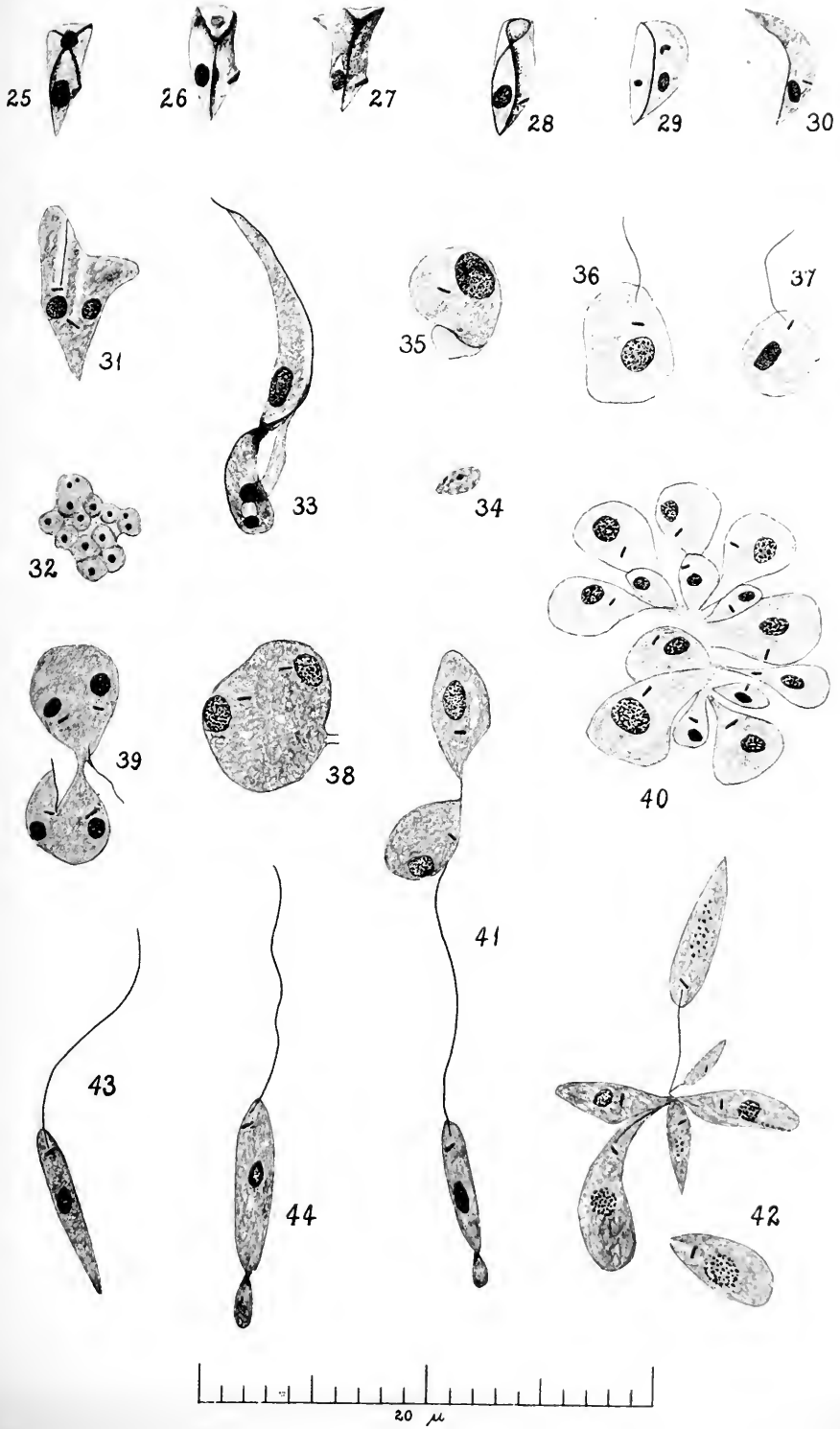
FIG. 78.—Ovate form of *H. lineata* possessing a very long free flagellum. ×2,000.

FIG. 79.—Sickle-shaped form of *H. lineata*. Note the long slender body with sharp posterior end and a long flagellum which passes along the body back to the blepharoplast in the posterior end. ×2,000.

FIG. 80.—Transitional form between the ovate and the sickle-shaped forms represented by Figs. 78 and 79. ×2,000.

PLATE I.

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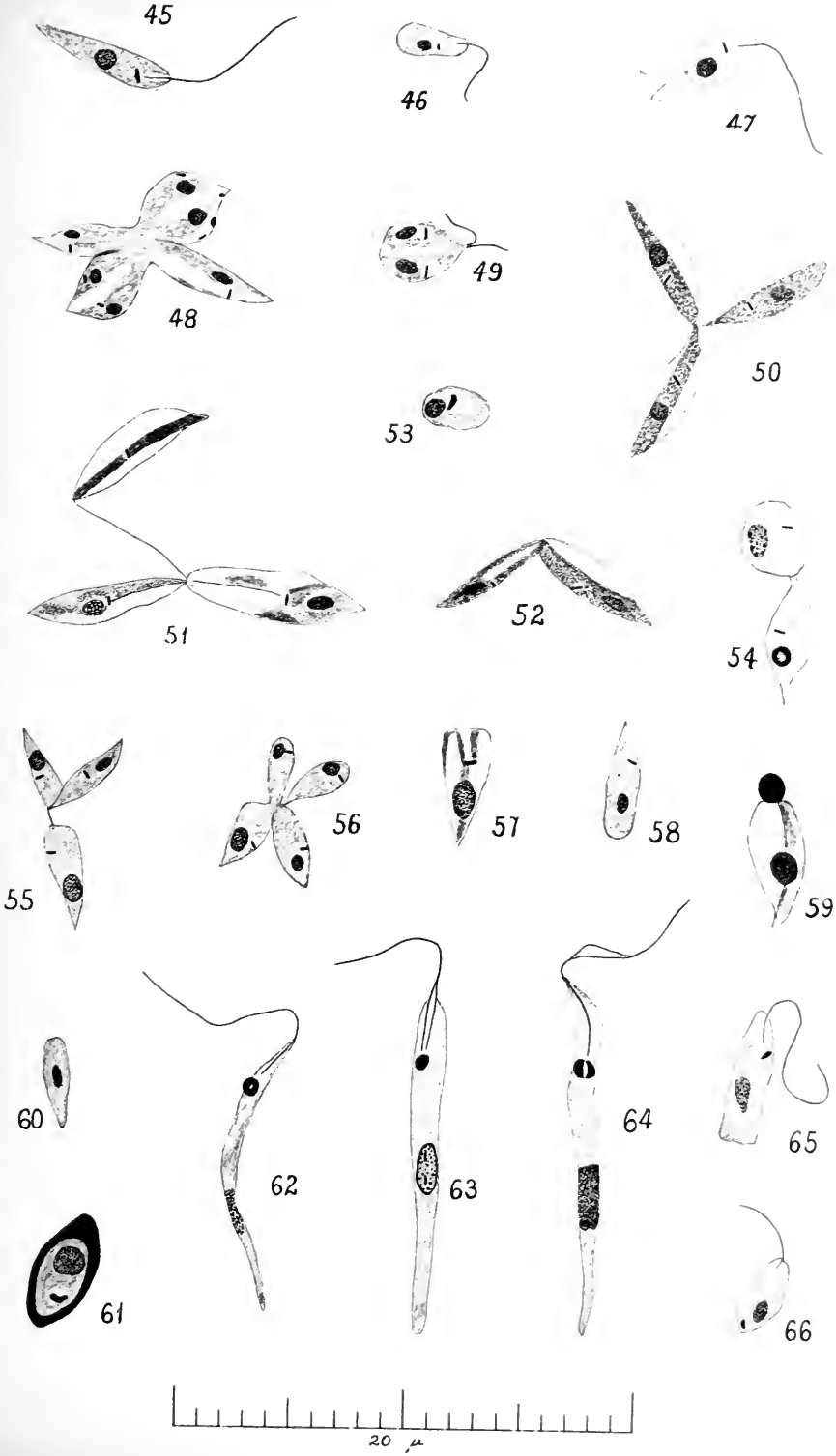
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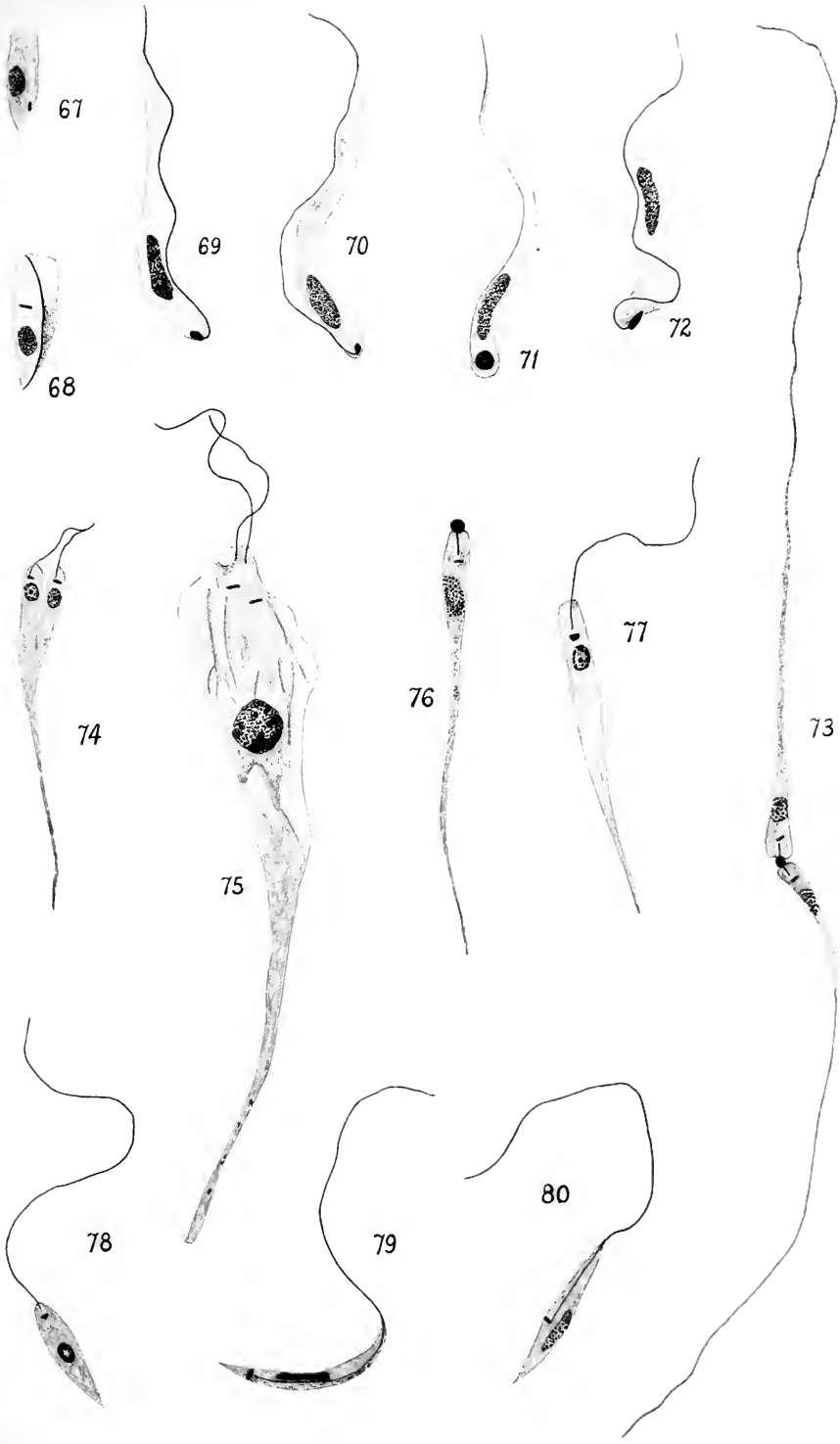
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THE INFLUENCE OF ETHER AND ETHER ANESTHESIA ON BACTERIOLYSIS, AGGLUTINATION, AND PHAGOCYTOSIS.*

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Coincident with the rapid increase of knowledge of biological chemistry chemical explanations are being sought for many of the phenomena of immunity. In order to study the effect of a fat-solvent drug on some of these phenomena the following investigation has been carried out. Ether was used because it provided an opportunity not only for observations in test-tubes but also for a study of the conditions in the human body by virtue of its general use as an anesthetic. Moreover, the idea presented itself that the action of the ether upon the various elements of the blood and other tissues might be intimately related to the occurrence of various post-operative lung complications, a study of which was suggested to the writer by Professor Arthur Dean Bevan. The phenomena investigated have been bacteriolysis, agglutination, and phagocytosis. The clinical cases were chosen from the surgical services of the Presbyterian Hospital. Squibb's anesthetic ether has been used.

EFFECT ON BACTERIOLYSIS.

No appreciable effect of the ether on the phenomenon of bacteriolysis was revealed, as shown in the following tables. Experiments were conducted by adding ether directly to normal and immune serum and by subjecting both normal and immune serum to its action *in vivo* by inhalation. The technic employed was the usual one of counting the number of colonies on agar plates after incubating the mixtures of serum and bacteria for variable periods. The dilutions in the control tubes were made with 0.85 NaCl solution and in the others with salt solution containing ether. Sterile corks were used in the tubes instead of cotton plugs in order

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to minimize the evaporation of the ether while in the incubator; 24-hour agar cultures of the typhoid bacillus were used because of the ease with which this organism undergoes lysis. Varying amounts of ether up to 1 per cent were tested in the different experiments, but no higher percentage was tested because of the fear of destroying the bacteria by the drug itself. The following tables are representative of the results obtained:

1.—10 c.c. of normal rabbit blood were removed from the heart, defibrinated, and centrifuged; 2 c.c. of serum were put into a small test-tube and 0.05 c.c. of 10 per cent ether in 0.85 per cent NaCl solution was added to it, making the amount of ether present in the serum 0.125 per cent. In the experiment this serum is designated as "etherized" serum. To another tube 2 c.c. of serum were added to which 0.05 c.c. of 0.85 per cent NaCl solution without ether was added to be comparable with serum to which NaCl containing ether was added. This control serum is designated as "normal" serum.

TABLE 1.

	Normal Serum						Etherized Serum					
	Serum 1 c.c. NaCl 0	Serum 0.5 c.c. NaCl 0.5 c.c.	Serum 0.25 c.c. NaCl 0.75 c.c.	Serum 0.125 c.c. NaCl 0.875 c.c.	Serum 0.0625 c.c. NaCl 0.9375 c.c.	Serum 0 NaCl 1 c.c.	Serum 1 c.c. NaCl 0	Serum 0.5 c.c. NaCl 0.5 c.c.	Serum 0.25 c.c. NaCl 0.75 c.c.	Serum 0.125 c.c. NaCl 0.875 c.c.	Serum 0.0625 c.c. NaCl 0.9375 c.c.	Serum 0 NaCl 1 c.c.
Immediate	424	640	640	648	768	1,000	500	400	480	464	520	720
After 1 hr	26	176	420	496	480	496	120	156	212	536	960	480
After 5 hrs	0	0	0	0	4	400	1	6	0	5	280	490
After 15 hrs	0	0	3	140	1,312	600	0	0	0	1,632	3,392	900

2.—5 c.c. of blood were removed from the heart of a normal rabbit which was then killed by inhalation of 5 c.c. of ether over a period of 15 minutes. Immediately after the death 5 c.c. blood were again removed from the heart. The serum obtained before death is designated as A_1; and that obtained immediately after death is designated as A_2. The experiment was undertaken immediately after collecting serum A_2.

TABLE 2.

	Rabbit A_1				Rabbit A_2			
	Serum 0.5 c.c. NaCl 0	Serum 0.25 c.c. NaCl 0.25 c.c.	Serum 0.1 c.c. NaCl 0.4 c.c.	Serum 0 NaCl 0.5 c.c.	Serum 0.5 c.c. NaCl 0	Serum 0.25 c.c. NaCl 0.25 c.c.	Serum 0.1 c.c. NaCl 0.4 c.c.	Serum 0 NaCl 0.5 c.c.
Immediately	904	1,320	1,440	880	912	1,062	760	808
After 2 hrs	34	50	112	900	68	136	152	900
After 5 hrs	3	1	14	1,000	4	0	30	1,000
After 14 hrs	0	0	0	1,200	0	0	0	1,500

3.—Human serum from a typhoid patient was used in this experiment. That portion designated as "immune serum with ether" contained 1 per cent ether in the first tube. The control serum contained 1 per cent 0.85 NaCl solution in the first tube. The sign ∞ indicates a countless number.

TABLE 3.

IMMUNE SERUM WITH ETHER.

	1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256	1:512	NaCl
Immediately...	128	60	20	160	80	400	640	960	800	640
After 3 hrs.....	0	0	1	4	8	560	800	1,000	480	720
After 20 hrs....	0	0	0	0	3,300	∞	∞	1,600	1,120	1,380
IMMUNE SERUM WITHOUT ETHER.										
Immediately...	204	168	52	260	396	600	664	744	704	640
After 3 hrs.....	0	0	0	0	80	320	464	800	720	800
After 20 hrs....	0	0	0	0	3,200	∞	∞	1,200	1,400	1,600

EFFECT ON AGGLUTINATION.

Here also ether seemed to have no appreciable effect. Experiments were carried out with both normal and immune typhoid serum and the typhoid bacillus. The dilutions were made with 0.85 NaCl solution. Both the macroscopic and microscopic methods were employed. Sterile corks were used instead of cotton plugs in order to prevent evaporation so far as possible. The amount of ether used in the different experiments varied from 0.1 per cent to 2 per cent in the first tube of a series. The following experiments are typical of the results obtained with the macroscopic method.

1.—Immune typhoid serum was used. Ether was added to the first tube of the series designated as "immune serum with ether," in an amount equal to 2 per cent. The first tube of the control series contained 2 per cent 0.85 NaCl solution.

TABLE 4.

IMMUNE SERUM WITHOUT ETHER.

	1:10	1:20	1:40	1:80	1:160	1:320
After 4 hrs.....	Clumped	Clumped	Clumped	Clumped	Clumped	Partially Clumped
After 20 hrs.....	"	"	"	"	"	Clumped
IMMUNE SERUM WITH ETHER.						
After 4 hrs.....	Clumped	Clumped	Clumped	Clumped	Clumped	Clumped
After 20 hrs.....	"	"	"	"	"	"

2.—This experiment was conducted similarly to that represented in the preceding table. Here, however, 1 per cent ether was used instead of 2 per cent.

TABLE 5.

IMMUNE SERUM WITHOUT ETHER.

	1:10	1:20	1:40	1:80	1:160	1:320	1:640
After 4 hrs.....	Clumped	Clumped	Clumped	Clumped	Partial Clumping	Partial Clumping	No Clumping
After 24 hrs.....	"	"	"	"	Clumped	"	"

IMMUNE SERUM WITH ETHER.

After 4 hrs.....	Clumped	Clumped	Clumped	Clumped	Clumped	Partial Clumping	Partial Clumping
After 24 hrs.....	"	"	"	"	"	Clumped	"

EFFECT ON PHAGOCYTOSIS.

In this series of experiments very decided effects were obtained. The usual technic was employed in estimating phagocytosis. In general the bacterial suspensions were made from 24-hour agar cultures; and unless otherwise stated normal human leukocytes were used. The mixtures were incubated for 15 minutes at 37° C. The following table illustrates the effect *in vitro* of ether on phagocytosis.

Ether was added directly to fresh human serum in varying proportions and the opsonic power determined. The organism used was an attenuated streptococcus. The figures represent the average number of bacteria in each of 100 leukocytes counted.

TABLE 6.

Normal human serum			.....	5.59
"	"	"	+ 1 per cent ether.....	3.81
"	"	"	+ 1 " " NaCl.....	4.92
"	"	"	.....	2.85
"	"	"	+ 2 per cent ether.....	0.70
"	"	"	+ 2 " " NaCl.....	2.00
"	"	"	.....	3.62
"	"	"	+ 2.5 per cent ether.....	1.15
"	"	"	+ 2.5 " " NaCl.....	2.90
"	"	"	.....	2.53
"	"	"	+ 5 per cent ether.....	0.66
"	"	"	+ 5 " " NaCl.....	2.12

In the table it is seen that ether *in vitro* diminishes phagocytosis of streptococci, using normal serum. This diminution is less when 1 per cent ether is present than when greater amounts are used.

Because of these results a number of observations were made to determine whether or not phagocytosis as determined by the opsonic index of an individual would be affected by an ordinary

ether anesthesia. For this purpose the indices to the streptococcus of 12 individuals were taken before anesthesia and at frequent intervals afterward. The determinations were made in the usual way. The charts given below represent the opsonic index curves in several of these observations. An effort was made to choose cases which were devoid of conditions which might complicate results, such as infection, shock, hemorrhage, anemia, etc. Hence, only those cases were studied which involved comparatively simple operative procedures on apparently fairly healthy young adults. The absence of infection was determined by: (1) absence of fever,

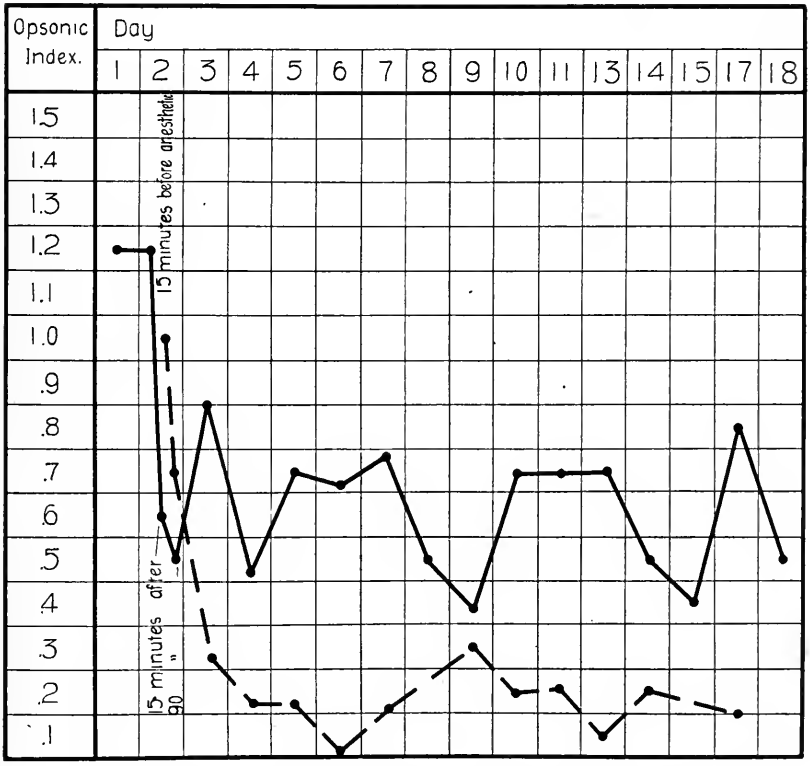

CHART 1.—Operation for bilateral inguinal hernia. Anesthesia administered for 45 minutes. Amount of ether used, 300 gm. The solid line represents the opsonic index taken in the usual way with normal leukocytes. The broken line represents the index using the patient's leukocytes instead of normal leukocytes. No observations were made after the 18th day until the 33d day. At the latter time the index was 1.1 with normal leukocytes and 0.9 with the patient's leukocytes. The index taken on "Day 1" was 24 hours before operation. On "Day 2" observations were made immediately before, 15 minutes after, and 90 minutes after respectively. This chart illustrates the longest period of depression of the index which was observed in the series.

(2) normal leukocyte counts, (3) sterility of cultures made from the wound secretion from 24 to 48 hours after operation. It may then reasonably be assumed that the results obtained are due at least in great part to the effect of the anesthesia.

In order to rule out the possibility that the results illustrated above might have been due to conditions involved in the operative procedures other than the anesthesia, three control experiments were made on normal rabbits by subjecting them solely to ether

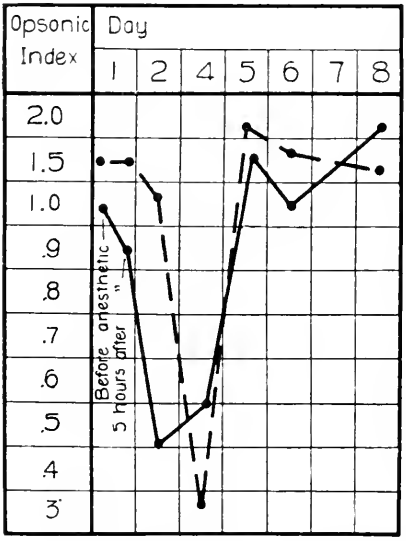

CHART 2.—Decompression operation for epilepsy. Duration, 15 minutes. Amount of ether used, 100 gm. The solid line shows the index curve with normal leukocytes; and the broken line represents the curve with the patient's leukocytes instead of normal leukocytes.

anesthesia without operation of any sort. The results obtained agree closely with those already observed in the human subject.

We see then from the above experiments that: (1) both ether *in vitro* and ordinary ether anesthesia lower markedly the phagocytic power of blood to streptococcus; (2) that this period of depression is variable, probably depending on several factors, such as the rapidity of excretion of the ether after stopping its administration, etc.; (3) that the curves of the index determinations when made with the patient's leukocytes show a greater drop than when normal leukocytes are used.

Since only the streptococcus was used it seemed to be of interest to determine whether this reduction is specific for phagocytosis of streptococci or whether the same phenomenon would hold with regard to other organisms. For this purpose observations were made in the same manner as those mentioned above, in a case of operation for inguinal hernia in a young man otherwise normal. The opsonic index of his serum to the streptococcus,

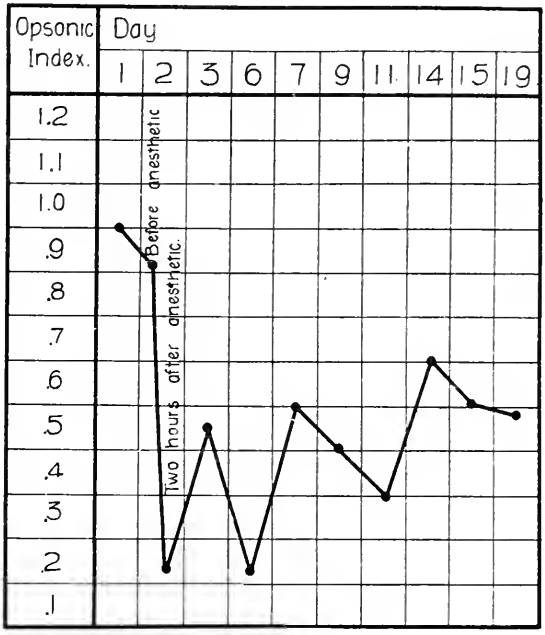

CHART 3.—Wiring of patella. Anesthetic administered for 30 minutes. Amount of ether, 200 gm. The indices were made with normal leukocytes.

pneumococcus, staphylococcus, *B. coli*, and *B. typhosus* was determined immediately before the administration of the ether and on several days afterward as shown in Chart 4. Before making the determinations in each instance both the patient's serum and the normal pool were heated in order partly to destroy complement, because of the unreliability of making opsonic determinations with the colon and typhoid bacillus when serum is used whose bacteriolytic power has not been at least partially

destroyed. Normal leukocytes were used, and the mixtures were incubated for 20 minutes. The duration of the administration of ether was 30 minutes.

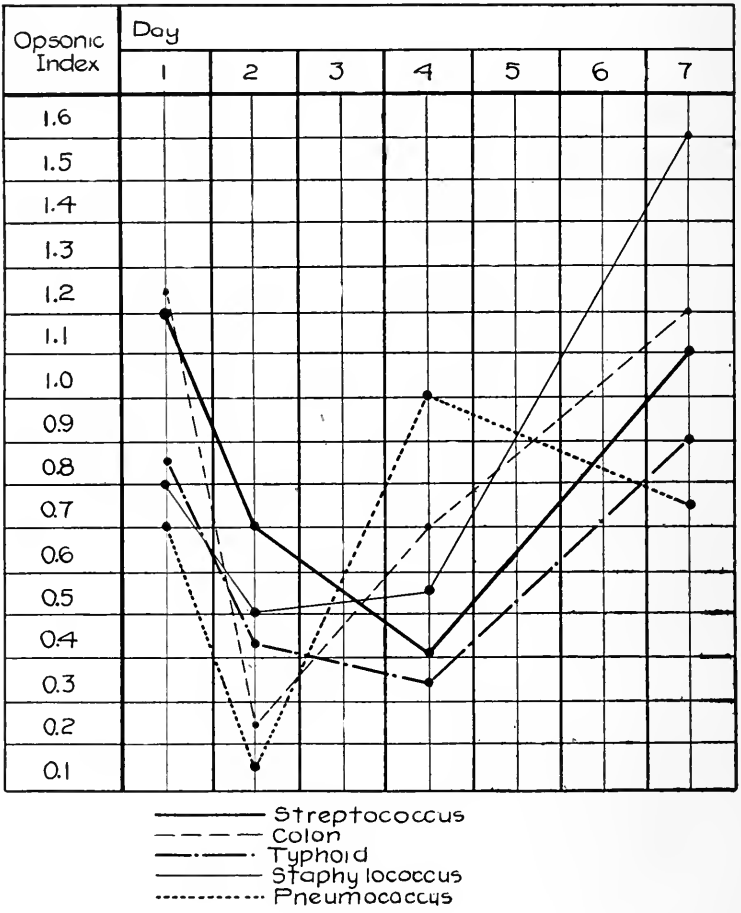

CHART 4.

Chart 5 represents a series of determinations of the opsonic index to the streptococcus and typhoid bacillus using unheated serum from a supposedly normal rabbit before and after being subjected to ether anesthesia. Normal human leukocytes were used. The solid line represents the indices to the streptococcus

and the broken line those to the typhoid bacillus. The duration of the anesthesia was 30 minutes. Why the rabbit's index to the typhoid bacillus was so high is difficult to explain. However, it may have been due either to an actual increase in typho-opsonin in the rabbit's serum or to an entirely different reason—viz., that the normal pool was more lytic for the bacillus than the animal's

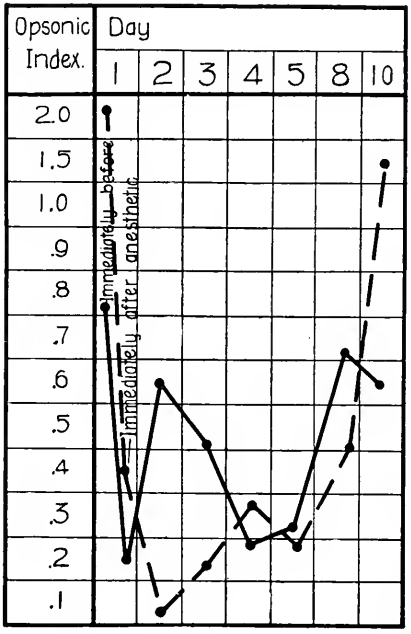

CHART 5.

serum and hence that fewer bacilli were seen in the preparations made from the normal pool.[1]

WHAT IS THE NATURE OF THE PHENOMENON?

Having found that ether reduces the phagocytic power of blood the question arose as to what part of the phagocytic system is affected, whether opsonin, leukocytes, or bacteria. The mere fact that the opsonic index is lowered does not mean necessarily that opsonin is affected, for the action may be only on the leu-

[1] The fact that a highly lytic serum may show an apparently low opsonic power has been demonstrated beautifully with reference to colon bacilli by D. J. Davis: "Immune Bodies in Urinary Infections with Colon Bacilli and Treatment by Inoculation," *Jour. Infect. Dis.*, 1909, 6, p. 224.

kocytes, the serum acting as a carrier of the anesthetic to them. The fact that when the patient's leukocytes were used there was a more pronounced reduction of the phagocytic power than when normal leukocytes were used argues for the assumption that there was some direct effect of the drug at least on the leukocytes. With this idea in view experiments were performed the object of which was to determine if possible what effect ether has upon the phagocytic activity of the white corpuscles with particular reference to their ameboid motion. Work on this point is still in progress, and the complete results will be published at a later date. However, by watching on a warm stage washed leukocytes in a suspension of carmine particles, a tentative conclusion was reached that ether inhibits the ameboid motion of the leukocytes. The results pertaining to the lowering of phagocytic power of the leukocytes *in vivo* are to be expected when it is considered that ether after being absorbed in the lungs is transported by means of the systemic circulation and that in all probability the leukocytes as well as every cell of the body are anesthetized to a more or less degree.

The next step was to determine whether or not opsonin is affected alone without reference to the leukocytes. Since bacteria are capable of being "sensitized" for phagocytosis by being placed in serum for several minutes at incubator temperature so that even if washed free from serum they remain readily phagocytable if allowed to come in contact with washed leukocytes in salt solution, it seemed as if this would afford a ready means of studying any possible effect of the ether on the serum. Accordingly the following experiment was performed: An identical amount of a suspension of streptococci was added to each of a series of tubes containing the same amount of carefully measured normal human serum. To the serum in one set of tubes was added ether in varying proportions. To the serum in the control set of tubes was added an amount of 0.85 NaCl solution equal in each case to the amount of ether which had been added to the corresponding tube. All the tubes were then incubated for one hour and later centrifuged. The supernatant fluid above the bacteria was withdrawn and the organisms were washed once in salt solution, and then resuspended in fresh salt solution. The duration of the

centrifuging process in first throwing down the bacteria and then in washing them was one hour. During this time only cotton plugs were in the tubes. Opsonic determinations were made after adding these washed streptococci to washed normal human leukocytes and incubating the mixtures for 15 minutes. The table below shows that apparently ether has in each case greatly inhibited "sensitization" and that when a large amount (25 per cent) has been used practically no sensitization has occurred. The figures represent the average number of bacteria in each of 50 leukocytes counted.

Streptococcus	sensitized	in	normal	serum	+	2	per cent	ether	2.96
"	"	"	"	"	+	2	"	" NaCl....	4.52
"	"	"	"	"	+	10	"	" ether	1.56
"	"	"	"	"	+	10	"	" NaCl....	4.38
"	"	"	"	"	+	25	"	" ether	0.20
"	"	"	"	"	+	25	"	" NaCl....	3.74

The conclusion that opsonification is inhibited by ether seems warranted in the light of these observations. But whether or not we can conclude that opsonin itself is affected directly by ether will depend on whether or not we can exclude in the interpretation of the results the following possibilities: (1) are the bacteria themselves affected by ether in such a way that they resist opsonification? or (2) is it certain that by the technic described all the ether which is at least not united with the bacteria has been removed? Concerning the first of these questions no experimental work has been undertaken in the present study, but Paul Th. Müller[1] concludes that there is no important difference in phagocytosis between bacteria extracted with ether and those not extracted. Regarding the second question, when we consider the volatility of ether and the fact that during the process of centrifugalization a considerable amount of the free ether must surely have passed off through the cotton plugs, we are led to conclude that at most only a very small amount of the drug could remain in the serum of which most, if not all, must surely have been removed by the washing, or diluted to a negligible amount by the second suspension of the organisms in fresh NaCl solution. In addition to all this is the

[1] "Einige Versuche über die Rolle der Bacterienlipoide bei der Phagocytose," *Ztschr. f. Immunitätsforschung u. exper. Therapie*, 1908, 1, p. 61.

fact that only a small amount of the final bacterial suspension was used in making the opsonic determinations so that we can conceive that only an infinitely small proportion of the original percentage of the ether could be possibly carried over in the suspension and thus have a possible direct action upon the leukocytes. Furthermore, even if we admit that the technic was insufficient to remove all the ether, we can hardly conceive of enough remaining to cause so marked a difference as is noted when only 2 per cent of ether is used, in the light of the results seen in Table 6, where we are dealing with known quantities of the drug. We are practically forced then to the conclusion that ether has in some way affected opsonin.

Granting that opsonin is composed of both a thermolabile and a thermostable element, the next logical step was to determine if possible whether one or both of these elements were effected. Up to this time no satisfactory conclusions can be made regarding this point.

WHAT IS THE CAUSE OF THE EFFECT OF ETHER ON PHAGOCYTOSIS?

That the ether enters into no stable chemical combination with opsonin is shown by the fact that its influence may be easily done away with by bubbling a current of air through serum to which the drug has been added, by which means supposedly the ether is all removed from the serum. The following experiment, which illustrates this point, was performed: 3 c.c. of normal human serum were divided equally among three tubes. To each of two tubes ether was added in an amount sufficient to make the serum contain 2.5 per cent. Through one of these tubes containing the serum-ether mixture a current of air was passed for 40 minutes by means of a suction pump attached to a water faucet. At the expiration of that time opsonic determinations to the streptococcus were made in the usual way, using normal human leukocytes. The figures represent the average number of streptococci in each of 100 leukocytes counted:

Normal human serum	3.62
" " " +2.5 per cent ether	1.15
" " " +2.5 " " " + air	3.01

It is apparent then that the inhibitory action of the ether upon phagocytosis has been removed by means of an air stream, a fact which could hardly occur if the ether had entered into a stable combination with the serum.

In attempting to explain the cause of the effect of ether on phagocytosis the interesting hypotheses of Meyer and Overton regarding the action of the fat-solvent group of anesthetics (chloroform, ether, and alcohol) seemed to afford a possible explanation. Meyer[1] and later Overton,[2] working independently, arrived at about the same conclusions. Meyer sums up his theories in the following sentences:

1. All chemical substances, not indifferent, which are soluble in fat and fat-like bodies must be narcotic for living protoplasm in so far as they are diffusible.

2. The action will be first and strongest on those cells in whose chemical structure those fat-like substances prevail and are especially the carriers of the cell functions: in the first place then in the nerve cells.

3. The proportionate working strength of such narcotics must be dependent on their "mechanical affinity," on the one hand, for fat-like substances; on the other hand, for the remaining body constituents, particularly water; consequently on the diffusion-coefficient which determines their diffusion in a mixture of water and fat-like substances.

In view of these considerations and because of the presence of lipoids not only in the corpuscular elements of the blood but also in the serum, the idea presented itself that possibly the cause of the effect of ether on phagocytosis was in some way concerned with its solution in lipoids. Accordingly, then, experiments were performed whose object was to attempt if possible the restoration of the phagocytic power by the addition of a lipoid to serum which already contained ether. For this purpose lecithin was chosen.[3]

[1] Hans Meyer, "Zur Theorie der Alcoholnarkose, Erste Mittheilung: Welche Eigenschaft der Anaesthetica bedingt ihre narcotische Wirkung?" *Arch. f. exper. Path. u. Pharmakol.*, 1899, 42, p. 109. Fritz Baum, "Zweite Mittheilung: Ein physikalisch-chemischer Beitrag zur Theorie der Narcotica," *ibid.*, 1899, 42, p. 119.

[2] *Studien über die Narkose*, Jena, 1901.

[3] Both Merck's ovo-lecithin and a lecithin prepared from agfa and kindly furnished me by Dr. Preston Kyes were used in the experiments. No appreciable difference in the action of the two was observed.

1.—The manner of conducting the experiments was as follows: Human normal serum was divided equally among 3 tubes. To two of these tubes ether was added in a certain definite quantity. To one of these two tubes lecithin was then added and emulsified in the serum by stirring. Opsonic determinations were then made, using normal human leukocytes. The figures indicate the average number of streptococci per leukocyte.

TABLE 7.

Normal human serum	2.12
" " " + 2 per cent ether	0.74
" " " + 2 " " " + 0.2 per cent lecithin	1.52
Normal human serum	5.59
" " " + 1 per cent ether	3.81
" " " + 1 " " " + 0.2 per cent lecithin	5.25
Normal rabbit serum	3.68
" " " + 0.5 per cent ether	1.88
" " " + 0.5 " " " + 0.2 per cent lecithin	3.80

2.—In this experiment the serum used was obtained from the patient represented in Chart 2 on the second day. 50 leukocytes were counted.

Patient's serum + normal leukocytes	2.40
" " + patient's leukocytes	3.94
" " + 0.02 per cent lecithin + normal leukocytes	4.58
" " + 0.02 " " " + patient's "	6.40

3.—This experiment was conducted in a manner similar to the preceding one. Serum was taken from a patient operated upon for inguinal hernia with ether anesthesia whose chart of index curves is not given. The serum and leukocytes were collected during the period of depression of the index. 50 leukocytes were counted.

Patient's serum + normal leukocytes	1.62
" " + " " + 0.1 per cent lecithin	3.80

4.—In this experiment serum was taken from a rabbit both immediately before and immediately after anesthesia and 0.2 per cent lecithin was added to a part of it. Normal human leukocytes were used, and the determinations were made with 50 leukocytes.

Rabbit's serum before anesthesia	2.12
" " after "	0.74
" " " " + 0.2 per cent lecithin	1.52

It seems evident then in the light of these experiments that the addition of lecithin to serum containing ether is capable of at least partly restoring the phagocytic power. The question then arises, will ether saturated with lecithin cause any reduction of the phagocytosis? The following experiment is illustrative of several experiments performed to determine this point. The figures

represent the average number of streptococci in each of 150 leukocytes counted.

TABLE 8.

1 c.c. normal human serum	2.85
0.9 c.c. " " " + 0.1 c.c. of 20 per cent mixture of ether in NaCl (making amount ether present equal to 2 per cent)	0.70
0.9 c.c. " " " + 0.1 c.c. NaCl	2.00
0.9 c.c. " " " + 0.1 c.c. of saturated solution of lecithin in ether	2.02

The conclusion seems justified then that ether which is saturated with lecithin is practically inert and alters phagocytosis only in so far as it is a diluent of the serum.

Briefly summarized the results of these last experiments are: (1) that lecithin when added to blood containing ether is capable of restoring its phagocytic power more or less completely; and (2) that ether previously saturated with lecithin is unable to reduce phagocytosis except as it is a diluent of the serum.

These observations, however, do not warrant the conclusion that ether lowers the phagocytic power of the blood by means of a combination with the fats or lipoids of either the serum or leukocytes. For in a mixture of fluids of different solvent powers for a third substance the dissolved substance is divided between them in direct proportion to its solubility in the two fluids. Therefore, in the serum-ether-lecithin system the ether which is very much more soluble in lecithin than in water, of which the serum is in greatest part made up, will be held almost wholly in the lecithin. When the ether-lecithin mixture is put into the serum there will be practically no ether allowed to pass into the serum, and hence no effect. Nevertheless, the suggestion is strong that the effect of the ether on phagocytosis may be related to a change produced in the lipoids of the blood by the drug. For instance, it seems by no means unlikely that ether is capable of penetrating into the leukocytes and by disturbing the equilibrium of their colloid solution through a solution of their lipoids produce such changes in surface tension as will result in an alteration of activity. Moreover, since lipoids are more easily soluble in ether than in water it seems reason-

able to suppose that at least some of the lipoids of the serum may be drawn from their watery medium to the ether, and therefore that the presence of ether in the serum results in a physical change which may explain our experimental results.

Of particular interest at this point is the work of other investigators relating to the effect of other fat-solvents upon the phenomena of immunity. The well-known effect of alcoholism in predisposing to pneumonia seems perhaps to be related to this problem, particularly since by the work of Rosenow[1] and others the type of immunity in this disease appears to be largely phagocytic. Rubin[2] has shown that hypodermic injections of alcohol, ether, and chloroform in rabbits in amounts equivalent to $1\frac{1}{2}$ to 2 gm. to the kilo of rabbit weight, except with chloroform the dose of which was 1 gm. per kilo, in general reduced the leukocyte counts and rendered the animals more susceptible to injections of pneumococci and streptococci, and also that *in vitro* alcohol and chloroform diminished phagocytosis of pneumococci, streptococci, and staphylococci. Deléarde[3] observed that it was difficult to immunize animals against rabies, tetanus, and anthrax after they had been made alcoholic experimentally. Laitinien[4] found that the administration of alcohol greatly hastened death in animals with experimental acute infections and rendered them much more difficult to immunize against diphtheria toxin. Abbot[5] concluded that the normal resistance of rabbits to infection with *Streptococcus pyogenes* is markedly diminished through the influence of alcohol when given daily to the stage of acute intoxication. A similar but less marked diminution of resistance to infection by the colon bacillus occurs in rabbits subjected to the same influence. Zimin[6]

[1] "Studies in Pneumonia and Pneumococcus Infections," *Jour. Infect. Dis.*, 1904, 1, p. 280.

[2] "The Influence of Alcohol, Ether, and Chloroform on Natural Immunity in Its Relation to Leukocytosis and Phagocytosis," *ibid.*, 1, p. 425.

"The Influence of Alcohol and Chloroform on Phagocytosis *in vitro*," *Jour. Am. Med. Assoc.*, 1907, 48, p. 1432.

[3] "Contribution à l'étude de l'alcoolisme expérimental et de son influence sur l'immunité," *Ann. de l'Inst. Pasteur*, 1897, 11, p. 837.

[4] "Ueber den Einfluss des Alkohols auf die Empfindlichkeit des thierischen Körpers für Infectionsstoffe," *Ztschr. f. Hyg.*, 1900, 34, p. 206.

[5] "The Influence of Acute Alcoholism on the Normal Vital Resistance of Rabbits to Infection," *Jour. Exper. Med.*, 1896, 1, p. 447.

[6] Monograph published in Russian from Tomsk Bacteriological Institute. Abstracted in *Med. Rec.*, New York, 1908, 74, p. 492.

studied the effect of ether and chloroform narcosis on phagocytosis and the bactericidal power of blood. By injecting *Proteus vulgaris* intraperitoneally into guinea-pigs he found in control animals an abundant actively phagocytic exudate with no free bacilli present in 24 to 36 hours after the injection. Of the anesthetized animals over one-half died. And in the others phagocytosis was not nearly so marked. Studies on the bactericidal properties of the blood of anesthetized and control animals gave inconstant results. Recently A. W. Kruschilin[1] by the injection into rabbits of *Staphylococcus aureus* and the spores of subtilis and anthrax bacilli has studied the effect of alcohol given intravenously in sublethal doses before the injection of the bacteria upon the destruction of these organisms by the blood *in vitro*. The technic consisted of taking cultures from the blood after inoculation at different times and comparing the number of colonies from those animals which had received alcohol with controls that had received no alcohol. In practically every case the destruction of the bacteria *in vivo* occurred much more rapidly in the control animals. Also fatal results occurred much more rapidly in alcoholized rabbits than in the controls. In animals purposely killed a short time after the injection there was always found a greater number of bacteria in the organs of the alcoholized rabbits than in the controls.

Of interest in this connection is the work of other investigators showing the probable relation of the fats and lipoids to certain other phenomena concerned in immunity. In 1902 Abbott and Bergey[2] found that the serum of rabbits which had received daily doses of alcohol for 25 days was less hemolytic for alien corpuscles than normal rabbit serum. They concluded that this action was due to a reduction of hemolytic complement in the circulating blood. Kyes[3] demonstrated that the hemolytic constituent of

[1] "Ueber die Wirkung des Alkohols auf die Tätigkeit der Phagocyten," *Ztschr. f. Immunitätsforschung u. exper. Therapie*, 1909, 1, p. 407.

[2] "The Influence of Alcoholic Intoxication upon Certain Factors Concerned in the Phenomenon of Hemolysis," *Univ. Penn. Med. Bull.*, 1902, 15, p. 186.

[3] "Ueber die Wirkungsweise des Cobragifts," *Berl. klin. Wchnschr.*, 1902, 38.
Kyes and Sachs, "Zur Kenntnis der Cobragift aktivierenden Substanzen," *ibid.*, 1903, 2.
Kyes, "Ueber die Isolierung von Schlangengiftlecithiden," *ibid.*, 1903, 42.
Kyes, "Lecithin und Schlangengifte," *Ztschr. f. physiol. chem.*, 1904, 41, p. 273.
Kyes, "Venom Hemolysis," *Jour. Infect. Dis.*, 1910, 7, p. 181.

cobra venom could be complemented very actively by lecithin. Later Morgenroth and Carpi[1] found a similar phenomenon in connection with bee poison. Neuberg[2] showed that fat-splitting substances have definite hemolytic properties, and, conversely, that the fluids containing animal, bacterial, and vegetable hemolysins contain lipolytic substances, hence presumably that the hemolysins are lipolytic. Woelfel[3] was able to extract with alcohol hemolytic substances from blood serum. Friedemann[4] and Wohlgemuth[5] demonstrated the presence in pancreatic juice of a hemolysin which could be activated by lecithin; and Noguchi[6] has found a similar hemolysin in the pancreas itself. Because he has found that soaps extracted from serum and organs act as hemolytic complements Noguchi[7] has suggested the possibility that at least some of the serum and cellular complements may be salts of the higher fatty acids with weak organic acids. Hektoen and Ruediger[8] have shown that calcium, barium, and strontium ions inactivate the complement concerned in the lysis of rabbit's corpuscles and of typhoid bacilli. As pointed out by Wells[9] these precipitate fatty acids.

Bassenge[10] has ascribed to lecithin a definite bacteriolytic property for the typhoid bacillus; but as shown by Sleeswyk[11] this property is apparently concerned with acid impurities. Vay[12] concludes

[1] "Ueber ein Toxolecithid des Bienengiftes," *Berl. klin. Wchnschr.*, 1906, 43, p. 1424.

[2] Neuberg and Rosenberg, "Lipolyse, Agglutination und Hämolyse," *ibid.*, 1907, 44, p. 54.

Neuberg and Reicher, "Lipolyse, Agglutination und Hämolyse," *Biochem. Ztschr.*, 1907, 4, p. 281; also *Munch. med. Wchnschr.*, 1907, 54, p. 1725.

[3] "Identification of Alcohol-soluble Hemolysins in Blood Serum," *Jour. Infect. Dis.*, 1905, 2, p. 97.

[4] "Ueber ein komplexes Hämolysin der Bauchspeicheldruse," *Deutsch. med. Wchnschr.*, 1907, 33, p. 585.

[5] "Untersuchungen über den Pankreassaft des Menschen," 4. Mitteilung, "Ueber ein in ihm enthaltenes komplexes Hämolysin und über die Darstellung des Lecithids," *Biochem. Ztschr.*, 1907, 4, p. 271.

[6] "Ueber eine lipolytische Form der Hämolyse," *Biochem. Ztschr.*, 1907, 6, p. 185.

[7] "Ueber gewisse chemische Komplementsubstanzen," *ibid.*, 1907, 6, p. 327.

"On Extracellular and Intracellular Venom Activators of the Blood, with Especial Reference to Lecithin and Fatty Acid and Their Compounds," *Jour. Exper. Med.*, 1907, 9, p. 436.

[8] "The Antilytic Action of Salt Solutions and Other Substances," *Jour. Infect. Dis.*, 1904, 1, p. 379.

[9] "The Present Status of Our Knowledge of the Chemistry of the Processes of Immunity," *Arch. Int. Med.*, 1908, 2, p. 262.

[10] "Ueber eine bakteriologisch interessante Eigenschaft des Lecithins," *Deutsch. med. Wchnschr.*, 1908, 34, p. 139.

[11] "Ueber die angebliche bakteriolytische Eigenschaft des Lecithins und über die Immunisierung mittels Lecithin," *Deutsch. med. Wchnschr.*, 1908, 34, p. 2263.

[12] "Ueber die immunisierende Wirkung von Lecithin—auszügen aus Pestbazillen," *Deutsch. med. Wchnschr.*, 1908, 34, p. 2265.

that lecithin in 1 and 10 per cent emulsions increases the power of agglutinating pest bacilli. Pick and Schwarz[1] found that by injections into animals of typhoid bacilli in 1 per cent lecithin emulsion a relatively high agglutinating power of the serum could be obtained in very short times. The same was true also when body lipoids (particularly leukocyte and serum lipoids) were used after being obtained by alcohol extraction.

Without going into the literature further we may conclude, then, that there is sufficient evidence already at hand to assume that the fats and lipoids play an important rôle in at least some of the immune reactions.

With the idea in view that possibly by treating serum with ether an extract could be obtained which would act as opsonic complement in reactivating heated serum, the following experiment was performed:

15 c.c. of normal rabbit serum were extracted with twice the volume of ether for 3 hours at room temperature. The ether was then removed and evaporated, after which the residue was stirred up in 1 c.c. of NaCl solution and added to heated serum. Opsonic determinations were made in the usual way, using normal human leukocytes and streptococcus. The figures represent the total phagocytosis in 50 leukocytes.

Normal serum	350
Heated normal serum	36
" " " + ether extract	30

It is evident then that ether extract of serum is unable to act as opsonic complement.

Will lecithin alone act as opsonin or as opsonic complement?

To determine the first of these points a number of experiments were performed with varying amounts of lecithin emulsified in NaCl solution. No appreciable amount of phagocytosis could be demonstrated. Table 9 (page 166) serves as a type of the results obtained.

To determine the latter point, viz., whether lecithin will act as opsonic complement, it was emulsified in varying proportions in normal human serum which had been inactivated by heat at 50° C. for 45 minutes. The experiment shown by Table 10 illustrates the negative results obtained.

[1] "Ueber die Beeinflussung der Antigenwirkung durch Lecithin und Organlipoide und deren Beteiligung am Immunisierungsprozess," *Biochem. Ztschr.*, 1909, 15, p. 453.

TABLE 9.

Normal human serum	6.40
0.85 NaCl	0.48
" " + 10 per cent lecithin	0.82
" " + 5 " " "	0.92
" " + 2 " " "	1.00
" " + 1 " " "	0.92
" " + $\frac{1}{2}$ " " "	0.95
" " + $\frac{1}{4}$ " " "	0.65
" " + $\frac{1}{8}$ " " "	0.58
" " + $\frac{1}{16}$ " " "	0.88
" " + $\frac{1}{32}$ " " "	0.75
" " + $\frac{1}{64}$ " " "	0.60
" " + $\frac{1}{128}$ " " "	0.55
" " + $\frac{1}{512}$ " " "	0.65
" " + $\frac{1}{1024}$ " " "	0.90
	1.00

TABLE 10.

The figures represent the average number of bacteria in each of 50 leukocytes counted.

Normal human serum	10.28
Heated normal human serum	0.36
" " " " + $\frac{1}{4}$ per cent lecithin	0.69
" " " " + $\frac{1}{2}$ " " "	0.76
" " " " + 1 " " "	0.42
" " " " + 2 " " "	0.85

Will lecithin when given in vivo after anesthesia restore the phagocytic power of the blood?

Since in the previous test-tube experiments we have seen that lecithin when added to blood whose phagocytic power has been reduced by ether will restore that power practically to a normal condition the question naturally arises as to whether the same results can be obtained *in vivo* after an anesthesia. Accordingly then a series of experiments was carried out as follows: A number of rabbits were anesthetized for varying periods of time; and immediately after stopping the anesthesia one-half of the series received an injection subcutaneously of an emulsion of lecithin in 0.85 NaCl solution. The other half of the series which served as controls received subcutaneously an equal volume of sterile NaCl solution. The lecithin was sterilized before the injections by heat for one hour at 58° C. Aseptic precautions were used when making the injections, and the site of the skin puncture in each case

was sealed with collodion. Opsonic determinations to the streptococcus were made with serum collected immediately before and immediately after the administration of the ether and then at varying periods afterward. The lecithin rabbit and its control in each instance received the same amount of anesthetic. In general rabbits of nearly the same weight were tested against each other. Where the results are expressed in terms of an index, the determinations were made by comparing the phagocytic counts of the rabbits with those of a normal human serum made with the same suspension of bacteria and leukocytes and incubated for the same length of time. Normal human leukocytes were used. The results, as will be seen in Chart 6 and the accompanying experiments, indicate that the injection of lecithin has resulted in a rather prompt restoration of the animal's phagocytic power to its preanesthetic state; whereas the controls, in general, have overcome the depression only gradually.

Two rabbits of 1,540 and 1,525 gm. weight respectively were anesthetized simultaneously with 50 c.c. of ether each over a period of 35 minutes. Immediately after stopping the inhalation of the anesthetic, serum was collected from a marginal vein of each rabbit for opsonic determinations. Rabbit A then received subcutaneously 0.1 gm. of lecithin emulsified in 2 c.c. of sterile physiological salt solution. Rabbit B at the same time was given 2 c.c. of sterile salt solution. The observations noted on the chart (page 168) represent three determinations made on the first day (before, immediately after, and four hours after the administration of the ether respectively), and one determination on each of the following three days. The solid line shows the index curve of Rabbit A, and the broken line that of Rabbit B.

Other experiments made in connection with this point are shown as follows:

1.—This experiment was conducted in a manner similar to the preceding one. Two normal rabbits of the same weight were anesthetized with 50 c.c. of ether over a period of 30 minutes. Rabbit A received 0.3 gram of lecithin in 3 c.c. of NaCl immediately after the anesthesia, and Rabbit B was injected with 3 c.c. of NaCl containing no lecithin. The figures represent the total phagocytosis.

	Before Anesthesia	Immediately after Anesthesia	24 Hours after Anesthesia	48 Hours after Anesthesia
A	350	184	178	396
B	372	140	62	130
Norman human pool	450	450	188	458

2.—Two normal rabbits of approximately the same weight, 50 c.c. of ether used with each during 40 minutes. Rabbit A received 0.5 gram of lecithin in 2 c.c. NaCl. Rabbit B received the same volume of NaCl.

	Before Anesthesia	Immediately after Anesthesia	5½ Hours after Anesthesia
Rabbit A	276	159	246
" B	258	132	132

3.—Fifty c.c. of ether administered to each for 30 minutes. Rabbit A was injected with 0.1 gram lecithin in 1 c.c. NaCl. Rabbit B received 1 c.c. NaCl.

	Before Anesthesia	Immediately after Anesthesia	4 Hours after Anesthesia	24 Hours after Anesthesia
Rabbit A	225	78	212	357
Rabbit B	248	108	187	351
Normal human pool	250	250	250	328

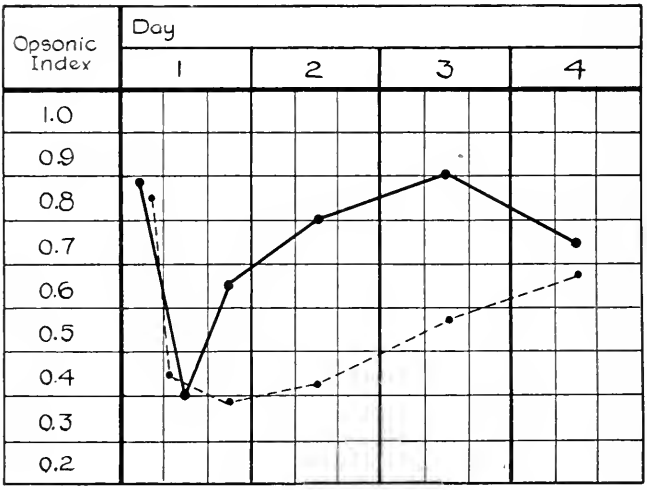

CHART 6.

In Experiments 1 and 3 the apparently marked variation in phagocytosis in all tubes which is observed after 24 hours, as compared with that obtained on the previous day with normal serum, is due merely to the fact that fresh bacterial and leukocytic suspensions were used on this day which probably varied from those of the previous day in density of both bacteria and leukocytes. On the first day when observations were made several times with each of the rabbits' sera only one determination was made with the normal human pool to compare with them; therefore the figures representing the pool are repeated to serve as a more ready means of comparison.

The explanation of how lecithin acts when given in this way is beset with many difficulties. That it is absorbed in a few hours and thereby enters the circulation to act in approximately the same manner as when in a test-tube it is stirred up in serum already containing ether, seems very unlikely in the light of the work of Henderson and Crofutt.[1] For these authors found that fats and oils were absorbed very slowly when injected subcutaneously and that usually at least several weeks were required for their disappearance. Our explanation is purely hypothetical but it seems reasonable to suppose that if when the fats of the body are nearly or completely saturated with ether, a condition which presumably exists during anesthesia, additional fat is added so that it may be within easy reach of the circulating fluids, there must be some withdrawal of the ether out of those watery media into the easily soluble fat. The effect of such a withdrawal would be then that a certain appreciable amount of ether would be removed from the serum and stored in the additional fat. This condition should consequently have two results: (1) that a certain amount of excess ether in the serum which is not held by the tissue cells because of the already existing saturation of the latter would be attracted into the fat so that temporarily at least it would be prevented from affecting any cell which had already become rid of its ether through ordinary means—as for example by becoming volatilized in the the lung and being exhaled; (2) because of the fact that ether may be stored up in the subcutaneous lecithin to be eliminated gradually, that lecithin may actually serve to hasten the elimination of the ether from the cells of both the fixed tissues and the blood. Somewhat in accord with this idea are observations reported by Nerking[2] in a preliminary communication. He has found that an intravenous injection of a suspension of lecithin shortens or even totally suppresses anesthesia. In our own work intravenous injections were not used because of the danger of not having a sufficiently fine emulsion to avoid pulmonary embolism.

The question also of how so small an amount as 0.1 gram of lecithin is capable of producing such marked changes is difficult

[1] "Observations on the Fate of Oil Injected Subcutaneously," *Am. Jour. Physiol.*, 1905, 14, p. 193.

[2] "Narkose und Lecithin (vorläufige Mitteilungen)," *München med. Wchnschr.*, 1908, 60, p. 1733.

to explain. But if, for example, we are dealing with a rabbit of 1,300 grams weight, the amount of blood contained in the animal is roughly one-thirteenth of its body weight, or 100 grams. Hoppe-Seyler's analyses of blood-plasma show that for one thousand parts of plasma there are but 1.2 parts of fat. In other words, in 100 gm. of plasma there are 0.12 gm. of fat. The 0.1 gm. of lecithin then used in the experiments would approximate the total amount of fat present in the blood of the animal and should be sufficient to exert considerable influence upon the ether present in the blood stream.

THE EFFECT OF OLIVE OIL WHEN ADDED TO ETHERIZED SERUM.

Since in the experiments dealing with lecithin we were concerned with a fat-like substance which exists almost universally in the body, it seemed of interest to determine if some fat of radically different nature would produce the same results. Accordingly the action of a purely vegetable fat was investigated and for this purpose olive oil was chosen. Experiments were conducted in a manner very similar to those with the lecithin. The results showed that, like lecithin, olive oil was capable of restoring the phagocytic power of blood in which it had been diminished by the addition of ether. One difference, however, was manifest, viz., that larger quantities of olive oil were required to produce the same results as those obtained with smaller amounts of lecithin. For the sake of brevity only one typical table is shown. The figures represent the total phagocytosis of streptococci using normal human leukocytes. The mixtures were incubated for 15 minutes:

TABLE 10.

Mixtures	Phagocytosis
Normal human serum	210
" " " + ether (2 per cent)	60
" " " + NaCl (2 per cent)	196
" " " + ether (2 per cent) + lecithin (0.1 per cent)	272
" " " + " 2 " " + olive oil (1 per cent)	202
" " " + " 2 " " + olive oil (2 per cent)	199

In view of these findings the question arose whether similar results could be obtained by giving oil *in vivo* after an ether anes-

thesia, and an effort was made to use the oil in such a manner that it could easily be employed on operative patients with no likelihood of dangerous results. For this reason the subcutaneous injection of comparatively large amounts of oil seemed impractical because of the increased danger of infection, etc. W. B. Müller[1] states that ether is excreted into the stomach after narcosis with this drug. Therefore it seemed reasonable to suppose that the introduction of the oil into the gastro-intestinal canal might produce the results in one or both of two ways: either by being at least partially absorbed and thus gaining access to the systemic circulation, or in some such way as was suggested in the attempt to explain the action of lecithin when given subcutaneously, viz., by extracting ether from the body fluids. Consequently it was decided to give the oil per rectum since this means seemed to furnish the simplest and easiest way of giving it immediately after the anesthesia. In regard to the question of how much fat can be absorbed by the large intestine there is great difference of opinion. Although Platenga,[2] Munk and Rosenstein,[2] and Deucher[2] found in their experiments that only comparatively small amounts were absorbed by the large intestine, yet, on the contrary, H. J. Hamburger[2] in a very extensive study concluded that the large intestine was able to absorb fully as much fat as the small bowel under favorable conditions. Edsall and Miller[3] in a study of two cases extending over a period of six days during which time the patients were fed entirely by rectum and received their fats chiefly as milk and eggs found that in one of these cases 33.4 per cent of the fat was absorbed and in the other 13.6 per cent.

The results of the experiments on phagocytosis using olive oil per rectum immediately after anesthesia are as follows:

Rabbits A (1,500 gm.) and B (1,475 gm.) were anesthetized, using 75 c.c. of ether for each over a period of 45 minutes. Serum was collected from each immediately before, immediately after, 3 hours after, and 24 hours after anesthesia. Rabbit A, however, received 25 c.c. of olive oil and Rabbit B 25 c.c. of NaCl solution per rectum immediately after the cessation of the administration of the ether. The figures

[1] *Narkologie*, 1, p. 338 (Berlin: R. Trenkel, 1908).

[2] Cit. by Hamburger, "Versuche über die Resorption von Fett und Seife im Dickdarm," *Arch. f. Physiol.*, 1909, p. 433.

[3] "A Study of Two Cases Nourished Exclusively per Rectum," *Univ. Penn. Med. Bull.*, Philadelphia, 1902, 15, p. 414.

in this and the following experiments represent total phagocytosis of streptococci in 100 washed normal human leukocytes.

TABLE 11.

Mixtures	Phagocytosis
Normal human pool	349
Rabbit A before ether	280
" " immediately after ether	85
" " 3 hours after ether and after receiving oil	183
Rabbit B before ether	303
" " immediately after ether	130
" " 3 hours after ether and after receiving NaCl	101
Rabbit A 24 hours after ether	133
Rabbit B " " " "	110

SIMPLE INGUINAL HERNIA OPERATION.

Duration of anesthesia was 30 minutes, at the end of which time 150 c.c. of olive oil were given per rectum.

Mixtures	Phagocytosis
Normal human pool	415
Patient's serum before ether	373
" " immediately after ether	129
" " 3 hours after ether and after oil	332

SIMPLE INGUINAL HERNIA.

Duration of anesthesia, 40 minutes; 150 c.c. of olive oil given per rectum.

Mixtures	Phagocytosis
Normal human pool	375
Patient's serum before ether	450
" " immediately after ether	187
" " 5 hours after ether and after oil	350

ENUCLEATION OF CEREBELLAR TUMOR.

Duration of anesthesia, 45 minutes; 150 c.c. of olive oil given.

Mixtures	Phagocytosis
Normal human pool	250
Patient's serum before ether	322
" " immediately after ether	137
" " 5 hours after ether and after oil	360

AMPUTATION OF LEG FOR ARTERIO-SCLEROTIC GANGRENE.

Duration of anesthesia, 20 minutes; 150 c.c. of olive oil given.

Mixtures	Phagocytosis
Normal human pool	305
Patient's serum before ether	308
" " immediately after ether	179
" " 4 hours after ether and after oil	300

TABLE 11.—*Continued.*

ENUCLEATION OF CYST OF FACE. ANESTHESIA FOR 30 MINUTES.

Mixtures	Phagocytosis
Normal human pool	248
Patient's serum before ether	273
" " immediately after ether	156
" " 2 hours after oil	235

HYSTERECTOMY FOR FIBROIDS. ANESTHESIA FOR 1 HOUR.

Mixtures	Phagocytosis
Normal human pool	210
Patient's serum before ether	184
" " immediately after ether	67
" " 3 hours after oil	168

SIMPLE INGUINAL HERNIA. ANESTHESIA FOR 25 MINUTES.

Mixtures	Phagocytosis
Normal human pool	326
Patient's serum before ether	358
" " immediately after ether	98
" " 3 hours after oil	319
" " 24 hours after oil	345
Normal human pool of day following anesthesia	355

TRANSPLANTATION OF TENDONS IN OLD BURN OF HAND. ANESTHESIA FOR 48 MINUTES.

Mixtures	Phagocytosis
Normal human pool	155
Patient's serum before ether	314
" " immediately after ether	187
" " 2 hours after oil	356

Control experiments were conducted on a series of operative patients under nearly parallel conditions except that in these 150 c.c. of physiological salt solution were given per rectum instead of oil. The following tables are typical of the results obtained:

SIMPLE INGUINAL HERNIA. ANESTHESIA FOR 30 MINUTES.

Mixtures	Phagocytosis
Normal human pool	420
Patient's serum before ether	460
" " immediately after ether	205
" " 4 hours after NaCl	121

SIMPLE INGUINAL HERNIA. ANESTHESIA FOR 35 MINUTES.

Mixtures	Phagocytosis
Normal human pool	280
Patient's serum before ether	276
" " immediately after ether	196
" " $3\frac{1}{2}$ hours after NaCl	182

TABLE 11.—*Continued.*

HYSTERECTOMY FOR FIBROIDS. ANESTHESIA FOR 1 HOUR.

Mixtures	Phagocytosis
Normal human pool	265
Patient's serum before ether	250
" " immediately after ether	198
" " 5 hours after NaCl	185

In a brief summary of these last experiments we find then that apparently the phagocytic power of blood which has been inhibited by the action of ether is at least partially restored to its preanesthetic condition within a few hours by the administration of olive oil per rectum although ordinarily as shown by Charts 1–5 the period of depression of phagocytosis extends over several days. Controls using the same amount of NaCl solution as of olive oil show no rise of phagocytosis. Whatever importance this phenomenon may have from a practical standpoint in combating the effect of ether anesthesia upon phagocytosis cannot be discussed in this article. It remains for a larger number of experiments to be performed with observation particularly, perhaps, on cases with infection.

SUMMARY AND CONCLUSIONS.

In my experiments the phenomena of bacteriolysis and agglutination were unaffected by ether.

Ether as given for anesthetic purposes reduces the property in human and rabbit blood of promoting phagocytosis of streptococcus, pneumococcus, staphylococcus, colon, and typhoid bacilli. This effect is easily studied *in vitro*.

The period of depression of phagocytosis after an ether anesthesia is variable, probably depending on many factors.

This reduction is apparently due to a direct action of the ether on the serum and the leukocytes.

There is not sufficient evidence at hand to determine which of the elements of the opsonic body of the serum is affected, whether the complement or the thermostable element.

The explanation of this action of ether is not clear, but the experiments suggest the possibility of its being due to the fat-solvent power of ether.

The ether seems not to be in any stable combination with the serum. Its inhibitory effect on phagocytosis may be removed by passing a current of air through the serum.

Lecithin in small amounts *in vitro* and also when given subcutaneously counteracts the inhibitory effects of ether. When ether is previously saturated with lecithin it has no marked inhibitory effect on phagocytosis and acts only as a diluent of the serum.

Lecithin is incapable of acting as opsonin in salt solution or of reactivating serum whose complement has been destroyed by heat.

Ether extract of serum fails to reactivate opsonin of heated serum.

Olive oil *in vitro* and *in vivo* when given per rectum seems to exert the same effect as lecithin in counteracting the inhibition of phagocytosis produced by ether, but to a less degree.

NOTE.—An abstract of this work was published in *Jour. Am. Med. Assoc.*, 1910, 54, p. 1043.

THE INFLUENCE OF FRESH AND AUTOLYZED ORGAN EXTRACTS ON EXPERIMENTAL TUBERCULOSIS.*

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The answer to the question why tubercle bacilli of the bovine type have never been found as the sole agent of tuberculous infection of the human lungs has not been satisfactorily found. It has been assumed that all human pulmonary infection caused by the tubercle bacillus occurs by way of the respiratory tract, and that infection by this path is usually from a pre-existing human source, and consequently the type of organism recovered from these cases is of the human type. There are, however, certain weaknesses in this assumption which justify a doubt as to its validity. It is generally conceded today that tubercle bacilli of the bovine type have the power to infect the human body (Weber and Taute, Oehlecker, British Commission, Th. Smith and Brown, C. W. Duval, Park, W. H. Lewis, A. Hess, Hohlfeld, Moss). There is some question as to the mode of entrance of the bovine type of bacillus to the body, although such entrance is possible, probably by the tonsil (Th. Smith), through the gastro-intestinal tract (Hess, Ravenel, Moss), through injury (Ravenel), or by the respiratory tract (Vagedes). Whatever the portal of entry, they infect mainly children, and once in the human body cause tuberculous lesions chiefly in glands, bones, and meninges. It is quite reasonable to suppose that, having once gained an entrance to the body, bovine bacilli have the same chance of reaching the lung tissues as those tissues where they chiefly lodge and produce disease. In fact, if as Ravenel, Schlossman, and Engel have shown, tubercle bacilli pass readily into the thoracic duct from the intestinal tract, and if the work of Calmette and Guerin, Bongert, Heymann, and Whitla contains any truth, the first capillary system reached by the bacilli is that of the lungs. They would be retained there for a longer or shorter period with the possibility of producing tubercles. Not only the pulmonary capillary system, but also

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those of the spleen and liver would act in a similar manner, as experiments by Ellinger, Besançon and Labbé, Oehlecker, Neumann and Wittgenstein tend to show. There is, however, as indicated, the possibility of lung infection and yet the bovine bacillus has never been recovered as the sole infecting agent from the human lung, the seat of tuberculosis.

The suggestion is frequently made that the tubercle bacillus is changed in type by the long sojourn in the body. Two potent objections make this seem unlikely: First, the strong adherence to type which persists in artificial cultivation (Th. Smith) as well as on the sojourn in the animal body (Weber, Baldwin). Second, that the bovine bacillus has been recovered not infrequently from tuberculosis glands in adults (Lewis, Park, Hess). However, this still leaves certain phases of the question open. Artificial culture cannot answer all the demands of growth in the animal body, and we have many striking examples of necessary caution in drawing conclusions from this source of evidence; for example, the changes occurring during animal passage and artificial cultivation in the other great enemy of lung tissue, the pneumococcus (Rosenow). It is also possible that the tubercle bacilli recovered from glands in adults may be due to a more or less recent infection. Or it may be possible that certain tissues of the body have the power of altering a microorganism while others possess no such power. (See Bail, anthrax bacillus, Tsuda, typhoid bacillus.) Two apparently successful attempts to alter the type of virulence of tubercle bacilli are those of Eber, who, after three years of effort, apparently changed the human into the bovine, and those by Olaf Bang, of changing bovine tubercle bacilli into the avian type. Here, however, the possibility of the animal having contracted tuberculosis of another type after the experimental injection must be borne in mind.

Recent experiments by Calmette and Massol seem to bear on the question of a closer relation than is usually allowed between the bovine and the human type of bacilli. They found that serum from animals immunized against the bacillus of the bovine type had the power of precipitating extracts of bacilli of bovine and human types alike. The criticism may fairly be offered here that

the precipitates may have resulted from certain albuminoids remaining in the extract from the culture medium.

The sensitiveness of tuberculous body cells alike to tuberculin of bovine and human bacillary origin is not admissible here as argument, as such a body may be the seat of infections by both types of bacilli.

The frequency with which children are found to be the hosts of tuberculous infection (51.8 per cent, etc.; Hohlfeld, Shaw, von Pirquet), however, has some bearing on the point, because we know that a fair percentage of these infections are bovine in type (20–25 per cent). This means that, in at least some instances, the infecting organisms which may remain latent for many years may later produce tuberculosis in the body.

Numerous experiments by Rabinowitch, Weichselbaum, Bartel, and others on the virulence of tubercle bacilli in apparently normal or more or less altered organs, have shown how difficult it is to judge the question of virulence of bacilli which have remained latent in the different tissues of the body for various lengths of time.

The question still remains open whether in a child infected previously by the bovine type of bacillus and which later develops the human form of tuberculosis, the new manifestations are to be attributed to a new infection with the human type, or whether the bovine bacilli have in the meantime assumed the characteristic features of the human form.

Recent observations have shown the important rôle of the different tissues of the body in changing the virulence and morphologic characters of the tubercle bacillus. After investigations bearing on the influence of normal blood serum on experimental tuberculosis (White and Graham), we concluded to study the influence of organ extracts on bovine bacilli. It might be possible by such work to account in some measure for the failure to find the bovine bacillus in cases of human pulmonary infection. We approached the question from the side of the possibility of lung tissue having some power of altering the organism used. The experiments here reported deal only with the alteration in the virulence of the bovine organism for rabbits.

TECHNIC.

The extracts were prepared from organs removed from rabbits under aseptic precautions. The organs were washed with sterile salt solution through the vessels to remove the blood as much as possible. They were then cut and reduced in a mortar to a pulp (with or without sterile glass pearls or sand) according to the consistency of the organ used. The material thus obtained was put into a sterile flask and shaken; 0.5 c.c. of this mixture was drawn into a sterile syringe and mixt with a definite weight of emulsion of tubercle bacilli. The balance of the material was placed in the incubator for autolysis for one to three days. In the different series 0.5 c.c. of the autolyzed mixture was taken up in a sterile syringe and mixt with a constant definite weight of bovine bacilli in emulsion. The bovine bacilli used were made with three-weeks'-old subcultures of the strain (Bl) obtained through the kindness of Dr. Baldwin of Saranac Lake. The tubercle bacilli for each series were weighed in total quantity for the number of animals used in the series. This quantity was then triturated in a sterile mortar with sterile saline solution, so that each 0.5 c.c. of the emulsion corresponded to 0.003 gm. of moist weight of the original culture. Before each injection the emulsion was triturated and immediately the quantity corresponding to 0.003 gm. of bacilli was drawn into the sterile syringe. To this was added 0.5 c.c. of the fresh or autolyzed organ extract. These suspensions of bacilli and extracts after thorough mixture were then placed in the incubator at 38° C. and allowed to remain for one hour. Each mixture was then injected intraperitoneally into a rabbit.

All extracts were used both before and after autolysis. As seen from series C and D, 1 per cent thymol was added to the organ extracts, both fresh and autolyzed. In the course of our experiments, which extended over 16 months, we obtained in 5 series results that were not uniform. Certain of the results, however, were so striking that a report seems justified.

We were familiar at the start with the interesting work of Bartel and Neumann on the influence of lymphatic extracts on infection of animals with tubercle bacilli. Since our work was commenced the publications of Deycke and Much, Loewenstein, Rabinowitch, Levierato, Weichselbaum, Fontes, Wittgenstein, Trudeau, and Krause have appeared. In our later series we used as a comparison bacilli treated with other extracts (of kidneys, spleen, and nervous tissue) as well as lung and liver extracts. In series E we inclosed the bacillary emulsion in thin capsules of celloidin which were imbedded in the different, washed organs and left for autolysis at 38° C. for 36 hours. After this time the content of the capsule was drawn up in a sterile syringe and injected intraperitoneally.

EXPERIMENTS.

The most striking thing in series A (see Table 1) is the extreme difference between the infection in the autolyzed lung and liver animals and the other animals. The difference between the four other animals was not very marked. It will be noticed that the control lost steadily in weight as did also the fresh liver extract and the fresh lung extract animals. The autolyzed lung animal gained steadily in weight. The autolyzed liver animal lost in all 50 gms.; while the normal serum animal gained 40 gms.

Series B[1] is an exact repetition of series A. The six rabbits used were not all

[1] We wish to acknowledge the work of Dr. D. L. Graham in carrying out the first two series of experiments.

TABLE 1.
SERIES "A."

	A 6 Control 0.003 gm. Bovine Tubercle Bacilli +0.5 c.c. Salt Solution	A 5 0.003 gm. Bovine Tubercle Bacilli +0.5 c.c. Rabbit Serum	A 4 0.003 gm. Bovine Tubercle Bacilli +0.5 c.c. Fresh Liver Extract	A 3 0.003 gm. Bovine Tubercle Bacilli +0.5 c.c. Fresh Lung Extract	A 2 0.003 gm. Bovine Tubercle Bacilli +0.5 c.c. Autolyzed Liver Extract	A 1 0.003 gm. Bovine Tubercle Bacilli +0.5 c.c. Autolyzed Lung Extract
Date of injection	June 21, 1909	June 21, 1909	June 21, 1909	June 21, 1909	June 21, 1909	June 21, 1909
Weight on injection	1,350 gms.	1,310 gms.	1,260 gms.	1,320 gms.	1,260 gms.	1,320 gms.
Life after injection	21 days, died	24 days, killed	24 days, killed	23 days, died	25 days, killed	24 days, killed
Weight at death	900 gms.	1,350 gms.	870 gms.	820 gms.	1,210 gms.	1,385 gms.
Number of organs involved	7	5	10	7	4	2
Tumor formation	2+	+	+	+	0	0
Miliary tubercles	Innumerable	Innumerable	Innumerable	Innumerable	Many single	Few scattered tubercles in omentun and small intestine
Lung involvement	Innumerable	Moderate	Innumerable	Moderate	Moderate	

TABLE 2.
SERIES "B."

	B 12 Control 0.003 gm. Bovine Tubercle Bacilli +0.5 c.c. Salt Solution	B 11 0.003 gm. Bovine Tubercle Bacilli +0.5 c.c. Normal Rabbit Serum	B 10 0.003 gm. Bovine Tubercle Bacilli +0.5 c.c. Fresh Liver Extract	B 9 0.003 gm. Bovine Tubercle Bacilli +0.5 c.c. Fresh Lung Extract	B 8 0.003 gm. Bovine Tubercle Bacilli +0.5 c.c. Autolyzed Liver Extract	B 7 0.003 gm. Bovine Tubercle Bacilli +0.5 c.c. Autolyzed Lung Extract
Date of injection	June 21, 1909	June 21, 1909	June 21, 1909	June 21, 1909	June 21, 1909	June 21, 1909
Weight on injection	1,370 gms.	1,870 gms.	990 gms.	1,195 gms.	720 gms.	1,095 gms.
Life after injection	19 days, killed	19 days, killed	19 days, killed	19 days, killed	6 days, died	19 days, killed
Weight at death	1,050 gms.	1,920 gms.	620 gms.	820 gms.	480 gms.	1,415 gms.
Number of organs involved with tuberculosis	9	7	7	10	Died too soon for comparison	0
Tumor formation	+	2+	+	+	.....	0
Miliary tubercles	Innumerable	Innumerable	Less than control	Innumerable	.....	0
Lung involvement	Innumerable	Less than control but conglomerate tubercles	Less than control	Innumerable	.....	0

of the same litter and were of different weights. In this series the difference between the autolyzed lung animal and all others of the series is more striking, in that the animal in question presented absolutely no sign of tuberculous infection. The autolyzed liver animal died too soon to offer any comparison. The other animals do not

differ materially from each other. The control and the fresh lung and liver extract animals lost steadily in weight. The autolyzed lung extract animal lost 95 gms. and then gained 415 gms. The normal serum animal gained in all 50 gms.

Series C consisted of ten rabbits. Four only are given in Table 3, the remaining six for reasons given below constitute series D. In this series the clumps of bacilli in the emulsion were large and difficult to separate, and the suspension was consequently centrifugalized. This probably resulted in a smaller dose of bacilli for each animal. There was some suspicion of the purity of the culture which had been growing through the summer. Whatever the explanation, the animals of the whole series did not develop the expected degree of sickness.

TABLE 3.
SERIES "C."

	C 1 0.003 gm. Bovine Tubercle Bacilli +0.5 c.c. Salt Solution	C 2 0.003 gm. Bovine Tubercle Bacilli +0.5 c.c. Thymol Autolyzed Lung Extract	C 4 0.003 gm. Bovine Tubercle Bacilli +0.5 c.c. Autolyzed Saline Lung Extract	C 15 0.003 gm. Bovine Tubercle Bacilli +0.5 c.c. Fresh Liver Extract
Date of injection...	November 16, 1909	November 18, 1909	November 18, 1909	November 16, 1909
Weight on injection.	2,030 gms.	1,460 gms.	1,680 gms.	1,410 gms.
Life after injection .	35 days, killed	179 days, killed	2 days, died	168 days, killed
Weight at death....	2,090 gms.	2,600 gms.	1,700 gms.	2,330 gms.
Number of organs involved.....	3	1	Died too soon for comparison	1
Miliary tubercles ..	Few tubercles on great omentum, diaphragm, and lungs	Fibrinous plaque on the peritoneum. No tubercles	.....	0
Lung involvement .	Many tubercles	0	.....	2 tubercles

In this series the lung autolyzed animal died from unknown cause at the end of two days. The lung thymolized autolyzed animal presented less infection than the control animal, as did also the fresh liver extract animal.

The control animal lost 380 gms. and then returned to 60 gms. more than its first weight. The autolyzed thymol lung extract animal had two pregnancies. It gained steadily in weight until one week following its parturition, and then steadily declined.

The fresh liver animal had one pregnancy and gained steadily in weight with the exception of the parturition period.

Series D consisted of six animals of series C and one control. The six animals of this series had received the following injections: Tubercle bacilli treated with extract of lung in salt solution, fresh extract, autolyzed lung extract in salt solution, lung thymol, fresh extract, autolyzed thymol liver extract, fresh thymol liver extract, and autolyzed liver extract in salt solution. These six animals and the one control were injected with 0.003 gm. pure bovine culture 120 days after the injection as indicated in series C, Table 3.

Of the reinjected animals (see Table 4), the autolyzed liver extract animal developed less tuberculosis than the control. It gained in weight in the period following the first injection, then declined steadily and died 6 days earlier than its control. It lost 600 gms. after the reinjection as compared with the 780 gms. lost by the control.

The autolyzed thymol liver extract animal developed less tuberculosis than the control animal, and died 8 days earlier. It gained steadily in weight until the reinjection and then lost 620 gms. The thymol fresh liver extract animal developed as much tuberculosis as the control; was killed one day later; it had one pregnancy and lost after the reinjection 270 gms.

TABLE 4.
SERIES "D."

	Control 0.003 gm. Bovine Tubercle Bacilli +0.5 c.c. Salt Solution	D 5 0.003 gm. Bovine Tubercle Bacilli +0.5 c.c. Fresh Lung Extract	D 13 0.003 gm. Bovine Tubercle Bacilli +0.5 c.c. Autolyzed Lung Extract	D 16 0.003 gm. Bovine Tubercle Bacilli +0.5 c.c. Thymol Fresh Lung Extract	D 18 0.003 gm. Bovine Tubercle Bacilli +0.5 c.c. Autolyzed Thymol Liver Extract	D 17 0.003 gm. Bovine Tubercle Bacilli +0.5 c.c. Thymol Fresh Liver Extract	D 20 0.003 gm. Bovine Tubercle Bacilli +0.5 c.c. Autolyzed Liver Extract
Date of first injection	March 17, 1910	Nov. 16, 1909	Nov. 18, 1909	Nov. 16, 1909	Nov. 18, 1909	Nov. 16, 1909	Nov. 18, 1909
Weight on injection	1,950 gms.	2,000 gms.	1,900 gms.	1,450 gms.	1,130 gms.	1,030 gms.	1,230 gms.
Date of second injection	.....	March 17, 1910	March 17, 1910	March 17, 1910	March 17, 1910	March 17, 1910	March 17, 1910
Weight on second injection	.....	2,700 gms.	2,170 gms.	2,500 gms.	1,970 gms.	1,980 gms.	1,960 gms.
Life after injection	35 days, died	162 days, killed	148 days, died	146 days, died	147 days, died	162 days, killed	146 days, died
Weight at death	1,170 gms.	2,370 gms.	1,830 gms.	1,860 gms.	1,350 gms.	1,710 gms.	1,300 gms.
Number of organs involved	18	14	14	15	13	18	14
Tumor formation	1	2	2	2	4	3	3
Miliary tubercles	Innumerable	Less than control	Innumerable on diaphragm, lungs, and large omentum	Innumerable in most organs. A little less in liver, diaphragm, pericardium, and sex organs	Innumerable, except in peritoneum, kidneys, and mesentery	Innumerable. No marked difference from control animal	Innumerable, except in large intestine and thoracic glands where less
Lung involvement	Innumerable	Less than control	Innumerable	Innumerable	Innumerable	Innumerable	Innumerable

The thymol fresh lung extract animal developed less tuberculosis than the control; died 9 days earlier; had one abortion after reinjection, and lost 640 gms. The saline autolyzed lung extract animal developed less tuberculosis than the control; died 2 weeks earlier and lost 340 gms. After the reinjection, the saline fresh lung extract animal developed less tuberculosis than the control; lived 7 days longer, and lost 330 gms.

If any conclusion be justified from this series, it would seem that the previous injection rendered the animals less resistant to the reinfection, for while all developed less tuberculosis and lost less in weight, yet they succumbed more readily to the infection.

Series E consisted of 5 animals; the suspension of 0.003 gm. of tubercle bacilli was inclosed in a thin celloidin capsule and imbedded in the freshly removed and washed organs (lung, liver, kidney, and spleen) which were allowed to undergo

autolysis, in a moist chamber, in the incubator at 38° C. for 36 hours. After autolysis, the suspension of tubercle bacilli was withdrawn from each capsule in a sterile syringe and injected intraperitoneally in a rabbit. The control received 0.003 gm. of tubercle bacilli which had been enclosed in a celloidin capsule in a moist chamber in the incubator for the same length of time. This was then withdrawn with a sterile syringe and injected intraperitoneally. In this series the amount extracted from the spleen and kidney capsule was less than that from the control and the lung and liver capsules.

We feel that owing to the fact that the amount of bacillary emulsion withdrawn from the capsule was not the same for each animal, no definite comparison of the weight of bacilli introduced could be made. This experiment was unsatisfactory; nevertheless, it is striking that none of the animals which received tubercle bacilli after being subjected to the action of autolyzing organs developed as much tuberculosis as the control, and especially that the lung capsule rabbit was the only one which gained in weight.

TABLE 5.
SERIES "E."

	Control Content of Control Capsule Corresponding to 0.003 gm. Bovine Tubercle Bacilli	E Lg. Content of Lung Capsule 0.2 c.c. Diluted with 0.4 c.c. Sterile Salt Solution	E Lv. Content of Capsule Kept in Autolyzing Liver+0.15 c.c. Bovine Bacilli+0.4 c.c. Salt Solution	E K. Content of Capsule from Autolyzing Kidney+0.015 c.c. Bovine Bacilli+0.2 c.c. Sterile Salt Solution	E Sp. Content of Capsule from Autolyzing Spleen+0.2 c.c. Sterile Saline Solution
Date of injection	February 5, 1910	February 5, 1910	February 5, 1910	February 5, 1910	February 5, 1910
Weight on injection	2,550 gms.	2,360 gms.	2,330 gms.	2,240 gms.	2,330 gms.
Life after injection	40 days, killed	40 days, killed	40 days, killed	40 days, killed	104 days, killed
Weight at death	2,330 gms.	2,530 gms.	2,200 gms.	2,140 gms.	2,260 gms.
Number of organs involved	15	8	6	2	8
Tumor formation	2	0	0	0	0
Miliary tubercles	Innumerable	Much less than control	Few tubercles in omentum, small intestine, diaphragm, and lungs	Few tubercles only in kidneys and spleen	Moderate amount
Lung involvement	Innumerable	Very few tubercles	Very few tubercles	None	Few tubercles

In series E we used 8 animals and one control. This series was similar to series A and B with the exception that the fresh and autolyzed extracts of spleen and brain were added. The organs which were used for the extracts in this series were taken from an animal two years old. The animals which were injected with the mixture of bacilli and organ extract were young, varying between two and four months. The extracts were allowed to autolyze for 24 hours only. These three points offer the only explanation we can give for the difference between this experiment and the previous ones, and we would suggest that extracts from an animal of old age, autolyzed for a shorter time, injected into very young animals, coupled with the difference of susceptibility to tuberculous infection at different ages, may offer the reason for the different result.

Both brain extract animals, which were not much different from each other however, were approximately the same as the control animal. For some reason this

TABLE 6.
SERIES "F."

	Control. 0.003 gm. Bovine Tubercle Bacilli +0.5 c.c. Salt Solution	0.003 gm. Bovine Tubercle Bacilli +0.5 c.c. Fresh Lung Extract	0.003 gm. Bovine Tubercle Bacilli +0.5 c.c. Autolyzed Lung Extract	0.003 gm. Bovine Tubercle Bacilli +0.5 c.c. Fresh Liver Extract	0.003 gm. Bovine Tubercle Bacilli +0.5 c.c. Autolyzed Liver Extract	0.003 gm. Bovine Tubercle Bacilli +0.5 c.c. Fresh Spleen Extract	0.003 gm. Bovine Tubercle Bacilli +0.5 c.c. Autolyzed Spleen Extract	0.003 gm. Bovine Tubercle Bacilli +0.5 c.c. Fresh Brain Extract	0.003 gm. Bovine Tubercle Bacilli +0.5 c.c. Autolyzed Brain Extract
Date of injection	April 4, 1910	April 4, 1910	April 5, 1910	April 4, 1910	April 5, 1910	April 4, 1910	April 5, 1910	April 4, 1910	April 5, 1910
Weight on injection	2,880 gms.	1,810 gms.	2,050 gms.	1,100 gms.	1,050 gms..	1,300 gms.	1,020 gms.	1,000 gms.	970 gms.
Life after injection	39 days, killed	40 days, killed	37 days, died	31 days, died	44 days, killed	43 days, killed	43 days, killed	45 days, killed	44 days, killed
Weight at death	2,080 gms.	1,545 gms.	1,130 gms.	920 gms.	1,810 gms.	1,720 gms.	1,060 gms.	1,800 gms.	1,530 gms.
Number of organs involved.	14	16	16	14	5	1 Abscess point of injection	16	11	14
Tumor formation	2	2	2	3	1	0	1	1	2
Miliary tubercles	Innumerable	Much less than control	Innumerable	Innumerable	Very few	0	Innumerable, even more than control	Innumerable, a little less than control	Innumerable, not much different from control
Lung involvement	Innumerable	Innumerable	Innumerable	Innumerable	Few conglomerate	0	Innumerable	Innumerable, not so many as in control	Less than control

does not bear out fully the experiments of Deycke and Much. The autolyzed lung and fresh liver animals died spontaneously two and eight days earlier than the control. The autolyzed liver extract, the fresh spleen extract, the autolyzed spleen extract and both brain extract animals gained steadily in weight. The control animal, both lung extract and fresh liver extract animals steadily declined. The animals that gained in weight, with the exception of the fresh liver animal, were about one month and a half younger than the other three animals.

GENERAL SUMMARY.

As a result of our experiments it would seem that under certain conditions extracts of autolyzed rabbits' lungs contain a factor which when incubated with bovine tubercle bacilli changes their virulence (series A, B, C, D).

The explanation of this phenomenon is difficult with our present knowledge. It is apparently not due to a dissolution of the bacilli, as smears made from the mixture of bacilli and extract after incubation show the bacilli very little changed in form and staining power. It may be due to the reaction of the extract which is alkaline from the beginning to as late as three weeks following the beginning of autolysis. As pointed out by Koch and later by Rabinowitch, an alkaline medium has a marked influence on the staining properties and virulence of tubercle bacilli. We do not feel, however, that the alkalinity of the extract is the sole factor to be considered in explanation of these results because the extracts of other organs during autolysis became alkaline (spleen and brain), and yet these did not produce the same reduction in virulence of the tubercle bacilli although the condition of the preparation was the same for all extracts made by us.

Another possible explanation of the influence of the autolyzed lung extract may be found in the presence of lipoid bodies in the lung (lecithin, neurin, and cholin). This would bring the experiments into the same category as the experiments of Deycke and Much, who believe that the influence of brain substance on tubercle bacilli in their experiments was due to the presence of neurin and cholin in the extracts. This latter statement, however, is doubted by Loewenstein, F. Jessen, and Rabinowitch, who ascribe the changes found by Deycke and Much to error in the technic and to the alkalinity of the solution as pointed out by

Koch in his original monograph. Lecithin, however, is probably present during the lung autolysis and we were able to demonstrate cholin crystals in our lung extracts (autolyzed for 3 days) by the method of Rosenheim.

Another possible explanation might be found in the action of nucleinic acids directly on the tubercle bacilli or indirectly by stimulating the lymphocytes of the body to a more vigorous defense (Neumann, citing von Bartel, Bacherach, and Stein).

Another explanation, which, however, does not seem to us very likely, may be found in the action of fatty acids and of soaps. The experiments of Noguchi have shown the influence in certain concentrations of oleic soaps on the virulence and growth of tubercle bacilli. The fats contained in lung tissue may undergo autolysis and be split into fatty acids and glycerin, and combined with the alkaline radicals may act on the tubercle bacilli. The alkaline reaction throughout the autolysis of lung tissue, however, would not support this view.

Another possible explanation might be found in the action of specific enzymes contained in autolyzed lung tissue and the action of these on the tubercle bacilli. Although Opie and Barker and Berger have demonstrated a lipase in lymphocytes, and Magnus Levi in the liver, the presence of such a lipolytic ferment in lung tissue has not been observed. Simon in working with autolyzed lung tissue found a fair amount of neutral fat in large and small drops. Bartel obtained his favorable results also with boiled organ extracts. A series of heating experiments now in progress in this laboratory render the enzyme explanation unlikely as does also the fact that we could find little change in smears made after the action of the extract of autolyzed lung on the bacilli.

It may be possible that the change in virulence is due to the presence of other symbiotic bacteria which grow in the tissue during autolysis. The experiments of Rosenbach, who observed that tubercle bacilli may lose in virulence if grown together with trichophyton, lend some support to this view. Further, Vaudremer has lately published observations on the influence of *Penicillium glaucum* and pyocyaneus in diminishing the toxic power

of tuberculin. Applied to our experiments, however, it seems unlikely since the other organisms were present in the extracts of all autolyzed organs alike.

Provided that these experiments are applicable to all animals it is interesting that the lung tissue, which is the seat of maximum attack of the bacillus during the life of the animal, should develop during autolysis a substance which has so marked an influence in reducing the virulence of the germ for other animals of the same species. Cadeac and Mallet found the tubercle bacilli still virulent after 102 days in lung tissue that had been desiccated and powdered and after 17 days in lungs that had been powdered and decomposed. Schottelius found virulent tubercle bacilli in lungs that had been buried for $2\frac{1}{2}$ years. Petri found virulent bacilli after 22 and 96 days in rabbits that had been buried in wooden and tin coffins. Loesener obtained tubercle bacilli which would produce general tuberculosis from lungs that had been buried for 60 days, and local tuberculosis from lungs that had been buried for 95 days. Bartel and Hartel, however, found that bovine tubercle bacilli after desiccation in rabbits' lungs for 9 days lost their virulence.

The length of time allowed for autolysis would seem to be a factor of great importance since the phenomenon was not obtained in lung tissue which was allowed to autolyze for 24 hours only, but was most marked after autolysis had progressed for three days. The autolysis of lung tissue follows a different and a slower course than the autolysis of other organs, as shown by Jacoby and Johannsen. The latter found that all organs, except the lungs, are able to reduce an aqueous solution of methylene blue.

The results obtained with the extracts of autolyzed liver are in accord with the investigations of Bartel and Stein and Neumann and Wittgenstein, who were able to reduce by these extracts the virulence of tubercle bacilli. Our results with liver extract, however, are not to be compared with the results in our lung animals. In this connection it is to be noted that the liver extract is acid from the beginning (Jacoby, Magnus Levi, Salkowsky, Bartel, Schryver), due to the presence of acids (lactic acid and, from the odor of our extracts, probably of butyric and acetic acid), thus

differing markedly from the lung extract which was alkaline throughout.

The two experiments with the extracts of the spleen and brain are not enough to justify any conclusions. The brain animals, while they increased steadily in weight, did not confirm the favorable results of Deycke and Much, as there were almost as many conglomerated and single tubercles found as in the control animal. The acid brain medium suggested by Ficker for the cultivation of tubercle bacilli would not support the view of diminished virulence of tubercle bacilli by the action of brain extract. The reaction of the brain extract at the beginning of autolysis was almost neutral and during the three days of autolysis became alkaline, though the tubercle bacilli subjected to the influence of this extract did not show great changes in their character or virulence.

LITERATURE.

BACHRACH AND BARTEL. *Wien. klin. Wchnschr.*, 1907, 20, p. 1040.
BAIL. *Centralbl. f. Bakt.*, 1908, Orig., 46, p. 488.
BALDWIN. *Jour. Med. Res.*, 1910, 22, p. 194.
BANG. *Centralbl. f. Bakt.*, 1908, Orig., 46, p. 461.
BARTEL. *Probleme der Tuberculosefrage*, 1909, p. 51 (Deuticke, Wien).
———. *Wien. klin. Wchnschr.*, 1907, 20, p. 1321.
——— AND NEUMANN. *Centralbl. f. Bakt.*, 1909, Orig., 48, p. 657.
——— AND STEIN. *Centralbl. f. Bakt.*, 1905, Orig., 38, p. 154.
BERGEL. *Muench. Med. Wchnschr.*, 1909, 56, p. 64.
BESANÇON ET LABBÉ. *Arch. de méd. expériment.*, 1898, 10, p. 318.
BONGERT. *Ref. a. d. 8. Internat. Congress Veterin.*, Budapest, 1905.
CADEAC ET MALET. *Lyon méd.*, 1888, 58, p. 229.
CALMETTE ET MASSOL. *Compt. rend.*, 1910, 149, p. 760.
DEYCKE. *Muench. med. Wchnschr.*, 1910, 57, p. 633.
DUVAL. *Jour. expér. méd.*, 1909, 11, p. 403.
EBER. *Muench. med. Wchnschr.*, 1910, 57, p. 115.
ELLINGER. *Deut. med. Wchnschr.*, 1900, 26, p. 580.
FICKER. *Centralbl. f. Bakt.*, 1900, 27, p. 504.
FONTES. *Centralbl. f. Bakt.*, 1909, Orig., 49, p. 317.
HESS. *Jour. Amer. Med. Assn.*, 1909, 52, p. 1011.
HEYMANN. *Ztschr. f. Hyg.*, 1909, 62, p. 3.
HOHLFELD. *Muench. med. Wchnschr.*, 1910, 57, p. 235.
JACOBY. *Ztschr. f. Physiol. Chem.*, 1910, 33, p. 156.
———. *Beiträge zur chem. physiol. u. path.*, 1903, 3, p. 446.
JESSEN AND L. RABINOWITCH. *Centralbl. f. Bakt.*, 1910, Orig., 54, p. 454.
———. II, *Interim Report of Roy. Commissioners*, 1907, Part I.

JOHANNSEN. *Arbeit. a. d. path. Instit. Tübingen*, 1905, 5, p. 326.
KOCH. *Deut. med. Wchnschr.*, 1897, 23, p. 14.
LEVIERATO. *Reform. medic.*, 1909, 25, p. 286, cit. *Ztschr. f. Immunit. Forschg.*, Part II, 1909, 70, p. 235.
———. *Gazd. Ospedal*, 1910, No. 11.
LEWIS, P. A. *Jour. Exp. Med.*, 1910, 12, p. 82.
LOESENER. *Arbeit. a. d. kais. Gesundhts. Amt*, 1898, p. 448.
LOEWENSTEIN. *Centralbl. f. Bakt.*, 1910, Orig., 53, p. 541.
MAGNUS LEVY. *Beiträge zur chem. physiol. u. path.*, 1902, 2, p. 261.
MOSS, W. L. *Johns Hopkins Hosp. Bull.*, 1909, 20, p. 39.
MUCH, H. *Muench. med. Wchnschr.*, 1909, 56, p. 1825.
NEUMANN AND WETTGENSTEIN. *Beitr. z. Klin. d. Tuberculose*, 1909, 13, p. 145.
NOGUCHI. *Internat. Congress Tuberculosis, Washington*, p. 246.
OEHLECKER. *Tb. Arbeit. a. d. Gesundhts. Amt*, 1907, 6, p. 88.
———. *Ibid.*, 1907, 7, p. 65.
OPIE AND BARKER. *Jour. Exp. Med.*, 1908, 10, p. 645.
PARK, cit. by SHAW. *Jour. Amer. Med. Assn.*, 1909, 53, p. 1807.
PETRI. *Arb. a. d. kais. Gesundhts. Amt*, 1891, 7, p. 1.
PIRQUET, C. v. *Jour. Amer. Med. Assn.*, 1909, 52, p. 675.
RABINOWITCH, L. *Berlin. klin. Wchnschr.*, 1907, 44, p. 315.
RAVENEL. *Jour. Amer. Med. Sci.*, 1907, 134, p. 467, and *Jour. Med. Res.*, 1903, 10, p. 460.
ROSENBACH. *Deut. med. Wchnschr.*, 1910, 36, p. 1513.
ROSENHEIM. *Jour. Physiol.*, 1905, 33, p. 220.
ROSENOW, E. C. *Jour. Infect. Dis.*, 1910, 7, p. 411.
SALKOWSKI. *Deut. Klin. a. Eingange d. XX. Jahrhunderts*, 11.
SHAW, H. K. *Jour. Amer. Med. Assn.*, 1909, 53, p. 1805.
SCHLOSSMANN AND ENGEL, cit. by RAVENEL. *Jour. Med. Res.*
SCHOTTELIUS. *62. Vers. deutsch. Naturforsch. u. Aerzte*, Heidelberg, 1890.
SHRYVER. *Biochem. Jour.*, 1906, 1, p. 123.
SIMON. *Deut. Arch. f. klin. Mediz.*, 1901, 70, p. 601.
SMITH AND BROWN, TH. *Jour. Med. Res.*, 1907, 55, p. 435.
———. *Trans. Assn. Amer. Phys.*, 1898, 13, p. 417.
TRUDEAU AND KRAUSE. *Jour. Med. Res.*, 1910, 22, p. 277.
TSUDA. *Centralbl. f. Bakt.*, 1910, Orig., 46, p. 6.
VAGEDES. *Ztschr. f. Hyg.*, 1908, 28, p. 276.
VAUDREMER. *Annal. d. l'Instit. Pasteur*, 1910, 24, p. 189.
WARD, E. P. *Ztschr. f. Tuberculose*, 1910, 14, p. 151.
WEBER AND TAUTE. *Tb. Arbeit. a. d. Gesundhts. Amt*, 1907, p. 15.
———. *Ibid.*, 1908, pp. 27–50.
WEICHSELBAUM AND BARTEL. *Wien. klin. Wchnschr.*, 1905, 18, p. 241.
———. *Ibid.*, 1907, 20, p. 1115.
WHITE AND GRAHAM. *Jour. Med. Res.*, 1909, 20, p. 67.
WHITLAW. *Brit. Med. Jour.*, 1908, 2, p. 61.

THE IMMUNOLOGICAL REACTIONS OF OIDIOMYCOSIS (BLASTOMYCOSIS) IN THE GUINEA-PIG.*

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The object of this work was to examine the mode of resistance of guinea-pigs to oidiomycetic infection; to determine whether it might be possible to increase this resistance by immunization, and, if so, to investigate the factors upon which this increase in resisting power depended.

LITERATURE.

For a full discussion of the relation of oidioid and blastomycetoid organisms to human and animal diseases and for literature as to their relative pathogenicity for various animals, the reader is referred to the work of Ricketts,[11] Brown,[25] Hektoen,[26] Montgomery and Ormsby,[27] and Spiethoff.[19] The papers considered here are those which have dealt somewhat specifically with the question of the mode of resistance of animals to infection with mold fungi, and with the demonstration of antibodies in the infected animals.

Metchnikoff[1] (1884) demonstrated that phagocytosis is the chief means of defense of the daphnia against infection with a mold fungus christened by him "Monospora bicuspidata." This was followed by a number of articles by Ribbert[2] (1887) who was inclined to believe that mold fungi (Schimmelpilze) and pathogenic schizomycetes injected into rabbits are ingested by leukocytes within which they undergo intracellular digestion. Charrin and Ostrowsky[4] (1896) observed that immunized animals were but moderately resistant to reinfection with *Oidium albicans*. Roger[5] (1896) obtained considerable resistance to *Oidium albicans* by vaccination. Rabbits given repeated intravenous injections of sublethal doses became able to resist infection by double the lethal dose. In normal serum the organisms grew quite readily; in immune serum they were first agglutinated, then became hyaline, the capsule disintegrated, and later attempts to obtain cultures showed that the oidia were dead. Schattenfroh[6] (1896), using a non-pathogenic yeast, found that the sera of animals were not bactericidal but that peritoneal exudates were markedly so. He concluded that the germicidal power of peritoneal exudates depends on phagocytosis. Gilkinet[7] (1897) believed from the results of his experiments, (1) that beer yeasts (*Saccharomyces cerevisiae*) introduced into rabbits either intravenously or subcutaneously produce

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neither local nor general symptoms; (2) that such yeasts do not multiply in living tissues; (3) that they are destroyed within a very short time, and cannot be discovered in the bodies of the animals by any method; (4) that this destruction is brought about by the substances in the plasma and is not a function of the body temperature, of chemical reaction, or of the absence of nutritive substances, but is a specific, unknown property of the organic fluids; (5) that this destructive property is not dependent upon the formed elements of the blood, but is exercised in the same degree by all the body liquids; and, finally, (6) that this property is itself destroyed by heating at 55° C. He found that yeast mixed with ox serum or rabbit serum and kept at 36° C. for from two to four days lost its power of growth on suitable media, but that if such mixtures were kept at room temperature for corresponding periods, the yeasts were unharmed. He placed porous tubes containing blastomycetes in the peritoneal cavities of three rabbits. The first rabbit was examined after four days; the yeast cells were not much changed, and were not in contact with leukocytes; in the second rabbit, killed after nine days, and in the third rabbit, killed after twelve days, there were but few normal yeast forms and apparently all were dead, since cultures were negative. Leukocytes were not in contact with the organism. The author produced edema of the rabbit's leg by tight bandaging; such edematous fluid, free from leukocytes, killed yeasts in three days at 37° C. Jona[8] (1897) gave rabbits intraperitoneal, intravenous, and subcutaneous injections of a non-pathogenic yeast, *Saccharomyces apiculatus*. He concluded that in all cases the organisms were destroyed within a few hours through the influence of the body fluids. Organisms injected into the peritoneal cavity, or subcutaneously, did not make their way into the blood. Obici[9] (1898) found that after repeated injections of the toxin (filtered broth culture) and small quantities of the spores, an immunity was established in rabbits, against *Aspergillus fumigatus*. This immunity, however, was incomplete, and vanished in a short time. He did not believe, with Ribbert, that phagocytosis was of such paramount importance in the defense of the animal body against this infection, as he saw evidences of degeneration in organisms in the absence of phagocytic cells. Skchiwan[10] (1899) experimented on guinea-pigs and rabbits with *Saccharomyces tumefaciens* of Curtis. He concluded that the body fluids are not bactericidal since the organisms protected from the leukocytes by celloidin sacs grew well on artificial media after being in the peritoneal cavity of the animal for four days. On the other hand, yeasts injected directly into the peritoneal cavity were phagocyted by certain cells and digested. The phagocyted yeasts lost their staining power in from two to four days, as well as their power to grow when planted on suitable media. The leukocytes formed rosette-like masses about the organisms. At first polymorphonuclear leukocytes were found in such masses; later, the groups consisted entirely of macrophages. Malvoz[13] (1901) experimented with six strains of yeast, some pathogenic and some non-pathogenic. The pathogenic cultures he obtained from Sanfelice, Plimmer, Curtis, and one he isolated himself from an epithelioma, calling it *Blastomyces E* (BE) as a convenient laboratory name. His non-pathogenic cultures consisted of a strain of *Saccharomyces ellipsoideus* and a powerfully fermentative organism which he called the yeast of Huy. Rabbits were given two weekly injections of the organism of Huy, BE, and Sanfelice over a period of months, and the agglutinative properties of the sera were then tested. The specific serum agglutinated the organism of Huy in a dilution of 1:50; organism BE, 1:90; organism Sanfelice, 1:5. The specific serum for the organism of Huy agglutinated *S. ellipsoideus* in the same titre as the organism of Huy itself; more

weakly the organism of Plimmer and organism BE; and the organism of Curtis not at all. The specific serum for organism BE agglutinated organism of Huy very slightly. The organisms of Huy, Sanfelice, and organism BE grew well in normal and immune serum, and grew on artificial media when transplanted after 1 to 24 hours. The author concluded that in this case either bactericidal antibodies were absent or the organisms were protected by their capsule. He suggests that the Bordet-Gengou reaction might be of service in deciding the presence or absence of such antibodies.

Ricketts[11] (1901) says: "Professor Hektoen has found that the undiluted serum of a dog which had received successive inoculations of the organism from Case I (cutaneous oidiomycosis) causes gradual clumping of the organism diffused in bouillon. Several hours elapse before the fullest extent of clumping possible is reached. Organisms from other cases show only a slight degree of clumping with the same serum. All organisms grew well in the serum and the production of mycelium was especially noticeable. Normal dog's serum caused no clumping of any of the organisms; abundant mycelium is produced in all cases.

"The organism from the case reported by Hyde, Hektoen and Bevan was repeatedly inoculated into the abdominal cavity of a rabbit. It was found that the animal's serum would cause fairly distinct agglutination of the organism inoculated."

Sanfelice[3] (1896) discovered that heating for 30" at 60° C. destroyed the virulence of his *Saccharomyces neoformans* for guinea-pigs, but did not destroy its power to grow on artificial media. He endeavored to immunize guinea-pigs against this organism (1) by the injection of organisms whose virulence had been destroyed by heating to 60° C. for 30"; (2) by repeated injections of filtered broth cultures of various ages; and (3) by the injection of the serum of guinea-pigs and of dogs which had received repeated injections of either the heated organism or the filtered culture, or had recovered from the infection induced by a dose of virulent organisms. He was wholly unsuccessful.

In 1902 Sanfelice[15] was able to immunize dogs, cats, and rabbits to *Saccharomyces neoformans*, Plimmer's yeast, and to a non-pathogenic yeast isolated from the air, so that the animals could withstand, without symptoms, an intravenous injection of quantities of the first two organisms which caused the death of normal, control animals. He had no difficulty in demonstrating the presence of a specific amboceptor in the serum of such immune animals by the fixation test. He was unable to demonstrate the antibody in actively infected animals which subsequently died of the disease. Unfortunately he never took the trouble to control his fixation experiments by testing the effects of his antigen—emulsion of yeast—or of his antibodies—inactivated immune and normal sera—upon the hemolytic system which he used—serum of rabbits immune to fowl corpuscles—a fact which detracts considerably from the value of his results. He says that Malvoz[12] demonstrated antiblastomycetic amboceptors by the fixation reaction in 1901. Sanfelice believed that the immune sera caused a change in the yeast cells exposed to their action such that the organisms assumed an appearance identical with the so-called Russell's fuchsin bodies of malignant tumors.

Wlaeff[16] (1902) immunized geese and donkeys to a yeastlike organism which he isolated from the ascitic fluid of a patient whose disease had been diagnosed as inoperable abdominal cancer with ascites. A piece of the growth, removed at an exploratory operation, was diagnosed by Cornil as a typical cylindrical-celled carcinoma. The sera of the immunized animals agglutinated and dissolved the specific organisms. Wlaeff claimed that, following repeated injections of such sera, the ascitic fluid of this

cancer patient likewise developed the property of agglutinating and dissolving the blastomycetes and that the cancer improved as the result of such treatment.

Fabozzi[18] (1905) concluded from his experiments that *Saccharomyces neoformans* never causes the development of tumors, and is finally destroyed by phagocytes.

Christensen and Hektoen[21] (1906) say: "The character of the lesions of blastomycosis, the accumulation of leukocytes, the formation of giant cells, and the phagocytosis of blastomycetes—indicates that this is an infection in which phagocytosis is an important means of defense and healing. Certain preliminary test-tube experiments showed that phagocytosis of blastomycetes is favored by the presence of normal serum, and the idea arose that it might be possible to stimulate the greater formation, in cases of blastomycosis, of the body that promotes phagocytosis (opsonin), as well as the other antibodies, by the injection of blastomycetic substances in a readily absorbable form. It was thought that the resistant character of the microorganisms, coupled with their inclosure in cellular exudate and granulation tissue, possibly prevents the absorption in proper quantities of the substances necessary to call forth strong immunizing reactions. Hence, in order to hasten, if possible, the reactions that favor healing, we injected in each of our cases a sterile blastomycetic vaccine prepared by Dr. H. T. Ricketts of the corresponding organism. Unfortunately the patients left the hospital at a time when no conclusions of value could be drawn as to the results of the vaccine."

Marco del Pont[23] (1907), using *Endomyces albicans*, grew broth cultures with increasing doses, first, of normal, and later, of immunized rabbit's serum. The development of a mycelium indicated that the medium was unfavorable. After the 60th transfer the yeast grew normally in undiluted immune serum. It retained this faculty. The immunity was specific; yeast habituated to rabbit serum did not grow in the serum of the dog, goat, or rat. The immunized fungus had lost its virulence. The author thought that the immunity to the serum might be due to the development of a mucilaginous capsule by the organism; he also thought that a substance anti-sensibilisatrice might be produced by the microorganism.

Widal, Abrami, Joltrain, Brissaud, and Weill[29] (1910) have found that the blood serum from cases of sporotrichosis possesses the power of agglutinating the spores—but not the cell bodies—of the *Sporotrichum schenchii* in dilutions of from 1:300 to 1:800. The agglutinability of the spores varies with the age of the culture and with the kind of medium, but not with the strain of the organism (10 strains were compared). The Bordet-Gengou reaction was constant. Among 168 normal subjects and subjects suffering from other diseases, they found an occasional agglutination or fixation reaction, but both never occurred in the same individual. The two reactions, therefore, control each other. Experimentally, two dogs and four rabbits were used. In dogs it was easy to produce infection with the organisms, and the agglutination and fixation reactions were constant and marked. In rabbits, intravenous injections of large quantities failed to cause infection. The agglutinative power of the serum increased appreciably; it varied from 1:10 to 1:30 in the different rabbits before injection, and rose, following injection, as high as 1:3,000 in one case and to but 1:300 in another. The reaction of fixation, which was tested parallel with the agglutination tests, gave "very incongruous results." No further details of these results are stated.

They found that the agglutination and fixation reactions disappeared in man after the infection was conquered. The fixation reaction disappeared more rapidly

than the other. They concluded that, so long as both are found, an active lesion is indicated; the disease is not really cured.

Co-reactions.—The organisms tested which did not give co-agglutination and co-fixation reactions with immune sporothrix serum and vice versa were the trichophytons, the organisms of Erythrasma, Favus, and of animal aspergillosis. The reactions of co-fixation and co-agglutination were obtained with organisms isolated from cases of actinomycosis and thrush (*Oidium albicans*). The serum from the case of infection with *Oidium albicans* agglutinated the spores of the sporothrix 3 times as strongly as it did the oidium itself, which was agglutinated only in dilutions of 1:10 to 1:50. The co-fixation reaction was positive with the following cultures of yeast: the organism of Curtis, of Blanchard, of Plimmer, *O. luteum*, *S. granulatus*, *S. lithogenes*, and *S. caprae*, using sera from cases of sporotrichosis, actinomycosis, and thrush. The organism of actinomycosis itself was not agglutinated by the specific serum.

Rothe[28] (1909), noting the preliminary reports of Widal,[29] tested the agglutinating power of the blood serum from two cases of actinomycosis upon the spores of the sporothrix and found them positive in dilutions of 1:160 and 1:200 respectively.

MATERIALS AND TECHNIC.

The organism used was obtained in 1905 by Dr. Rosenow from a case of oidiomycosis in the Presbyterian Hospital of Chicago. It had, therefore, been growing on artificial media for three years before the present work was begun. The stock culture was grown on a 1 per cent glucose, 1 per cent acid agar, and also in nutrient broth of similar sugar and acid content. On acid-glucose-agar, at room temperature, the organism grows in the form of long hyphae which burrow peripheralward through the superficial layers of the agar. No aerial hyphae are developed excepting in those cultures which have been started in the incubator and are later placed at room temperature. The characteristics of these hyphae will be discussed later.

In acid-glucose-broth, at room temperature, the organism grows in the form of fluffy balls, the size which the latter may reach apparently being limited only by the volume of the broth, the capacity of the retaining vessels, and the number of colonies which develop. There seems to be no production of gas. In old cultures a heavy membrane forms on the surface of the broth, and the medium, though remaining clear, assumes a dark amber color. Under the low power of the microscope in a hanging-drop preparation kept at room temperature and first observed October 20, 1908, one of these "fluffy balls" appeared as a mass of mycelial threads arranged in two zones, a central mass of tangled threads and granu-

lar material too thick for light to pierce and so not discernible in detail, and a peripheral zone of radiating threads. These threads presented a rather homogenous appearance, were quite refractive to light, and possessed a slightly greenish color by transmitted light. Their central ends were lost in the central mass; their peripheral ends were bluntly rounded. Branches were few and these few were found toward the base of the radiating threads; the branches were equal in diameter to the parent stem, were of uniform thickness throughout their length, for the most part, and were given off, sometimes at right angles, but usually at an angle of about 45°. The contour of the angle was not that of a sharp corner, but rounded. No segmentation of the hyphae nor lateral conidia were observed. No distinct cell wall appeared. On October 22, two days later, the following notes were made: Hyphae have at least doubled in length; three zones can now be distinguished in the colony: (1) a central, practically opaque zone; (2) a middle zone of branched, interlacing hyphae bearing lateral conidia; most of these hyphae show marked but somewhat irregular segmentation; and (3) a peripheral zone similar to the one described on October 20. The lateral conidia arise by short stalks from alternate sides of the hyphae, one conidium to each segment. In some cases no structures are discernible within the conidia; at other times they seem to be filled with sharply defined spherules. The segments consist of oblong, clear areas separated from each other by discs, cubes, and cylinders of a homogenous, highly refractive, greenish substance very similar in appearance to the material in the young hyphae described two days before. The peripheral ends of the hyphae and those intermediate portions in which the segmentation is but slightly marked contain scattered small, spherical, highly refractive bodies which seem to be attached to the inner side of the double-contoured cell wall which has now become visible. The hyphae are reaching far out into the vaseline by which the cover glass is attached.

November 29.—No change in method of development.

In agar hanging-drop preparations kept at room temperature essentially the same appearances are obtained as in the broth. Sometimes the lateral conidia appear to be filled with sharply out-

lined, homogenous spherules. If such conidia are kept under observation for a few weeks, the spherules can be seen to gradually fuse into larger and larger masses until finally the conidial content consists of a single homogenous mass. A somewhat similar condition has been described by Bowen and Wolbach.[20] In the case described by these authors, bodies were found on agar tubes, which were filled with refractive spherules. When these bodies were placed on fresh media the spherules fused before mycelial formation began.

On acid-glucose-agar at 37° C., the organism grew exclusively in the budding form, that is, like yeast, for about fourteen months, when it rather suddenly—in the course of a month or six weeks—developed a propensity for growing in the hyphal form to which it has clung tenaciously since. While growing in the budding form the colonies which formed on the agar were soft, yellowish white, raised, and button-shaped, consisting of a flat central area surrounded by a thickened, rounded border. They were circular in outline and from 1–7 mm. in diameter. The individual cells were from 10–30 μ in diameter.

With the assumption of the hyphal form of growth, the organisms became prone to the development of aerial hyphae. This tendency was especially noticeable in cultures kept at about 34.5° C.; 36–37° C. and room temperature (about 19–20° C.) appeared to be very much less favorable to their production, excepting in cultures, as mentioned above, which had been transferred from the incubator to room temperature. To the naked eye, the hyphae—developed at 34.5° C.—appear as very delicate stalks perhaps a millimeter in height (estimated), white by transmitted light, and having a tendency to assume a silvery sheen by reflected light. In the mass, they present a dead white, somewhat fuzzy appearance, resembling a piece of high grade, heavy filter-paper. About the edges of the growth, where the hyphae are less numerous, the cultures have a "woolly" appearance. In this condition the culture is less adherent to the medium than at earlier stages—the hyphae begin to appear about 10 days to two weeks after inoculation—and may be peeled off in a single layer taking with it pieces of agar. At a few scattered points hyphae may be seen which are three or

four times the ordinary length and project above the general level at odd angles, like the poles from the top of an Indian wigwam—probably the "porcupine" appearance mentioned by Hamburger.[22]

Under the microscope the hyphae appear as slender, hollow rods, 1 to 2 μ in diameter, with a very delicate wall. They contain a very fine granular substance in addition to quite highly refractive, homogenous, greenish spherules which vary in diameter from a fraction of a micron to one micron. Sometimes the hyphae are segmented and bear lateral and terminal conidia, as well as occasional enlargements of the individual segments, in the course of the tube itself. The conidia and other swellings are from 5 to 10 μ in diameter and contain, usually, one, and occasionally 3 or 4 spherules, similar in appearance to those noted in the bodies of the hyphae, but of considerably larger size, 4 to 7 μ in diameter. In the larger conidia, the double-contoured membrane which forms their wall is very conspicuous. Some of the largest conidia are empty; their walls are nearly 1 μ in thickness and present breaks in their continuity. Free in the suspending fluid, 0.85 per cent NaCl solution, about the hyphae under examination, are numbers of spherules exactly similar in appearance to those described within conidia. Their origin from the latter seems probable. They frequently present a marked Brownian movement.

In acid-glucose-broth cultures a granular sediment forms in the course of a week or ten days, the overlying fluid remaining clear. On shaking, the tube becomes diffusely clouded but resumes its original appearance after standing a short time. Microscopically the typical budding yeast forms appear. Occasionally elongated forms suggesting hyphae are found. The same change has overtaken the broth cultures that has been mentioned as occurring in the agar cultures, that is, the organisms no longer grow in the budding form but have reverted to the growth characteristic of room temperature, namely, the mycelial form. Whether or not it will be possible to induce the organism to resume the former mode of growth at incubator temperature (37° C.) is a question which must be left for further observation.

No attempt has been made to determine the finer details of the biology and morphology of this organism, since it is believed

that enough has been said to warrant the assumption that we are dealing with an organism which undoubtedly belongs to that genus of the pathogenic mold fungi placed by Ricketts,[11] provisionally, among the oidia, and which therefore may be considered a fair sample of the parasites responsible for oidiomycosis (blastomycosis) in man.

Besides the organism itself, a so-called oidiomycetic "extract" was made use of in the following experiments. This "extract" was made according to a method which has been in use in this laboratory for a number of years, and which is as follows:

The organism, in order to obtain considerable quantities, is planted on a large covered plate or in flat-sided, wide-mouthed bottles. When a good growth has appeared, which may take from three weeks to a month, it is scraped off by means of a glass or platinum hoe and placed in a desiccator where it is allowed to dry. The dried material is weighed, then placed in a porcelain-ball mill together with an equal volume of sterile sand and about 10 c.c. of sterile 0.85 per cent salt solution and ground for a couple of hours. Salt solution in small quantities, 5 c.c., is then added at short intervals until the volume of fluid is equal to about 50 c.c. The liquid is drawn off, centrifugated, the supernated fluid poured into a sterile bottle or other suitable receptacle, and the solid portion, which still contains large numbers of yeast cells, is returned to the mill and reground. This process is repeated until microscopical examination fails to reveal unbroken cells in the centrifugated sediment. The various fluid portions are added together and the total volume made up to such a point that every 100 c.c. represents one gram of the dried organism. As a preservative, 0.5 per cent carbolic acid or 0.3 per cent chloroform is then added. As a further precaution the "extract" is kept in the ice-box. If one wishes a fresh extract the moist organisms may be used. It has been shown by experiment that the yeast loses about $\frac{1}{12}$ of its weight when dried. Therefore if one grinds 12 grams of fresh, moist organisms and wishes to make a 1 per cent solution comparable to the preserved extracts, he makes the total volume of extract 100 c.c. This, of course, is not a method of great accuracy, owing to the variations in the amount of water which the moist organisms contain, but it seems fairly satisfactory.

Upon standing any length of time a white, amorphous precipitate separates out of the extract and settles to the bottom. It does not go into solution again when mixed with the overlying fluid. The extract itself has a yellowish, opalescent appearance, which tends to clear somewhat on shaking with ether. It possesses a markedly yeasty smell. The following rough qualitative analysis was made on "Ext. BlR 1/19/09." The results are typical of those observed in all cases examined.

To 10 c.c. of the extract were added 30 c.c. of absolute alcohol, and the mixture was allowed to stand in the ice-box for 30 minutes. At the expiration of that time, a fairly heavy, whitish, flocculent precipitate had formed, which showed a tendency to remain suspended in the liquid in large and small loose clumps. The material was filtered. The filtrate was evaporated to dryness at a temperature not over 65° C. and yielded a yellow, gummy-looking material which possessed a marked sweetish, yeasty smell. This material was allowed to stand over night at room temperature.

The precipitate on the filter paper was washed with 30 c.c. of distilled water in which it seemed to be readily taken up, giving the water a whitish, cloudy appearance. It had no characteristic odor. Under the microscope, the aqueous solution showed a fine, granular débris, but no crystalline suspension; the residue from the evaporated alcoholic extract was amorphous and of a yellow tinge.

The aqueous solution was tested for dextrin, starch, and reducing sugars with negative results. The protein color and coagulation tests resulted as follows:

Heller's nitric acid test	negative
Boiling with glacial acetic	"
Millon's reagent	"
Xanthroproteic test	"
Adamkiewicz	+very sharp
Biuret	? very slight if any
Liebermann	negative
Hopkins-Cole	+very sharp
H_2SO_4 (conc.)+sugar	negative

Similar tests applied to the whole extract gave similar results.

The yellow residue from the alcoholic extract was soluble in ether and chloroform and was not precipitated from the chloroform solution by acetone. It gave a negative Salkowski's test for cholesterol. Further analysis was not attempted.

No experiments were undertaken with killed organisms, the living organisms and the extract alone being used in the immunological work.

As a matter of routine, injections of living organisms were made into the peritoneal cavity; the pleural cavity was used a few times. Intravenous injections are inconvenient in the guinea-pig, while subcutaneous injections are open to the objection that the resulting subcutaneous nodules early cause necrosis and sloughing of the overlying skin, thus not only giving splendid opportunities for secondary infections but tending to scatter virulent mold fungi about the cages, which was regarded as dangerous.

The extract was injected subcutaneously or intraperitoneally according to the seeming needs of the particular experiment in which it was being used.

OIDIOMYCOSIS (BLASTOMYCOSIS) IN THE GUINEA-PIG.

Dosage.—In a series of experiments carried out about three years previously with this same organism, Dr. Ricketts had determined roughly its virulence for guinea-pigs. He found that 0.3 of a gram of an agar plate culture, grown at incubator temperature, injected into the peritoneal cavity of a 375–400 gm. guinea-pig

would cause death in about 35 days, with generalized oidiomycosis, especially in the abdominal and thoracic viscera.

In view of the prolonged course of the disease, even following the injection of such large doses as that mentioned, it did not seem so essential for present purposes to determine a uniformly fatal dose as to find out what dosage would give a constant clinical picture, and, at the same time, would not require a month or six weeks for development. With this in view a series of 42 guinea-pigs were injected with quantities of moist organisms from agar culture, the dosage varying from 0.5 to 0.0001 of a gram. Each pig was examined daily with reference to weight, temperature, and to changes which might be revealed by inspection or palpation. The following general conclusion was drawn: There is no constant symptom by means of which one may diagnose oidiomycosis in the guinea-pig during life. The weight is an unreliable guide, it varies with the abundance of food and the length of time which elapses between feeding and the taking of weights. Again, even if the animal develops an apparently fatal infection, the weight before death may approximately equal that before inoculation, or, if it does decrease, the major portion of the loss occurs during the last week or so of life. The temperature is also unsatisfactory. In the majority of cases when the oidiomycosis cannot be doubted, the temperature runs an absolutely normal course. When it does follow an abnormally high curve, it is difficult to exclude secondary infection, or the possible effect of occlusion of excretory ducts (that is, ureter, seminal vessels, urethra).

If one compares the effects in males and females, however, he finds that there is, symptomatically at least, one marked difference. In males with doses as low as 0.001 gm. one usually can detect small nodules (0.5–2 mm. in diameter) in the testicles within seven to ten days after inoculation; nodules can practically always be found with doses of 0.01 gm. within five to seven days. With doses of 0.1 gm. or over, nodules not only occur regularly but they are of good size and the infection may have a fatal outcome. In the female pig there is absolutely no sure way of diagnosing oidiomycosis during life except to open the abdominal cavity and inspect the contents. One might think of deep pal-

pation, but how distinguish nodules from feces? We have found it practically impossible in most cases. Of great assistance in determining the severity of the infection—and this may be applied to both sexes alike—is the "look" and "feel" of the animals. By the term "feel" well is meant that when the animal is handled the muscles are found to have their normal firmness and tone. In fresh guinea-pigs which have not been handled, the tense, hard, wiry feeling of the muscles is very noticeable. After the animals have been manipulated daily for a week or so, they evidently become used to it and relax readily when they are picked up. In this state the muscles are soft and pliable but have not lost their tone. If such pigs become the victims of a chronic wasting disease, this normal muscular tone is lost; the muscles become decidedly flabby to the touch so that the animal feels like nothing so much as the time-honored "dish-rag." This "feel" may be present before the animal's weight has fallen off appreciably. The animal usually appears thin and we say it looks sick, but this appearance is due more to the staring coat and the "hunched-up" attitude which the animal assumes than to a real emaciation. These symptoms, of course, are characteristic of cachectic conditions in general and are not specific. It should be mentioned that the development of nodules at the point of inoculation noted in some of the pigs was regarded as the result of faulty technic and could be avoided to a considerable extent by rinsing the needle in water before making the injection.

For the remainder of this work, in view of the above facts, the taking of temperatures, the recording of weights, and the use of female guinea-pigs were eliminated excepting in special cases. Male pigs were used as a matter of routine and their condition was adjudged by the results of careful palpation of the testicles and by their general "look" and "feel." 0.1 gm. of moist agar culture was adopted as the standard, surely infective dose.

The difference in the results of intraperitoneal inoculation in male and female guinea-pigs is very marked from the symptomatological standpoint. Take guinea-pig 75 and guinea-pig 81, for example. Each received the same dose from the same culture on the same day. Guinea-pig 75 (male) became weak and flabby

to the touch with a staring coat and enormously swollen scrotum which finally ulcerated. Guinea-pig 81 grew fat and sleek and it was only at autopsy that signs of the infection could be demonstrated. Some of the possible reasons for this difference will be discussed later as will also the question of localization of infection, and, in a general way, the course of the disease and possible treatment.

ACTIVE IMMUNIZATION.

In the endeavor to establish an active immunity in guinea-pigs, use was made of the living organism and of the extract. In the case of the living organism, the method pursued for the greater part of the time was to reinoculate a pig as soon as he had made an apparently complete recovery from his previous infection, and then to study the development of the new infection as regards (1) the time of the appearance of nodules in the testicles, their size, consistency, and the rate at which they disappeared, (2) the development of palpable nodules in the abdominal cavity, and (3) the "look" and "feel" of the animal. The results of such observations had led to the belief that it made practically no difference how many times an animal was subjected to oidiomycetic infection and recovered, he always retained his original susceptibility. In order to put this conclusion to a thorough test the following experiment was performed. All the living pigs which had recovered from previous injections were gathered together with ten normal pigs of about the same average size and all were given an equal dose of living oidiomycetes (approximately 0.1 gm.) and the results carefully watched. The previous history of the so-called "immune" animals is given in Table 1.

On comparing the results of the inoculation in the immune and the control guinea-pigs, some slight, but quite well marked differences were noted. First, regarding the course of the infection, it is a fact worthy of remark, that in so far as the male pigs of the two series are concerned, the immune animals made a decidedly more rapid recovery than the controls, as judged by the rate of disappearance of the nodule from the testicles. The female pigs offer no accessible basis for comparison. If we examine the

temperature charts of the immune and normal animals before and after injection we again find differences. The average temperature for the immunized animals for the three days prior to the inoculation on which temperatures were observed, that is, the 19th, 20th and 23d, is 103.29°+ F.; that for the control animals is 103.31°+ F. The average of the observed temperatures of the immunized animals on the days immediately following the injection (the 24th, 25th, and 27th) is 104.27°+ F.; that of the control

TABLE 1.

HISTORY OF "IMMUNE" GUINEA-PIGS.

GUINEA-PIG NO.	NO. TIMES PREVIOUSLY INOCULATED	AVERAGE INTERVAL BETWEEN INOCULATIONS	TIME ELAPSED SINCE LAST INOCULATED	DOSAGE (IN GM.)			
				1st Dose	2d Dose	3d Dose	4th Dose
1.....	3	61 days	68 days	0.1	0.1	0.1	...
72.....	4	24 "	68 "	0.005	0.1	0.1	0.1
75.....	1	.. "	166 "	0.5	...	...	..
77.....	4	24 "	68 "	0.001	0.1	0.1	0.1
80.....	4	24 "	68 "	0.0001	0.1	0.1	0.1
81.....	3	33 "	68 "	0.5	0.1	0.1	...
83.....	4	24 "	68 "	0.1	0.5	0.1	0.1
92.....	2	29 "	68 "	0.1	0.1	...	...
47.....	2	24 "	240 "	0.1	0.1	...	...
93.....	2	25 "	50 "	0.1	0.1	...	...
94.....	2	25 "	50 "	0.1	0.1	...	...
95.....	2	25 "	50 "	0.1	0.1	...	...
69.....	3	32 "	68 "	0.1	0.1	0.1	...

guinea-pigs for the same period is 103.49°+ F., or practically 104.3° F. in the one case and 103.5° in the other—a difference of nearly 1° F. To be sure, this difference is small, but in view of the fact that the temperatures of the two groups of pigs were almost identical before inoculation, while following the inoculation the rise was confined almost entirely to one group, it would seem that one would be justified in considering such a rise a positive reaction of the animal organism against the injected oidiomycetes. Apparently, then, a low grade of immunity is developed in guinea-pigs by the intraperitoneal inoculation of living organisms. As symptomatological evidence of the immunity—which seems to be emphatically a relative immunity—we have the more rapid disappearance of the lesions following infective doses and a small but fairly decided rise in temperature on the days immediately following the inoculation. These reactions may be manifest as long as 240 days after the last inoculation (guinea-pig 49)

or, allowing the animal 40 days in which to recover from the immunizing inoculation, a liberal margin, the acquired power to react against renewed infection in a guinea-pig which has recovered from two moderate injections may persist at least 200 days.

A large series of guinea-pigs were given repeated injections of the extract. Of this series but four pigs were tested by the injection of living organisms. Their histories follow:

TABLE 2.

No. of Guinea-Pig	No. of Injections	Interval between First and Last Injection	Interval between Last Injection and Day Tested	Average Volume of Injection	Route
13	23	178 days	63 days	About 1.5 c.c.	Intraperitoneal
37	14	105 "	77 "	2 c.c.	"
38	14	105 "	77 "	2 "	"
56	12	91 "	63 "	2 "	"

Each of these four pigs, together with each of seven control animals, was given 0.1 gm. BIR, intraperitoneally. All of the control animals developed severe infections, large nodules in the testicles, weakness, staring coat, etc.; and one of them died, post-mortem examination revealing generalized oidiomycosis. Of the immune animals, but one developed testicular lesions—a small, solitary nodule; all presented rather large, fairly hard, intra-abdominal masses, but no other symptoms. The subsequent history of the immune pigs follows. Guinea-pig 13 was bled to death on December 3, 1909, 102 days after injection. At autopsy the seminal vessels and portions of the small intestines were found adherent to a fibrous nodule on the anterior abdominal wall, within which was fluid detritus containing oidiomycetic forms; cultures were negative. Microscopic sections show oidiomycetes. No other abnormalities.

Guinea-pig 37 died January 19, 1910, 149 days after injection. At autopsy a mass of dense fibrous adhesions were found about the point of inoculation. In the center of this mass was a cavity 1 cm. in length and 0.5 cm. in width and about 1 to 2 mm. deep in which was fluid detritus. Testicles were normal. No oidiomycetes were found, microscopically, in the contents of the nodule. The cause of death was pneumonia and pericarditis. No oidiomycetes were recovered in cultures.

Guinea-pig 38 was found dead on March 14, 1910, 203 days after injection. Autopsy showed pneumonia and old fibrous adhesions about the point of injection. Testicles were normal.

Guinea-pig 56 was killed March 14, 1910, 203 days after injection. Autopsy disclosed an oval fibrous sac about 1 cm. in greatest length attached to the anterior abdominal wall. Adherent to the sac were the seminal vesicles and portions of the small intestines. Within the sac was fluid detritus; microscopically forms very similar to oidiomycetes were observed; unfortunately these bodies were soluble in ether. Cultures were negative as regards oidiomycetes.

One cannot argue from these experiments that the prolonged immunization with the "extract" resulted in the development of such a high grade of immunity as would lead to the absolute walling off of infective doses of organisms at or near the point of inoculation, for it is not at all certain that those inoculations may not have been made into previously existing adhesions, resulting from the numerous immunizing injections, which could not be detected by external examination. On the other hand, in view of the fact that most of the control animals developed marked infection, one of them dying, and that the nodules which appeared in the testicles of one of the immune animals vanished with considerable celerity, it seems justifiable to assume that a certain degree of immunity, at least, was manifested. Another point of interest which will be considered later was the finding of morphologically typical oidiomycetes in stained sections from guinea-pig 13. Apparently this pig's immunity, whatever it may have been, was not of a markedly lytic type.

EXPERIMENT TO TEST THE EFFECT OF THE INJECTION OF "EXTRACT" INTO INFECTED ANIMALS.

Eggers has shown (*vide infra*) that repeated intraperitoneal injections of oidiomycetic extract seem to decrease the resistance of guinea-pigs to accidental infection. He has also mentioned the fact that the animals so treated suffer a considerable loss of weight. It has been noticed in the present experiments that this loss of weight in guinea-pigs receiving 2 c.c. intraperitoneally

every fourth day may be extreme, the animals become flabby to the touch and appear markedly cachectic. They slowly recover when the injections are discontinued. Bearing in mind the above findings, it became of interest to test infected animals with graduated doses of the extract. The results, as shown in the following experiments, appear to be of possible importance.

A series of 10 guinea-pigs which had recovered from spotted fever eight months to a year before were given intraperitoneal injections of 0.1 gm. of oidiomycetes from an agar slant culture on January 21, 1909. As soon as nodules became palpable in the testicles, five of these pigs were given subcutaneous injections of extract ("BlR 1/19/09," $CHCl_3$ preservative) as indicated below, the other five animals being used as controls.

Guinea-pig 45: January 21, 1909, control pig; wt.=680 gm. 0.1 gm. intraperitoneally; January 25, nodules palpable in testicles; February 8, nodules in testicles about 1 cm. in diameter; March 1, testicles normal.

Guinea-pig 46: January 21, 1909, control pig; wt.=775 gm. 0.1 gm. intraperitoneally. Result like guinea-pig 45.

Guinea-pig 47: control pig; wt.=515 gm.; January 25, slight induration of testicles; February 8, same; true nodules never developed; March 1, testicles normal.

Guinea-pig 48: control pig; wt.=515 gm.; January 25, slight induration of testicles; February 8, testicles contain nodules, some of which are about 1 cm. in diameter; March 1, testicles normal.

Guinea-pig 49: control pig; wt.=955 gm.; January 25, small nodules can be felt in the testicles; February 8, large nodules are palpable in the testicles; March 1, testicles normal.

Guinea-pig 50: January 21, 1909, wt.=690 gm. 0.1 gm. oidiomycetes intraperitoneally. Received subcutaneous injection of 0.5 c.c. extract January 25, 30, February 4, 9, 15, 23; January 25, nodules palpable in testicles. Small nodule at point of inoculation drained and sterilized with 95 per cent carbolic acid and absolute alcohol; wound healed rapidly. Pus contained large numbers of oidiomycetes. February 8, small nodules in testicles; February 28, animal fully recovered.

Guinea-pig 51: January 21, 1909, wt.=680 gm. 0.1 gm. oidiomycetes intraperitoneally. Received 0.5 c.c. extract subcutaneously January 25, 30, February 4, 9, 15, and 23; January 25, nodules in testicles; February 8, nodules in testicles nearly as large as those in control pigs; March 1, animal fully recovered.

Guinea-pig 52: January 21, 1909, wt.=690 gm. 0.1 gm. oidiomycetes intraperitoneally. Received 0.5 c.c. extract subcutaneously January 25, 27, 29, February 1, 3, 5, 8, 10, 12, 15, 17, 23, and 25; January 25, slight hardening of tip of right testicle noted; February 8, nodules in testicles fully as large as the largest among the control pigs; February 25, on this date the nodules had almost disappeared so that the injections were discontinued.

Guinea-pig 53: January 21, 1909, wt.=725 gm. 0.1 gm. oidiomycetes intraperitoneally. Received 0.5 c.c. extract subcutaneously January 25, 27, and 29;

January 25, nodules palpable in testicles; February 1, found dead. Autopsy: The abdominal parietes, testicles, seminal vesicles, and diaphragm are peppered with nodules varying in diameter from 0.5 mm. to 4 mm. A few similar nodules appear on the surfaces of the spleen and liver. In the great omentum along the greater curvature of the stomach is a thick fibrinous mass; a mass of cheesy pus 1 cm. in diameter and 0.5 cm. in thickness surrounded by a hyperaemic zone occurs on the anterior abdominal wall at the point of inoculation. A few grayish patches, 1 to 3 mm. in greatest width, appear beneath the epicardium and seem to involve the heart muscles. The lungs do not appear to be so collapsible as normal; they appear to be congested; no gross nodules. Cultures from the various nodules show oidiomycetes in pure culture. Histological examination of the lungs shows that there is no edema nor exudation.

Guinea-pig 54: January 21, 1909, wt.=610 gm. 0.1 gm. oidiomycetes intraperitoneally. Received 0.5 c.c. extract subcutaneously February 1, 3, 5, 8, 10, and 12; February 1, nodules palpable in testicles; February 8, fairly large nodules in testicles; February 15, found dead. Autopsy: The right testicle is transformed into a sac of pus containing oidiomycetes in large numbers. Scattered nodules 1 to 5 mm. in diameter are found on liver, spleen, mesentery, left testicle, and abdominal wall. A nodule in the intermuscular fascia of the anterior abdominal wall is 1 cm. in diameter and 2 to 3 mm. in thickness, and contains an enormous number of budding oidiomycetes. The left lung is very much enlarged and occupies three-fourths of the thoracic cavity; it is hard, non-collapsible, dark in color, with hemorrhagic spots. Heart and right lung appear normal. Material from various nodules smeared on glucose agar slants yielded oidiomycetes in pure culture. Histologically the left lung appears to be in a state of pneumonic consolidation.

This experiment suggests that in the extract we may have a means of modifying to a large extent the course of an oidiomycetic infection in guinea-pigs. Those animals which received 0.5 c.c. of the extract subcutaneously every fifth day seemed to suffer a milder infection than the control pigs and to make, possibly, a somewhat more speedy recovery; of those animals receiving 0.5 c.c. extract subcutaneously every second day, one—guinea-pig 42—paralleled very closely the control pigs in this infection; guinea-pig 53 died in 11 days of generalized oidiomycosis, and guinea-pig 54 died 25 days after injection, autopsy showing extensive oidiomycosis of the abdominal parietes and viscera with a terminal lobar pneumonia; 0.5 c.c. of the extract injected subcutaneously every second day into infected guinea-pigs may be toxic; 0.5 c.c. of the extract injected subcutaneously every fifth day into similarly infected guinea-pigs apparently is not toxic and seemingly increases the animal's resistance to the disease. Broad generalizations from a single experiment such as this are, of course,

unwarranted, but it seems possible that further work along the lines indicated by these results may lead to the development of a technic which will enable us to treat effectively oidiomycetic infections.

EXPERIMENTS IN ANAPHYLAXIS.

In testing further the properties of the "extract," an attempt was made to elicit with it the phenomenon of anaphylaxis in guinea-pigs. It was with such an end in view that the following experiments were performed. The temperature of the animals was observed only in cases where specific mention of that fact is made.

Experiment 1.—Guinea-pig 1 received an intraperitoneal injection of 1 c.c. of "Extract BlH_2," which had been preserved in 0.5 per cent phenol for about 13 months, on October 27, 1908. Sixteen days later 5 c.c. of the "extract" were injected intraperitoneally. Result: no convulsions nor tremors of any kind; pig alive and well 24 hours later.

Experiment 2.—Guinea-pig 17 was treated with doses of "Extract BlH_2," similar to those given guinea-pig 1. An interval of 27 days separated the two injections. Results: Negative, similar to Experiment 1.

Experiment 3.—Guinea-pig 17 received an intraperitoneal injection of 0.05 gm. of organism BlR on November 12, 1908. Twelve days later the animal was found to have lost 90 gm. in weight; both testicles had become swollen and hard. Five c.c. of "Extract BlH_2" were injected intraperitoneally. There ensued a temporary fall of about 8° F. in temperature which returned to normal within 24 hours. No further symptoms appeared. The animal thereafter gained weight.

Experiment 4.—Guinea-pig 31 was given 5 c.c. of centrifugated "Extract BlR" (preservative 0.5 per cent phenol) intraperitoneally on January 4, 1909. The injection was repeated on January 15, without the production of symptoms.

Experiment 5.—Guinea-pig 40 received 0.2 c.c. "Ext. BlR" (preservative 0.5 per cent phenol) intraperitoneally on January 15, 1909. Guinea-pig 41 received 0.5 c.c. "Ext. BlR" intraperitoneally on January 15. Guinea-pig 42 received 1.0 c.c. "Ext. BlR" intraperitoneally on January 15. Guinea-pig 43 received 1.5 c.c. "Ext. BlR" intraperitoneally on January 15. Guinea-pig 44 received 2.0 c.c. "Ext. BlR" intraperitoneally on January 15. On January 26—interval of 11 days—guinea-pigs 40 and 43 received 5 c.c. of Ext. BlR intraperitoneally—no reaction. On January 28 guinea-pig 41 and guinea-pig 42 with a normal control pig received 7 c.c. Ext. BlR intraperitoneally. Negative results. Guinea-pig 44 died of pneumonia before he was tested.

Experiment 6.—Guinea-pig 55, wt. 660 gm., received 0.1 c.c. Ext. BlR (preservative $CHCl_3$ 0.3 per cent) intraperitoneally January 22, 1909. Guinea-pig 56, wt. 795 gm., received 0.7 c.c. Ext. BlR intraperitoneally. Guinea-pig 57, wt. 830 gm., received 1.5 c.c. Ext. BlR intraperitoneally. Guinea-pig 58, wt. 680 gm., received 3.0 c.c. Ext. BlR intraperitoneally. After an interval of 14 days, each of these four animals and each of two normal control pigs were given an

intraperitoneal injection of 5 c.c. of the extract. Only one animal presented any untoward symptoms and one of the control animals died in about 12 hours. Postmortem examination revealed an acute fibrinous peritonitis.

Experiment 7.—Each of three small guinea-pigs averaging about 250–300 gm. in weight was given 0.3 c.c. of Ext. BlR intraperitoneally on February 26, 1909. The chloroform used as a preservative had been allowed to evaporate before the extract was used. After an interval of 17 days each of the above animals and two normal pigs of similar size received 5 c.c. of chloroform free extract intraperitoneally. All of the animals appeared to be sick immediately following the injection. The three sensitized pigs were found dead next morning. One of the control animals died a week later without apparent cause; the other remained in normal condition.

Experiment 8.—A couple of grams of moist oidiomycetes were ground in the porcelain-ball mill for three hours, and then taken up with NaCl solution (0.85 per cent). Four c.c. of this fresh extract were injected into each of two medium sized guinea-pigs. Twenty-six days later, each of these two animals and two normal pigs were given 10 c.c. of a fresh extract intraperitoneally. The two sensitized animals were found dead the following morning; one control pig died two days later, the other a week later. This extract yielded the usual white, flocculent precipitate upon the addition of absolute alcohol.

Experiment 9.—Fresh moist organisms were ground in a mortar with sterile sand in 0.85 per cent NaCl solution containing 0.2 per cent of normal NaOH. The total volume of the ground material was made up to 25 c.c. by the addition of the salt solution containing 0.2 per cent of normal NaOH. Five c.c. of the total was sediment. The fluid, when injected, was yellowish white and opaque. Guinea-pig (*a*): wt. 140 gm., received 0.1 c.c. intraperitoneally. Guinea-pig (*b*): wt. 215 gm., received 0.3 c.c. intraperitoneally. Guinea-pig (*c*): wt. 225 gm., received 0.5 c.c. intraperitoneally.

Eighteen days later guinea-pig (*b*) and a normal guinea-pig of similar size were given an intraperitoneal injection of the original fresh extract, which had been kept in the ice-box, warmed to 37° C. The extract at this time yielded the usual precipitate with absolute alcohol.

Immune Pig (*b*): Temp. before injection, 10:30 A.M., 102° F. Injection 10:50 A.M.
Normal Pig: " " " " " 100.6° F. " " "
Immune Pig (*b*): Temp. 10:55, 100.2° F.
Normal Pig: " " 99.8° F.

12:15	1:45	2:45	3:45	4:45	5:45	10:30 A.M.
99.8° F.	98.2° F.	97° F.	97.6° F.	98° F.	99.2° F.	102.6° F.
96.8° F.	97.6° F.	96.8° F.	97.3° F.	98.2° F.	98.1° F.	100.6° F.

Two days after this, an interval of 20 days after sensitization, guinea-pigs (*a*) and (*c*) and each of two control animals of a size approximating that of pigs (*a*) and (*c*) were given intraperitoneally 5 c.c. of a freshly prepared extract, warmed to 37° C., similar to that originally employed. Injection at 12:20 P.M.

Temp. at	11:50	12:45	1:50	2:30	4:35	11:00 A.M.
Guinea-Pig (*a*):	102° F.	101.4° F.	98.8° F.	94.4° F.	96.4° F.	102° F.
Control:	100.4° F.	94° F.	96.4° F.	95.4° F.	96° F.	102° F.
Guinea-Pig (*c*):	101.0° F.	100.4° F.	97.8° F.	94.2° F.	94° F.	104° F.
Control:	101° F.	100.2° F.	96.8° F.	94.6° F.	94.4° F.	100.8° F.

In addition to the fall in temperature, pigs (*b*) and (*c*), and, to a lesser extent, (*a*), developed a tense, swollen, and apparently painful abdomen which subsided as the temperature rose to normal. There was no suggestion of such a condition among the control pigs.

From the above experiments, the conclusions seem justified that, (1) the extract is toxic alike for normal, sensitized, and infected animals as indicated by the sharp fall in temperature following intraperitoneal injection of considerable quantities. (2) It seems possible to demonstrate the phenomenon of anaphylaxis by the use of freshly prepared extract, or of extract freed from its preservative ($CHCl_3$), the reaction manifesting itself either by the death of the animal within 12 to 20 hours, or in the development of a sharp intraperitoneal reaction within two or three hours following the intoxicating injection, which subsides within 24 hours. Large doses may kill the control animals; in such cases, the death of the sensitized animals usually precedes that of the normal animals.

PASSIVE IMMUNIZATION.

In view of the seemingly low grade of active immunity which guinea-pigs developed against oidiomycetes, it appeared that efforts to confer a passive immunity could be more profitably expended along other lines so that no such experiments were undertaken. In Dr. H. T. Ricketts' notes on his work on oidiomycosis, which he kindly placed at my disposal, the following experiment is reported:

Rabbit 44, was immunized against BlR as follows:

November 19, 1905, 1 tube of glucose-agar culture (killed) subcutaneously. Local abscess produced.

November 26, 1905, 1 tube of glucose-agar culture (killed) intraperitoneally.
December 4, 1905, 1½ " " " " " " "
December 12, 1905, 1½ " " " " " " "
December 19, 1905, 1½ " " " " " " "
December 26, 1905, 1½ " " " " " " "
January 7, 1906, 2 " " " " " " "
January 17, 1906, 2 " " " " " " "
January 23, 1906, 1 gram living agar culture intraperitoneally.
February 9, 1906, 1 " " " " "
February 16, 1906, 12 c.c. of blood drawn. This blood was defibrinated, centrifugated, and the serum used in the following experiment:

Experiment 10.—February 16, 1906. Weighed amounts of moist agar culture were mixed with varying quantities of "immune" rabbit serum and injected into the peritoneal cavity of a series of guinea-pigs. Control experiments were carried out with normal rabbit serum and with the untreated organisms.

TABLE 3.

	Date	Wt. in Grams	Result
Guinea-pig 1 received 0.3 gm. Bl + 2 c.c. im. serum, intraperitoneally on 2/16.	2/16/06 3/ 7/06 3/16/06 3/24/06	390 395 360 270	Result: Death on 3/24 from generalized oidiomycosis; interval of 36 days. Total loss of wt.= 120 gm.
Guinea-pig 2 received 0.3 gm. Bl+1 c.c. im. ser.+1 c.c. NaCl sol. (0.85 per cent) intraperitoneally on 2/16.	2/16/06 3/ 7/06 3/16/06 3/24/06	375 370 340 245	Result: Death on 3/24 from generalized oidiomycosis; interval of 36 days. Total loss of weight =130 gm.
Guinea-pig 3 received 0.3 gm. Bl+0.5 c.c. im. ser.+1.5 c.c. NaCl sol. (0.85 per cent) intraperitoneally on 2/16.	2/16/06 3/ 7/06 3/16/06 3/23/06	378 350 285 ?	Result: Death on 3/23, from generalized oidiomycosis; interval of 35 days.
Guinea-pig 4 received 0.3 gm. Bl+2 c.c. normal serum, intraperitoneally on 2/16.	2/16/06 3/ 7/06 3/11/06	400 400 ?	Result: Death on 3/11, from generalized oidiomycosis; interval of 23 days.
Guinea-pig 5 received 0.3 gm. Bl+1 c.c. normal serum+1 c.c. NaCl sol. (0.85 per cent) intraperitoneally on 2/16.	2/16/06 3/ 7/06 3/18/06	400 375 270	Result: Death on 3/18 from generalized oidiomycosis; interval of 30 days. Total loss of weight =130 gm.
Guinea-pig 6 received 0.3 gm. Bl+0.5 c.c. normal serum+ 1.5 c.c. NaCl sol. (0.85 per cent) intraperitoneally on 2/16.	2/16/06 3/ 7/06 3/16/06	425 410 380	Result: Animal lost.
Guinea-pig 7 received 0.3 gm. Bl+2 c.c. NaCl sol. (0.85 per cent) intraperitoneally 2/16.	2/16/06 3/ 7/06 3/16/06 3/29/06	386 365 325 230	Result: Death from generalized oidiomycosis on 3/29. Interval of 41 days. Total loss of weight =156 gm.
Guinea-pig 8 received dose similar to that of guinea-pig 7.	2/16/06	390	Result: Accidentally killed 2/22/06, six days after injection.

One cannot argue from these results that the "immune" serum conferred an immunity of any degree. It should be remembered in this connection, however, that rabbit serum is somewhat toxic for guinea-pig leukocytes, a property which might mask the protective power of such a serum. There seems to be a suggestion of a reaction of this kind in the results of the above experiment, but more work must be done before conclusions may be drawn.

IMMUNOLOGICAL REACTIONS.

Some preliminary work along this line, carried out with the organism "BlR," was done in 1905 by Dr. H. T. Ricketts, and continued during the year 1906–7 by Dr. H. E. Eggers. Dr. Ricketts performed the following experiment: A guinea-pig was immunized against the oidiomycetes by the repeated injection of

killed organisms and of extract as is shown in the accompanying table.

GUINEA-PIG 1.

Date	Weight	Inoculated with
February 7		0.1 gm. killed agar culture intraperitoneally
February 16		0.1 " " " " "
February 27		0.1 " " " " "
March 9		2 c.c. of extract subcutaneously
March 16	390 gm.	2 " " " "
March 25		2 " " " "
March 30	315 "	Killed on account of loss of weight, and serum used for precipitation tests.

PRECIPITATION TESTS. APRIL 4, 1906. BLASTOMYCES R—EXTRACT II. SERUM FROM IMMUNIZED GUINEA-PIG 1.

I. Original Extract II, after prolonged centrifugation to remove all sediment, was used as antigen. The fluid was whitish-opalescent.

Extract	Immune Serum	Normal Serum	NaCl (0.85 per cent) Sol.	Result
0.5 c.c.	0.1 c.c.	0.0 c.c.	0.4 c.c.	Distinct precipitate
0.5 "	0.2 "	0.0 "	0.3 "	Marked "
0.5 "	0.3 "	0.0 "	0.2 "	Fairly heavy "
0.5 "	0.0 "	0.1 "	0.4 "	No "
0.5 "	0.0 "	0.2 "	0.3 "	" "
0.5 "	0.0 "	0.3 "	0.2 "	" "

II. Absolute alcohol precipitate of Extract II, freed of alcohol and redissolved in NaCl (0.85 per cent) solution. Fluid was slightly opalescent but much less so than the centrifugated extract.

Redissolved Precipitate	Immune Serum	Normal Serum	NaCl Sol. (0.85 per cent)	Result
0.5 c.c.	0.1 c.c.	0.0 c.c.	0.4 c.c.	Distinct precipitate
0.5 "	0.2 "	0.0 "	0.3 "	Marked "
0.5 "	0.3 "	0.0 "	0.2 "	Fairly heavy "
0.5 "	0.0 "	0.1 "	0.4 "	No "
0.5 "	0.0 "	0.2 "	0.3 "	" "
0.5 "	0.0 "	0.3 "	0.2 "	" "

III. Clear, colorless, non-opalescent filtrate of Extract II used as precipitogen.

Filtrate	Immune Serum	Normal Serum	NaCl Sol. (0.85 per cent)	Result
0.5 c.c.	0.1 c.c.	0.0 c.c.	0.4 c.c.	No precipitate
0.5 "	0.2 "	0.0 "	0.3 "	Very faint precipitate
0.5 "	0.3 "	0.0 "	0.2 "	Small, but distinct precip.
0.5 "	0.0 "	0.1 "	0.4 "	No precipitate
0.5 "	0.0 "	0.2 "	0.3 "	" "
0.5 "	0.0 "	0.3 "	0.2 "	" "

Normal serum alone = no precipitate.
Immune serum alone = no precipitate.
Centrifugated extract alone = no precipitate.
Redissolved alcoholic precipitate alone = no precipitate.
Filtrate alone = no precipitate.

The records of work done by Dr. H. S. Eggers were lost before the present work was begun. Dr. Eggers has, however, written a summary of his work from memory which is introduced at this point with his permission:

The following work on attempted immunization to blastomycosis was carried on, on guinea-pigs, rabbits, and goats.

The plan was followed of injecting into the animals, at periods of from seven to eight days, an extract prepared from the dried blastomyces by grinding. Before injection care was taken to shake the material thoroughly to secure suspension of the solid fragments of the organisms.

Using this suspension, work was begun on 12 guinea-pigs and 9 rabbits. 0.5 c.c. of the material was injected into the guinea-pigs, 1 c.c. into the rabbits. Injections were for the most part intraperitoneal; they were repeated every seven to eight days. The condition of the animals was controlled by weighing. If any considerable loss of weight followed an injection, one, occasionally more, periods were passed over without injection. In the case of some of the animals a sharp reaction followed the first few injections; several died within two or three days. Postmortem examination revealed acute parenchymatous changes, particularly in the liver and kidneys. Other animals were substituted for the ones so lost. Several of the rabbits and guinea-pigs became pregnant during the course of the work; such animals were injected subcutaneously while pregnant.

These injections were carried on from early in October, 1906, until January, 1907. At this time serum from several of the rabbits was tested with the homologous extract for a precipitation reaction. A positive reaction, not given beyond a dilution of 1:20, was found at this time with one animal. The serum of another one, which had shortly previously given birth to young, as well as the serum of the young was tested, both being negative. With the animal showing the slight positive serum reaction the injections were kept up for a month; no increase in reaction resulted, and shortly after this the rabbit died of a meningitis.

The periodic injection was carried on longer, the doses being increased until the guinea-pigs received 1 c.c. at a time and the rabbits two. Evidently their vitality was considerably lowered by the repeated injections, as a considerable number, of the rabbits especially, died of secondary infections—meningeal for the most part. Although the injections of the few remaining rabbits were continued until June, 1907, in no case was I successful in getting a precipitation reaction beyond a dilution of 1:20. The rabbits showing even this reaction were too few to enable any decisive test as to their degree of immunization to be made.

Early in April, 1907, that is, six months after injection had been begun, six of the eight guinea-pigs that had been injected a sufficient number of times to warrant expectation of results were injected intraperitoneally with varying doses of a freshly prepared suspension of blastomyces, the doses being 0.25, 0.5, 1, 2, 3, and 4 c.c. respectively. Six normal animals were injected with similar doses. In no case did any of the animals die with any of the findings of blastomycosis. The general lowered resistance of the previously injected animals was betrayed by the fact that for the most part they were survived by the normal animals.

Late in October, 1906, weekly injections of a goat were begun, the animal receiving at the start 3 c.c. of the extract subcutaneously, later increased gradually to 5 c.c.

The serum of this animal, tested late in January, was found to give a positive precipitin reaction with the homologous extract in a dilution of 1:25. With the extract of another strain of the organisms—"H II"—it gave a precipitate in a dilution of 1:12.5; with the third strain no reaction at all occurred. Injections were continued, until the animal was noticed to be becoming thin and feeble. It finally succumbed late in February; postmortem examination revealed a bronchopneumonia, other tissues negative. The serum at the time of its death reacted in the same manner and in similar dilutions to the results just given.

Work was at once begun with another goat, the injections, of 3 c.c. each, being made subcutaneously at eight day intervals. This was kept up until the beginning of June, three months, at which time precipitin reactions similar in every way to those obtained with the preceding animal were found.

As the formation of antibodies with this method of repeated small injections was evidently rather slight, a second goat was obtained, and injected with larger and ascending doses at somewhat greater intervals, 10 or 11 days. The first dose, of 20 c.c. of extract, given subcutaneously, produced a marked reaction, the animal being quite sick for three or four days afterward. Local reaction at the site of injection was extremely slight. The next dose of 30 c.c. was again followed by a marked reaction, the third dose of 40 c.c. by considerably less.

The same plan was followed on rabbits and guinea-pigs. Before this time the writer left Chicago for the summer, and the injections were made without the possession of facilities for testing the serum reactions of the animals. The change was disastrous to the rabbits, as all of them died of an acute enteric affection. The quantity of extract on hand did not warrant work being begun on new rabbits. The guinea-pigs were injected successively with doses of 1, 3, 5, and 7 c.c. After two doses of this last amount had been given, it was dropped back to 2 c.c. per dose; the dosage of the goat, after reaching 40 c.c., was dropped back to 10. In the case of the guinea-pigs, the reaction from the larger doses used here was no more marked than from all the first few smaller doses used previously.

While out of the city, the first of the two goats, which was still being injected with the repeated small doses of extract, died; postmortem examination showed a condition of apparently long-continued pylorospasm, caused by the lodging of a pin in the gastric mucosa.

The remaining animals, the second goat and the guinea-pigs, were brought back to the city. At this time the writer was obliged to discontinue the work.

In the fall and winter of 1908–9 the work on precipitins was continued, guinea-pigs being used as the experimental animals. The results are tabulated below. Some of the animals were immunized by injections of the extract of another organism than BlR, known in the laboratory as Bl H II.

The experiment of Dr. Ricketts with the centrifugated, filtered, and redissolved alcoholic precipitate of the extract was repeated, using the serum of guinea-pig 5 as the immune serum, with positive results in all cases. Thereafter the centrifugated extract was used exclusively as the precipitogen in the precipitation experiments.

The technic used in the work was as follows: Blood was drawn, usually from the guinea-pig's heart, defibrinated, and centrifugated, and varying quantities of the elcar serum added to a constant amount of centrifugated extract in small test-tubes, and the mixtures made up to a uniform volume by the addition of NaCl solution (0.85 per cent). The tubes were then incubated at 37° C. for two hours, placed in the ice-chest, and results observed after 24 hours. Control tubes of normal serum plus extract in dilutions similar to those of the immune serum, and of immune serum and NaCl solution, normal serum and NaCl solution, and extract plus NaCl solution, were always prepared as a part of each experiment. It was noticed that occasionally after 72 to 96 hours in the ice-chest precipitates formed in tubes, which at the end of 24 hours were negative. As the control tubes remained clear and without sediment, and as no proofs of contamination could be obtained by cultural methods or by direct microscopical examination, it was assumed that such precipitates probably represented a specific reaction and they were therefore noted in subsequent experiments. Once in a while, the serum of normal animals formed a precipitate with the extract. Of the 22 normal animals tested, 2 or approximately 10 per cent gave positive reactions, one in a dilution of 1:3 after 24 hours and the other in a dilution of 1:10 on the 4th day.

In Table 4 the figures 1:7, 1:10, etc., represent the highest dilutions at which precipitates appeared; a dash (—) indicates that no observations were made and "0" indicates absence of precipitate.

Six animals were tested more than once. Their history appears in Table 5.

Of the 32 immune sera tested, 22 reacted positively in from 24 to 96 hours. Nine were absolutely negative in so far as they were observed. Of the sera of those animals which were immunized by the injection of extract alone, 8 reacted positively in 24 hours, 0 in 48 hours, 7 in 72–96 hours in one or another of the tests, and 4 were negative; of the sera of those which received the organisms alone, 4 reacted positively in 24 hours, 0 in 48 hours, 1 in 72–96 hours, and 4 were negative; of the sera of the animals which received both extract and organisms, 2 were positive in 24 hours and 1 was

TABLE 4.
PRECIPITINS.

GUINEA-PIG NO.	NO. INJECTIONS OF		NO. OF DAYS INTERVAL SINCE LAST INJECTION	AVERAGE DOSE C.C.	NO. INJECTION LIVING ORGANISMS	DOSE GM.	NO. OF DAYS INTERVAL SINCE RECOVERY FROM LAST INJECTION	RESULTS		
	Ext. H II	Ext. BIR						24 Hrs.	48 Hrs.	72–96 Hrs.
1.....	0	4	4	2	0	—	—	0	—	—
5*.....	4	0	37	1	0	—	—	1:20	—	—
8.....	9	8	13	H II 1 BIR 2	0	—	—	1:20	—	—
10.....	5	0	28	2	0	—	—	0	—	—
I.....	0	0	—	—	3	0.1	28	0	—	—
17.....	0	0	—	—	1	0.05	about 26	0	0	0
22.....	0	0	—	—	1	0.01	20	0	—	—
31.....	0	2	24	5	0	—	—	0	0	1:20
32.....	0	5	6	2	0	—	—	0	—	—
36.....	0	8	14	2	0	—	—	0	0	1:20
39.....	0	4	11	2	0	—	—	0	—	—
43.....	0	9	15	2	—	—	—	1:20	—	—
49.....	0	0	—	—	2	aver. 0.1	about 19	0	0	1:20
50.....	0	6	119	5 subcu.	1	0.1	119	1:7	1:10	1:20
51.....	0	6	122	5 subcu.	1	0.1	122	1:5	1:10	1:20
52†.....	0	13	133	5 subcu.	1	0.1	about 133	0	0	? (0)
56.....	0	9	48	3	0	—	—	—	—	1:7
57.....	0	9	48	3	0	—	—	0	—	1:10
58.....	0	8	48	3	0	—	—	0	—	1:10
75.....	0	0	—	—	2	1st dose 0.5 2d 0.1	about 60	0	0	0
83.....	0	0	—	—	5	2d 0.5 others 0.1 each	about 60	0	0	0
92.....	0	0	—	—	3	aver. 0.1	"	‡1:10	—	—
95.....	0	0	—	—	3	"	"	‡1:10	—	—
II.....	0	2	28	0.1 5	0	—	—	0	0	0
III.....	0	2	2	1st dose 20 c.c. 2d 10 c.c.	0	—	—	0	—	1:10

* Not tested in higher dilution.
† Blood drawn July 13 but not tested until July 22.
‡ Ext. H_2 used as precipitogen.

TABLE 5.

GUINEA PIG NO.	TOTAL NO. INJECTIONS BEFORE						NO. OF DAYS INTERVAL BETWEEN TIME OF LAST INJECTION OR INFECTION AND			RESULTS		
	1st Test		2d Test		3d Test							
	Ext.	Liv. Orgs.	Ext.	Liv. Orgs.	Ext.	Liv. Orgs.	1st Test	2d Test	3d Test	1st Test	2d Test	3d Test
13*....	17	0	23	0	23	2	13	3	100	1:20	1:20	0
35.....	8	0	14	0	—	—	14	6	—	1:20 after 3 da.	1:5 after 4 da.	—
37.....	8	0	14	0	—	—	11	5	—	1:20	1:1 only after 4 days	—
38.....	8	0	11	0	—	—	13	3	—	0	1:20	—
55.....	12	0	12	0	—	—	6	60	—	1:20	1:5 after 48 hrs.	—
81.....	0	4	0	4	—	—	27	60	—	1:20	1:10	—

*At autopsy of guinea-pig 13 (date of 3d test) oidiomycetes were found in a nodule in the abdominal cavity. Cultures were negative. See p. 204.

negative. There does not seem to be any very constant relation between the number of injections, the interval between the time of the last injection and that of the test, and the results obtained; nor does the quantity of the living organisms introduced seem to be of paramount importance. About all that one seems justified in concluding from the above experiments, is (1) that the serum of some normal guinea-pigs will cause a precipitate when mixed in proper proportions with oidiomycetic extract, and (2) that immunization with the extract, infection with living organisms, or combined infection with oidiomycetes and immunization with extracts results in the development of precipitating substances in the serum of about 70 per cent of the animals so treated (the percentages in the above experiments were: extract alone 79 per cent, organisms alone 50 per cent, organisms plus extract [small quantities were used] 67 per cent). These figures suggest also that living organisms plus small quantities of extract, too small to produce noticeable symptoms themselves, may be much more effective in calling forth antibodies than living organisms alone and so rather tend to support the deductions made from the results of the experiment described on p. 207, according to which it was suggested that the extract may have some value as a curative agent. As a diagnostic procedure, with guinea-pigs at any rate, the precipitation reaction would have to be controlled by symptomatological findings; a negative reaction would mean nothing, a positive reaction would be suggestive but not conclusive because of the fact noted above that normal guinea-pig serum may precipitate with the extract in dilution as high as 1:10, after 96 hours in the ice-chest, at least.

According to Dr. Eggers' work, the results with rabbits are even more variable than with guinea-pigs. The goat seems to give a more constant reaction, but even in that case the highest dilution at which a precipitate formed was 1:25, which is certainly not evidence of a very powerful serum.

AGGLUTININS.

At the same time that the precipitating powers of the various sera were being tested, parallel experiments on their agglutinating

power were attempted. Emulsions of the whole organism in 0.85 per cent NaCl were used in these experiments. The serum dilutions were as follows: For methods (*a*) and (*b*)—see below—whole serum 1 part + emulsion 1 part; 50 per cent serum 1 part + emulsion 1 part; 10 per cent serum 1 part + emulsion 1 part. For method (*c*) the dilutions were:

Serum	Emulsion	NaCl
0.05 c.c.	0.5 c.c.	0.45 c.c.
0.1 "	0.5 "	0.4 "
0.15 "	0.5 "	0.35 "
0.2 "	0.5 "	0.3 "
0.25 "	0.5 "	0.25 "
0.3 "	0.5 "	0.2 "
0.35 "	0.5 "	0.15 "
0.4 "	0.5 "	0.1 "
0.45 "	0.5 "	0.05 "
0.5 "	0.5 "	0.0 "
1.0 "	1. loop of moist organisms	0.0 "

Three different methods were tried: (*a*) hanging drop in a hollow-ground slide, (*b*) quantities of 1 to 2 c.c. in watch glasses, which could be observed with ease both with the unaided eye and with the microscope, and (*c*) the materials were placed in narrow test-tubes with the idea that something might be learned from possible variations in the rate with which the oidiomycetes settled out of the suspension. In each experiment the following mixtures were made: (1) immune serum plus emulsion, (2) normal serum plus emulsion, (3) salt solution plus emulsion. These mixtures were incubated at 37° C. for from 1 to 12 hours and then allowed to stand at room temperature until observations were discontinued.

The results were practically identical with the normal and immune sera in all the experiments. It was found that if the organisms were allowed to stand in contact with the serum, normal or immune, for a few hours, a beautiful agglutination reaction, as judged by the naked eye, occurred. This is especially noticeable in the watch-glass preparations. Under the microscope, however, the clumps which had attracted the attention resolved themselves not into masses of agglutinated oidiomycetes, but into groups of oidiomycetes which had developed hyphae, and it was to the appearance of this mycelial network that the apparent agglutination was due.

Regarding the rate at which the organisms settled out of suspension in the tubes, no differences between the normal and immune sera could be determined. The salt-solution tube sedimented first, the other tubes sedimented at about the same rate, and the organisms, like those used in the other methods, sprouted in a few hours.

It is true that sometimes, especially with the hanging-drop and the watch-glass methods, there appeared to be a tendency for the organisms to gather into a few, large, loosely constructed groups, but this occurred with normal serum as well as immune, and almost as frequently with the salt solution alone, and the question arose whether it might not be the expression of an imperfect trituration of the organism in the preparation of the emulsion. Certainly no specific agglutination of oidiomycetes by "immune" sera which did or did not yield a precipitate with the extract was observed.

Possibly the experiment might be more successful if the organisms were first partially ground and if the fragments thus obtained were used; the spherules described within the conidia and the hyphae might also be used.

LYTIC AND BACTERICIDAL SUBSTANCES.

It had been observed in the agglutination experiments that oidiomycetes were able to develop hyphae when suspended in undiluted or diluted normal and immune serum, and that sometimes organisms which had been subjected to the action of such fluids for three or four days grew when planted on agar. It was therefore argued that neither normal nor immune sera possessed lytic or bactericidal properties. To put the matter to a test a number of experiments, of the following nature, were performed.

The sera of guinea-pigs 11 and 55 (see Tables 1 and 2) were mixed with constant quantities of oidiomycetic emulsion in dilutions similar to those used in method (*c*) of the agglutination tests at the same time that those tests were made. These mixtures were incubated for 30 minutes, placed in the ice-chest over night, and then allowed to stand at room temperature for three days. At the expiration of that time the entire contents of each tube was

transferred to an agar slant, which was kept in a horizontal position for 30 minutes in order to allow the organisms to settle on the agar surface, and then placed in an upright position in the incubator with as little agitation as possible. Observation five days later (June 30, 1909) showed growth on the agar surface in all tubes, both above and beneath the surface of the liquid. The colonies growing beneath the surface were in general much smaller than those above the fluid, and gave the impression that they were growing much more slowly. On July 6 the following notes were made: Heavy growth on agar slope above fluid in all tubes excepting tube containing 0.8 c.c. serum (guinea-pig 55) and tube containing 0.2 c.c. serum (guinea-pig 11); no evidence of further growth beneath the surface of the fluid. In tubes containing 0.3 c.c., 0.6 c.c., 0.7 c.c., and 1.1 c.c. serum of guinea-pig 55 and that containing 0.5 c.c. serum of guinea-pig 11, practically all of the water of condensation and introduced fluid has evaporated. In such cases a heavy growth extends to the bottom of the slope. July 8, no further growth beneath the surface of the fluid in any case; otherwise conditions are unchanged. Growth is abundant in all tubes excepting tubes 0.2 c.c. and 0.5 c.c. (guinea-pig 11) and tubes 0.4 c.c. and 0.8 c.c. (guinea-pig 55), in which the growths are but fair.

The sera of guinea-pigs 50, 51, and 52, together with the sera of three normal pigs, were next tested in a manner similar to the above, with the difference that in these cases the tubes were not placed in the ice-chest, that control tubes of emulsion plus salt solution were run and the mixtures were incubated for 48 hours before being transferred to the agar slope. In these cases the results with normal and immune sera were about the same. In all tubes containing serum, growth was slight or did not occur at all; while in the control salt-solution tubes growth was abundant.

It would seem from these experiments that normal and immune guinea-pig serum may have the power of impairing the vitality of oidiomycetes, as suggested by Gilkinet for beer yeasts when subjected to the action of rabbit serum. However, these experiments are regarded as merely of a preliminary nature, and it is hoped that it may be possible to test the above conclusion thoroughly at a later date.

DEVIATION OF COMPLEMENT.

It cannot as yet be stated positively whether or not normal or immune guinea-pig serum will deviate the complement according to the Bordet-Gengou technic. The few sera which have been tested, six recently immunized and one normal, have given absolutely negative results. The experiments have been carried on side by side with successful efforts to demonstrate a specific antibody in the serum of guinea-pigs immunized to a bacillus of the hog cholera group, so that in one way at least the work has been abundantly controlled. On the other hand, the positive results reported by other workers with various yeasts and molds make it still seem possible that the technic used and not the oidiomycetes may be at fault. One thing which has been noticed is that 0.1 c.c. of a moderately turbid emulsion of oidiomycetes, or 0.1 c.c of a freshly made extract, or 0.15 c.c. of redissolved alcoholic precipitate of the extract, is usually sufficient to inactivate completely the complement in 0.01 c.c. of guinea-pig serum which has stood in the ice-chest over night. This is, of course, but the expression of the property, common to all yeastlike forms, of absorbing or destroying complement. With this fact in mind, however, it is to be regretted that those who have reported positive results with various mold fungi have neglected to give the full protocol of even one experiment. It might be of interest to compare the complement-binding power of various pathogenic and non-pathogenic yeast plants; possibly, by this means, some further idea of the factors which determine their power of resistance to destruction by the animal body might be gained.

CUTANEOUS AND OPHTHALMIC REACTIONS.

From the results of the skin and conjunctival tuberculin tests as applied in man, it appeared of interest to experiment in an analogous way with the oidiomycetic extract on infected guinea-pigs.

Cutaneous reaction.—The method of applying this test was as follows: A space on the abdomen about 3 cm. square was shaved and washed with $HgCl_2$ (1:1000) and alcohol (80 per cent). After drying, four small areas, forming the corners of a square of about 1 cm. in breadth, were scarified with the blade of a blunt knife

which had been dipped into the following materials: for area No. 1, the extract of organism BlH_2; for area No. 2, the extract of organism BlR; for area No. 3, 0.5 per cent carbolic acid. For area No. 4, the dry blade alone was used. 0.5 per cent carbolic acid was applied because the extracts had been preserved in that strength of carbolic acid. In cases in which chloroform had been used as a preservative, a 0.3 per cent aqueous solution of chloroform was substituted for the phenol. In cases in which fresh extract alone was used, the carbolic acid and chloroform controls were omitted.

Experiments on animals suffering from light, moderate, and severe infection resulted uniformly and negatively. It appeared to make no difference at what time with reference to the infection (early, middle, or late) the tests were applied; the results never varied. Repeated scarification of the same areas after intervals of from 3 to 14 days called forth no reaction.

Ophthalmic reaction.—The technic used in these experiments was as follows: To 10 c.c. of the extract was added an equal volume of absolute alcohol. The resulting precipitate was removed by filtration, washed in absolute alcohol, and dried in a partial vacuum over sulphuric acid. The dried material was redissolved in salt solution (0.85 per cent NaCl). A drop of this solution was instilled into the eye of the animal to be tested, the dose being repeated in the same and in the opposite eye after an interval of from 3 to 10 days. The results were always negative. The fresh extract and the extract preserved in phenol and in chloroform were also used, care being taken to control with corresponding aqueous solution of carbolic acid and chloroform, with similar negative results.

Apparently then cutaneous and ophthalmic reactions against the extract do not occur in guinea-pigs infected with oidiomycosis.

THE FATE OF THE OIDIOMYCETES WHEN INJECTED INTO THE PERITONEAL CAVITY OF THE GUINEA-PIG.

Changes in the peritoneal fluids of recently injected animals.—The technic and results of this study are best illustrated by the report of the following typical experiment.

Guinea-pig 86 (normal) was given an intraperitoneal injection of 3 c.c. of a rather dense emulsion in salt solution (0.85 per cent) of a nine day agar culture of oidiomyces at 11:10 A.M., April 8, 1909. By means of a fine glass tube, specimens of peritoneal fluid were drawn off at approximately hourly intervals. Some of this material was placed upon a slide, a cover glass applied, and sealed with paraffin. The specimen was then examined microscopically without further delay. Cover glass smears were made of other portions and stained with the Giemsa mixture. These preparations were examined at leisure. The guinea-pig was kept on his back for a few minutes before fluid was withdrawn, but was allowed to run free in his cage between times.

12:00 M.: Two specimens of clear, slightly viscid fluid were obtained. One, slide 1, was incubated for 30 minutes, the other, slide 2, was examined immediately.

Slide 2: Quite a number of red cells; a very small number of leukocytes, and these mostly mononuclear; no oidiomycetes found; red cells probably due to trauma incident to the insertion of the glass pipette, since this occasioned a slight hemorrhage. Slide 1 is similar to slide 2 in all respects.

Stained smear.—Very few polymorphonuclear leukocytes, about in the same proportion to the number of red cells as in the blood; the vast majority of the leukocytes are mononuclear cells; some are clearly small lymphocytes; most of them are large mononuclear leukocytes, some approaching a distinctly endothelial type as judged by the abundance of their cytoplasm. Many of the leukocytes appear to be disintegrating and losing their staining power. One organism is present. It is free. The cytoplasm takes an irregular bright blue stain; the capsule appears as a bright, sharply outlined, shiny rim, with merely the suggestion of a bluish tinge.

1:20 P.M. Slide 3: Still many red cells; many times more leukocytes, seemingly mostly mononuclears, a few organisms, some apparently free, most of them surrounded by from three to forty leukocytes; in some cases the organism has been ingested by a single leukocyte while the other leukocytes crowd around; in other cases ingestion has seemingly not occurred, though two or three layers of leukocytes inclose the organism. The latter appear to be unchanged.

Stained smear.—Mononuclear cells still predominate, although the proportion of polymorphonuclear cells has increased. The leukocytes are frequently clumped, in some cases about organisms. The leukocytes in these clumps seem to be mostly mononuclears; a few polynuclears occur.

2:20 P.M. Slide 4: There are about the same number of red cells as in slide 3, but many more leukocytes. Leukocytes occur singly and in large and small groups, the tendency being toward the formation of grape-bunch-like masses of cells so large and compact that it is impossible to make out individual cells excepting at the periphery. Sometimes one can distinguish organisms down deep in these masses; frequently cannot make sure of the identity of anything there; no organisms found in very small masses or in single leukocytes. In one instance an oidiomycete can be seen within a single leukocyte, near the periphery of one of the large masses; other organisms are present within that mass, but one cannot tell whether they have actually been ingested or not. No organisms lying free. Exudate is more viscid than before, but does not seem to coagulate readily. The oidia are apparently unchanged.

Stained smear.—The majority of the leukocytes approach the transitional type; there are quite a number of polynuclears; cells grouped about organisms are mostly large mononuclear to transitional in type.

3:20 P.M. Slide 5: Exudate is very abundant and viscid; cloudy and granular.

Microscopically very similar to slide 4, with the exception of the greatly increased number of leukocytes. Many of the organisms can be seen definitely phagocyted as described in 4. All organisms which can be well defined are found to be completely engulfed by one to three leukocytes, generally one, the rest of the leukocytes in the mass simply forming a many layered capsule about the organism and its phagocytes. Exudate does not clot in 30 minutes.

Stained smear.—Large numbers of polynuclear cells, otherwise similar to 4. Cytoplasm of cells grouped about oidiomycetes is fused into a single mass in some cases; in others the cells are simply connected by strands of cytoplasm, giving a vacuolated appearance to the region between the cells.

4:20 P.M. Slide 6: Exudate is abundant. It is still richer in leukocytes than before; there are quite a number of phagocyted organisms. The leukocytes do not seem to be bunched so strikingly as in previous slides; the oidia are inclosed within from 1 to 10 or a dozen leukocytes; the leukocytes are more compact about the organisms than formerly, and instead of appearing like a bunch of grapes, that is, grouped rather loosely about phagocyted organisms, the leukocytic mass now has the appearance of segmenting frog eggs, all of the cells fitting closely to each other. None of the very large bunches of leukocytes are seen which were so striking in slides 3, 4, and 5. Exudate does not coagulate readily.

Stained smear.—Polynuclears predominate largely, and seem to be the phagocytes.

5:20 P.M. Slide 7: Large clumps of leukocytes about oidiomycetes do not appear. Frequently a single organism may be found within one leukocyte; almost never are more than five or six leukocytes concerned, excepting where a number of organisms occur together, in which case the whole mass may be fairly large, though the number of leukocytes per organism is apparently not what it was in earlier slides. The organisms appear to be unchanged.

Stained smear.—Similar to 6. The nuclei of the leukocytes appear to be undergoing a process of karyorrhexis.

6:20 P.M. Slide 8: Similar to the above in the main. One important difference, i.e., leukocytes immediately surrounding organisms seem to be breaking up; they are losing their distinctness of outline and the granules are spreading out diffusely into the surrounding zones. Oidia are all budding, this condition seeming to be a little more marked than in earlier specimens of the exudate.

Stained smear.—Similar to 7, more leukocytic changes. It is hard to determine whether the phagocytic cells are mononuclear or polymorphonuclear; it seems best to regard them as mononuclear, although doubtful.

March 9, 1909. 9:30 A.M. Slide 9: Leukocytes grouped about organisms seem to be fragmenting. Leukocytes are scattered evenly over the field. Aggregations of 20–30 about an organism, especially a budding form, are still to be found; those leukocytes in the center of such masses seem to be more or less fused with each other. Oidiomycetes contain variable numbers, generally inconsiderable (10–20), of small, somewhat highly refractive, spherical bodies, arranged as a rule near the periphery of the cell within the capsule. Frequently they are scattered irregularly through the cell. Many of the leukocytes in diverse parts of the field contain very similar granules. Sometimes organisms appear to be full of such bodies.

Stained smear.—In a number of instances it can be seen that the phagocytosis is undoubtedly by large mononuclear cells. One group is especially noticeable;

it consists of a budding organism inclosed by four leukocytes. The leukocytes are arranged radially about the organism. The peripheral three-quarters of the cytoplasm of each cell is independent and distinct, the inner fourth, however, has merged with a corresponding part of the other three to surround the oidium by a uniform cytoplasmic mass. The disposition of the cells reminds one a little of the structure of a four-leaf clover. The nuclei of the leukocytes stain purple; the cytoplasm, which is moderately vacuolated, a robin's-egg blue. The intracapsular portion of the organisms takes an irregularly distributed, deep to light blue stain; the capsule is clear, homogeneous, and of a very light blue color.

10:30 A.M. Slide 10: Cells, unstained, appear like large mononuclears for the most part. Groups of leukocytes are sometimes found arranged radially about an indistinct, somewhat homogeneous central mass in which it seems one can make out leukocytic shadows. The cells at the periphery of such masses are more or less cone-shaped, apex inward, nucleus at the base, peripheralward. The apex is drawn out into a narrow projection which loses itself in the central area. Budding oidia are found packed full of granules; then again non-budding forms occur which contain a very indistinct, faintly granular, slightly refractive material. Organisms are still engulfed by leukocytes, though it looks sometimes, especially in the case of the budding, strongly granular forms, as though the leukocytes were beginning to break away from the organism and its immediate phagocyte. Again we find budding, markedly granular organisms in the center of such leukocytic masses as are described above. Granules may or may not be present in the buds of such organisms. Phagocyted oidia are observed in which the cytoplasm seems to have become homogeneous and is divided into two or three segments.

Stained smear.—Sometimes the nucleus and cytoplasm of the leukocytes are markedly vacuolated, and the cell appears to be disintegrating. In a few instances the intracapsular portion of the oidia stains much less deeply than usual while the immediate leukocytes are quite well preserved. Polymorphonuclear cells are much more numerous than the other varieties of leukocytes, but appear to take no part in the phagocytosis. Typical small lymphocytes are very few in number. The large mononuclear cells with a great deal of cytoplasm form the great bulk of mononuclear leukocytes.

2:30 P.M. Slide 11: Essentially similar to 10. One organism is present which has an unusually thick capsule (2–3 times ordinary thickness). The outside of the capsule is smooth, the inside irregular. This organism has one small bud. Other oidiomycetes in the same mass appear normal.

Stained smear.—Phagocytosis is not exclusively by mononuclear leukocytes; a few polymorphs may be included in the plasmodial masses.

5:00 P.M. Slide 12: Leukocytic groups occur about organisms as in the two preceding slides. Fusion of leukocytes and extrusion of granules—breaking up of leukocytes?—is, if anything, more marked. As many as seven budding organisms are found in one leukocytic mass. Oidia seem to be budding and multiplying since we now find two, three, or four cells grouped together, sometimes in chains—mother cell, daughter cell?—sometimes in pairs nearly always giving evidence of budding. One organism is found with two buds. Also find free organisms for the first time since the second hour. One of these is rather thick-walled and is packed with 25–30 spherical, homogeneous bodies. The spherules are not so numerous but that each can be distinguished.

Stained smear.—Similar to 11. Phagocytic cells seem to be breaking up.

April 9, 1909. 8:20 A.M. Slide 13: Many free organisms. Also many large accumulations of leukocytes in the midst of which groups of organisms, two to six in a group, occur. Exudate is relatively scanty.

Stained smear.—Similar to 12. Mononuclear cells seem to be relatively more numerous than in last few specimens.

April 12, 1909. 11:30 A.M. Slide 14: (Pig seems somewhat asthenic. Tissues are swollen at abdominal wound. Wound is closed but bleeds readily. Small, pinhead nodules are palpable in right testicle. Left testicle is hardened; nodules similar to those in the right testicle are found.) Exudate is very scanty. One small drop obtained which shows very marked concentration of leukocytes about organisms, giving an appearance very similar to typical giant cells of the foreign body type.

Stained smear.—Mononuclear cells only, big, endothelial-like cells for the most part. They occur in huge masses, surrounding numerous organisms. All leukocytes in these masses seem to be losing their staining power; cytoplasm and nucleus is vacuolated.

This specimen was the last that could be obtained. Slide 14 was allowed to stand at room temperature. Examined the next day, it was found that long hyphae had grown out from many of the organisms which were inclosed in giant cells.

The guinea-pig was etherized two days after the making of slide 14. At autopsy the peritoneum, visceral and parietal, was dotted with grayish nodules from the size of minute points to nodules having a diameter of from 5 to 7 mm. The omentum was practically a mass of such nodules. The testicular surfaces also presented numerous grayish nodules, the largest being about 1 mm. in breadth. There was no excess of fluid in the peritoneal cavity. The nodules at first sight seemed to be retroperitoneal. Attempts to remove them showed that most could be peeled off without a great deal of difficulty, leaving a roughened opaque surface, while a few were considerably more adherent and, when removed, left a roughened opaque surface on which could be seen an occasional bleeding point. On cutting the nodules, some, especially the larger ones, were found to have soft centers; smears of this material showed polymorphonuclear leukocytes and oidiomycetes. Microscopically the nodules fixed in Zenker's fluid, imbedded in celloidin and stained with hematoxylin and eosin, were found to consist of masses of fibrin and leukocytes. The leukocytes were mostly of the large mononuclear, endothelial-like type, although in some isolated spots polymorphonuclear leukocytes predominated. Oidiomycetes were present in large numbers, usually in the center

of the nodule, and inclosed usually within cells having from 1 to 20 oval, well stained, peripherally placed nuclei. As a rule the nuclei numbered from 3 to 8.

The abundance of the cytoplasm of such cells was in direct proportion to the number of nuclei and took a marked uniform eosin stain with suggestion of a very finely granular structure. Sometimes the organisms were apparently free in the nodule; in such cases they formed the center of a small, circular, sharply circumscribed accumulation of polymorphonuclear leukocytes. There were no evidences of necrosis. The oidiomycetes themselves presented a variety of appearances. The budding forms frequently occupied the center of the small abscesses just mentioned; they presented a brightly shining, unstained, double-contoured external membrane, within which was an irregularly distributed, blue-stained, somewhat granular material which seemed, however, to form a lining layer just within the limiting membrane and not to be diffused to any great extent throughout the cell. In other organisms, the double-contoured membrane was unchanged and the material within the cell still took a blue stain; the latter, however, had seemingly shrunk away from the outer wall at all but one point, leaving a clear, unstained, new-moon shaped space. In still other organisms, this material had seemingly shrunk still more and was beginning to take an eosin stain; in others, it was beginning to lose its granular structure and to assume a homogeneous appearance, taking a sharp eosin stain. In such organisms as this, the central portion had sometimes become completely separated from the cell membrane so that the new-moon shaped space had grown into a complete circle; in other organisms, an equally pink-stained, homogeneous central portion was still in contact with the external membrane at one point, leading one to imagine that possibly the complete separation just described might be more apparent than real owing to the plane in which the organism had been cut.

Quite frequently no sign of "shrinking" was observed, the cells being a homogeneous pink without other modification. In those organisms which took the blue stain, it appeared as though the unstained portions within the capsule represented the homo-

geneous spherules described in the fresh specimens while the stained parts represented what might be termed the interspherular substance. A somewhat similar series of changes occurring in yeast *intra vitam* has been described by Potron.[17]

The peritoneum extended intact beneath most of the nodules. The cells of the peritoneum, however, had assumed a marked cuboidal or rounded shape and seemed to have broken contact with each other. In a few instances a typical organization was proceeding from the sub-peritoneal tissues into the overlying oidiomycetes containing exudate.

The kidneys were normal, as were the other organs. It will be remembered that some of the organisms in the peritoneal exudate of slide 14 grew. Oidiomycetes developed in pure culture from material taken from nodules at autopsy.

It does not appear to be necessary to report further experiments in detail. We shall summarize the course of events in the peritoneal cavity of a guinea-pig following the injection of an emulsion of living oidiomycetes in 0.85 per cent NaCl solution, basing our summary on numerous experiments such as the foregoing, on the autopsy findings of pigs dying or killed during the course of an experimental infection, and on observations made at exploratory operations, as follows: There is first a great transudation of fluid poor in fibrinogen and leukocytes into the peritoneal cavity. The few leukocytes present are mostly large mononuclears, though lymphocytes and polymorphonuclear leukocytes occur. After one or two hours the leukocytes begin to accumulate rapidly, and at the end of three or four hours, the peritoneal fluid, at first thin and watery, becomes very cloudy and quite viscid. This cloudiness and viscidity gradually increase as the fluid becomes more and more scanty, until finally, after three or four days, no more fluid can be obtained. If one opens the abdominal cavity of a guinea-pig 24 hours after the injection of a large dose of oidiomycetes, he finds an acute, diffuse, suppurative, and fibrinous peritonitis. The peritoneum is covered with a thick layer of turbid, sticky fluid in which are occasional yellowish-gray clumps. These clumps are soft and may be easily lifted from the peritoneal surface; in fact, they can hardly be said to be adherent.

The microscopical appearance of this exudate has been described above. The clumps consist of accumulations of phagocytic cells and groups of cells—the "rosettes" of Skchiwan—supported by a scanty fibrinous meshwork. After four days very little fluid will be found; the peritoneum may have a dull grayish tinge or may be about normal in appearance; it surely, however, will present numerous raised, convex, grayish nodules 0.5 to 5 mm. in diameter and sometimes whitish patches as much as 1 cm. in greatest extent, but these latter are not usual. As a rule, nodules and patches are easily detachable, leaving a dull, rough surface which sometimes may be slightly granular. They consist of masses of single large mononuclear leukocytes, some containing organisms, of polymorphonuclear leukocytes and masses of the phagocytic "rosettes," in a fibrinous meshwork. As the observations are extended to include lesions at longer and longer intervals following the injection, it is found that the nodules, beginning about the fifth day, become more and more difficult to remove, and, when removed, become more and more prone to leave a bleeding surface. Some seem to sink down into the tissue upon which they at first rested; the peritoneum grows over them; they become less and less prominent, and finally disappear entirely in the course of 15 to 30 days. Other nodules—and this seems to be especially true of nodules in the testicles following the intraperitoneal injection of 0.1 gm. of organism or more—commonly grow progressively larger for from 4 to 10 days, become somewhat soft and doughy, and then, in favorable cases, gradually harden, decrease in size and disappear entirely within 40 days. In unfavorable cases the process of enlargement and softening continues until, as in guinea-pig 75 (see p. 201), the scrotum becomes greatly distended, the overlying skin becomes involved, and the lesion finds an external opening. Microscopically, in the first type, blood-vessels are observed to grow into the "rosette" masses from underlying tissues, connective tissue is laid down, the nodules become young connective-tissue growths, and presently, the "rosettes" formed from free, phagocytic macrophages are transformed into morphologically typical Langhans giant cells of inflammatory granulation tissue merely by this replacement of their supporting fibrinous reticulum

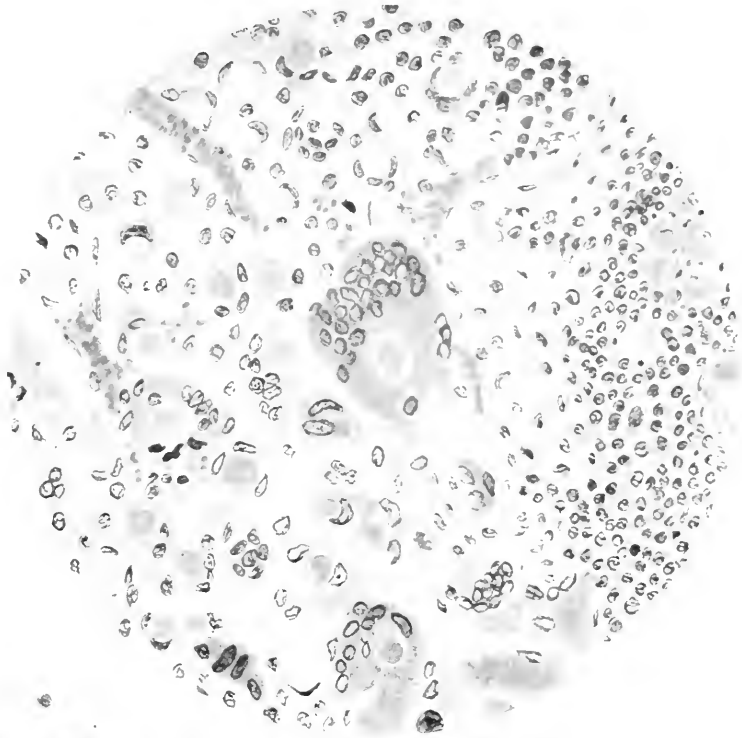
by a mesh-work of capillaries and their accompanying connective-tissue cells (see Fig. 5).

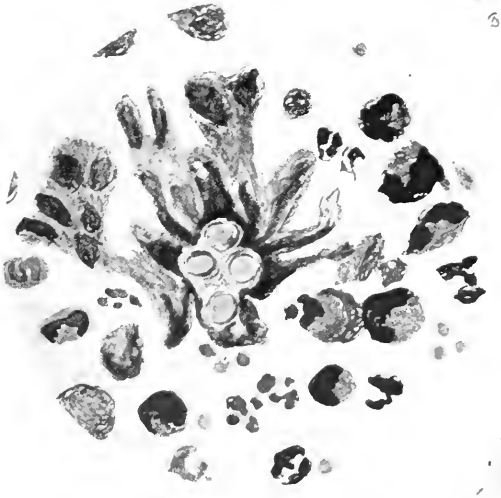
In the case of very large nodules such as those which formed in the peritoneal cavities of guinea-pigs 13, 37, 38, and 56 (see p. 204) the connective tissue growth may be so extensive as to cut off its own blood supply, thick-walled cysts being formed which contain a pus-like fluid. This material is not pus, however, but consists of cellular detritus, and, in the earlier stage, contains oidiomycetes showing various degrees of degeneration. In stained sections of this necrotic material from guinea-pig 13, autopsied 100 days after the last injection, one finds oidiomycetes which are practically normal in appearance, others have assumed appearances similar to those described in the nodules of guinea-pig 86, while others have gone still farther in that the cell membrane has either become thickened, homogeneous, eosin-stained, irregular in outline, or in other instances has disappeared leaving merely the inner homogeneous, eosin-stained spheres. Microscopical examination of the unstained detritus reveals bodies very similar to organisms in appearance but which are soluble in ether. In specimens from other nodules of similar age, guinea-pig 56, for instance, autopsied 116 days after last injection, the ether-soluble bodies are the only things which occur that in any way resemble oidiomycetes. Whether or not they bear any direct relation to the organisms has not been determined.

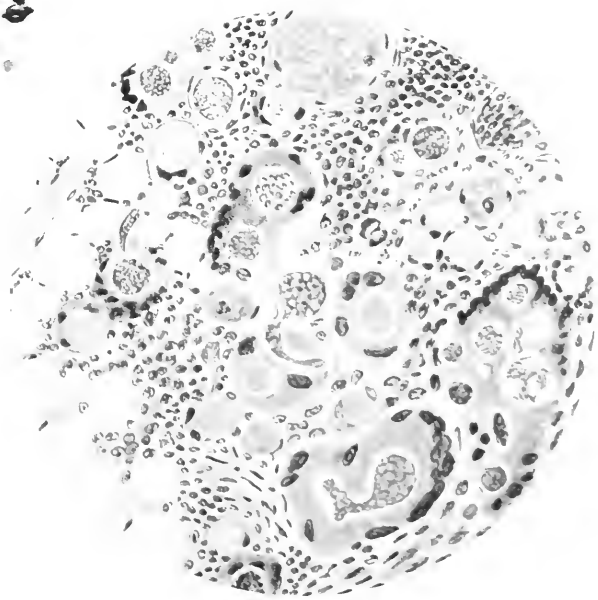
In the case of those nodules which become progressively larger and tend to soften, the bulk of the leukocytes consists of polymorphonuclears. In such masses, organisms commonly occur free; frequently they remain inclosed in Langhans giant cells which thus appear in stained sections as eosin-stained islets in the mass of blue-lobed nuclei. Such nodules occur to some extent in the early stages of all cases, but in the later stages of fatal cases only.

Mention has been made of nodules "in the testicles." This statement is not strictly correct since most nodules palpated "in the testicles" are merely in the peritoneal and fibrous tunics. Even in the most advanced cases the testicle itself may be and

PLATE 5.







generally is unharmed, as in guinea-pig 75 (see p. 201) in which at autopsy the testicles were found normal.

Besides the peritoneal lesions, macroscopic nodules have been found in the liver and in the lungs. In the liver they appear as white to grayish red patches 1–5 mm. in cross-section, rather sharply demarkated. In the lungs they appear as translucent droplets 0.5–2 mm. in diameter, occurring both sub-pleurally and on the cut surface. The animals seem to have died before larger nodules had time to develop.

Under the microscope in sections stained with hematoxylin and eosin, typical liver nodules appear as follows:

Section of the liver of guinea-pig 53 (for history, see p. 206; see also Plate 5). Slight passive congestion; slight central fatty infiltration; there is one area with a diameter of 4–6 liver lobules in which the parenchyma cells seem to have been replaced by widely dilated, erythrocyte-filled sinuses, and oidiomycetes inclosed in cells having from 1 to 20 nuclei. The multinucleated cells resemble closely foreign-body giant cells, excepting that the nuclei tend to be more rounded than those commonly seen in the latter cells, and, when oval in shape, their long axis tends to run parallel with the surface of the cell rather than centralward. This area is sharply marked off from the liver tissue by a narrow layer of parallel, wavy, pink-stained fibers, peripheral to which is quite a marked infiltration of mononuclear cells, mostly of the lymphoid type, though many are cells with abundant cytoplasm. The neighboring parenchyma cells are normal in most cases, although occasional islets of liver cells in which are found all stages of degeneration, even to that of complete necrosis, appear in the border tissue.

In the lungs, the nodules occur principally in the neighborhood of the bronchi or larger blood-vessels. They consist of dense accumulations of round and epithelioid cells with an organism at rare intervals; no necrosis; no edema; no exudation into alveoli. Aside from the nodules the lungs are normal.

PHAGOCYTOSIS IN VITRO.

The leukocytes.—Guinea-pigs were given intraperitoneal injections of a thick suspension of sterile aleuronat in 0.85 per cent NaCl solution. From examination of the exudate at hourly intervals it was found that, after a lapse of from 12 to 15 hours, the exudate was very rich in leukocytes and was still sufficient in quantity to be easily obtained. Of the leukocytes, from 25–50

per cent were large mononuclears, the remainder being mostly polymorphonuclear neutrophiles and eosinophiles. Both the fresh exudate and "washed" leukocytes were used. The "washed" leukocytes were obtained by washing the fresh exudate first in sodium citrate solution and then in one or two changes of physiological salt solution according to the ordinary opsonic technic. The suspension was then made up to the original volume of the exudate by the addition of 0.85 per cent NaCl solution. The fresh exudate clotted readily, so that, in using it, rapid work was necessary.

Exudate serum.—This was obtained by allowing the fresh exudate to clot spontaneously in test-tubes, centrifuging immediately, and removing the serum by means of a glass pipette.

The oidiomycetes were placed in a bottle of normal salt solution and shaken vigorously in a shaking machine for several hours before being used, in order to break up any clumps which might be present. An emulsion of the organisms in salt solution was then made which contained approximately 5,000 organisms per c.mm. In order to do this, counts were made of the organisms in the emulsion in the shaking bottle by means of the Thoma-Zeiss hemocytometer, and this emulsion was then diluted to the volume required as shown by the excess of organisms over the 5,000 per c.mm. which was desired.

Method.—The observations were made on hanging-drop preparations. The various materials were mixed on clean cover-glasses in triplicate, in the dilutions called for by the protocol, mounted on hollow ground slides, and the cover-slip sealed to the slides with paraffin. The preparations were incubated at 37° C. and observed after 30 minutes, after 3 hours, and after 24 hours. No stain was employed.

The first attempt was directed to determining (1) whether or not phagocytosis would occur *in vitro*, using (*a*) the fresh exudate and (*b*) washed leukocytes, and (2) the possible influence of guinea-pig serum upon the process.

Several preliminary experiments of which the following is a typical example were performed:

TABLE 6.
EXPERIMENT IN PHAGOCYTOSIS.

Slide No.	Organisms (Units of Emulsion)	Fresh Exudate (Units)	Washed Leukocytes (Units)	Normal Blood Serum (Units)	Exudate Serum (Units)	NaCl (0.85 per cent) (Units)
0.....	1	1	0	20	0	0
1.....	1	1	0	10	0	0
2.....	1	0	1	0	0	1
3.....	1	1	0	8	0	2
4.....	1	0	1	0	1	0
5.....	1	1	0	6	0	4
6.....	1	0	1	1	0	0
7.....	1	1	0	4	0	6
8.....	1	1	0	2	0	8
9.....	1	1	0	0	0	10
10.....	1	1	0	0	0	0

The final examination of these slides after 24 hours at 37° C. disclosed the following conditions:

Slide 0: Well mixed; many red cells; leukocytes occur singly but also very frequently in groups of two to a dozen or more—most commonly three or four cells per group. Organisms may or may not, infrequently not, be found within these groups. The rule appears to be for the leukocytes to pay no attention to the oidiomycetes. Very rarely a single leukocyte may be seen which has partially or totally engulfed an organism.

Slide 1: Organisms occurring singly are seen only within leukocytes. Organisms occurring in clumps are always surrounded and, to be perceived by careful focusing, engulfed by leukocytes. Leukocytic clumps are found in the absence of organisms, but not to the extent observed in slides 2, 4, and 6 (see below). Many large leukocytes with abundant cytoplasm occur; they are the actively phagocytic cells. There are many red blood corpuscles, but very few of them have been ingested.

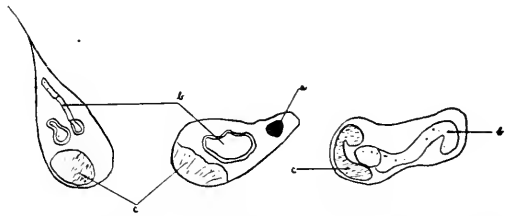


FIG. 1.—Types of phagocytic cells: *a*, erythrocytes; *b*, organisms; *c*, cell nucleus.

Slide 2: Leukocytes and organisms are quite numerous. Both types of cells, leukocytes and oidiomycetes, occur singly, by twos and threes, and in large groups, the organisms to a greater extent than the leukocytes. The cells are well intermixed and frequently approximate each other, but this is not marked enough to suggest phagocytosis or even positive chemiotaxis.

Slide 3: Very similar to slide 1, excepting that there is more extensive ingestion of red cells. In some cases leukocytes are merely grouped about organisms, in others there is not only grouping, but phagocytosis.

Slide 4: Similar to slide 2, excepting that two leukocytes are present which appear to be phagocytic. Each has partly surrounded an elongated blastomycete.

Slide 5: Many red blood cells are present. A good many of them have been phagocyted. Organisms are present in fair numbers and occur for the most part

within single, or plasmodial masses of, leukocytes. It is noticeable that the leukocytes which take up the red blood corpuscles also take up the organisms. It is common to find a leukocyte which contains 2, 3, or even 4 red cells in addition to an organism. Such large cells seem to be less numerous here than in slide 3. They assume bizarre shapes—greatly elongated, spindle forms, star-forms, and various other irregular outlines, with long, slender projections. This is especially noticeable about the edges where the drop is thin.

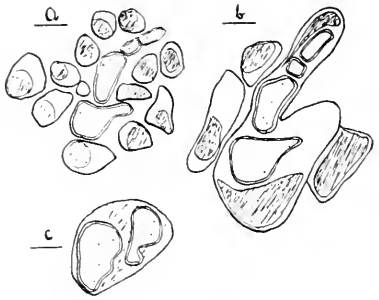


FIG. 2.—Sketches of two strikingly similar oidiomycetic groups to illustrate the difference between (*a*) mere grouping of leukocytes, and (*b*) grouping with phagocytosis; (*c*) shows a leukocyte containing two organisms. These sketches also illustrate the type of cell which is actively phagocytic.

Slide 6: Same general appearance as slide 4.

Slide 7: Fewer cells of all kinds: otherwise similar to slide 5.

Slide 8: Cells of all kinds seem to be still fewer than in slide 7. Absolutely free organisms are quire rare; on the other hand, ingestion is not always complete and many of the groupings suggest that shown in Fig. 2. This appears to be due in some degree to a dearth of the big leukocytes, since where they occur ingestion is as complete, as a rule (there are exceptions), as their bulk makes possible, as is suggested in the accompanying sketch.

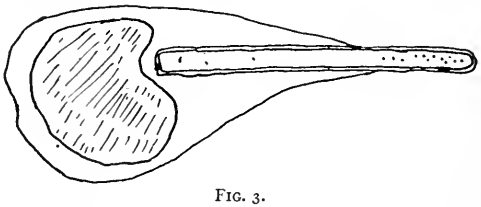


FIG. 3.

Red blood corpuscles have been phagocyted quite freely but by no means to the degree, relatively, that is evident in the case of the oidiomycetes.

Slide 9: Complete ingestion of single and budding organisms occurs with fair frequency; on the other hand, in

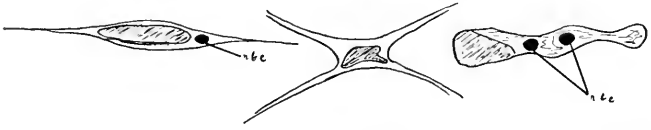


FIG. 4.

FIG. 5.

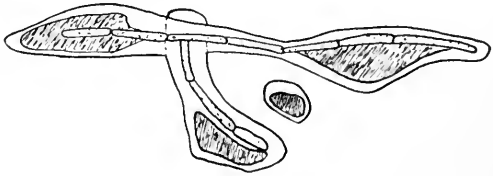


FIG. 6.

many instances the leukocytes seem to be entirely indifferent to the oidia. In plasmodial masses which are in relation to organisms there is generally more or less com-

plete phagocytosis; contrarywise, places are not hard to find where mere grouping, with little or no phagocytosis, occurs.

Slide 10: Conditions here are very similar to those in slide 0. If anything, phagocytosis may be slightly less marked, something of a hair-spitting distinction, however.

From this experiment it appeared (1) that the leukocytes of the fresh exudate, either without the addition of normal serum or when mixed with excessive quantities of normal serum, do not phagocyte oidiomycetes as well as such leukocytes plus moderate quantities of serum; (2) that washed leukocytes do not phagocyte oidiomycetes readily, when suspended in simple salt solution, when suspended in salt solution plus normal serum, or salt solution plus exudate serum; (3) that phagocytosis is carried on in the fresh exudate *in vitro* by cells of the same type as those which are responsible for the major part of the work in the peritoneal cavity.

An extensive series of further experiments was planned with the idea of testing thoroughly the above deductions. Unfortunately, the adoption of the mycelial mode of growth by the organism interrupted the work while it was still far from being complete, so that the results reported below must be regarded as purely tentative.

The summary of events follows: (1) It appears that the leukocytes of the undiluted fresh exudate are very actively phagocytic; complete phagocytosis is the rule within 30 minutes at 37° C.; that is, as a rule, every organism in the preparation will have been ingested within that space of time. The leukocytes commonly form a plasmodial mass about the oidiomycetes, the mass being surrounded by a narrow clear zone of fairly uniform width which separates it from the surrounding leukocytes. If the exudate is diluted by salt solution, normal serum, immune serum or exudate serum, the degree of phagocytosis seems to decline, roughly, with the increase of the dilution. In other words, the more widely the organisms are separated from the leukocytes, the less apt is phagocytosis to occur. The organisms do not seem to be strongly chemiotactic in the sense that leukocytes will be attracted to them from a distance, the ingestion of the oidiomycetes by the leukocytes seems to be a sort of contact phagocytosis. The sera

do not encourage phagocytosis to any greater extent than does physiological salt solution.

(2) As was indicated in the earlier work, washed leukocytes are very inconstant with respect to their ability to phagocyte oidiomycetes. The addition of the various sera mentioned above does not help matters. It was thought that perhaps the manipulation to which they were subjected injured the leukocytes in some way and an effort was made to obviate this as much as possible by decreasing the number of washings and the length of time in the centrifuge, by keeping the solutions at 37° C., etc., but without effect. Once in a while such leukocytes ingested organisms, but, in so far as could be determined, with neither rhyme nor reason. As these leukocytes were capable of ingesting carmine granules as well as an occasional organism it appeared that they had not lost their powers entirely; why, then, this failure to engulf oidiomycetes as actively as the leukocytes in the fresh exudate? Several explanations were suggested. The technic may have been unsuited to the materials with which the work was being done. Savtchenko[14] found that the mononuclear cells of the peritoneal exudate of guinea-pigs have a tendency to clump and not to phagocyte after centrifugation in citrate solution. Brisco[24] has made a similar observation with respect to the alveolar cells of the lung. It was noted that the ingested carmine granules were much smaller than the oidiomycetes. Possibly the leukocytes had been injured sufficiently to deprive them of the ability to envelop such giants as the yeasts cells but still retained vitality enough to engulf smaller particles. Again, the fresh exudate was quite viscid and fibrin threads formed within it in a very short time after the mixtures were made. Possibly the viscidity of the medium and the fibrin network were the factors which enabled the leukocytes to extend themselves over the organisms. It would be interesting, in this connection, to observe the effects of the addition of a solution of gelatin upon the phagocytic activity of the washed leukocytes. This might also be the key to the effects of dilution on phagocytosis in the fresh exudate.

(3) The proposition that phagocytosis is carried on in the fresh exudate *in vitro* by cells of the same type as those which are respon-

sible for the major part of the work in the peritoneal cavity is borne out by further observations. This does not mean that, either within the peritoneal cavity, or out of it, all the work is done by the macrophages. Such is not the case; polymorphonuclear leukocytes frequently take part in the work, and sometimes are the only leukocytes present in the plasmodial masses. They, of course, are too small to ingest any but the smallest oidiomycetes. Nevertheless, they are active in surrounding and "hemming in" the organisms. On the other hand, there is no room for doubt that the bulk of the work is done by the macrophages.

In three experiments, normal and immune serum and sodium citrate solution seemed actually to inhibit phagocytosis in fresh exudate, while the leukocytes in the salt-solution dilution of the same exudate were actively phagocytic. No explanations of these results have suggested themselves.

Fresh normal rabbit serum was mixed with guinea-pig peritoneal exudate. The leukocytes in the exudate became spherical in shape, and were strongly agglutinated within 30 minutes at 37° C. The observations were not carried further. Needless to say, no phagocytosis occurred in such preparations.

These experiments seem to indicate that opsonins are, at least, not of great importance in the phagocytosis of the oidiomycetes used in this work by the leukocytes of the peritoneal exudates of normal and immune guinea-pigs.

SUMMARY AND CONCLUSIONS.

Oidiomycosis in the guinea-pig, following intraperitoneal inoculation, is characterized, in fatal cases in male animals, by a gradually developing cachexia accompanied as a rule by a steady loss of weight which is especially marked during the last three or four days of life and by the development, in pigs of 400 gm. or over, of palpable nodules in the testicles in from three to seven days after inoculation. In males weighing 400 gm. or less the nodules in the testicles are inconstant, and, when present, may be difficult to palpate owing to the fact that they occur frequently about the upper pole of the testicle and about the neck of the scrotal sac—points which may be difficult to feel in small animals. The appearance of palpable nodules in the skin or anterior abdomi-

nal wall has been noted but may be avoided in a great measure by rinsing the outside of the needle before making the injection. In female guinea-pigs symptoms are usually entirely lacking; they survive intraperitoneal doses which kill the males, a point which should be borne in mind when testing the pathogenicity of yeasts for guinea-pigs. The scrotal sac of the male pig seems to afford a *locus minoris resistentiae* to which the females have no counterpart. The reasons for the lower resistance of this particular corner of the peritoneal-lined cavity are not apparent. Postmortem examination of animals dying of the disease reveals multiple grayish nodules from 0.1 to 10 mm. in diameter on all peritoneal surfaces. All such nodules contain oidiomycetes, as may be demonstrated in microscopic sections and in cultures. Most nodules have softened centers, especially the large nodules which are found in the testicles, in which oidiomycetes and great numbers of polymorphonuclear leukocytes occur. The lungs and liver may also present small areas of cellular infiltration about oidiomycetes. There are no constant changes in the various organs.

Recovery from an infection is accompanied by a low grade of immunity which manifests itself in a somewhat more speedy recovery from subsequent infections, and by the development of a slight temperature for a few days immediately following reinoculations.

Repeated injections of an "extract" of oidiomycetes lead to the development of an immunity in guinea-pigs which is characterized mainly by the more rapid walling off of organisms injected into the peritoneal cavity, and by a more rapid disappearance of the lesions which appear in the testicles.

The sera of guinea-pigs immunized by repeated intraperitoneal injections of living organisms or of an extract of organisms develop precipitating substances against the "extract" in from 50 to 79 per cent of the cases, which may be manifest in a dilution as high as 1:20. Normal sera occasionally precipitate with the "extract" in lower dilutions. Substances which will agglutinate oidiomycetes suspended in dilutions of the sera are not formed. Specific amboceptors also do not appear to be developed. Opsonins have not been demonstrated. Prolonged exposure of oidiomycetes to immune or normal serum at 37° C. seems to impair the vitality of

the organisms—judging by their diminished power of growth when transferred to suitable media—as compared with that of similarly tested control tubes containing organisms suspended in physiological salt solution. The reaction of anaphylaxis may, under suitable conditions, be obtained with the "extract" in guinea-pigs. The "extract" is toxic; prolonged administration by the intraperitoneal route results in cachexia and death usually by secondary infection; judiciously used, it may have a favorable effect upon the course of an oidiomycetic infection.

Oidiomycetes injected into the peritoneal cavity of guinea-pigs are rapidly taken by leukocytes, macrophages principally, which form plasmodial masses and become attached to the peritoneal surface at first by fibrinous, later by fibrous, adhesions, the mass of cells grouped immediately about the organisms presently assuming the structure of typical Langhans giant cells. In cases ending in recovery, the organisms may grow for a time, but eventually degenerate and disappear; the nodule within which they were, being absorbed with them. In fatal cases the nodules increase in size and become soft; many of the organisms degenerate, but others multiply; the inclosing giant cell disintegrates and there ensues an infiltration of polymorphonuclear leukocytes *pari passu* with the enlargement and softening of the nodule. Sometimes the organisms are surrounded by polymorphonuclear cells from the beginning; such a mass may in turn be inclosed by layers of macrophages. Just what such a condition may mean as regards the prognosis of the lesion has not been determined.

The organisms do not tend to penetrate the peritoneal surface. It seems probable that they leave the peritoneal cavity, only in case there has been actual tearing of the lining tissues. Oidiomycetic nodules were described in the liver and lungs of a few animals. The belief seems to be justified that such generalization of the infection followed the introduction of the organisms into the circulation by mechanical means, because the condition occurred almost solely (there were two exceptions) in animals in whose peritoneum one could detect tears, as in the animals used for the study of phagocytosis *in vivo*.

The mode of defense of guinea-pigs against oidiomycetes in-

jected into the peritoneal cavity appears to consist, firstly, in phagocytosis and intracellular digestion; secondly, in a walling off and encapsulation of the phagocyted organisms by connective tissue; and, thirdly, upon a somewhat ill-defined, and possibly questionable, unfavorable influence of the serum upon the vitality of the organism. These agencies act but slowly; oidiomycetes may retain their power to grow on artificial media for days, and their characteristic staining properties for weeks, in the inflammatory nodules of supposedly immune pigs. Specific antibodies are but poorly developed.

The author is indebted to Dr. Howard T. Ricketts for advice and assistance, and for the use of unpublished experiments, portions of which have been incorporated in the body of this paper; to Dr. H. G. Wells for advice and assistance, and to Mr. William F. Peterson who has contributed generously of both time and energy to the promotion of the latter part of this work.

REFERENCES.

1. METSCHNIKOFF. *Virchow's Archiv*, 1884, 96, p. 177.
2. RIBBERT. *Der Untergang pathogener Schimmelpilze im Körper*, Bonn, 1887.
3. SANFELICE. *Centralbl. für Bakt.*, Abt. 1, Orig., 1896, 20, p. 219.
4. CHARRIN AND OSTROWSKY. *Comptes rendus Soc. de Biol.*, 1896, 48, p. 743.
5. ROGER. *Comptes rendus Soc. de Biol.*, 1896, 48, p. 728.
6. SCHATTENFROH. *Arch. für Hyg.*, 1896, 27, p. 234.
7. GILKINET. *Arch. de méd. expér.*, 1897, 9, p. 881.
8. JONA. *Centralbl. für Bakt.*, Abt. 1, Orig., 1897, 21, p. 147.
9. OBICI. *Zeiglers Beiträge*, 1898, 23, p. 197.
10. SKCHIWAN. *Ann. de l'Inst. Pasteur*, 1899, 13, p. 770.
11. RICKETTS. *Jour. Med. Res.*, 1901, 6, p. 377.
12. MALVOZ. *Ann. de la soc. méd.-chir. de Liège*, 1901, quoted from Sanfelice (15).
13. MALVOZ. *Centralbl. für Bakt.*, Abt. 1, Orig., 1901, 29, p. 668.
14. SAVTCHENKO. *Ann. de l'Inst. Pasteur*, 1902, 16, p. 106.
15. SANFELICE. *Centralbl. für Bakt.*, Abt. 1, Orig., 1902, 32, p. 360.
16. WLAEFF. *Compt. rendus Soc. de Biol.*, 1902, 54, p. 412.
17. POTRON. *Blastomycetes dans les tissues.* Thèse, Nancy, 1903.
18. FABOZZI. *Centralbl. für Bakt.*, Abt. 1, Ref., 1906, 39 (Abstract), p. 282.
19. SPIETHOFF. *Jahrbücher der Hamb. Staatskrankenanstalten*, 1903–4, 9, Heft II, p. 167.
20. BOWEN AND WOLBACH. *Jour. Med. Res.*, 1906, 15, p. 167.
21. CHRISTENSEN AND HEKTOEN. *Jour. Amer. Med. Assoc.*, 1906, 47, p. 247.
22. HAMBURGER. *Jour. Infect. Dis.*, 1907, 4, p. 201.
23. MARCO DEL PONT. *Bull. de l'Inst. Pasteur*, 1907, 5, p. 46.
24. BRISCO. *Jour. Path. and Bact.*, 1907, 12, p. 66.
25. BROWN. *Jour. Amer. Med. Assoc.*, 1907, 48, p. 743.
26. HEKTOEN. *Jour. Amer. Med. Assoc.*, 1907, 49, p. 1071.
27. MONTGOMERY AND ORMSBY. *Archives Int. Med.*, 1908, 2, p. 1.
28. ROTHE. *Deut. med. Wchnschr.*, 1910, 36, p. 30.
29. WIDAL, ABRAMI, JOLTRAIN, BRISSAUD, ET WEILL. *Ann. de l'Inst. Pasteur*, 1910, 24, p. 1.

CLASSIFICATION OF THE B. COLI GROUP.*

DANIEL D. JACKSON.

The term *B. coli* as an indication of fecal contamination in water and milk has been so often misapplied that the result has been much confusion and the frequent misinterpretation of bacterial examinations.

It has been the custom of many bacteriologists to throw out of sanitary consideration all bacteria which do not absolutely conform to the so-called "typical" *B. coli.* There are many known varieties, all of fecal origin and closely related to "typical" *B. coli*, which will be described in this paper and there are many more possible varieties which probably exist and undoubtedly will be discovered in the future. Any of these varieties when they occur in water or milk have a sanitary significance and, because of their close relationship, all should be included in the *B. coli* group.

The fermentative reactions have been chosen as a means of classification, not only because of the ease with which these organisms are thus separated from those of other groups, but because of the facility with which each variety may be separated from the others.

B. COLI GROUP.

The general characteristics common to this group are: Fermentation with gas production with dextrose and lactose, short bacillus with rounded ends, non-spore-forming, facultative anaerobic, gives positive test with esculin, grows at 20° C. on gelatin and at 37° C. on agar, non-liquefying in fourteen days on gelatin.

The group consists of four species:

B. communior (Durham)
B. communis (Escherich)
B. aerogenes (Escherich)
B. acidi-lactici (Hueppe)

The relative frequency with which the species of the *B. coli* group have been isolated is shown as follows:

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	MacConkey: Number of Strains from Feces		Graham-Smith: Number of Strains from Flies		Winslow and Walker: Number of Strains from Feces	
B. communior	110	23%	15	43%	7	28%
B. communis	178	37%	6	17%	15	60%
B. aerogenes	72	15%	4	11%	1	4%
B. acidi-lactici	120	25%	10	29%	2	8%
	480	100%	35	100%	25	100%

The first two species are separated from the second two by their gas production with dulcite and the first of each of these two groups may be separated from the second by its gas production with saccharose.

Each of these species may be separated into four possible varieties in accordance with their gas production with mannite and raffinose. Three varieties each of the first and fourth species are now known, two varieties of the third, and all four possible varieties of the second group have been found. These varieties are classified by letters, as, for instance, *B. communis, A, B, C,* and *D.*

Under these varieties are classified subvarieties as in the case of *B. communior*, A_1, and A_2.

Graphically the separation of the species and varieties is brought about as shown in diagram on following page.

In this diagram 21 varieties of *B. coli* are given, four of which are as yet unknown. Out of the 17 varieties the author has cultures of 15 varieties. The other two, *B. communis D* and *B. acidi-lactici D*, are described by Winslow and Walker.[1]

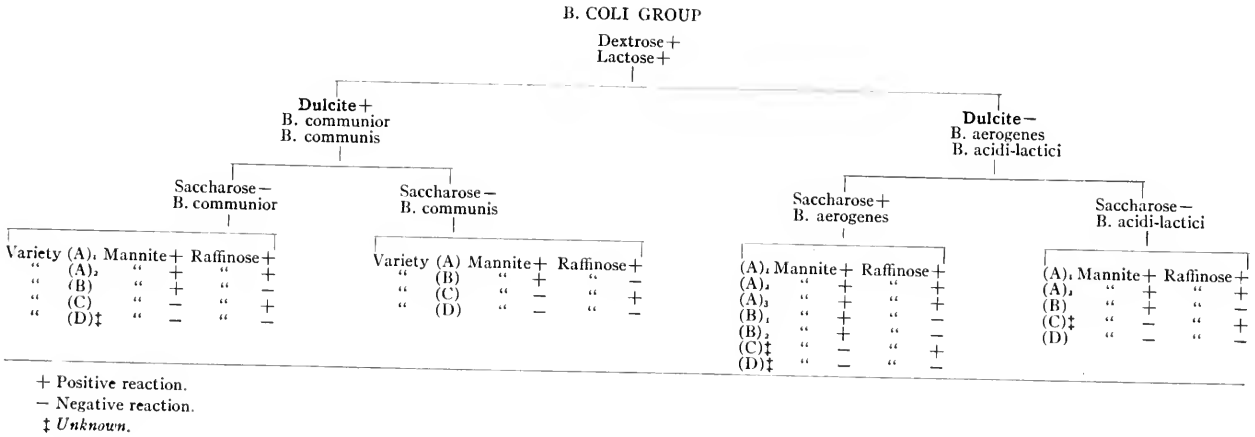
It will be seen that the classification admits of indefinite expansion as other subvarieties are found. The following is a description of the characteristics of the known members of the *B. coli* group.

B. COMMUNIOR (DURHAM).

Variety A_1.—Fermentation with gas production with dextrose, lactose, dulcite, saccharose, mannite, and raffinose. Milk coagulated, nitrate reduced, motile, and indol positive.

Isolated by West of the Philadelphia Filter Laboratories from

[1] Winslow and Walker, *Science*, 1907, 26, p. 675.



\+ Positive reaction.
− Negative reaction.
‡ *Unknown.*

contaminated river water and checked by the author; also 7 strains isolated by Winslow from feces; by Dr. Avery of the Hoagland Laboratories, Brooklyn, from two cases of chronic cystitis, and from an abscess associated with streptococci in a chronic case of cellulitis; also isolated by the author from contaminated water and from feces.

This appears to be the most common variety of communior. While raffinose determinations were not made, it is probably the same variety as isolated by MacConkey[1] in 110 strains and by Graham-Smith[2] in 15 strains taken from fecal matter.

Variety A_2.—Fermentation the same as A_1. Motile, reduces nitrate. Differs from A_1 in not producing indol.

Variety B.—Ferments with gas production with dextrose, lactose, dulcite, saccharose, and mannite, but forms no gas with raffinose.

Also distinguished by no coagulation in milk even after heating and by slow formation of gas in dulcite. This latter test usually takes three days for the gas formation to become active. Motile, indol positive, nitrate reduced.

Variety found by Melia, of Mt. Prospect Laboratory, in 5 strains from human feces, also received from Dr. Avery of the Hoagland Laboratories, Brooklyn, and isolated from urine from a case of cystitis.

Variety C.—Fermentation with gas production with dextrose, lactose, dulcite, saccharose, and raffinose. Forms no gas with mannite. Milk coagulated, nitrate reduced, motile, and indol positive.

Culture obtained from Dr. J. R. Fraser, McGill University, Montreal.

B. COMMUNIS (ESCHERICH).

Variety A.—Fermentation with gas production with dextrose, lactose, dulcite, mannite, and raffinose. No gas formation with saccharose. Motile, indol slight, nitrate reduced.

Isolated by Cerrolo at the Bellevue Hospital.

Isolated by Avery from a case of cystitis.

[1] MacConkey, *Jour. Hyg.*, 1905, 5, p. 333.

[2] Graham-Smith, *Report of Local Government Board* (London, 1909).

Variety B.—Fermentation with gas production with dextrose, lactose, dulcite, and mannite. No gas production with saccharose and raffinose. Milk coagulated, nitrate reduced, motile, and indol positive.

This appears to be the most common variety of *B. communis*. Isolated by Avery and checked by the author from a case of urinary cystitis, by Winslow from feces, and by the author from feces, water, and milk.

Variety C.—Fermentation with gas production with dextrose, lactose, dulcite, and raffinose. No gas production with saccharose or mannite. Nitrate reduced, indol positive, motile.

Isolated by Winslow from feces.

Isolated by Melia from Brooklyn Water Supply.

Variety D.—Fermentation with gas production with dextrose, lactose, and dulcite. No gas production with saccharose, mannite, or raffinose. Nitrate reduced, indol positive. Isolated by Winslow and Walker from feces.

B. AEROGENES (ESCHERICH).

Variety A_1.—Fermentation with production of gas with dextrose, lactose, saccharose, mannite, and raffinose. No gas production with dulcite. Indol positive, nitrate reduced, motility negative. Viscous growth on agar and in lactose bile. In the latter it can be drawn out into a long, thin string.

Culture obtained by the author from Bellevue Hospital, also isolated by Dr. White of the Hoagland Laboratories from a urinary fistula, by Winslow from feces, and by the author from the Brooklyn Water Supply.

Variety A_2.—Fermentations the same as A_1. Motile, indol negative, nitrate reduction positive. Differs from A_1 in being less viscid or stringy when touched with the needle; in being motile, and indol negative.

Isolated by Avery from a case of chronic urethritis and from a a case of cellulitis associated with *B. pyocyaneus* and *Streptococcus pyogenes*.

After the identification of this variety a vaccine was made which was specific for this particular variety of infection, whereas the

vaccine previously made from *B. communis B* apparently had no curative effect.

Variety A_3.—Fermentations and all tests with one exception same as A_2. It liquefies after 26 days.

Isolated from contaminated well water by Phelps and Hammond.[1] Differs from A_2 in being slightly liquefying in gelatin stab after about twenty-six days. The total gas and percentage of CO_2 is high when grown in dextrose broth and particularly in liver broth. This species has been at times grouped with *B. cloacae* (Jordan), but the former never fails to produce gas with lactose, while typical *B. cloacae* apparently always gives negative results, when dextrose-free lactose solutions are used. Another marked distinction is that true *B. cloacae* after rejuvenating is always strongly liquefying, while *B. aerogenes* A_3 never liquefies before twenty days, even after careful rejuvenation over long periods.

Variety B_1.—Forms gas with dextrose, lactose, saccharose, and mannite but no gas with dulcite and raffinose. Non-motile, indol negative, nitrate reduced. Viscous growth on agar and in lactose bile. May be drawn out into a thin string by using a platinum needle.

Culture obtained from Kral's Laboratory; also isolated by the author from water and milk. This is probably the most common variety of *B. aerogenes*.

Variety B_2.—Differs from B_1 in being motile, indol positive, and non-viscous in lactose bile.

Isolated by Melia in two strains from feces.

B. ACIDI-LACTICI (HUEPPE).

Variety A_1.—Fermentation with gas production with dextrose, lactose, mannite, and raffinose. No gas production with dulcite and saccharose. Non-motile, indol positive, nitrate reduction positive.

Isolated by Hueppe from milk.

Variety A_2.—Fermentation same as A_1. Indol positive, nitrate reduction positive. Differs from A_1 in being motile.

Isolated by West from contaminated river water.

[1] Phelps and Hammond, *Am. Jour. Pub. Hyg.*, 1909, n.s. 5, p. 545.

Variety B.—Fermentation with gas production with dextrose, lactose, and mannite. No gas production with dulcite, saccharose, or raffinose. Milk coagulated, nitrate reduced, motile, and indol positive. Isolated by Melia in nine strains from human feces. Often exceeding in numbers all other varieties of bacteria in feces.

Culture also obtained from Dr. J. R. Fraser, McGill University, Montreal. This appears to be the most common variety of *B. acidi-lactici*.

Variety D.—Gas production with dextrose and lactose. No gas production with dulcite, saccharose, mannite, or raffinose. Indol positive, nitrate reduced.

Isolated by Winslow from feces.

The accompanying table shows the distinguishing characteristics which separate the individual members of the *B. coli* group. The four unknown varieties indicated will probably be discovered later.

B. COLI GROUP.

	Dextrose	Lactose	Dulcite	Saccharose	Mannite	Raffinose	Motility	Indol	Nitrate Reduction	Liquefaction Gelatin in 14 Days	Coagulation of Milk
B. communior A_1	+	+	+	+	+	+	+	+	+	−	+
B. " A_2	+	+	+	+	+	+	+	−	+	−	+
B. " B	+	+	+	+	+	−	+	+	+	−	−
B. " C	+	+	+	+	−	+	+	+	+	−	+
B. " D±	+	+	+	+	−	−	··	··	··	−	··
B. communis A	+	+	+	−	+	+	+	slight	+	−	+
B. " B	+	+	+	−	+	−	+	+	+	−	+
B. " C	+	+	+	−	−	+	+	+	+	−	+
B. " D	+	+	+	−	−	−	··	+	+	−	+
B. aerogenes A_1	+	+	−	+	+	+	−	+	+	−	+
B. " A_2	+	+	−	+	+	+	+	−	+	−	+
B. " A_3	+	+	−	+	+	+	+	−	+	positive after 26 days	+
B. " B_1	+	+	−	+	+	−	−	−	+	−	+
B. " B_2	+	+	−	+	+	−	+	+	+	−	+
B. " C±	+	+	−	+	−	+	··	··	··	−	··
B. " D±	+	+	−	+	−	−	··	··	··	−	··
B. acidi-lactici A_1	+	+	−	−	+	+	−	+	+	−	+
B. " A_2	+	+	−	−	+	+	+	+	+	−	+
B. " B	+	+	−	−	+	−	+	+	+	−	+
B. " C±	+	+	−	−	−	+	··	··	··	−	··
B. " D	+	+	−	−	−	−	··	+	+	−	+

+ Positive reaction. − Negative reaction. ± Unknown varieties.

SUMMARY.

I. A study of this classification shows that 13 out of 17 known varieties of *B. coli* have been isolated from feces or diseased con-

ditions, and that 7 of these varieties have been isolated from water. Of the 7 varieties isolated from water, 4 would conform to so-called "typical" *B. coli* in spite of the fact that they are here grouped under three distinct species, *B. communior*, *B. communis*, and *B. acidi-lactici*. It is evident that the so-called "typical" *B. coli* does not exist as such, but that the entire group is typical of fecal contamination when water or milk examinations are to be considered.

II. All the known members of this group give positive gas tests with lactose bile while no other known species gives such a test except *B. welchii*, a pathogenic bacterium also of fecal origin. This may be readily distinguished from the *B. coli* group by its appearance under the microscope after growing in lactose bile, when long strings of bacteria considerably larger than those of the *B. coli* group are shown. Also unlike all members of the *B. coli* group *B. welchii* gives a negative test with esculin solution. It usually gives more rapid and active gas production in lactose bile than does *B. coli*. It is also distinguished by being obligate anaerobic.

III. The importance of this classification from a medical point of view is shown by the fact that a vaccine made from *B. communis B* was not effective in cases of urethritis and cellulitis when the infection was from *B. aerogenes* A_2. It is evident that different members of the *B. coli* group may not be used indiscriminately for the production of vaccine, but that the variety of the *B. coli* causing the infection should be known and should be the one chosen for this purpose. The above classification readily facilitates the identification of any specific variety.

IV. The classification of bacteria into main groups according to motility[1, 2] widely separates the most closely allied forms. Winslow[3] has discarded this classification for the Coccaceae and called attention to the fact "that this property is not correlated with any other character—arising independently in forms exactly resembling non-motile forms in every other respect." Classification by motility would widely separate three of the varieties of *B. aerogenes* herein given from the other two known varieties

[1] Migula, *System der Bakterien*, Jena, 1897–1900.
[2] Chester, *A Manual of Determinative Bacteriolgy*, New York, 1901.
[3] *Systematic Relationships of the Coccaceae*, p. 51, New York, 1908.

whereas their descriptions show an unusually strong natural relationship. A classification based first on form and grouping of cells, second on the relation of their growth to air, third on their fermentive characteristics and, finally, on general cultural and morphological characteristics and biochemical reactions, would bring allied species and varieties into closely related groups. Carrying out this idea the next group to be classified would be the facultative anaerobic bacilli which ferment dextrose with gas production but do not produce gas in lactose. Then would follow a classification into groups of those facultative anaerobic bacteria which produce acid but no gas when grown in the various sugar media. Just as in qualitative chemistry allied elements are brought together into groups by the reactions which they produce, so in qualitative bacteriology species and varieties having natural relationship may be brought together into groups by a classification based on their fermentive characteristics.

A METHOD FOR DETERMINING THE GERMICIDAL VALUE AND PENETRATING POWER OF LIQUID DISINFECTANTS.*

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The ever-increasing use of disinfectants, following, logically, the interest manifested by communities and individuals in the control and suppression of communicable diseases, has been accompanied by a remarkable development in the manufacture of these substances.

Coincident with this increased use and production of disinfecting agents there has arisen a natural demand on the part of the public for information regarding the relative merits of these various germicides, and various attempts have been made in the past to formulate methods which should furnish information relating to their germicidal powers. Prior to the publication of the Rideal-Walker method no satisfactory test had been evolved which would give concordant results in the hands of different workers.

The lack of success which has attended these previous investigations appears to have been due to the non-appreciation of the influence of certain factors and details which should be integral parts of such procedures, and which when properly controlled should lead to constancy in results. Of the various methods which have been proposed and employed, that known as the Rideal-Walker coefficient or, as it was first termed, the carbolic coefficient, takes these factors most fully into consideration, and the constancy of results attainable with this method depends in no small degree on the recognition and control of these variable elements.

Among the most important factors considered in the method are: (1) the time of exposure of the test organism to the germicide; (2) the age of the culture employed; (3) the choice and reaction of the medium in which the test organisms are grown; (4) the temperature of incubation; (5) the temperature of medication; (6) the use of a

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24-hour-old typhoid culture in standard broth; (7) the proportion of culture to disinfectant.

The Rideal-Walker coefficient is a method for determining by direct comparison the relative germicidal values of different disinfectants upon naked germs in the absence of more than minimal amounts of organic matter. Carbolic acid is the standard to which the disinfecting power of the various germicides is referred. This method is of value in determining the bactericidal power of only one class of germicides—those employed in the disinfection of surfaces and substances contaminated by pathogenic organisms; it is not applicable for testing the efficiency of gaseous disinfectants. The procedure, therefore, is definitely limited in application to that group of germicides which are employed as solutions or emulsions for the disinfection of contaminated liquids or solids. Among the more commonly met with instances in which these liquid or emulsified disinfectants are employed are for alvine discharges, fomites, urinary discharges, for privies and such surfaces as floors, walls of stables, and so on.

A brief consideration of the conditions bringing about this type of contamination will reveal the fact that purely superficial infection will be an exception rather than the rule, and consequently the ability to penetrate is one of the most important properties that an efficient germicide of this class can possess.

The method here outlined takes into consideration the salient features of the Rideal-Walker method, and in addition furnishes a means for estimating the relative powers of penetration of these liquid disinfectants. The method consists essentially in the exposure of test organisms in infected cylinders of agar of definite diameter, to the germicide for definite periods of time. At stated intervals the agar cylinders are removed from the disinfecting solutions and washed with sterile water. A plug of agar about 3 mm. in diameter is then removed from the cylinder, or cylinders, by means of quill tubing. This plug of agar is taken from the center of the agar cylinder, along its long axis. It is placed in sterile broth and incubated at the body temperature for several days, during which time observations are made to determine the presence or absence of growth. By comparing the results obtained with a

given disinfectant and those obtained under the same conditions with carbolic acid as a standard, a ratio may be established which expresses in terms of carbolic acid the combined germicidal and penetrating power of the bactericidal substance in question.

It is essential to discuss briefly the various steps which led to the method which has been outlined above.

The first attempt to measure the penetrating power of disinfectants consisted in pouring the germicide upon the surface of a solidified agar shake culture maintained in an upright position. The disinfectant was allowed to remain on the top of the agar for a definite time, then poured off, and the tube inverted to permit the excess to drain away. This procedure gave no definite results.

The next step was a modification of the foregoing in which dextrose agar and a gas-forming bacillus were used. Differences in penetrating power could be discerned, but accurate comparisons were impossible. This appeared to be due, partly at least, to the fact that but one surface of the agar was exposed to the solution. This method did, however, show very definitely the advantage of gas production as an indicator in those cases in which the germicide failed to penetrate completely.

In order to take advantage of this gas production and at the same time to allow penetration from all directions, agar containing dextrose, and infected with colon bacilli, was poured into long cylindrical glass tubes (sterile of course) of definite diameter, and allowed to harden thoroughly. The agar was then forced out and cut into lengths slightly greater than the diameter. These fell directly into the germicidal solutions, the time at which they entered being noted. At the end of stated intervals some of these cylinders were removed, washed, and placed in sterile water in which they were incubated at body temperature. They were examined daily for several days to observe growth.

Any development of the colon bacilli was demonstrated by the production of gas as shown by the splitting of the agar. The method proved to be satisfactory for showing the combined inhibitory and penetrating power of these disinfectants, but it soon became apparent that a distinction must be drawn between the power of inhibition and the actual killing of the test organisms.

It is obvious that the penetrating germicide will reach those organisms in the centers of the cylinders last; hence, if a core be removed from the agar cylinder along its long axis in the center, and this core or plug be placed in dextrose broth, a production of gas will indicate that the germs in the center of the agar cylinder were not killed by the germicide, and consequently that the germicide was not efficacious. It is essential that there shall be a considerable volume of broth in proportion to the size of the core of agar, to dilute the disinfectant which has penetrated the ends of the core when the agar was exposed to the germicide.

The last modification adopted consisted of using plain agar, and *B. coli* as a test organism. The plugs or cores were placed in dextrose broth for incubation, and the development of gas showed that the test organisms were not killed.

The cultures used in the experiments were made from 24-hour-old growths in standard broth: 10 c.c. of this broth culture were added to each liter of agar, and the two were thoroughly mixed. This infected agar was formed into a long cylinder by pouring it into long sterile tubes, and allowing it to harden in the vertical position. The ends of the tube were closed with sterile rubber stoppers.

The diameter of the tubes in which the infected agar is hardened is a point of great importance, since the germicides must penetrate a distance equal to one-half the diameter of the cylinders of agar to kill the organisms in the center. The diameter finally selected was 15 mm.; tubes ranging from 8 to 30 mm., however, were tried before deciding upon the 15 mm. tube. This size of tube was found to be particularly satisfactory in those experiments in which it was desired to bring out both the inhibitory and the killing times of the germicides. The zone between the inhibitory and the killing power is less marked in the 8 mm. tubes, and is greatly drawn out in the larger sizes. With the larger agar tubes, again, it was found that a much longer exposure to the germicides was necessary to actually kill the test organisms.

After the tubes of infected agar were hardened, it was possible to push out the agar in the form of a long cylinder, and as the agar cylinder emerged from the glass tube, it was cut into lengths a little

longer than the diameter of the agar cylinder and these short lengths in turn were dropped, as they were cut, into the disinfectant solutions. A sufficient number of such cylinders were dropped into each solution to provide a separate one for each time interval in which it was desired to make a test. At the end of each time interval, a cylinder of agar was removed, washed with sterile water (or water free from gas-forming organisms), and a core about 3 mm. in diameter was removed from the center along the long axis, with a piece of quill tubing, and placed at once in dextrose broth. The dextrose tubes (fermentation tubes) were then incubated at body temperature, and gas formation was looked for upon several successive days.

The time intervals should be a matter of hours rather than of minutes, inasmuch as shorter intervals than an hour seldom showed more than an inhibitory action.

Several technical details need further comment: The agar cylinders that have been exposed to the germicidal solutions are most conveniently handled in holders made of wire gauze, bent up at one end in such a manner as to form a cradle of appropriate size. The pieces of gauze from which these holders are made should be at least 6 inches long and 1 inch wide, to allow for a handle on each. Several of these holders are sterilized in boiling water and kept for use.

The cores of agar removed from the cylinders by means of the quill tubing are readily forced into the fermentation tubes prior to incubation simply by blowing on the free end. By so doing the cores will readily slip from the tubing into the fermentation tubes.

The advantage of using *B. coli* for a test organism is a twofold one: the organism is very readily obtained, and the gas formation in dextrose or lactose broth furnishes a ready method of distinguishing it from accidental contaminating organisms, should such occur. Almost any organism, however, can be used as a test organism to suit special cases.

The temperature at which the exposure to the disinfectants is made should be 20° C.—a temperature readily gained and maintained in the ordinary laboratory.

One decided advantage of this method, aside from those men-

tioned, is the fact that it is possible to add almost any organic substance (or even feces) to the agar before it is allowed to harden. This addition may be made at a temperature below the coagulating point of most albuminous bodies.

It should be stated that there are two possible objections to the procedure: In the first place it may be argued that agar is a substance not met with in nature, and that the results obtained might conceivably not be applicable in practice. The other objection is that an error due to unavoidable densities of various samples of agar might render the results inconstant.

To the first objection it can be stated that it would be very difficult to find any one substance that would fulfil all requirements; the fact that various organic materials can be added as desired makes the method one of general if not of universal application. The procedure, furthermore, is designed to test the relative penetrating and germicidal values of disinfectants, and for this purpose it appears to be of real value.

The density of the agar can be determined, if necessary, with a modification of the well known Vicat needle, as suggested by Whipple.

In spite of the rather lengthy description necessary to elucidate the workings of this method, it is in reality simpler and more readily carried out than the Rideal-Walker coefficient.

In its present form, this procedure is not regarded by the writers as a perfect method; doubtless modifications and additions will be introduced which will add much to its completeness. On the other hand, the inference must not be drawn that it is an untried method. The results obtained have been reasonably constant, and the trials sufficiently numerous to justify its presentation.

In the appended table, several features are worthy of note.

Formaldehyde, even in relatively weak solutions, appears to be the most efficient of the germicides tested. Carbolic acid, in corresponding dilutions, is less efficient.

The chloride of lime tested here was bought in the open market, and was found to be deficient in chlorine.

The emulsions, of which hyco and sulphonaphthol are examples, are especially noteworthy. These germicides are without doubt

efficient when acting upon naked germs, and consequently give high Rideal-Walker coefficients, but are relatively useless when their penetrability is considered. The most logical explanation of this fact is that the very nature of their physical state—emulsions—is incompatible with penetrating power.

In conclusion, the writers wish to express their heartiest appreciation to Dr. Rosenau for his many helpful suggestions. It is fair to state that many of the most essential features of the procedure outlined above are the result of his friendly criticisms.

The procedure finally adopted was as follows:

1. Prepare a 24-hour culture of *B. coli* in plain broth of standard composition and reaction.

2. Add 10 c.c. of this standard culture to one liter of agar [1.5 per cent agar] and mix thoroughly.

3. Pour the infected agar into sterile tubes of convenient length [1 meter] and of exactly 1.5 cm. diameter and allow to harden, after closing the ends with sterile rubber stoppers.

4. Allow to harden at 20° C.

5. Prepare dilutions of the desired disinfectants and a standard 5 per cent carbolic acid solution. The latter is the standard to which the other disinfectants are referred.

6. Place the disinfectants so prepared in sterile beakers, allowing 50 c.c. for each agar cylinder (described below).

7. Remove stoppers from the infected agar tubes, and permit the contents to run out slowly as a long cylinder; with a sterile knife cut off portions by transverse cuts of 2 cm. length, and allow these smaller cylinders to fall directly into the disinfectant solutions, one cylinder for each time interval selected.

8. Note the temperature of the solutions. They should be kept at exactly 20° C. during the experiment.

9. Note the time at which the cylinders were dropped into the disinfectant solutions.

10. At the end of stated intervals (usually at hourly intervals for preliminary tests) remove one cylinder from each solution with one of the sterile holders mentioned above, wash it thoroughly with sterile water, and remove a core from the center with a sterile piece of quill tubing (3 mm. bore).

11. Place these cores in lactose fermentation tubes, after they are properly labeled, and incubate at 37° C. for several days, making daily observations.

12. Compare the killing times of the various solutions tested with that obtained for carbolic acid, and determine the carbolic coefficient of germicidal and penetrating powers combined.

The appended table shows the results obtained with several well known germicides. They illustrate the general method of stating results. The results presented here represent preliminary tests;

final tests are made by studying the killing power in shorter time intervals, utilizing the data obtained in the first examinations.

TABLE SHOWING COMBINED GERMICIDAL AND PENETRATING POWERS OF SOME COMMON DISINFECTANTS.

Agar 1.5 per cent. 72-hour incubation at body temperature. Temperature of exposure, 20°.

DISINFECTANTS	DILUTION	TIME OF EXPOSURE		
		1 hour	3 hours	5 hours
Carbolic acid	5 per cent	+	+	−
Carbolic acid	1 " "	+	+	+
Formalin	4 " "	−	−	−
Formalin	1 " "	+	−	−
Formalin	0.25 " "	+	+	−
$HgCL_2$	0.1 " "	+	+	+
$HgCL_2$	1 " "	+	−	−
Chloride of Lime	10 " "	+	+	−
Chloride of Lime	4 " "	+	+	+
Hyco	2 " "	+	+	+
Cresol	1 " "	+	+	+
Sulphonaphthol	2 " "	+	+	+

+ = growth.
− = no growth.

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INVESTIGATIONS ON THE PURIFICATION OF BOSTON SEWAGE.*

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I. PURIFICATION OF BOSTON SEWAGE ON TRICKLING FILTERS (1907–9).

1. *Description of experiments.*—Earlier investigations carried out at the Sewage Experiment Station of the Massachusetts Institute of Technology (Winslow and Phelps, 1906; Winslow and Phelps, 1907) have led to the conclusion that sewage of the character of Boston sewage could be purified most satisfactorily and economically by treatment on trickling beds without further preliminary treatment than that afforded by screens and grit chambers. The beds used in the previous work were 8 feet in depth and the filling material was $1\frac{1}{2}$–2-inch broken stone. The studies now reported were mainly directed toward the solution of the question of the best depth for trickling beds to treat Boston sewage and the best size of filling material to use in their construction.

The experimental filters were situated at the Albany Street Experiment Station and the applied sewage was drawn from the

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9-foot trunk sewer of the Boston Main Drainage Works at a point where there is a flow of some 50 million gallons a day from a contributing population of 250,000 (Winslow and Phelps, 1906). It was raised by a 4×6 Warren duplex pump, and roughly settled by passage through a grit chamber, 19 inches in diameter and 16 inches deep, fitted with a screen having bars $\frac{1}{2}$ inch apart, to a small tank on the ground floor. The sewage was then lifted without further sedimentation by a $\frac{3}{4}$-inch centrifugal pump to a distributing tank in the upper story of the tank house. This distributing tank was 6×4 feet in plan and 3 feet deep, and a constant head was maintained in it by wasting over a 36-inch weir at one end. From the opposite end of the tank, sewage flowed to the trickling filters through two brass orifices whose vertical positions were adjustable. No sludge was removed from this tank, any sludge which temporarily settled out being stirred up and allowed to pass to the filters. The liquid applied to the beds was therefore crude sewage except for the action of the small detritus tank described above and except for the comminution which always attends the passage of sewage through small pipes and pumps.

The filters themselves were outside the tank house in the open air. The total surface was 200 square feet, divided into four units, each 5×10 feet. For a foundation 4×4 spruce sills were buried in the ground and upon them a 1-inch hemlock floor was laid and covered with Portland cement mortar. Half-inch radiating channels in the concrete directed the flow of the effluent to $1\frac{1}{2}$-inch outlet pipes. The sides of the filters were of spruce planks with $\frac{1}{2}$–2-inch openings between them, supported by 4×4-inch studs braced on all sides and tied at the tops by $\frac{1}{2}$-inch iron rods.

The first of the four filter units (A) was filled to a depth of 7 feet with 1–$1\frac{1}{2}$-inch broken stone resting on about 1 foot of 3–12-inch boulders. The second (B) was of the same construction except that the stone was coarser, $1\frac{1}{2}$–2 inches in diameter. This stone was the same that had been used for two years in the previous experiments (Winslow and Phelps, 1907). The third filter (C) was just like B in construction, but only 5 feet deep instead of 8. Filter D was at first constructed like C except that the surface foot was of finer material, $\frac{1}{2}$-inch stone. This fine filter clogged so badly, how-

ever, that after four months' use it was dug out and replaced by a bed built of brick somewhat after the fashion of a Dibdin slate bed. Ordinary building bricks, approximately $2\times4\times8$ inches, were laid so as to overlap as little as possible on the corners, forming a bed with nearly half its total capacity as open space.

The effluent from the four filter units passed by $1\frac{1}{2}$-inch pipes into the tank house and there the effluents from A and B and from C and D were mixed and sedimented in two secondary sedimentation tanks for the removal of suspended solids. Each tank was a simple inverted cone with a diameter of 7 feet 2 inches at the top and a depth of 4 feet. At the center of each tank a vertical funnel 6 inches in diameter extended to within 15 inches of the bottom. The filter effluents were discharged into the top of this funnel and passed out into the tank through lateral openings near the bottom. They ascended with decreasing velocity and overflowed at four points through skimming-troughs at the surface. The storage period in the tanks was about 2 hours.

Sewage was applied to Filters A and B from a single gravity distributor of the type devised at the experiment station and described in earlier publications (Winslow and Phelps, 1907). The sewage was conducted to the center of the double bed in a 3-inch wooden trough from which it dropped through a $\frac{3}{4}$-inch opening in the bottom of the trough on to a concave disc from which it splashed upward and outward. The splash cup was 3 inches in diameter and its concavity had a 6-inch radius. It was supported 1 foot above the surface of the bed and 3 feet below the bottom of the trough. Filters C and D were dosed from below with a Columbus sprinkler nozzle having a $\frac{9}{16}$-inch orifice and a 90° cone above for spreading the spray. This nozzle was dosed intermittently from a siphon tank under a head varying between 4 feet and 3 feet and taking $1\frac{1}{2}$ minutes to discharge and 2 minutes to fill.

Samples of sewage and effluents were collected day and night at intervals of three hours, chloroformed, mixed, and analyzed at the end of the week. Chemical determinations were made by the standard methods of the American Public Health Association, modified in respect to nitrate and free ammonia as discussed in our previous paper (Winslow and Phelps, 1907). Daily samples of all

effluents were tested for stability by the methylene blue test and the results are expressed in terms of "relative stability" as defined in an earlier paper (Phelps, 1909).

2. *General results of trickling filtration.*—All four filter units were operated during the course of the experiments, October, 1907, to May, 1909, at a rate of about 1,500,000 gallons per acre per day. The actual net figures as determined by ratings of the mixed effluents from each pair were 1,280,000 gallons per acre per day for Filters A and B and 1,320,000 gallons per acre per day for Filters C and D.

In regard to the practical working of the beds there were important differences corresponding to the size of the filling material. While Filter D was operated on the first plan, with a 1-foot layer of half-inch material on the surface, it clogged badly. It is clear that half-inch material cannot be used for the treatment of crude sewage like that at Boston. A (1–1½-inch stone) and B and C (1½–2-inch stone) all clogged somewhat during the first winter, but the clogging of A was the most serious. On four occasions it was necessary to rake up a few inches of stone from Filter A to the corners of the bed, and the same thing was done to B twice. The surface of Filter C on the other hand required no attention. It was evident that the more perfect dosing from the Columbus nozzle was a distinct advantage. After the first winter all the beds cleared up considerably and no material was actually removed from any of them. The surface of Filter A remained more or less clogged, however, and it was evident that filtering material under 1½ inches would not operate successfully with crude Boston sewage.

Since the experiments began in the autumn, there was a long period of ripening before the filters were really doing effective work. The quarterly averages in Table 1 show that none of the effluents had a relative stability over 35 for the first six months of operation. With the warm weather of spring all but Effluent D began to improve, reaching a stability of 50–70, and in the autumn quarter Effluents A and B attained a stability over 80. When the filters were once well ripened good conditions were maintained through the winter and spring of 1909. The free ammonia values show the same relation. For Effluent A the free ammonia averaged 13.4 for

the first three quarters and 6.8 for the last four. For Effluent B the figures were, respectively, 13.0 and 7.9. Nitrites appeared in considerable amounts only in the spring and summer of 1908; and after the nitrite-forming bacteria had established themselves the nitrate-formers began their work. The whole cycle of ripening exactly paralleled that recorded in the experiments of the previous two-year periods (Winslow and Phelps, 1907).

The average results for the sewage, the four trickling effluents and the two sedimentation effluents for the last year of operation, are brought together in Table 2. Quarterly averages for the whole 20 months are given in Table 1; but a fairer idea of the actual working of the process may be gained by excluding the preliminary period of ripening.

It appears from the table that all the filters effected an appreciable though not a large reduction in suspended solids. Whatever reduction did occur may probably be accounted for by storage in the beds, as other experiments have shown that trickling beds do not effect a marked diminution of suspended matter. A distinct change from volatile to fixed solids is evident, however, every effluent showing a considerable reduction in volatile suspended matter and an increase in fixed suspended solids. Organic nitrogen is only slightly reduced as compared with the sewage value but free ammonia shows a reduction of 24 per cent for Filter D, 37 per cent for Filter C, 46 per cent for Filter B, and 54 per cent for Filter A. Nitrites in all the effluents average from 0.5 to 0.8 parts and nitrates from 4.2 for Filter D to 6.6 for Filter B. Oxygen consumed values show for the two best filters, A and B, a reduction of 32 per cent in the total oxygen consumed and 34 per cent in soluble oxygen consumed; as determined in fifteen minutes in the cold, the reduction was about the same for the total oxygen consumed (31 per cent), but distinctly better for the soluble portion (40 per cent). Oxygenation was fairly satisfactory in all the effluents, the average value of 2.2 parts for the crude sewage being increased to from 5.6 to 8.1 parts.

The analytical data show comparatively slight differences, aside from the tests for organic stability. Effluent D has a considerably larger proportion of suspended solids, as would of course be expected from its coarse construction. There are two significant

TABLE 1.

Analyses of Sewage and Effluents, 1907–9. Quarterly Averages.

Average of Months	Parts per Million																Relative Stability	Temp. Fahr.
	Turbidity	Sediment	Suspended Solids			Nitrogen as						Oxygen Consumed				Dissolved Oxygen		
						Total	Organic		Free NH_3	N_2O_3	N_2O_5	30′ Boiling		15′ Cold				
			Total	Vol.	Fixed		Total	Sol.				Total	Sol.	Total	Sol.			
CRUDE SEWAGE.																		
Oct., Nov., Dec., 1907	246	106	149	85	64	27.0	6.5	2.5	18.0	0.0	0.0	65	47	11.6	7.6	4.0	..	53
Jan., Feb., Mar., 1908	203	57	212	152	60	31.7	10.6	3.5	17.6	0.0	0.0	74	48	11.8	7.5	6.1	..	44
Apr., May, June, 1908	204	55	171	126	45	38.4	12.7	7.3	18.4	0.0	0.0	68	54	14.1	10.3	1.6	..	59
July, Aug., Sept., 1908	183	68	147	98	49	28.8	8.7	4.3	13.8	0.0	0.0	50	42	12.4	8.0	0.0	..	71
Oct., Nov., Dec., 1908	262	109	147	98	49	29.9	9.5	5.1	15.2	0.0	0.1	57	41	11.4	8.1	3.1	..	55
Jan., Feb., Mar., 1909	300	156	142	112	30	35.3	11.7	9.8	12.6	0.2	1.0	53	26	10.3	6.8	3.8	..	43
Apr., May, 1909	317	125	98	96	2	34.7	9.2	7.1	17.1	0.1	1.2	69	43	13.2	9.3	2.1	..	51
EFFLUENT A.																		
Oct., Nov., Dec., 1907	146	50	65	62	3	23.6	4.9	2.6	14.6	0.0	1.5	39	34	6.3	5.4	7.9	30	45
Jan., Feb., Mar., 1908	146	47	128	92	36	27.9	7.3	2.7	15.4	0.2	2.3	49	37	8.4	6.6	9.2	30	36
Apr., May, June, 1908	200	118	299	169	130	34.1	11.8	5.5	10.3	0.7	5.8	57	37	14.2	6.5	8.0	68	55
July, Aug., Sept., 1908	94	53	95	57	38	21.3	6.4	4.3	5.9	0.7	4.0	36	29	7.3	5.5	7.4	88	68
Oct., Nov., Dec., 1908	105	68	93	58	35	25.4	6.8	3.4	4.9	0.6	6.7	32	23	5.9	3.5	8.5	95	48
Jan., Feb., Mar., 1909	129	89	96	72	24	27.4	7.1	4.1	7.7	0.3	8.2	25	16	6.6	4.0	8.7	80	39
Apr., May, 1909	293	246	161	114	47	37.2	15.6	8.4	8.8	0.4	4.0	62	32	13.1	6.5	7.7	81	48
EFFLUENT B.																		
Oct., Nov., Dec., 1907	138	48	116	57	59	25.2	5.6	4.2	12.2	0.2	3.0	40	33	7.1	5.3	7.8	32	46
Jan., Feb., Mar., 1908	125	38	177	137	40	27.6	6.0	3.1	13.9	0.1	4.5	42	32	7.2	5.5	9.4	30	36
Apr., May, June, 1908	201	124	315	198	117	35.5	11.0	5.4	13.0	0.6	5.5	63	39	14.6	7.2	7.7	63	54
July, Aug., Sept., 1908	113	72	89	58	31	22.4	6.0	4.1	8.1	0.8	3.4	39	32	8.5	6.2	6.9	81	69
Oct., Nov., Dec., 1908	123	87	97	66	31	25.1	7.5	2.9	7.3	0.8	6.6	35	25	6.7	3.9	7.3	87	49
Jan., Feb., Mar., 1909	155	111	100	66	34	27.0	6.7	4.3	6.7	0.5	8.8	29	14	6.6	3.5	8.2	92	41
Apr., May, 1909	276	240	140	62	78	34.6	11.2	6.2	9.4	0.3	7.5	52	28	10.6	5.5	7.1	91	50

EFFLUENT C.

Oct., Nov., Dec., 1907...	149	53	112	58	54	22.8	5.3	2.2	13.6	0.2	2.5	38	30	7.3	5.6	7.8	28	46
Jan., Feb., Mar., 1908...	139	37	173	132	41	27.6	6.9	2.9	15.0	0.1	2.7	48	37	8.2	6.4	8.8	27	37
Apr., May, June, 1908...	202	109	292	194	98	40.3	13.5	7.0	14.8	0.5	4.5	66	40	15.4	8.0	5.8	46	55
July, Aug., Sept., 1908...	120	77	106	63	43	24.0	7.7	4.3	7.6	1.0	3.4	42	31	3.9	5.9	5.4	58	68
Oct., Nov., Dec., 1908...	144	97	95	68	27	23.9	6.7	3.2	7.8	0.7	5.5	36	26	7.7	4.8	6.8	75	47
Jan., Feb., Mar., 1909...	101	118	102	72	30	28.1	6.8	4.5	8.2	0.5	8.1	29	16	6.9	4.0	7.1	76	40
Apr., May, 1909.....	315	242	101	55	46	62.9	26.2	17.1	13.2	0.1	6.3	52	25	11.5	5.6	5.3	50	50

EFFLUENT D.

Oct., Nov., Dec., 1907, Jan., Feb., 1908.....	136	36	57	40	17	27.4	4.8	3.3	14.9	0.5	3.9	44	39	8.0	6.4	8.2	33	42

NEW FILTER D.

Mar.,Apr.,May,June,1908.	171	65	238	189	49	35.7	10.4	6.6	16.6	0.3	1.8	60	43	11.6	7.7	6.4	26	50
July, Aug., Sept., 1908...	133	68	117	71	46	24.1	7.2	5.5	7.3	1.3	2.8	43	33	9.7	7.1	5.0	41	67
Oct., Nov., Dec., 1908...	153	95	130	91	39	27.6	7.3	4.4	10.1	1.1	4.7	39	28	8.6	5.3	6.6	45	47
Jan., Feb., Mar., 1909...	173	107	107	78	29	31.7	9.2	5.5	11.7	0.8	4.5	29	19	7.7	4.9	6.8	29	39
Apr., May, 1909.....	246	167	118	70	48	35.4	9.5	5.0	15.7	0.2	5.0	54	30	11.7	6.7	4.0	37	50

EFFLUENTS A AND B SETTLED.

Oct., Nov., Dec., 1907...	120	37	68	37	31	22.8	3.7	1.9	14.6	0.1	2.5	35	34	5.2	3.6	...	45	..
Jan., Feb., Mar., 1908...	118	29	168	143	25	28.2	6.5	3.1	14.1	0.4	4.1	45	40	6.3	5.3	...	..	..
Apr., May, June, 1908...	109	46	87	71	16	27.3	5.9	3.4	11.9	0.7	5.4	42	35	7.5	5.3	...	..	..
July, Aug., Sept., 1908...	63	33	40	30	1	19.3	4.5	3.6	6.7	0.8	3.7	32	28	5.7	4.9	...	..	..
Oct., Nov., Dec., 1908...	84	59	52	35	17	19.5	3.6	1.9	6.9	0.7	6.4	27	22	4.9	3.7	...	91	..
Jan., Feb., Mar., 1909...	83	55	49	38	11	26.5	4.8	3.4	7.8	0.5	10.0	22	15	4.3	3.4	...	94	..
Apr., May, 1909.....	126	104	55	41	14	29.8	5.1	3.9	9.4	0.4	11.0	33	25	6.1	4.4	...	96	..

EFFLUENTS C AND D SETTLED.

Oct., Nov., Dec., 1907...	128	41	...	...	...	22.6	3.7	2.2	13.9	0.2	2.6	37	35	7.3	5.8	...	31	..
Jan., Feb., Mar., 1908...	125	27	114	91	23	28.1	4.8	2.9	15.9	0.3	4.2	47	41	7.4	6.3	...	27	..
Apr., May, June, 1908...	109	39	98	75	23	32.9	8.8	5.2	15.6	0.5	2.8	45	39	8.8	6.8	...	30	..
July, Aug., Sept., 1908...	59	33	54	44	10	23.1	7.0	4.8	7.0	1.2	3.1	30	30	6.2	5.3	...	59	..
Oct., Nov., Dec., 1908...	85	63	40	34	6	20.1	3.9	2.3	7.9	0.7	5.3	28	24	5.0	3.7	...	71	..
Jan., Feb., Mar., 1909...	83	55	49	38	11	26.9	4.8	3.4	7.8	0.5	10.4	22	15	4.3	3.3	...	59	..
Apr., May, 1909.....	127	104	55	41	14	30.0	5.1	3.9	9.4	0.4	11.2	37	25	6.1	4.4	...	40	..

TABLE 2.

AVERAGE ANALYSES OF SEWAGE TRICKLING EFFLUENTS AND SEDIMENTATION EFFLUENTS FOR THE ELEVEN MONTHS, JULY, 1908—MAY, 1909.

Parts per Million.

	Suspended Solids			Nitrogen as						Oxygen Consumed				Dissolved Oxygen	Relative Stability
					Organic					30′ Boiling		15′ Cold			
	Total	Vol.	Fixed	Total	Tot.	Sol.	NH_3	NO_2	NO_3	Tot.	Sol.	Tot.	Sol.		
Sewage	133	101	32	31.7	9.8	6.6	14.7	0.1	0.6	57	38	11.8	8.0	2.2	....
Eff. A	111	75	36	27.8	9.0	5.0	6.8	0.5	5.7	39	25	8.2	4.9	8.1	86
Eff. B	106	63	43	27.3	7.8	4.4	7.9	0.6	6.6	39	25	8.1	4.8	7.4	88
Eff. C	101	64	37	34.7	11.8	7.3	9.2	0.6	5.8	40	24	7.5	5.1	6.1	65
Eff. D	118	77	41	29.7	8.3	5.1	11.2	0.8	4.2	41	27	9.4	6.0	5.6	38
A–B settled	49	38	11	23.8	4.5	3.2	7.7	0.6	7.8	28	22	5.2	4.1	....	94
C–D settled	49	39	10	25.0	5.2	3.6	8.0	0.7	7.5	30	23	5.4	4.2	....	57

points about the chemical figures. The free ammonia values for effluents are distinctly affected by the size of material and depth of filter. The reductions in this constituent were 54 per cent, 46 per cent, 37 per cent, and 24 per cent respectively for A, B, C, and D. The other noticeable difference lies in the soluble oxygen consumed as determined in the cold. Instead of a purification of 40 per cent as shown by Filters A and B, and 36 per cent for Filter C, Filter D only decreased this constituent by 25 per cent. The oxygenation of Effluents C and D was distinctly less than for A and B.

The main difference between the effluents is, however, brought out only by a comparison of the figures for stability. The quarterly averages for this determination are brought together for comparison in Table 3 and are plotted in Figure 1. The data were obtained by daily tests by the methylene blue method and the relative stability number is derived from a scale in which a stability of 21 corresponds to decolorization in 1 day, 37 to decolorization in 2 days, 50 to decolorization in 3 days, 60 to decolorization in 4 days, 68 to decolorization in 5 days, 75 to decolorization in 6 days, 80 to decolorization in 7 days, 90 to decolorization in 10 days, and 96 to decolorization in 14 days, all at 70° F. (Phelps, 1909). Any value over 75 may be considered reasonably satisfactory.

During the first six months of operation in the autumn and winter of 1907–8 the bacteria did not begin active work in the filters and all the effluents were putrescible, losing their oxygen in less than 2 days.

In the spring of 1908, A and B, the 8-foot stone filters, began to improve, and for the last year of operation neither of them showed a quarterly average below 80. In other words these effluents stored

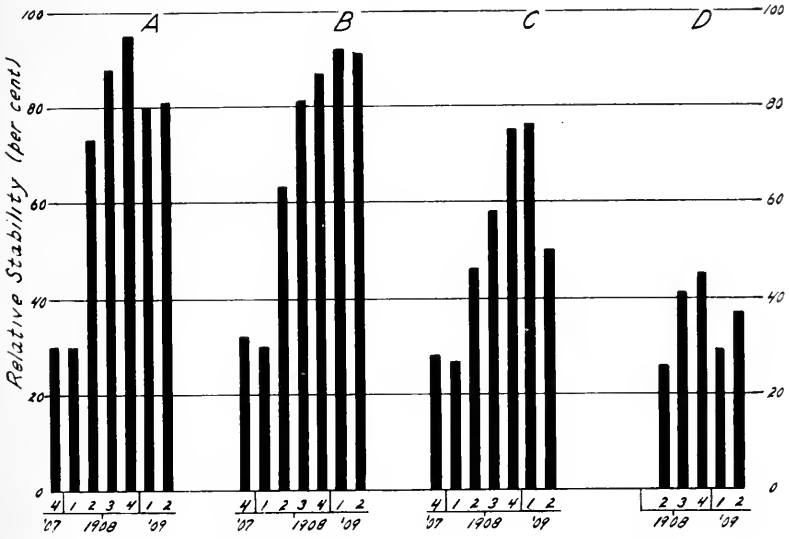

FIG. 1.—Relative stability, quarterly averages, filters A, B, C, and D.

in closely stoppered bottles contained oxygen enough to last for over a week, a condition which could scarcely cause a nuisance in a natural water course under any conceivable circumstances. There was little choice between these two effluents during this period, A being a little more stable in 1908 and B a little better in 1909.

TABLE 3.

RELATIVE STABILITY OF TRICKLING AND SEDIMENTATION EFFLUENTS.

Quarterly Averages.

	1907		1908			1909	
	Autumn	Winter	Spring	Summer	Autumn	Winter	Spring
Effluent A.....	30	30	68	88	95	80	81
Effluent B.....	32	30	63	81	87	92	91
Effluent C.....	28	27	46	58	75	76	50
Effluent D.....	..	29	25	41	45	29	37
A–B settled.....	45	..	..	..	91	94	96
C–D settled.....	31	27	36	59	71	59	40

Filter C, on the other hand, with only 5 feet of depth, gave distinctly inferior results. In every quarter its effluent was less stable

than those of the 8-foot beds. During the autumn and winter of 1908–9 it yielded an effluent of barely passable quality (75), but with the discharge of suspended solids in the spring, the stability fell again to 50. It seems clear that a bed of this character cannot be relied upon to yield a satisfactory effluent.

The open brick bed was unable to produce a stable effluent under the conditions of the experiment. Its stability figure varied between 30 and 45 when it was at its best, decolorizing in 2–3 days. It is somewhat remarkable perhaps that such a crude mechanism should have operated even as well as it did. The attainment of an effluent stable for 2 days even is a notable achievement for a process in which sewage runs rather rapidly over a structure of open brickwork with no filtering action whatever.

3. *Conclusions in regard to depth of beds and size of filtering materials.*—The first general conclusion to be drawn from these experiments concerns the size of the filtering material. The use of fine filling has been urged by many engineers in England. In testifying before the Royal Commission, Garfield, Ducat, and Corbett all fixed the size of stone for trickling beds at values less than one inch (Martin, 1905), and both Barwise (1904) and Raikes (1908) express similar opinions. This sort of material, however, requires very careful preliminary treatment. In America the comparative weakness of the sewage makes such fine material unnecessary and it is possible to use coarse beds and allow more suspended matter to pass upon them. Both at Reading and Columbus the filling material is over $1\frac{1}{4}$ inches in diameter.

The brief experience with Filter D when it was filled with $\frac{1}{2}$-inch stone showed that the treatment of crude Boston sewage on a bed of this kind was quite out of the question on account of the resultant clogging. A comparison of Filters A and B made it apparent that, with the particular sewage we were working with, $1\frac{1}{2}$ inches was the lower limit for practical efficiency. Filter A was of 1 to $1\frac{1}{2}$-inch, Filter B was of $1\frac{1}{2}$ to 2-inch stone. The analytical data, as indicated in Table 2, were practically identical for the two beds. In fact Filter B was a little the better in all respects, except as regards free ammonia and dissolved oxygen. Filter A however gave considerable trouble from clogging, while Filter B kept in good condition

with only a reasonable amount of care. $1\frac{1}{2}$ to 2-inch stone may therefore be considered the most suitable material for the purification of sewage like that of Boston.

Granting this premise a comparison of Filters B and C makes it possible to draw a fairly definite conclusion in regard to the relation between depth and efficiency in purification. Filter B had about 7 feet of effective filtering material over the underdrains, Filter C about 4 feet. Filter C had the advantage of somewhat better distribution from a siphon tank. Reference to Table 2 shows that in regard to suspended solids and oxygen consumed there was little or no choice between the beds. Organic nitrogen, particularly in the soluble form, was very much higher in Effluent C. Free ammonia was slightly higher and dissolved oxygen slightly lower. The main difference, however, was in the stability. The great delicacy of the methylene blue reaction is well shown here. Analytical differences between the two effluents are too slight to permit a very sharp distinction between them. Yet the methylene blue test shows that Effluent B had an average stability of over 80 for the last year of operation, and Effluent C rose to 75 only in the cold autumn and winter months, was under 60 in the summer, and fell to 50 in the spring of 1909. Thus the average reducing time for Effluent B was always over a week, while that for Effluent C fell for the spring quarter to 3 days, a decidedly unsatisfactory result.

It may be concluded then that beds of broken stone treating sewage like that with which we worked must be in the neighborhood of 7 feet deep (exclusive of underdrains) in order to insure an effluent having 80 per cent relative stability, a reasonable requirement under local conditions.

4. *The cycle of suspended solids.*—It was noted in our earlier work (Winslow and Phelps, 1907), as it has been observed at Birmingham, England (Watson, 1910), and elsewhere, that the suspended solids in trickling filter effluents exhibit a very marked and characteristic seasonal cycle. The results of the 1905–7 studies and of the present experiments are plotted together for comparison in Fig. 2. In each of the four years represented on the plot there is indicated a great increase in suspended solids during the spring

quarter, the total suspended solids being doubled or tripled in amount. The trickling bed accumulates suspended matter during nine months of the year and discharges its accumulation in the other three. The process begins with the first onset of warmer weather in March or April and culminates in May. The cycle appears to be as constant and regular as any other seasonal biological phenomenon, and it must be reckoned with practically in connection with the effect of a trickling effluent upon the body of water into which it is discharged.

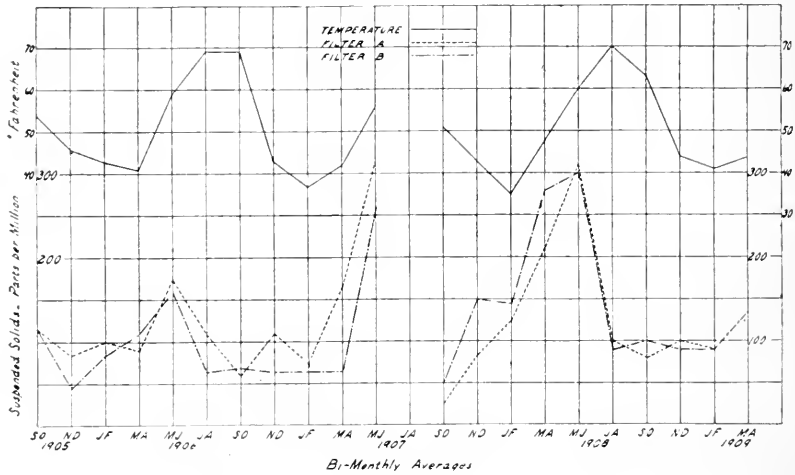

FIG. 2.—Relation between temperature and suspended solids in trickling filter effluents, Sewage Experiment Station of the Massachusetts Institute of Technology.

It must be noted, however, in connection with the character of this spring discharge that the increase in fixed solids is much greater than in the volatile constituents (see Table 1).

5. *Sedimentation of trickling effluents.*—In spite of the stability of the trickling effluents, the presence of more than 100 parts of suspended solids would be likely to cause obnoxious sludge deposits if the effluent were discharged into water where there was not a rather vigorous current. The effluents were therefore submitted to secondary sedimentation for a period of about two hours in the conical tanks described above.

The character of the resulting effluents is indicated in Table 2. Total suspended solids were reduced by 55 per cent, volatile suspended solids by 45 per cent, and fixed suspended solids by 72 per cent (comparing the average of Effluents A and B with the mixed settled effluent). A rather notable feature of the analyses is the marked reduction in soluble organic nitrogen, the appreciable reduction in soluble oxygen consumed, and the definite increase in nitrates. It is clear that during the brief storage period in the tanks the aerobic changes initiated in the filter were continued, leading to further organic purification, aside from the physical effect of sedimentation. This is an interesting indication of the excellent character of the trickling effluents themselves. The sedimented effluents were of course rendered more stable than before treatment, the figure for A and B rising from an average of 87 to 94 and for C and D from an average of 51 to 57.

The amount and character of the sludge removed from the secondary sedimentation tanks is indicated below in Table 4, for all seven quarters in the case of Effluents A and B and for the last three quarters in the case of Effluents C and D.

The volume of sludge produced varied from 3 to 12 cubic yards per million gallons of sewage treated, excluding the first two quarters, in which the discharge of suspended matter was of course comparatively small. The high figures, 6 to 12 cubic yards, occurred in the spring quarters, resulting from the annual discharge of suspended solids at that season.

The disposal of the sludge from this process remains of course a serious problem, as indeed is the case in all methods of sewage disposal. Experiments have been conducted at the station looking toward the disposal of this sludge along two principal lines. In one investigation the sludge was treated in a sort of intensive septic tank in the hope that liquefaction might reduce the amount to be handled. In another investigation the sludge was pressed and a study was made in regard to its possible fertilizing value. Neither line of work yielded encouraging results, and it appears probable that dumping at sea, digging into the land, or pressing and burning remain the most satisfactory methods of sludge disposal.

II. The Purification of Sewage by Intermittent Filtration at High Rates, after Preliminary Removal of Suspended Solids in a Biolytic Tank.

(With notes on some experiments by J. H. White.)

In Massachusetts, the home of the intermittent sand filter, it has been the general practice to purify sewage at comparatively low rates. The original studies at the Lawrence Experiment Station pointed to a rate of about 100,000 gallons per day as a probable maximum, and in actual operation few of the plants in the eastern states have ever reached this figure. The data recorded by the Massachusetts State Board of Health six years ago (Mass., 1904) were as follows: Stockbridge, 20,000 gallons per acre per day; Andover, Clinton, and Framingham, between 30,000 and 40,000; Brockton, Southbridge, and Spencer, between 40,000 and 50,000; Natick, 50,000; Hopedale and Pittsfield, between 60,000 and 70,000; Westboro, 70,000; Leicester, 80,000; Concord and Marlboro, between 90,000 and 100,000; Gardner and Worcester, between 120,000 and 130,000.

It is apparent however that no such limit as this is necessarily set by the essential nature of the process, so far at least as nitrification goes. A dose of 100,000 gallons an acre corresponds to a depth of less than four inches of sewage. With a clean bed of fairly coarse sand, well leveled and equipped with good distributors, such a dose disappears in half an hour and may be repeated once every six hours without interfering at all with nitrification. A few hours is ample for the bacterial processes of purification, as indicated by numerous laboratory experiments like those of Scott-Moncrieff (1899) at Ashtead and of Calmette and his associates at Lille (Rolants and Gallemand, 1901). The difficulty in the practical operation of intermittent filters at high rates arises from the clogging of the surface layers with suspended solids. The period for which the sewage remains on the surface of the beds is gradually prolonged as the clogging increases, until finally a new dose can be applied only after a long interval. In summer this condition can be avoided without any serious difficulty. If sewage is applied at a rate of 400,000 gallons instead of 100,000, sludge deposits on the surface accumulate four times as fast, while for

TABLE 4.

SLUDGE DATA.

AVERAGE OF MONTHS	TOTAL VOL. OF SLUDGE IN GALS.	SP. GRAVITY	GRAMS PER 100 C.C.							TOTAL VOLUME OF SEWAGE IN GALS.	SLUDGE PER MILLION GALS. OF SEWAGE IN CU. YDS.	POUNDS PER MILLION GALLONS OF SEWAGE						
			Suspended Solids			Organic Nitrogen		Organic Carbon by Permanganate				Suspended Solids			Organic Nitrogen		Organic Carbon by Permanganate	
			Total	Vol.	Fixed	Total	Susp.	Total	Susp.			Total	Vol.	Fixed	Total	Susp.	Total	Susp.
SLUDGE FROM COMBINED EFFLUENTS OF FILTERS A AND B.																		
Oct., Nov., Dec., 1907	60	1.02	8.44	4.78	3.66	...	0.165	....	0.357	240,000	1.29	188	111	77	....	3.5	....	5.6
Jan., Feb., Mar., 1908	102	1.03	9.04	5.38	3.66	...	0.181	....	0.391	252,000	2.59	275	169	106	....	5.8	....	13.1
April, May, June, 1908	406	1.03	9.30	2.11	7.19	...	0.213	....	0.450	296,000	6.37	1,275	497	778	....	24.0	....	48.1
July, Aug., Sept., 1908	176	1.03	8.22	3.54	4.63	...	0.31	....	0.51	221,000	3.90	522	223	299	....	18.0	....	29.8
Oct., Nov., Dec., 1908	246	1.04	8.55	4.36	4.19	0.41	0.40	0.55	0.54	220,000	3.78	545	277	268	25.3	25.0	36.5	36.2
Jan., Feb., Mar., 1909	401	1.05	6.57	3.78	2.89	0.31	0.30	0.42	0.40	389,000	5.27	490	274	216	26.8	26.1	43.1	39.0
Apr., May, 1909	440	1.05	....	....	....	0.29	0.29	0.67	0.67	172,000	12.64	....	...	....	36.3	36.3	84.0	84.0
SLUDGE FROM COMBINED EFFLUENTS OF FILTERS C AND D.																		
Oct., Nov., Dec., 1908	210	1.04	7.6	4.2	3.3	0.27	0.26	0.50	0.49	266,000	3.98	496	281	214	18.7	17.8	32.4	31.9
Jan., Feb., Mar., 1909	176	1.04	6.1	3.2	2.9	0.30	0.29	0.38	0.37	300,000	3.07	200	138	122	13.7	13.4	19.2	18.9
April, May, 1909	199	1.04	...	...	...	0.49	0.49	0.54	0.53	163,000	6.35	....	...	....	24.0	24.0	26.4	26.0

successful operation at the high rate the beds cannot be allowed to clog to the extent which would be quite permissible with daily dosing. It is simply necessary, however, to remove the sludge deposit more frequently from a smaller area; and it is probably quite as economical to scrape a ton of sludge from one acre as from two. It is in winter however that difficulty arises. The surface of the beds cannot be reached during the coldest weather, the beds clog, more area is required, and the whole plant must be designed for this condition of excessive strain. When crude sewage is applied to poorly leveled beds, being allowed to flow on as it will without dosing tanks to secure efficient distribution, there seems no hope of securing better results.

In the middle western states, however, intermittent filters have been constructed which appear to be of a more intensive and efficient type. Individual beds are small and carefully leveled; sewage is applied from dosing tanks automatically discharged at frequent and regular intervals; and the sewage is prepared for treatment by the preliminary removal of a considerable proportion of its suspended solids. If we are correct in our conclusion that the clogging of beds in winter is the factor which limits the efficiency of the intermittent filter, preliminary treatment would seem clearly indicated as a rational remedy. At several of the middle western plants rates of 400,000 gallons per acre per day are claimed under this system of operation. Notable examples of such filters are to be seen for instance in the town of Wauwatosa, Wis. The town plant (100,000 gallons) and the County Institutions plant (400,000 gallons) both include septic tanks for preliminary removal of suspended solids, both are dosed several times in the twenty-four hours from automatic dosing tanks, and both operate at a rate of 400,000 gallons per acre per day. The County plant when seen by one of the writers five years ago (Winslow, 1905) was in excellent condition and was yielding an effluent of good appearance. Unfortunately no analyses are obtainable from either of these plants. The septic tank and intermittent filter combination installed by Mr. F. A. Barbour (1905) at Saratoga, N.Y., is designed on the same general plan but is operated at a rate of only 60,000 gallons per acre per

day. Mr. Barbour believes, however, that this rate could be greatly increased if the amount of sewage demanded it.

In some of our earlier experiments (Winslow and Phelps, 1906), we made a study of the operation of sand filters at high rates, with unexpected success. The experimental filters were cypress tanks, 6 feet by 4 feet, filled with 2 feet of sand over 6 inches of gravel underdrainage. The sand had an effective size of 0.17 mm. and a uniformity coefficient of 3.5. The filters were in a covered shed and were thus protected from the severity of the weather, the quarterly average temperature never falling below 39° F. Crude sewage was applied to Filter 1, and septic effluent to Filters 24 and 25. Filter 1 was started in June, 1903, at a rate of 100,000 gallons per acre per day, but the rate was doubled in December and doubled again in June, 1904, being then maintained at 400,000 gallons till the close of the experiments in May, 1905. Filters 24 and 25 were put in operation in March, 1904, and operated consistently at 400,000 gallons per acre per day. The analytical results from all three filters were excellent, as indicated in Table 5. The care of the surface was not serious. Filter 1 was scraped three times and raked once, Filter 24 was raked twice, and Filter 25 was raked once and scraped once.

TABLE 5.

RESULTS OF INTERMITTENT FILTRATION AT HIGH RATES. INDOOR FILTERS. 1904–5.

Parts per Million.

	Filter 1	Filter 24	Filter 25
Nitrogen as Alb. NH_3	0.6	0.6	0.7
Nitrogen as Free NH_3	5.4	4.0	4.9
Nitrogen as Nitrites	0.3	0.3	0.2
Nitrogen as Nitrates	23.9	21.3	18.8
Oxygen consumed	6.8	6.5	6.4
Oxygen dissolved	6.3	7.4	7.5

When the new experimental plant for the Sanitary Research Laboratory was built at Old Harbor Point (Winslow and Phelps, 1910), we were anxious to make a test of the possibility of treating sewage on sand filters at high rates on a more practical scale. We therefore constructed an outdoor sand bed of about 500 square feet in area to be operated under normal weather conditions. A general view of the bed is shown in Fig. 3.

The sand bed is 21×22 feet in plan and was built on the old brick floor of a ruined building, once a portion of the garbage reduction plant, the walls and foundation of which were utilized in the construction of the filters. For underdrains, 4-inch half-round tile pipes were laid in four channels in the brick floor. The filtering layer is 3.5 feet deep of beach sand, from the immediate vicinity, with an effective size of 0.36 mm. and a uniformity coefficient of 1.9.

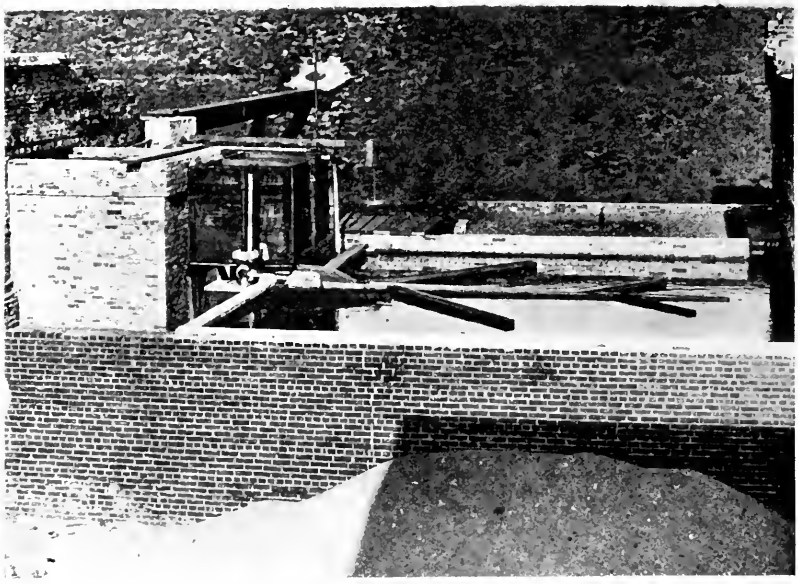

Fig. 3.—View of sand filter bed, Sewage Experiment Station of the Massachusetts Institute of Technology, 1909.

The sewage is distributed on the bed from a dosing tank holding 850 gallons, which at the theoretical rate would discharge about once in 5 hours, giving a rate of 4,250 gallons, or approximately 400,000 gallons per acre per day. The sewage is distributed on the bed by a system of wooden troughs, shown in Fig. 3, with 1-inch outlets, spaced 6 inches apart. The effluent from the sand filter passes from the underdrains to cement channels in an arched brick tunnel under the floor, which serves as a sampling chamber.

The tank used for the preliminary treatment of the sewage to

be applied to the sand filter is of a somewhat peculiar type, and to distinguish it from the ordinary septic tank we have called it a biolytic tank.

The actual removal of suspended solids is of course accomplished in almost all preliminary processes by the simple physical action of sedimentation. The proportion of suspended solids thus removed is usually between 50 and 65 per cent with American sewages (Fuller, 1909), although in England and Germany considerably higher results have been reported. The septic tank was originally designed not to improve the removal of suspended solids but to eliminate by liquefaction a portion of the material deposited. The early claims to the effect that 75 to 80 per cent of the deposited sludge could be thus liquefied have not, however, been generally substantiated. Most of the septic tanks which have been carefully studied, both in England and in the United States, have liquefied between 25 and 40 per cent of the suspended solids which they removed. At certain plants, as at Birmingham, England, values as low as 10 per cent have been recorded.

In considering the limitations of the septic process which cause it to stop so far short of complete decomposition it seemed probable that the accumulation of waste products from the process itself might be one of the principal factors in checking its progress. This is a common phenomenon in all bacteriological reactions, the removal of the end-products being almost invariably a necessary condition for continued activity. An experiment made some years ago at the Lawrence Experiment Station is suggestive of such an action, since it indicates that a shorter storage period tends to facilitate liquefaction and a shorter storage period means less accumulation of waste products. A small septic tank was dosed, not with sewage, but with the more concentrated sludge from settled sewage. For six months the storage period was from five to fifteen days, and sludge accumulated, filling up 60 per cent of the tank. The rate was then increased, so that the storage period was reduced to 49 hours, when the accumulated sludge decreased to 8 per cent and did not further increase for a year (Massachusetts, 1901). At Leeds it was found that a 72-hour septic period interfered with the solution of sludge (Leeds, 1905).

In some of our own earlier experiments (Winslow and Phelps, 1906) we found that sludge liquefaction was much more active with a 12-hour period than with a 24-hour period and more active with a 24-hour period than with one of 48 hours.

In the spring of 1909, at the suggestion of one of the writers, Mr. J. H. White made a laboratory study of this problem which tended to confirm these conclusions. Mr. White took two 3-liter bottles, A and B. Into A was poured 1,000 c.c. of sewage sludge and 1,000 c.c. of tap water, into B 1,000 c.c. of sludge alone. Bottle A was fitted with an inlet tube by which fresh tap water could be admitted through a stopcock, an outlet tube discharging by a siphon into a second stoppered bottle, a gas outlet through which the accumulated gas could be discharged, and a mercury gage to measure gas pressure. Bottle B was fitted with a gas outlet and pressure gage alone. Both were tightly sealed with sealing-wax and paraffin.

Daily observations were made of the temperature and pressure in each bottle and the gas was then allowed to escape. Each day 200 c.c. of fresh tap water was added to Bottle A and 200 c.c. of the contained liquid siphoned off. The sludge was analyzed at the beginning of the experiment and the contents of both bottles at the close; and weekly analyses were made of the effluent from Bottle A. Vigorous septic action took place in both cases, as manifested by the formation of scum and the production of gas bubbles. The fermentation in both bottles continued to the end of the experiment (33 days).

Mr. White calculated from his analyses the actual amount of various constituents in the original sludge, in the contents of Bottle A at the end plus the effluents from Bottle A, and in Bottle B at the end. The results, as shown in Table 6, indicate very clearly that the liquefying processes were markedly favored by the flushing out with fresh tap water. Bottle A yielded more than twice as much organic nitrogen in solution, free ammonia, and fixed solids in solution and nearly twice as much volatile solids in solution, as compared with Bottle B. It left, on the other hand, only 8.66 gms. of volatile solids in suspension against 14.20. Fixed solids in suspension were increased in the A effluent.

If our conclusions are justified and the limitations of the liquefying activity of the ordinary septic tank are due to the accumula-

tion of toxic waste products, the Hampton hydrolytic tank and the Imhoff deep tanks used in West Germany are designed on a principle that is not favorable to sludge liquefaction. In both these tanks the sludge is separated from the flowing sewage and stored in a liquefying chamber where it is subjected to intensive septic action with a minimum of opportunity for the removal of end-products. We have attempted to secure precisely the opposite result by using the deep conical tank with an inflow at the bottom and an outflow at the top, the sludge being constantly washed in a

TABLE 6.

RESULTS OF SEPTIC TREATMENT WITH AND WITHOUT FLUSHING.

(Experiments made by J. H. White.)

	Sludge before Treatment	Contents of Bottle A + Effluent	Contents of Bottle B
Nitrogen (parts per million):			
Total organic	1,300	1,504	1,200
Organic in solution	120	560	188
Free Ammonia	49	386	144
Solid (gms.):			
Total	35.20	34.64	31.20
Total in solution	6.00	13.74	7.40
In suspension	29.20	20.90	23.80
Volatile:			
Total	17.90	14.08	17.60
In solution	2.12	5.42	3.40
In suspension	15.78	8.66	14.20
Fixed:			
Total	17.30	20.56	13.60
In solution	3.88	8.32	4.00
In suspension	13.42	12.24	9.60

current of fresh sewage so that the products of decomposition may be removed. The tank is essentially like the Dortmund tank used for many years for sedimentation of sewage. Tanks of this general pattern are in operation with great success at Birmingham, England (Watson, 1910). So far as we are aware, however, all such tanks have hitherto been used for plain sedimentation, the sludge being removed too frequently to permit of septic action. In the case of our tank no sludge has been removed for a year and septic processes were obviously active. On the other hand, as the essential principle of the septic tank is recognized to be the retention of the sewage so as to promote anaerobic conditions as far as possible, while our aim is to limit these conditions, this tank is hardly a septic tank in the ordinary use of the term and might better be called a biolytic tank.

The tank itself is square at the top, and 7 feet across (see Fig. 4). The vertical sides extend downward for 2.5 ft., below which the walls converge to form a hopper. They have a slope of about 55 degrees with the horizontal. The capacity is 1,540 gals., and the flow period 8.5 hrs. The sewage enters through a 2-inch pipe about 9 inches from the bottom and the effluent is skimmed off at the surface by four 60-degree triangular metal weirs, placed at the

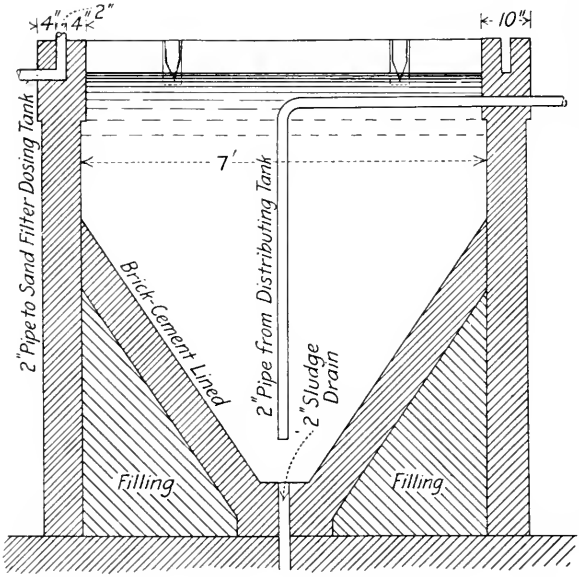

Fig. 4.—Cross-sectional view of biolytic tanks, Sewage Experiment Station of the Massachusetts Institute of Technology.

corners and discharging into 2-inch channels in the walls. Scum-boards protect these weirs. At the bottom of the tank is a 2-inch effluent drain for sludge.

The biolytic tank was first put in operation in July, 1909, and has since been in continual use, for six days in the week, and for seven days during the colder months. Samples are collected from all parts of the experimental plant every three hours, and the samples are mixed, chloroformed, and analyzed once a week. The analytical methods used include determinations of turbidity and

sediment, suspended matter, nitrogen as free ammonia, nitrites, nitrates and total Kjeldahl, oxygen consumed, total and dissolved, by both the 30-minute boiling and the 15-minute room temperature method.

The general character of the crude sewage is indicated by the monthly averages in Table 7. It represents the mixed sewage from the entire South Metropolitan District of Boston (about 90 million gallons a day), after screening through coarse bar screens at the city pumping plant and the removal of heavy grit in a small detritus chamber at our own station. The sewage of Boston is rather weak for the sewage of a large American city, particularly as regards suspended solids and free ammonia.

TABLE 7.

COMPOSITION OF CRUDE SEWAGE.

Monthly Average of Weekly Composite Samples.

MONTH 1909–10	TURBIDITY	SEDIMENT	SUSPENDED SOLIDS			NITROGEN AS						OXYGEN CONSUMED			
							Organic					30′ Boiling		15′ Cold	
			Total	Vol.	Fixed	Total	Total	Sol.	Free NH_3	NO_2	NO_3	Total	Sol.	Total	Sol.
August	305	150	156	130	26	29.5	13.1	6.7	16.2	.1	.1	73	53	19.3	12.0
September	420	280	212	145	67	30.0	14.8	9.3	14.8	.0	.0	68	39	17.4	8.9
October	335	220	139	109	30	24.0	9.0	3.8	14.9	.2	.1	70	49	15.8	12.9
November	340	220	184	139	45	27.0	9.5	6.1	17.5	.1	.3	76	47	17.1	9.6
December	315	190	112	74	38	27.0	14.0	7.3	12.1	.1	.3	56	40	14.2	8.1
January	290	160	114	76	38	18.0	7.7	5.3	8.7	.1	1.2	65	46	12.0	7.1
February	405	190	180	100	80	21.0	13.4	10.9	6.6	.1	.8	74	58	13.7	9.2
March	240	160	200	....	....	23.0	11.3	7.8	10.0	.1	1.2	59	35	12.9	8.9
April	340	230	150	100	50	30.0	15.5	13.3	13.3	.1	1.2	74	49	18.1	12.4
May	370	205	180	120	60	24.0	9.2	7.0	18.0	.1	.2	85	72	20.0	12.9
Average	365	200	163	110	48	25.3	11.7	7.7	13.2	.1	.5	70	49	16.0	10.2

Marked seasonal variations are apparent in free ammonia and in oxygen consumed as determined in the cold, both values falling during the winter months and rising as increasing temperatures facilitate bacterial action. Suspended solids are low during the freezing weather of December and January and rise again with the spring thaws.

The work of the biolytic tank in regard to the removal of sus-

pended solids may be estimated by comparing the analyses of its effluent for the corresponding period as they are given in Table 8.

TABLE 8.

COMPOSITION OF EFFLUENT FROM THE BIOLYTIC TANK. MONTHLY AVERAGES OF WEEKLY COMPOSITE SAMPLES.

Parts per Million.

MONTH 1909–10	TURBIDITY	SEDIMENT	SUSPENDED SOLIDS			NITROGEN AS						OXYGEN CONSUMED			
							ORGANIC					30′ BOILING		15′ COLD	
			TOTAL	VOL.	FIXED	TOTAL	TOTAL	SOL.	FREE NH_3	NO_2	NO_3	TOTAL	SOL.	TOTAL	SOL.
August	165	55	77	69	8	24	6.7	3.6	17.2	.0	.1	53	39	18.0	15.7
September	230	120	123	92	31	27	11.1	6.4	16.1	.0	.0	56	35	17.0	11.3
October	200	110	88	74	14	21	5.3	4.5	15.5	.0	.0	57	37	15.5	12.2
November	195	105	59	49	10	23	6.1	5.0	16.4	.0	.0	55	40	15.0	12.3
December	185	75	70	45	25	23	8.9	4.4	14.3	.0	.0	53	39	14.1	10.6
January	210	100	70	40	30	18	7.2	5.0	9.5	.2	.7	49	35	11.5	8.2
February	260	120	90	60	30	19	8.6	4.1	9.9	.1	.4	49	37	11.4	7.7
March	240	125	90	..	..	21	8.6	6.4	12.1	.1	.1	47	28	14.1	11.0
April	180	100	70	50	20	25	6.0	5.8	18.5	.0	.0	60	40	20.8	17.5
May	175	75	70	50	20	23	6.4	6.0	16.6	.0	.0	65	44	24.4	21.9
Average	204	98	81	58	21	22	7.5	5.1	14.6	.0	.1	54	37	16.2	12.8

Comparison of the final averages in Tables 7 and 8 shows the usual changes, characteristic of septic action. Free ammonia was increased from 13.2 parts to 14.6 parts; and of the unstable carbonaceous compounds determined by oxygen consumed in the cold the soluble portion was increased from 10.2 parts to 12.8 parts. Of the organic nitrogen, the suspended portion was reduced from 4.0 parts to 2.4 parts (40 per cent), and the dissolved portion from 7.7 parts to 5.1 parts (34 per cent). The oxygen consumed determined at 212° F. showed similar decrease from 21 to 17 (19 per cent) for the suspended portion and from 49 to 37 (24 per cent) for the dissolved portion.

The principal changes, however, were of course in the suspended matter. The sediment was reduced from 200 to 98 (51 per cent); the total solids from 163 to 81 (50 per cent); the volatile portion from 110 to 58 (47 per cent); and the fixed portion from 48 to 21 (56 per cent). These results correspond pretty well with those obtained from good septic tanks in actual practice, as indicated in Table 9, compiled from the final report of the British Royal Commission (1908) and from the results of certain American tanks.

TABLE 9.

REDUCTION OF SUSPENDED SOLIDS BY SEPTIC TREATMENT.

Plant	Per cent Reduction	Plant	Per cent Reduction
Andover, England	30	Hartley Wintney, England	54
Slaithwaite, England	34	Knowle, England	57
Worcester, Mass	35	Saratoga, N.Y.	65
Prestolee, England	42	Exeter, England	67
Caterham, England	47	Reading, Pa.	74
Columbus, Ohio	49	York, England	75
Accrington, England	50	Rochdale, England	86
Boston, Mass	50		

The variations at different plants are of course very great, since both the nature of the sewage and the construction of the tanks influence the end result. Seven of the other fourteen plants recorded, however, show a purification less than that effected by our tank, while the other seven yielded better results. This seems a good showing in view of the fact that the weakest point in a deep continuous-flow tank lies in its tendency to discharge too much of the suspended matter it receives.

In regard to the other requisite in a successful tank, the liquefaction of the deposited solids, the biolytic tank has proved notably successful. After eight months' use, in the third week of April, 30 gallons of sludge were withdrawn for analysis and on June 13, after ten months' operation, the tank was stirred up and emptied and a representative sample taken for analysis. The withdrawal of sludge and the examination of the tank were solely for the purpose of studying the process and were not conditioned by any obvious accumulation of sludge; and the weekly analyses showed no tendency to deterioration. In fact no accumulation of sludge was apparent by probing from the top of the tank.

From the average analyses of sewage and effluent, and from the analyses of sludge and tank contents, we have calculated the removal of suspended solids and organic nitrogen and the proportion of the deposited solids liquefied, in Table 10. The figures cover the whole period up to June 13 and therefore differ slightly from those given in Tables 7 and 8.

These figures show, as do those in Tables 7 and 8, a removal of about half the suspended solids in the crude sewage. The most important point however is brought out in the last line, which shows that 72 per cent of the total solids and 81 per cent of the volatile

TABLE 10.

EFFICIENCY OF BIOLYTIC TANK. AUGUST, 1909—JULY, 1910.

Parts per million.

	Suspended Solids			Suspended Organic Nitrogen
	Total	Volatile	Fixed	
Sewage	166	114	52	4.4
Tank effluent	77	50	27	2.4
Deposited in tank	89	64	25	2.0
Found in tank	25.2	12.6	12.6	.9
Per cent of total removed by tank	54	56	48	45
Per cent of deposited solids dissolved	72	81	49	52

solids deposited were eliminated by septic action. This result is a very favorable one, as indicated by Table 11, in which the figures for a number of English and American tanks are brought together for comparison (from Kinnicutt, Winslow, and Pratt, 1910).

TABLE 11.

LIQUEFYING EFFICIENCY OF SEPTIC TANKS.

Percentage of Deposited Solids Dissolved.

Place	Total Solids	Place	Total Solids	Organic Solids
Birmingham, England	10	London, England	41	71
Exeter, England*	25	Boston, Mass.†	42	81
Manchester, England	26	Glasgow, Scotland	50	..
Ilford, England	30	Hampton, England	..	58
Sheffield, England	30	Saratoga, N.Y.	69	..
Accrington, England	35	Boston, Mass.‡	72	81
Worcester, Mass.	39	Exeter, England§	80	..
Leeds, England	20–60			
Huddersfield, England	40			

* Royal Commission studies.

† 1905–7. Rectangular tanks.

‡ 1909–10. Hydrolytic tank.

§ Early reports from town.

Except for the very high reduction originally reported from Exeter, the efficiency of the biolytic tank in the liquefaction of total suspended solids appears to be much greater than that of most septic tanks of the ordinary form. The tank at Saratoga is the only one which equals it, and this tank has the advantage of receiving its chief burden in summer when the temperature is favorable to active bacterial development. In regard to volatile suspended solids the old rectangular tanks used at the Technology Station in 1905–7 were equally efficient. These tanks, however, were in a shed and thus protected from winter weather, and their vigorous liquefaction of volatile suspended solids was counterbalanced by an actual increase in fixed suspended solids.

Altogether it seems that the use of a conical tank with upward flow for septic treatment is justified by our results. The removal of suspended solids is about as good as that attained with a rectangular tank and the liquefaction of the deposited solids is distinctly better.

The effluent from the biolytic tank, as noted above, passed to a dosing tank of 850 gallons capacity, which if discharged once in five hours gives a rate on the sand bed of 4,250 gallons per day or approximately 400,000 gallons per acre per day.

The actual net rates on account of slackening in the flow of the septic tank and Sunday rests were considerably lower, as shown by months in Table 12.

TABLE 12.

SAND FILTER RATINGS.

Rates in gallons per acre per day.

1909–10	No. Gallons
Up to January 1	293,000
January	342,000
February	337,000
March	315,000
April	221,000
May	245,000

The general operation of the bed at this high rate was notably successful. The dose of sewage disappeared ordinarily in fifteen minutes. This period gradually increased however in the colder weather until sewage stood on the bed from one dosing to the next. It was merely necessary to rake the bed to relieve the clogging.

During ten months of operation no material was removed from the bed and there was no apparent tendency to clogging or other sign of deterioration. The surface was raked six times, on November 6, January 24, January 31, February 28, March 7, and April 9. On November 22 the bed was furrowed for the winter and on March 17 it was leveled again. Ice formation was complete over the furrows for periods of three or four days in the coldest weather. The warmth of the sewage beneath together with that from the sun soon broke the ice directly above the channels. No doubt the frequent application of sewage tended to protect the bed from the more severe effects of winter weather.

The sand bed will undoubtedly clog in the future and it will be

necessary to scrape its surface and remove sludge deposit as is done in all plants under actual operation. The important point however is that with the application of septicized effluent the clogging is so gradual that the accumulation of solids from ten months of operation did not affect its efficiency in any appreciable degree. Such being the case, the removal of sludge deposit during the cold winter months could easily be avoided, without reducing the intensive rate of operation.

The composition of the filter effluent is indicated by the results tabulated in Table 13.

TABLE 13.

COMPOSITION OF EFFLUENT FROM THE INTERMITTENT FILTER.

Monthly Averages of Weekly Composite Sample. Parts per million.

MONTH 1909-10	NITROGEN AS					OXYGEN CONSUMED		RELATIVE STABILITY
	Total	Organic	Free Ammonia	Nitrites	Nitrates	30′ Boiling	15′ Cold	
August	19	10.5	2.2	.2	6.2	12	2.0	96+
September	19	7.4	1.9	.1	9.5	16	1.9	96+
October	21	7.1	.4	.3	13.0	14	1.6	93
November	26	5.5	5.7	.4	14.0	17	2.4	96+
December	28	8.5	4.3	.3	15.3	16	2.5	95
January	26	5.2	5.7	.1	15.3	15	1.9	96+
February	22	5.1	4.2	.1	12.7	11	2.1	96+
March	28	3.3	4.0	.0	21.0	14	1.6	96+
April	48	4.0	1.5	.0	42.0	20	2.4	96+
May	45	2.3	1.1	.1	41.6	18	3.2	96+
Average	28	5.9	3.1	.2	19.1	15	2.2	96

The usual seasonable phenomena are indicated, free ammonia and organic nitrogen rising in November and December as the cold weather checks bacterial action. In March these organic constituents drop again, and the nitrates rise, reaching the enormous value of 40 parts and over in April and May. In these two months about 90 per cent of the total nitrogen discharged was in the nitric form.

A better idea of the work of the bed may be gained from Table 14, in which the effluent is compared with the applied liquor. In spite of the fact that the total nitrogen was 27 per cent more in the effluent than in the applied liquor (due perhaps in part to concentration by evaporation from the surface of the bed), the free ammonia showed a purification of 79 per cent, the oxygen consumed determined at 212° F. a purification of 72 per cent, and the more

unstable portion as determined in the cold a purification of 86 per cent. For the whole period 69 per cent of the nitrogen present in the effluent was in the mineral form.

TABLE 14.

EFFICIENCY OF INTERMITTENT FILTER.

Parts per million.

	NITROGEN AS					OXYGEN CONSUMED	
	Total	Organic	Free Ammonia	Nitrites	Nitrates	30′ Boiling	15′ Cold
Septic Effluents	22	7.5	14.6	0.0	0.1	54	16.2
Sand effluents	28	5.9	3.1	0.2	19.1	15	2.2
Percentage purification	21	...	79	...	....	72	86

The effluent from the bed was always of excellent appearance and free from turbidity and odor. Its stability is given in the table as 96. This corresponds to the maximum period of 14 days for which the methylene blue samples were kept (Phelps, 1909). As a matter of fact the true stability must have been between 96 and 100, probably nearer 100. Out of 41 weekly averages for stability 35 were 96, or the maximum; one week averaged 95, two weeks 94 each, one 93, one 92, and one 88. The last of these values corresponds to a reducing time of between 9 and 10 days, so that it is evident that the effluent was uniformly of a very high quality.

These experiments are being continued and will be more fully reported after a longer period. After ten months of operation however the results seem to indicate clearly that intermittent sand filters can be operated at high rates (400,000 gallons per acre per day) with marked success, if the beds are carefully constructed of fairly coarse sand and dosed at regular and frequent intervals with sewage from which suspended solids have been partially removed by preliminary treatment. Our experience suggests that preliminary treatment can with advantage be carried out in a deep tank with upward flow, the removal of suspended solids by such a tank being fairly efficient and the liquefaction of the deposited solids unusually high.

REFERENCES.

Barbour, F. A., 1905. "The Sewage Disposal Works at Saratoga, N.Y.," *Journal of the Association of Engineering Societies*, 34, p. 33.

Barnes, S., 1904. *The Purification of Sewage*, p. 122.

British Royal Commission on Sewage Disposal, 1908, 4th Report.

Fuller, G. W., 1909. "The Principles of Sewage Purification," *Engineering Record*, 59, p. 622.

Kinnicutt, L. P., Winslow, C.-E. A., and Pratt, R. W., 1910. *Sewage Disposal*, p. 145.

Leeds, 1905. *Report on the Experiments in Sewage Disposal Carried Out at the Knostrop Sewage Works, Leeds, from 1895 to 1905.* By Colonel T. W. Harding and W. H. Harrison.

Martin, A. J., 1905. *The Sewage Problem*, p. 214.

Massachusetts State Board of Health Report, 1903, p. 446; *ibid.*, 1901, *Annual Report for 1900*, p. 374; *ibid.*, 1904, *Annual Report for 1903*, p. 446.

Phelps, E. B., 1909. "The Disinfection of Sewage and Sewage Filter Effluents, with a Chapter on the Putrescibility and Stability of Sewage Effluents," *United States Geological Survey, Water-Supply Paper 226*, Washington. Also, *Contributions from the Sanitary Research Laboratory and Sewage Experiment Station of the Massachusetts Institute of Technology*, 1909, Vol. 5.

Raikes, H. P., 1908. *Sewage Disposal Works*, p. 166.

Rolants, E., and Gallemand, E.-A., 1901. "La nitrification dans les lits bactériens aérobies," *Revue d'hygiène*, 23, p. 968.

Scott-Moncrieff, W. D., 1899. "High Nitrification in Sewage Treatments," *Jour. Sanitary Inst.*, 20, p. 653.

Watson, J. D., 1910. (*a*) "Birmingham Sewage-Disposal Works," *Proc. Inst. Civil Engineers* (in press).

———, 1910. (*b*) "Birmingham Sewage-Disposal Works," *Proc. Inst. Civil Engineers* (in press).

Winslow, C.-E. A., 1905. "A Winter Visit to Some Sewage Disposal Plants in Ohio, Wisconsin, and Illinois," *Jour. Asso. of Engineering Societies*, 34, p. 335. Also, *Contributions from the Sanitary Research Laboratory and Sewage Experiment Station of the Massachusetts Institute of Technology*, Vol. 3.

Winslow, C.-E. A., and Phelps, E. B., 1906. "Experiments on the Purification of Boston Sewage, 1903–5," *Contributions from the Sanitary Research Laboratory and Sewage Experiment Station of the Massachusetts Institute of Technology*, 22, p. 126. Second ref., p. 125.

Winslow, C.-E. A., and Phelps, E. B., 1907. "Investigations on the Purification of Boston Sewage in Septic Tanks and Trickling Filters," *Technology Quarterly*, 20, pp. 387–452. Also, *Contributions from the Sanitary Research Laboratory and Sewage Experiment Station of the Massachusetts Institute of Technology*, 4, 1909.

Winslow, C.-E. A., and Phelps, E. B., 1910. "The New Sewage Experiment Station of the Massachusetts Institute of Technology," *Engineering News*, 63, p. 652.

LIVER BROTH: A MEDIUM FOR THE DETERMINATION OF GAS-FORMING BACTERIA IN WATER.*

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Various observers have noted that when dextrose broth is used for the bacterial examination of water it occasionally gives a positive test for *B. coli* in one and sometimes in two dilutions higher than does the lactose bile medium. This is particularly the case when waters of fairly good sanitary quality are tested. In tests on sewage and contaminated waters, however, the lactose bile has been proven to give positive results in higher dilutions than any other medium, the gas production being more vigorous and anomalies caused by the overgrowth of other forms very rare.

After numerous experiments, it has been found that the lactose bile medium is slightly inhibitive to *B. coli*, especially in attenuated form, so that any positive tests with this medium indicate recent and fresh contamination.

Dextrose broth, containing no inhibitive substance, favors to some extent the attenuated forms, but often overgrowth by other bacteria more numerous than *B. coli* renders the test either doubtful or negative.

This feature of dextrose broth has been overcome by the use of a broth made from fresh beef liver. The latter medium gives all the gas-producing bacteria present, attenuated or otherwise, including those forms which ferment dextrose but not lactose. It therefore has every advantage over the use of dextrose broth for general gas production and preliminary rejuvenation.

If one sugar medium alone is used in the sanitary examination of water, the lactose bile should be employed, as it gives relative results which are entirely comparable with each other on the actual sanitary condition of the waters under examination. If a further study of all gas-forming bacteria including attenuated forms is desirable, then liver broth should be employed in preference to the

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usual dextrose broth, as it gives a larger amount of attenuated forms, has better rejuvenating power, and gives fewer anomalies and greater and more rapid gas production. This medium has been in use at Mt. Prospect Laboratory for over two years and has proved its value in all of these respects.

The composition of the medium is as follows:

LIVER BROTH.

Beef liver	500.0 gms.
Peptone (Witte's)	10.0 "
Dextrose	10.0 "
Potassium Phosphate (K_2HPO_4)	1.0 "
Water	1,000.0 "

1. Chop 500 gms. of beef liver into small pieces and add 1,000 c.c. of distilled water. Weigh the infusion and container.

2. Boil slowly for two hours in a double boiler, starting cold, and stirring it occasionally.

3. Make up the loss in weight by evaporation and strain through a wire strainer.

4. To the filtrate add 10 gms. of peptone, 10 gms. of dextrose, and one gm. of potassium phosphate. Weigh the infusion and container.

5. After warming this mixture in a double boiler and stirring it for a few minutes to dissolve the ingredients, titrate with N/20 sodium hydrate, using phenolphthalein as an indicator, and neutralize with normal sodium hydrate.

6. Boil vigorously for 30 minutes in a double boiler, and five minutes over a free flame with constant stirring to prevent the caramelization of the dextrose.

7. Make up any loss in weight by evaporation and filter through cotton flannel and filter paper.

8. Tube and sterilize in an autoclave for 15 minutes at 120° C. (15 lbs.).

Other valuable liver media (for use in the identification of *B. sporogenes* and other species) are prepared as given below:

LIVER GELATIN.

1. Proceed as in steps 1, 2, 3 in preparing liver broth.

2. Cool the filtrate to 50° C. Add 10 per cent of sheet gelatin and stir a few minutes until dissolved.

3. Add one per cent of peptone, one per cent of dextrose, and one-tenth (0.1) per cent of potassium phosphate.

4. Stir until the ingredients are dissolved, keeping the temperature below 50° C., and then proceed as in steps 5, 6, 7, 8 in preparing liver broth.

LIVER AGAR.

1. Chop 500 gms. of beef liver into small pieces, add 500 c.c. of distilled water, and boil slowly for two hours, stirring occasionally.

2. Add five gms. of agar (dried at 105° C. for 30 minutes) to 500 c.c. of distilled water and digest for 30 minutes in an autoclave at 120° C. (15 lbs.).

3. After making up the loss by evaporation, strain the liver infusion through a wire strainer, add 500 c.c. of the filtrate to the agar solution and proceed as in steps 4, 5, 6, 7, 8 in preparing liver broth.

It is very important to note that liver broth should not be exposed to the high temperature attained in the autoclave any longer than 15 minutes, as prolonged heating above the boiling point causes caramelization of the carbohydrates, rendering the medium less delicate for bacterial development. For the rejuvenation of species, especially *B. sporogenes*, the addition of very small pieces of liver tissue which have been sterilized in Petri dishes in the autoclave for 15 minutes improves the rejuvenating properties of the medium. They should be added to the tubes after sterilization.

Bacterial growth being very rapid in this medium, preliminary rejuvenation at 37° C. should be concluded between six and 12 hours.

It was determined by the chemical analyses of several different lots of liver broth, before and after sterilization, that the beef liver as purchased in the market and occurring in the sterilized medium usually contains about one-tenth to two-tenths of one per cent of glycogen, to which it probably owes its principal rejuvenating properties. The authors are indebted to Mr. John E. Dowd, of the Mt. Prospect Laboratory, for numerous glycogen determinations.

The following table will show the comparative results obtained by planting samples of water directly into lactose bile, dextrose broth, and liver broth, as well as by preliminary rejuvenation in nutrient broth and liver broth, and then planting into lactose bile. In four cases all tubes showing gas formation with liver broth in higher dilution than with lactose bile were transplanted into lactose bile to determine whether the attenuated colon bacillus was among the gas-formers present.

The figures on p. 292 show that in waters where the good sanitary quality is in part due to storage, as in the city tap samples and No. 4 Pond, little or no gas was obtained in the lactose bile; considerable gas was often found in the dextrose broth, while in liver broth the growth was more vigorous, as is shown by the gas production in shorter periods, and in larger amounts as well as in higher dilutions.

Sample	Bacteria per c.c. at 20° C.	Hrs.	Lactose Bile: Per cent Gas						Dextrose Broth: Per cent Gas						Liver Broth: Per cent Gas						From Nutrient Broth into Lactose Bile: Per cent Gas						From Liver Broth into Lactose Bile: Per cent Gas					
			10 c.c	1 c.c	.1 c.c	.01 c.c	.001 c.c.	.0001 c.c.	10 c.c	1 c.c	.1 c.c	.01 c.c	.001 c.c.	.0001 c.c.	10 c.c	1 c.c	.1 c.c	.01 c.c	.001 c.c.	.0001 c.c.	10 c.c	1 c.c.	.1 c.c.	.01 c.c.	.001 c.c.	.0001 c.c.	10 c.c.	1 c.c.	.1 c.c.	.01 c.c.	.001 c.c.	.0001 c.c.
1. Deep Well	10	24	0	0	0	0	..	...	0	0	0	0	..	...	0	0	0	0	..	...	0	0	0	0	...	....	0	0	0	0	...	....
		48	0	0	0	0	..	...	0	0	0	0	..	...	0	0	0	0	..	...	0	0	0	0	...	....	0	0	0	0	...	....
		72	0	0	0	0	..	...	0	0	0	0	..	...	0	0	0	0	..	...	0	0	0	0	...	....	0	0	0	0	...	....
2. N.Y. City Tap	210	24	0	0	0	0	..	...	2	13	3	0	..	...	80	37	10	0	..	...	15	0	0	0	...	....	85	22	0	0	...	....
		48	0	0	0	0	..	...	13	32	18	0	..	...	80	45	21	0	..	...	18	0	0	0	...	....	85	28	85	0	...	....
		72	0	0	0	0	..	...	24	30	20	0	..	...	83	50	28	0	..	...	21	0	0	0	...	....	85	35	85	0	...	....
3. N.Y. City Tap	175	24	0	0	0	0	..	...	0	0	0	0	..	...	12	13	12	0	..	...	50	18	0	0	...	....	35	12	0	0	...	....
		48	0	0	0	0	..	...	20	0	0	0	..	...	33	45	30	0	..	...	53	38	40	0	...	....	65	20	8	0	...	....
		72	17	0	0	0	..	...	20	0	0	0	..	...	37	47	38	0	..	...	53	45	52	0	...	....	75	26	23	0	...	....
4. Pond	780	24	0	0	0	0	..	...	3	8	5	0	..	...	20	24	9	18	..	...	8	0	0	0	...	....	38	10	0	0	...	....
		48	0	0	0	0	..	...	10	20	11	0	..	...	38	35	85	32	..	...	33	0	0	0	...	....	42	18	0	0	...	....
		72	0	0	0	0	..	...	12	26	12	0	..	...	38	38	90	35	..	...	42	0	0	0	...	....	47	21	0	0	...	....
5. Pond	175	24	0	0	0	0	..	...	13	12	16	0	..	...	17	13	18	2	..	...	35	0	0	0	...	....	42	38	3	0	...	....
		48	28	23	0	0	..	...	38	28	32	0	..	...	40	15	77	26	..	...	48	35	0	0	...	....	56	46	10	0	...	....
		72	34	45	0	0	..	...	45	40	35	0	..	...	45	13	83	33	..	...	50	40	0	0	...	....	67	62	13	0	...	....
6. Polluted Brook	15,000	24	42	55	22	0	0	0	14	18	30	0	0	0	45	28	40	25	15	0	..	..	..	..	...	....	..	..	..	..	...	....
		48	56	75	47	43	0	0	35	32	56	57	10	0	55	33	50	35	32	0	..	..	..	..	...	....	..	..	..	..	...	....
		72	58	78	50	45	0	0	38	35	55	68	16	0	55	30	52	35	32	0	..	..	..	..	...	....	..	..	..	..	...	....

In No. 5 Pond, where the pollution is more recent, the lactose bile shows the extent of such pollution, while in the dextrose broth and liver broth the distinction is not so marked. The same is true with the more highly polluted water shown in No. 6.

The figures on attenuated coli obtained by rejuvenating before planting into lactose bile show that preliminary rejuvenation in liver broth gives higher results than nutrient broth.

In a long series of experiments with various kinds of waters, in which all tubes showing gas formation in liver broth in higher dilution than in lactose bile were transplanted into lactose bile, about 65 per cent were positive after this rejuvenation.

It was observed that gas production in the liver broth tubes was usually well advanced within 24 hours, thus giving a prompt indication of the presence of gas-forming bacteria.

The following experiment will give a fairly good idea of the value of liver broth as a rejuvenating medium for attenuated *B. coli*.

A sample of surface water containing 300 bacteria per c.c. and no gas-formers of any kind was inoculated with a pure culture of *B. coli*, and kept in a stoppered bottle in the refrigerator for about four months.

At various times during this period this mixture was planted into lactose bile, dextrose broth, and liver broth.

Gas formation was obtained in dilutions varying from .0001 c.c. to 10 c.c., until finally gas formation was negative with 10 c.c. of the water in lactose bile and dextrose broth, even after rejuvenation in nutrient broth. The liver broth continued to yield vigorous gas formation, within 24 hours after inoculation with one c.c. of the water, several weeks longer. All tubes showing gas with liver broth were transplanted into lactose bile, as before, and positive results obtained.

The value of the use of liver broth in a study of the gas-forming bacteria in water, in the presumptive evidence of their presence, and as a preliminary step in their isolation as compared with other media now in use, has been many times demonstrated in this laboratory. In work on milk, sewage, or feces it has been found to be of great assistance in isolating the various species of bacteria of intestinal origin.

Except in the case of spore-forming bacteria, transplanting from liver broth should usually be done between six and 12 hours after inoculation and incubation at 37° C., as development is generally so rapid that after that period the excessive amount of toxic products often causes considerable attenuation or inhibition.

During the course of our work on various species, it was observed that *B. sporogenes*, in addition to gas formation, gave a very offensive cheesy odor in liver broth, while other forms gave only a very slight odor.

This feature is a very good indication of its presence, and furnishes a simple means for its identification.

CONCLUSIONS.

The determination of gas-forming bacteria in water by means of lactose bile gives results which represent the relative degree of contamination of dangerous or recent origin. While of especial value in judging the degrees of pollution present, it does not show the presence of gas-formers other than the colon group and does not often indicate *B. coli* in an attenuated state.

For those observers who desire to determine the presence of all gas-formers, including *B. coli*, to the highest accuracy, the use of liver broth is recommended.

In the examination of water, dilutions of 0.1, 1.0, and 10 cubic centimeters of the sample may be inoculated into lactose bile and another series into liver broth.

Positive tests in the lactose bile indicate the degree of recent pollution with the colon group.

Gas formation in the liver broth indicates the degree of contamination with gas-forming bacteria both attenuated and vigorous.

Any difference in the results obtained in lactose bile compared with those found by transplanting within 12 hours from the liver broth into a second set of lactose bile tubes gives a fairly accurate idea of the amount of attenuated *B. coli* present. Negative results in both sets of bile tubes and positive results in the liver broth usually show that the gas-producing bacteria present are not of the colon group.

GUINEA-PIG TEST OF THE VIRULENCE OF DIPHTHERIA BACILLI.*†

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(From the Laboratory of the Philadelphia Hospital for Contagious Diseases.)

No method for testing the virulence of diphtheria bacilli is ideal, because it is impossible to conduct a practical test under the same conditions which the bacilli encounter on gaining entrance into the human body. Probably no one practical method can be considered absolutely reliable. The object is to secure the most favorable conditions for the production of toxin, and to test it on a suitable animal which does not possess any marked degree of natural immunity. Toward this end certain steps in technic have been advanced from time to time as favorable to a reliable test, and it is only by taking advantage of each step that one can secure a technic as satisfactory as we can expect in the present light of our imperfect knowledge of the formation of diphtheria toxins. While investigators report their cultures of diphtheria bacilli as virulent or nonvirulent, as the case may be, the majority do not give the technic whereby such conclusions were reached, and certainly the technic has much influence on the result. Consequently quite a number of workers have a hazy idea of this subject. It is the object of our paper briefly to review the more important tests with which we are familiar and to give one which has given us uniform satisfaction.

As a practical measure the guinea-pig test is used under the following conditions:

1. Cases harboring bacilli morphologically similar to the diphtheria bacillus over a long period of time. These cases include those who have recovered from clinical diphtheria, and contact cases. Quarantine is often inconvenient and irksome, both to the patient and the attending physician, especially when it is prolonged. Cases of clinical diphtheria often harbor virulent bacilli three and four weeks and even longer after the disappearance of every symp-

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tom, when the patient is healthy in every other respect. A test demonstrating this fact helps to pacify the patient and to convince him of the wisdom of measures tending to prevent the spread of infection. Again, persons after contact with cases of clinical diphtheria frequently harbor virulent bacilli, which are just as dangerous as the bacilli from the patient. On the other hand, some persons may harbor perfectly harmless non-virulent bacilli. In both these instances local treatment is of little avail in getting rid of the bacilli. It is impossible by microscopic examination alone to claim that any culture does or does not possess virulence. We know now that certain types are often found non-virulent, but in our experience we never have been able to foretell the result with confidence by microscopic examination alone. Nor can we recognize with certainty the pseudo-diphtheria bacillus, whether the term be applied to non-virulent diphtheria bacilli or to the organism known as Hofmann's bacillus. Since it never has been definitely proven that a non-virulent organism can become virulent, and since the weight of evidence is against such a view, we must not depend on microscopic examination which tells us no more than that bacilli morphologically similar to the diphtheria bacillus are present, but on a reliable animal test for the virulence of the bacilli.

2. Frequently the discharges from infected ears show a diphtheria-like bacillus. Such cases usually pursue a prolonged course and the question of virulence is a serious one, especially when the patient seeks admission into a hospital, school, etc. We have isolated the bacilli from over 100 such cases following scarlet fever in order to prepare autogenous vaccines; 57 per cent gave a diphtheria-like bacillus—in pure culture, 35 per cent, and in combination with another organism, 22 per cent. Twenty-two of these were tested for virulence with negative results. On the other hand, several of our most potent toxins were obtained from bacilli isolated from ear discharges in the diphtheria department. These cases of ear infection especially require the aid of test on guinea-pigs for proper management.

3. One should always be cautious in giving a positive diagnosis in cases of nasal infection, because the Hofmann's bacillus is frequently present. We have often found solid types of bacilli re-

sembling this bacillus to be virulent, and therefore we never feel justified in giving a negative report by microscopic examination alone and trusting that the bacilli are not virulent.

Any technic to be of value as a practical routine measure should give (1) a reliable and decisive result in the shortest time possible; (2) it should be the least expensive possible. At best the test on guinea-pigs is a costly one, and this factor is of much importance where the demand for tests is likely to be large.

In order to secure a strong toxin representative of the bacilli tested it is necessary to observe the following points: (1) The broth used for making the toxin should be distinctly alkaline to litmus, at least "0.5 per cent acid to phenolphthalein"; (2) it should contain a sugar, as 1 per cent glucose; (3) the culture should be grown at 37° C. for at least 48 hours. We believe that enough soluble toxins are generated in this time to make a reliable test, although there is no doubt but that in a large number of cases, stronger toxin results after seven days' growth. If the test can be made in a leisurely manner and especially if it is desired to obtain a strong toxin, as in the manufacture of antitoxin, it is well to secure a good surface growth by "educating" the bacilli and to test the product between the seventh and ninth day.

A crude test can be made by injecting an emulsion made by mixing a loopful of bacilli with sterile normal saline solution. The main objection to this method is the fact that the dose cannot be well regulated. If the pig dies, the assumption is that death was due to diptheria, and when injecting a second pig with toxin-antitoxin mixture, it is desirable to have some control of the dosage. In case the pig survived, it would be necessary to make a toxin and try that. In this way two animals would be used.

There is a difference of opinion concerning the value of filtered and unfiltered cultures. We have frequently seen the unfiltered culture kill a pig when the filtrate did not, both being made in exactly the same manner and given in the same dose. Antitoxin protected a second pig, each time showing that we were dealing with diphtheria toxin. Escherich[1] never obtained a negative result when using the filtrate in doses corresponding to 0.5 per cent

[1] Lehmann and Neumann, *Med. Hand Atlas*, Saunders & Co., 1901, p. 399.

of the body weight of the pig. In his virulent cultures, about 0.05 per cent sufficed. We believe in the use of unfiltered cultures after giving both methods extensive trial, for as Theobald Smith[1] has written: "We may, for convenience, regard the disease-producing power of diphtheria bacilli as made up of two elements: toxicity and virulence. The former represents the rate of accumulation of toxin in culture fluids, and is easily measured; the virulence on the other hand, which may be regarded as the behavior of diphtheria bacilli toward living tissue, is as yet an unknown quantity." If we use the filtered toxin, "but an instrument of pathological power is here dealt with, and under artificial conditions, since we do not know the nature of the nutritive fluid which the bacteria make use of on mucous membrane, nor as a consequence, whether the toxin production in bouillon is a true index of the production of toxin in mucous membrane."

We have employed the following technic in the Philadelphia Hospital for Contagious Diseases. We wish to state here, that it has been worked out in its essentials according to the technic of Rosenau,[2] in establishing the legal immunity unit for standardizing diphtheria antitoxin.

1. If under a microscope the bacillus has the morphology of *B. diphtheriae*, it is restained by Gram's method. If it is Gram negative (that is, does not retain the stain after having been washed with alcohol), we conclude that it is not a diphtheria bacillus, for no diphtheria bacillus has ever been found Gram negative. Should it be Gram positive, the organism is isolated.

2. Isolation is readily accomplished by "streaking" on a Petri dish of blood serum, or by inoculating the water of condensation in large tubes of gelatin-agar and passing the water once over the surface of the medium. Usually within 24 hours the colonies are well developed and can be picked out. The bacilli are stained again with methylene-blue and Gram to ascertain their purity and morphological characteristics.

3. When a pure culture is obtained it is planted in slightly alkaline (0.5 per cent to phenolphthalein) broth containing 1 per

[1] Nuttal and Graham, *Bacteriology of Diphtheria*, Cambridge University Press, 1908, p. 173.

[2] *Bull. No. 21*, Hyg. Lab., U.S. Public Health and Marine Hospital Service, Washington, pp. 35–72.

cent glucose and grown for 48 hours at 37.5° C. The tubes are slanted in order to expose as large a surface as possible to the oxygen in the tube. During this time soluble toxins are generated in sufficient quantities to kill a guinea-pig, supposing of course the bacillus to be a virulent diphtheria bacillus.

4. A healthy guinea-pig is selected, weighing about 250 and not over 300 gms. Rosenau states that "in general terms the guinea-pig as a vital factor in the standardization of antitoxin is quite as dependable as our ordinary weights and measures." The dose, 0.5 per cent of the body weight of the pig expressed in cubic centimeters, is accurately measured and poured into the barrel of the syringe. Thus a 250-gm. pig would receive 1.25 c.c. of (unfiltered) culture. We use and warmly recommend the syringe devised by Dr. Hitchens.[1] The syringe is thoroughly washed with absolute alcohol followed by sterile salt solution, and the tip of the needle plugged with sterile vaselin. In the side arm of the syringe enough sterile salt solution is poured to bring the total quantity of injection up to 4 c.c. An assistant holds the pig; the abdomen is dampened with alcohol and then sterile salt solution "and the needle introduced in the left flank from one-half to an inch above the supraspinous process of the ileum. The needle is introduced its whole length (1.25 inch), strictly subcutaneously toward the middle line. This part of the operation is arranged so that the point of the needle will be just about at the linea alba." The culture is then injected, the syringe turned in order that the salt solution may flow out of the side arm down the barrel of the syringe, and this is injected without removing either the needle or the bulb. It is very necessary to avoid entering the peritoneum or piercing the skin too superficially, and special care must be observed to avoid injecting the toxin into muscles, as emphasized by Meltzner and Auer.[2] By following these directions, several conditions are assured: (*a*) "The rate of absorption is always the same; (*b*) the pressure effects are always the same; (*c*) the relation of toxin to body juices is constant when the injection is made always in the same anatomical structures"; (*d*) by using percentage of body

[1] *Jour. Exp. Med.*, 1906, 8, p. 647.

[2] *Am. Med.*, 1905, 5, p. 75.

weight of culture to weight of animal, the dose is always the same, regardless of the size of the animal.

5. The animal is kept under daily observation four to five days. Symptoms of toxemia are usually manifest in 36 hours, in which case the pig is inclined to nestle in some corner, the hair bristled, appetite poor, etc. He usually dies in four to six days, or may recover. He may be sick for a day or so and recover, and later show signs of paralysis. If at the end of four to six days the animal is apparently healthy, the organism is said to be non-virulent. The late paralytic effects of toxone appear about the second to third week. If the animal dies within four to six days an autopsy is performed in order to establish the cause of death; the possibility of death from other infections, as pneumonia, tuberculosis, etc., must not be left out of consideration. The condition of the site of inoculation, of the spleen, the suprarenals, the pleurae, the heart, etc., is carefully observed. Smears and cultures are made from the site of inoculation, the pleural and peritoneal cavities, heart's blood, and spleen. If diphtheria-like bacilli are recovered, it is justifiable to conclude that they killed the animal; but it is not justifiable to conclude that they are diphtheria bacilli. To be sure of this, it is necessary to go a step further and inject a second pig protected with antitoxin.

6. A new sub-culture is made in the same manner and from the same source as the one used for the first test; the same dose is injected plus 250 units of antitoxin. The culture and antitoxin are measured out and mixed, and allowed to stand at room temperature for an hour before injection. This gives ample time for the toxins to be neutralized by the antitoxin. The remaining antitoxin immunizes the animal against toxins formed in the body by the living bacteria injected. The question of the amount of antitoxin needs no discussion. One unit neutralizes 100 times the least fatal dose of toxin, so that 250 units would immunize against 25,000 fatal doses, or more than 25 c.c. of virulent toxins. It is rarely necessary to inject more than 2.5 c.c. of culture, so that there still remains enough antitoxin to immunize against 11 times the amount injected. This is the final test. If the second pig lives, it is proof sufficient that the organisms were virulent diph-

theria bacilli. If it dies, they were not, and although they killed both pigs, they cannot produce diphtheria.

It may be well to add here a few words in reference to toxones. The identity of this substance is still unsettled, although Erlich's views are widely accepted. He believes that toxones constitute definite secretory products of the diphtheria bacillus, but differ from toxin in having a weaker affinity for antitoxin and in the toxophore group. Toxones do not produce death even in large doses, but are believed to cause the paralyses which frequently develop about the second or third week of the disease. We have seen paralyses develop in the guinea-pig at the end of the first week. Should a patient harboring diphtheria-like bacilli capable of producing paralyses be given a clean bill of health? In practically all cases if a second pig is protected with antitoxin, no paralyses occur. So far the assumption has been that only the toxins are capable of producing clinical diphtheria, and if the culture is free of toxic action, the organisms are considered sufficiently harmless to give the patient his freedom. Personally we have always regarded such strains with suspicion, for it seems that if the bacillus produces sufficient toxone to give paralysis, it is likely to be in some degree a toxin producer, although we have seen guinea-pigs recover after being slightly sick for a few hours or a day and yet be seized with typical paralyses about two weeks after the injection. The subject of toxones is still quite unsettled.

The following is a list of tests and results that have been made in the course of routine work in the Philadelphia Hospital for Contagious Diseases from February 15, 1910, to October 15, 1910.

Number of tests in which filtered toxin was given.....25

Result Positive..... 2
Result Negative.....23

Number of tests in which unfiltered culture was given.....67

Result Positive.....10
Result Negative.....57

Filtered and unfiltered cultures from the same case were given two separate pigs in 8 cases.

ON VARIATIONS IN THE PHAGOCYTIC AND COCCIDAL POWER OF THE BLOOD IN PNEUMONIA AND SCARLET FEVER.*

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It has been shown by various observers that the phagocytic activity of leukocytes may vary just as the opsonic power of the serum varies, and that it is necessary to examine both leukocytes and serum if one wishes to determine so far as possible the actual capacity of the blood for phagocytosis.

Rosenow[1] found that the leukocytes from pneumonia patients were perceptibly more actively phagocytic than normal leukocytes, as were also leukocytes from patients with puerperal sepsis and appendicitis. He points out that this is possibly due to the preponderance of young leukocytes. This increased phagocytic power of pneumonic leukocytes seemed to be without reference to the stage of the disease. Potter and Krumwiede[2] found that at the height of streptococcus and pneumococcus infections the phagocytic power of the leukocytes as compared with that of leukocytes of supposedly normal persons was diminished, and more noticeably so than the opsonic index of the serum, but that during recovery the phagocytic power rose well above the normal, and again distanced the opsonic index; during convalescence the phagocytic power of the leukocytes and the opsonic index gradually fell to normal or just below. They made similar observations in staphylococcus infections and tuberculosis. Shattock and Dudgeon[3] observed that washed pneumonic leukocytes were more active than washed normal leukocytes in the serum of three different patients (empyema, urinary fever, and pneumonia), the difference being most marked with pneumonic serum. Achard, Ramond, and Foix[4] found that the activity of the leukocytes and the opsonic

* Received for publication December 17, 1910.

[1] *Jour. Infect. Dis.*, 1906, 3, p. 683.

[2] *Jour. Am. Med. Assn.*, 1907, 49, p. 1815; *Jour. Infect. Dis.*, 1907, 4, p. 601.

[3] *Proc. Royal Soc.*, 1907, B, 80, p. 165.

[4] *Compt. rend. soc. biol.*, 1909, 66, p. 1031.

index, especially with reference to yeast cells, followed the same course in the acute infectious diseases (typhoid fever, pneumonia, scarlet fever, erysipelas, tuberculosis, and meningitis), being diminished at first and later increasing, except in cases of death, when both would be depressed. They obtained more striking results with yeast cells than with bacteria.

Rosenow[1] has studied also the changes that occur in the phagocytic activity of the leukocytes in cases of chronic or subacute pneumococcus and staphylococcus endocarditis; he found that increased phagocytic activity was not associated necessarily with an increase in the power to destroy the infecting bacteria.

In a case of recurrent and complicated erysipelas, Boughton[2] observed a specificity in the relation of serum, leukocytes, and bacteria to each other. He calls attention to the fact that so far these specific variations of serum and leukocytes with respect to the invading bacterium have been observed only in cases of chronic infection. He found that variations in the opsonic power of the serum might occur independently of variations of the phagocytic power of the leukocytes, but that such variations on the part of the leukocytes were not entirely independent of the variations of the serum. The changes in the serum appeared earlier and were more persistent and more marked than the changes in the leukocytes. He considered that the low phagocytic power of the leukocytes in their own serum might depend either upon the lack of some element necessary to phagocytosis or to some inhibitory substance specific for the leukocytes in question and neutralized by normal serum.

It has been shown that human serum has no bactericidal action on streptococci (Weaver and Ruediger,[3] Ruediger,[4] Meakins[5]) and that washed leukocytes alone have no bactericidal power (Ruediger), but Ruediger found that when leukocytes were added to the serum, the streptococci were destroyed. In an attack of post-scarlatinal nephritis, Ruediger observed that not only was the opsonin diminished but also the leukocytes had lost some of

[1] *Jour. Infect. Dis.*, 1909, 6, p. 245.
[2] *Ibid.*, 1910, 7, p. 111.
[3] *Medicine*, 1903, 9, p. 515; *Trans. Chi. Path. Soc.*, 1903, 5, p. 285.
[4] *Jour. Amer. Med. Assn.*, 1906, 46, p. 108.
[5] *Jour. Exp. Med.*, 1908, 11, p. 100.

their power to destroy streptococci. Rosenow[1] has demonstrated that neither normal (human) nor pneumonic serum has any pneumococcidal action, the pneumococcidal action of pneumonic or other leukocytic blood being the result of the combined action of the serum and leukocytes.

The following experiments have been made to determine (1) the changes, if any, in the phagocytic activity of the leukocytes during the course of pneumonia and scarlet fever; (2) the specificity or non-specificity of the changes in phagocytic activity; and (3) the relation between the phagocytic and destructive power of the leukocytes. Similar experiments were carried out also in a case of chronic rheumatism receiving injections of galactose-killed streptococci.

It was necessary to use sterile blood on account of the coccidal experiments. Hence sterilized tubes and solutions were used in collecting the blood from the ear, the lobe being first washed carefully with alcohol. There was rarely any contamination. Normal human serum and leukocytes were used as controls. In order to compare the phagocytic activity of the leukocytes of a patient with that of the leukocytes of a normal person, it is essential to use suspensions containing the same number of polymorphonuclear leukocytes. To obtain such suspensions, the number of polymorphonuclear leukocytes in each suspension is counted by means of a white blood counting apparatus and the suspensions are equalized by the addition of the required amount of normal salt solution.

The opsonic estimations were made in the usual way, using equal parts of serum, leukocytes, and bacterial suspension. The experiments were controlled from time to time by diluting the serum to the point of opsonic extinction. In this case both normal and patient's sera were first heated at 60° C. for 10 minutes. As in previous experiments of this kind the two methods were found now also to yield the same results.

In the regular routine, four specimens are prepared for each organism, namely, from mixtures of normal serum with normal leukocytes and with patient's leukocytes, and of patient's serum with normal leukocytes and patient's leukocytes. From the study

[1] *Jour. Infect. Dis.*, 1906, 3, p. 683.

of these specimens one can determine: (1) the opsonic index with normal and with patient's leukocytes; (2) the phagocytic activity of the patient's leukocytes (cytophagic index of Glynn and Cox[1]) by determining the relation of the phagocytic index of mixtures of normal serum and patient's leukocytes to that of mixtures of normal serum and normal leukocytes; (3) the phagocytic power of the whole blood (opsono-cytophagic index of Glynn and Cox), obtained by determining the relation between the phagocytic index of a mixture of the patient's serum and the patient's leukocytes and that of a mixture of normal serum and normal leukocytes.

Exactly the same mixtures are made to determine the destructive power of the patient's serum and leukocytes and of normal serum and normal leukocytes. In all cases accurately standardized suspensions of leukocytes must be used. For these experiments a very thin bacterial suspension is necessary, the suspension used for opsonic determinations being diluted about 30 times. Suspensions of leukocytes thicker than those used in opsonic work are required, an average of 20,000 leukocytes per cubic millimeter being sufficient. The mixtures of equal parts of serum, bacterial suspension, and standardized leukocytic suspension are incubated 18 hours, when a loopful of each mixture is plated, defibrinated blood (goat) being added to each tube of melted agar to more easily distinguish the colonies (see Fig. 1). The number of colonies is counted at the end of 24 hours' incubation at 37° C. By dividing the number of colonies on the plate made from the mixture containing normal serum and normal leukocytes by the number on the plate made from the mixture containing patient's serum and normal leukocytes, one gets a figure representing the destructive power of the patient's serum as compared with that of normal serum, normal leukocytes being used in both cases. This figure corresponds, then, to the opsonic index. By dividing the number of colonies on the plate made from a mixture of normal serum and leukocytes by the number of colonies on the plate made from the mixture containing normal serum and patient's leukocytes, one obtains a figure which represents the destructive power of the patient's leukocytes as compared with normal leukocytes, normal

[1] *Jour. Path. and Bact.*, 1909, 14, p. 90.

serum being used in each case. This figure corresponds to the cytophagic index. By again dividing the number of colonies on the plate from the mixture of normal serum and normal leukocytes by the number on the plate from the mixture of the patient's serum and patient's leukocytes, one obtains a figure representing the destructive power of the whole blood of the patient as compared with that of normal blood. This figure corresponds to the opsonocytophagic index.

Where there is much phagocytosis of red blood cells, as occurs with certain normal sera and certain pneumonic and scarlatinal leukocytes, the number of bacteria engulfed may be so diminished that correct phagocytic determinations are impossible.

In the case of pneumonia, opsonic estimations were made with the pneumococcus, streptococcus, and staphylococcus. Bactericidal experiments were made with the pneumococcus only. The pneumococcus employed was one cultivated from the blood of a pneumonia patient. A non-virulent strain is necessary, one which is readily phagocytable, especially for the bactericidal experiments. The staphylococcus was isolated from a furuncle and the streptococcus was a typical *Streptococcus pyogenes*.

The opsonic determinations in the case of the scarlet fever patients were made with the same streptococcus. In the majority of cases the homologous streptococcus and in some mixed cases the homologous diphtheria bacillus were also employed. The bactericidal experiments were made with streptococcus; in one case only was the homologous streptococcus also used. The results corresponded closely, whatever the streptococcus strain used (Chart 2).

In the case of chronic rheumatism the early opsonic estimations were made with the pneumococcus, staphylococcus, and homologous streptococcus, isolated from the excised tonsil by Dr. D. J. Davis. Later the streptococcus only was used. The bactericidal experiments were all made with the homologous streptococcus.

Nine cases of pneumonia in adult men were studied. No examinations were made before the fourth day of the disease because the patients did not enter the hospital earlier. Both before and after crisis the opsonic power of the serum was found to be normal in all of the mild cases except one, where it was slightly increased

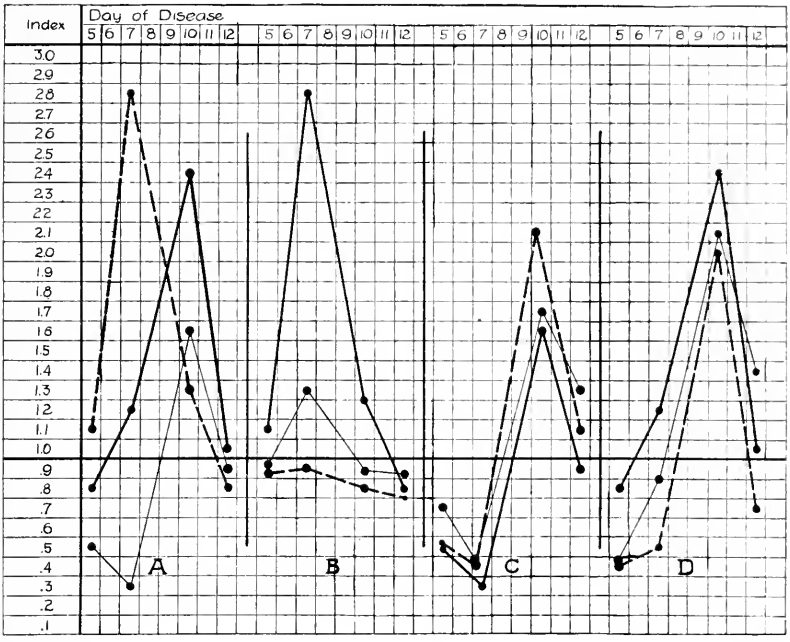

CHART 1.—Severe case of pneumonia (adult man).

A.

Heavy broken line = The pneumococcus opsonic power of patient's serum compared with that of normal serum (opsonic index).

Fine solid line = The phagocytic power of patient's leukocytes with respect to pneumococcus compared with that of normal leukocytes under the influence of normal serum (cytophagic index).

Heavy solid line = The phagocytic power of the patient's whole blood with respect to pneumococcus compared with that of normal blood (opsono-cytophagic index).

B.

The Opsonic Power of the Patient's Serum Compared with Normal Serum.

Heavy dotted line = The pneumococcus opsonic index.

Fine solid line = The staphylococcus opsonic index.

Heavy broken line = The streptococcus opsonic index.

C.

The Phagocytic Power of Patient's Leukocytes Compared with That of Normal Leukocytes under the Influence of Normal Serum.

Heavy solid line = The pneumococcus cytophagic index.

Fine solid line = The staphylococcus cytophagic index.

Heavy broken line = The streptococcus cytophagic index.

D.

The Phagocytic Power of the Patient's Whole Blood Compared with That of Normal Blood (Opsono-cytophagic Index).

Heavy solid line = The pneumococcus opsono-cytophagic index.

Fine solid line = The staphylococcus opsono-cytophagic index.

Heavy broken line = The streptococcus opsono-cytophagic index.

on the 15th day of the disease. In one fatal case examined before crisis, the opsonin also was normal. In another severe case (Chart 1-B)the opsonic power was normal early in the disease, but with improvement in the patient's condition it increased. In two other severe cases the opsonic power of the serum was below normal before crisis, but rose considerably afterward.

In these cases of pneumonia the phagocytic power of the leukocytes (cytophagic index) was also estimated. In the mild cases, both before and after crisis, it was found to be greater than normal, except once, in those cases in which only one estimation was made on the same patient. In one patient examined both before and after crisis, the phagocytic power of the leukocytes was found to be below normal before crisis and increased afterward. One fatal case showed normally active leukocytes. The other severe cases all showed diminished leukocytic activity during the height of the disease with an increased power as the patient improved (Chart 1-C).

Pneumococcidal experiments were made to determine if the destructive and phagocytic power corresponded. It was found that in the majority of experiments, increased or decreased phagocytosis meant increased or decreased destructive power.

The cases of pneumonia examined showed then specific variations in the opsonic power of the serum for the pneumococcus (Chart 1-B) but no specific variations in the phagocytic power of the leukocytes (Chart 1-C) or in the phagocytic power of the whole blood (opsonocytophagic index, Chart 1-D), the leukocytes showing the same variations in phagocytic activity when tested with the streptococcus and the staphylococcus as with the pneumococcus.

Thirteen cases of scarlet fever were studied in the same way. Four were mild, one moderately severe, and eight severe, three of these being fatal. In the four mild, uncomplicated cases, there was little or no change in the phagocytic activity of the leukocytes with respect to the streptococcus, staphylococcus, and pneumococcus, although the opsonic index was low at first, followed by a rise for the streptococcus as improvement occurred.

In the moderately severe case, after a slight rise, the opsonic index remained persistently low. The leukocytes at no time be-

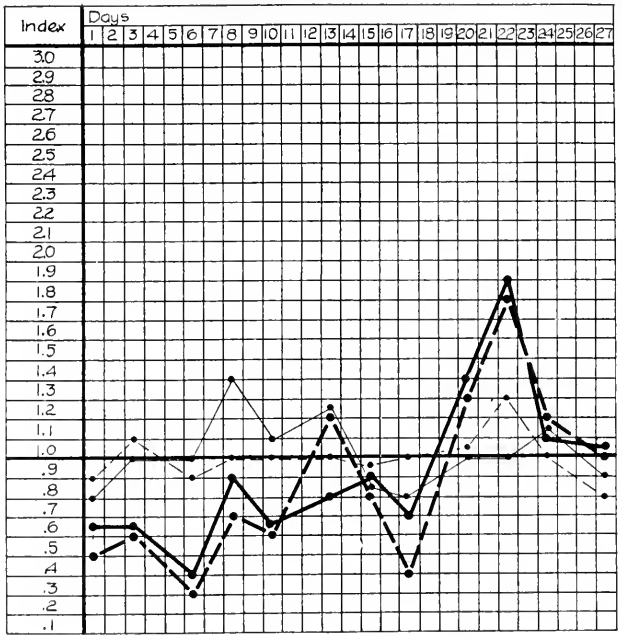

CHART 2.—Severe case of scarlet fever (adult man).

Heavy solid line = Opsonic index with streptococcus from patient's throat.
Heavy broken line = Opsonic index with heterologous streptococcus.
Fine broken line = Opsonic index with staphylococcus.
Fine solid line = Opsonic index with pneumococcus.

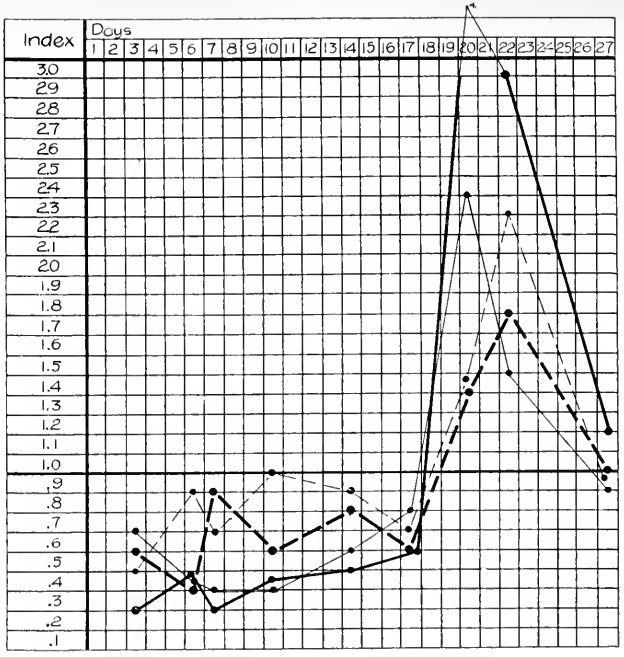

CHART 3.—Severe case of scarlet fever (adult man).

Heavy broken line = Opsonic index for homologous streptococcus with normal leukocytes.

Fine broken line = Opsonic index for homologous streptococcus with patient's leukocytes.

Fine solid line = Phagocytic power of patient's leukocytes with respect to homologous streptococcus as compared with that of normal leukocytes under the influence of normal serum (cytophagic index).

Heavy solid line = Phagocytic power of patient's whole blood with respect to homologous streptococcus as compared with that of normal blood (opsonocytophagic index).

came as active as normal. Nothing could be found to explain the low index. In the 30 experiments on this case, the destructive and phagocytic power of the serum and leukocytes followed the same general course.

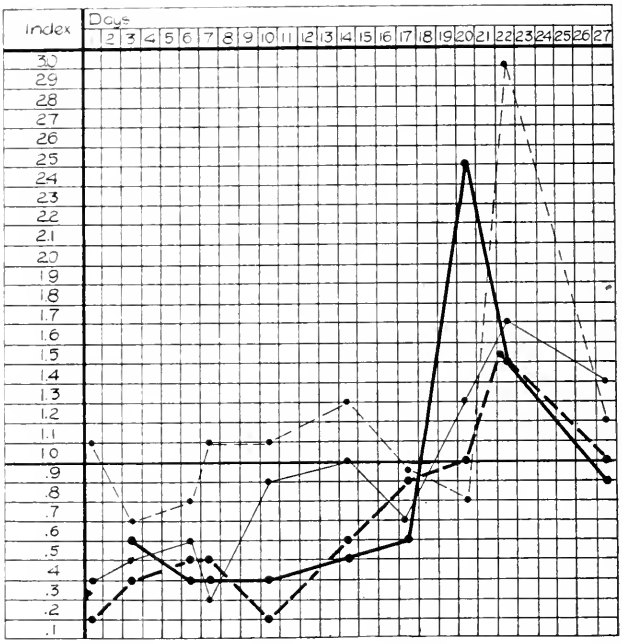

CHART 1.—Severe case of scarlet fever (adult man).

The Phagocytic Power of Patient's Leukocytes Compared with That of Normal Leukocytes under the Influence of Normal Serum (Cytophagic Index).

Heavy solid line = Homologous streptococcus index.
Fine solid line = Heterologous streptococcus index.
Heavy broken line = Pneumococcus index.
Fine broken line = Staphylococcus index.

In the eight severe cases the opsonic index was low to the invading streptococcus during the early stage, followed by an increase later on in the cases that recovered (Chart 2). The leukocytes also showed a diminished phagocytic activity early in the disease, with an increased activity as the patient's condition improved (Chart 3). These changes in the phagocytic activity were not specific for the

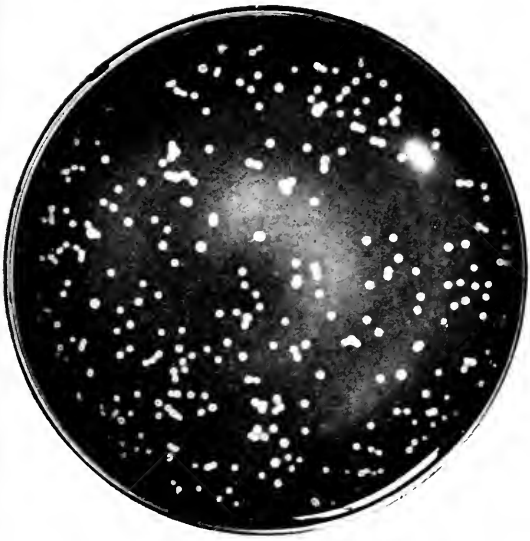

Plate 1.—Normal Serum and Normal Leukocytes.

Plate 2.—Patient's Serum and Normal Leukocytes.

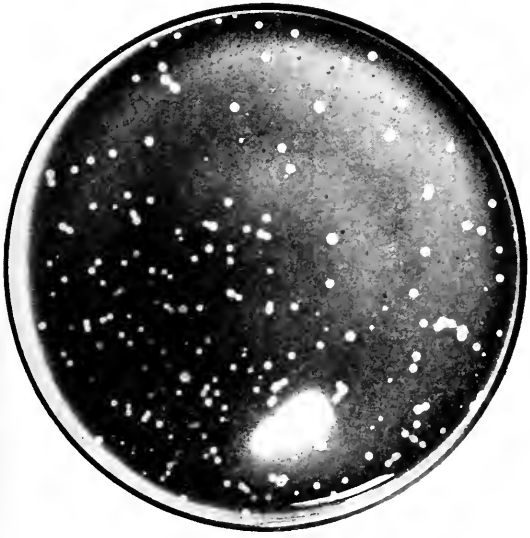

Plate 3.—Normal Serum and Patient's Leukocytes.

Plate 4.—Patient's Serum and Patient's Leukocytes.
On this particular day the opsonic power of the serum (opsonic index) and the phagocytic activity of the leukocytes (cytophagic index) were both above normal.

FIG. 1.—Photographs of blood-agar plates from mixtures of streptococci, leukocytes, and serum to show increased streptococcidal power in case of severe scarlet fever with complications.

invading organism, but were demonstrable also for the pneumococcus and staphylococcus (Chart 4). In a large majority of the experiments, the destructive and phagocytic power of the serum and the leukocytes corresponded quite closely.

One septic case of scarlet fever showed a persistently low opsonic index and low phagocytic power of the leukocytes (cytophagic index) with correspondingly low streptococcidal power, although the patient was apparently well. A diffuse streptococcus phlegmon of the whole arm which developed in a few days perhaps explained

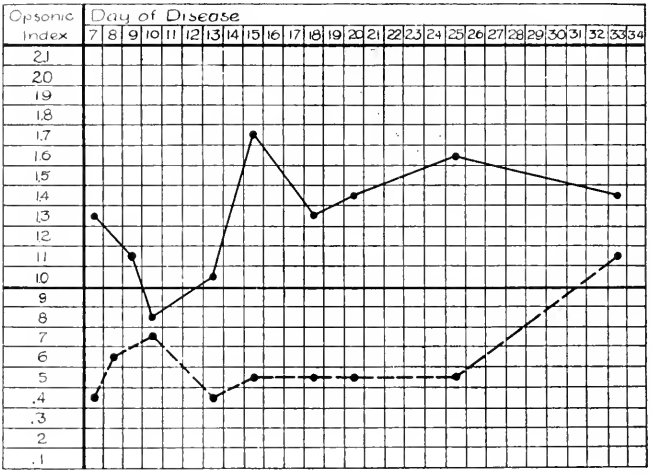

CHART 5.—Severe case of scarlet fever (adult woman).

Solid line = Streptococcus opsonic index with normal leukocytes.
Broken line = Streptococcus opsonic index with patient's leukocytes.

this condition. On recovery from this infection of the arm, the opsonic index rose above normal, but the phagocytic power of the leukocytes remained low (cytophagic index) and the destructive power of the serum and leukocytes stayed below normal. A severe nephritis now set in. When this subsided, the opsonic index and the cytophagic index both increased, as did also the streptococcidal power of the serum and the leukocytes, but with the appearance of an abscess on the arm the destructive power of the whole blood again decreased, although the phagocytic activity remained quite high. The results of these two experiments are in accord with

the observations of Rosenow in cases of chronic endocarditis, in which he found that the amount of phagocytosis and the destructive power did not go together.

In almost every case, both of pneumonia and of scarlet fever, the opsonic index was the same whether normal or homologous leuko-

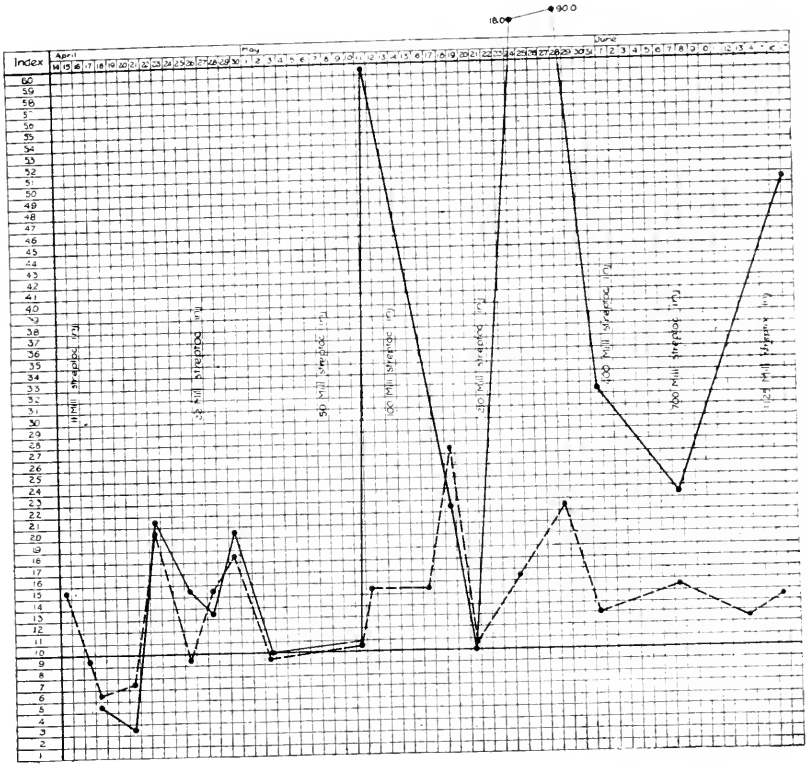

CHART 6.—Observations in the case of a boy, 7 years old, with chronic articular rheumatism, receiving injections of killed streptococcus cultures isolated from the tonsils.

Heavy broken line = Phagocytic power of the patient's leukocytes with respect to the streptococcus as compared with that of normal leukocytes under the influence of normal serum (cytophagic index).

Heavy solid line = Streptococcidal power of the patient's leukocytes as compared with that of normal leukocytes under the influence of normal serum.

cytes were employed (Chart 3). One scarlet fever patient, only, showed considerable variation in this respect (Chart 5).

The patient with chronic articular rheumatism (for the chance to study which I am indebted to Dr. John M. Dodson) showed an

increase in the amount of streptococcus opsonin and in the phagocytic activity of the leukocytes after each injection of the autogenous vaccine. Corresponding to this increase in opsonin and phagocytic activity there was generally also an increase in the destructive power of the serum and leukocytes. The streptococcidal power of the patient's serum and leukocytes when combined also increased with the elevation of the total phagocytic power of the blood (opsono-cytophagic index). The details are given in Table 1 and Chart 6.

TABLE 1.

A COMPARISON BETWEEN THE PHAGOCYTIC AND DESTRUCTIVE POWER FOR STREPTOCOCCUS OF THE SERUM AND LEUKOCYTES OF A PATIENT WITH CHRONIC RHEUMATISM AND OF NORMAL SERUM AND LEUKOCYTES.

DATE	Opsonic Power of Patient's Serum	Streptococcidal Power of Patient's Serum and Normal Leukocytes as Compared with that of Normal Serum and Normal Leukocytes	Phagocytic Power of Patient's Leukocytes in Normal Serum as Compared with that of Normal Leukocytes in Normal Serum	Streptococcidal Power of Patient's Leukocytes in Normal Serum as Compared with that of Normal Leukocytes in Normal Serum	Phagocytic Power of Patient's Blood as Compared with that of Normal Blood	Streptococcidal Power of Patient's Blood as Compared with that of Normal Blood
Apr. 18	0.5	4.0	0.6	0.5	0.2	0.1
Apr. 20	0.5	0.3	0.7	0.4	0.6	0.5
Apr. 22	0.8	2.0	2.0	2.0	1.9	3.0
Apr. 25	0.9	6.0	0.8	1.5	0.92	2.0
Apr. 27	1.6	3.0	1.5	1.2	1.5	1.2
May 2	0.9	1.3	0.95	0.9	0.9	1.0
May 9	1.3	6.0	1.0	0.8	1.2	600.0
May 11	1.0	2.5	1.5	6.0	1.4	200.0
May 18	2.7	1.0	2.7	2.2	4.4	4.4
May 20	0.84	0.46	1.0	1.0	1.0	14.0
May 24	2.2	3.2	1.6	18.0	2.4	732.0
May 28	1.2	3.6	2.2	90.0	1.68	360.0
May 31	1.7	58.0	1.24	32.0	1.7	58.0
June 7	1.38	0.94	1.46	2.3	1.32	1.8
June 15	1.4	2.0	1.4	5.0	1.4	11.0

SUMMARY.

The observations here recorded indicate: (1) As a rule there is an increase in the phagocytic power of the leukocytes in mild cases of pneumonia. (2) In mild and in uncomplicated cases of scarlet fever, there is no change in the phagocytic activity of the leukocytes. (3) In severe cases of pneumonia and scarlet fever, the phagocytic power of the leukocytes is generally diminished during the height of the disease, but when the patient improves

it rises above normal, to which it returns during convalescence. (4) Although the opsonic power of the serum is specific, there is no such specificity in the phagocytic power of the leukocytes so far as my results indicate. (5) So far as pneumonia and scarlet fever are concerned, variation in the opsonic power of the serum and in the phagocytic activity of the leukocytes as a rule is accompanied by a corresponding variation in the pneumococcidal or streptococcidal power of the serum and the leukocytes.

FURTHER STUDIES ON FUSIFORM BACILLI AND SPIRILLA.*

RUTH TUNNICLIFF.

(From the Memorial Institute for Infectious Diseases, Chicago.)

In a previous article[1] Dr. Weaver and I described three strains of fusiform bacilli which were isolated in pure culture from cases of ulceromembranous angina, ulceromembranous stomatitis, and diphtheria of the tonsil. These organisms were anaerobic and appeared to be the same as those described by Ellermann.[2]

In a later communication I[3] described three strains of fusiform bacilli isolated in pure culture from the normal mouth. These organisms were anaerobic and differed only slightly culturally from those described by Dr. Weaver and me. Morphologically I found that after growing a few days filaments and decidedly spiral forms appeared. The spiral forms resembled the spirilla found in the smears made from the mouth and found in conjunction with the fusiform bacillus in ulceromembranous angina (Vincent's angina), ulceromembranous gingivitis, and noma. This was in accord with the opinion of various observers (Seiffert,[4] Perthes,[5] Herrman,[6] and Neuof[7]) that the fusiform bacilli and spirilla are different forms of one organism. Several of these authors describe it as a cladothrix or streptothrix. Seiffert isolated in pure culture a cladothrix from two cases of noma. This organism produced threads, "spirilla," and branching forms of cladothrix. No branching could be seen in any of my cultures.

Ghon and Mucha[8] and Kaspar and Kern[9] isolated from abscesses anaerobic fusiform bacilli, which formed long filaments and were also extremely polymorphous.

* Received for publication December 15, 1910.

[1] *Jour. Infect. Dis.*, 1905, 2, p. 446.

[2] *Centralbl. f. Bakt.*, Abt. 1, Orig., 1904, 37, p. 729.

[3] *Jour. Infect. Dis.*, 1906, 3, p. 148.

[4] *Münch. Med. Wchnschr.*, 1901, 49, p. 1988.

[5] *Archiv. f. klin. Chir.*, 1899, 59, p. 111.

[6] *Archiv. f. Pediatrics*, 1905, 22, p. 817.

[7] *Amer. Jour. Med. Sci.*, 1910, 139, p. 705.

[8] *Centralbl. f. Bakt.*, Abt. 1, Orig., 1909, 49, p. 493.

[9] *Ibid.*, 1910, 55, p. 7.

Since isolating the fusiform bacilli and spirilla from the normal mouth, I have isolated in pure culture three strains from cases of ulceromembranous angina (Vincent's angina), one from a case of ulceromembranous gingivitis, and one from a case of noma of the face.[1]

The organisms from the cases of Vincent's angina were isolated on slants of ascites-agar (1:3), those from the cases of gingivitis and noma on blood-agar. The material was smeared on a series of slants. Usually in the second tube the colonies of fusiform bacilli could be found after three or four days' incubation at 37° C. The cultures were grown anaerobically according to the method of Wright, by saturating the cotton stopper with a strong solution of pyrogallic acid in a five per cent solution of sodium hydroxide, and closing the tube with a tightly fitting cork and sealing it with paraffin. The sub-cultures were made from single colonies.

The colonies are delicate and whitish and resemble colonies of streptococci, but are rather smoother and softer, especially when grown on blood-agar. When the colonies are quite isolated, they sometimes attain considerable size (0.3 cm. in diameter). The organisms are obligate anaerobes growing at 37° C. The cultures from the normal mouth grew slightly at room temperature and aerobically on dog's blood-agar after long isolation. The organisms show no progressive but considerable vibratory motion, the spirilla retaining their spiral form. They grow on ascites-agar, Loeffler's blood-serum, blood-agar (dog, sheep, goat, rabbit, and human), and horse and human serum-agar. The best spirilla are most frequently found in a medium of agar (5 c.c.) and 12 drops of equal parts of goat's or sheep's blood and two per cent sodium citrate solution in normal salt solution. An offensive odor is given off in all successful cultures. As the organisms die out rapidly it is necessary to transplant a large number of organisms to get a successful culture.

The organisms are extremely polymorphous. They are usually during the first 24 hours' growth delicate pointed rods 3–10 mikrons in length, straight or slightly bent. Spores are often seen during

[1] I am indebted to Dr. Thomas L. Gilmer for the opportunity of making cultures from the case of gingivitis and to Dr. Boughton and Dr. Hayhurst for assistance in obtaining material from the case of noma.

the first days of growth. Filaments of various lengths may appear in 24–48 hours or later. They are found most often in cultures on Loeffler's blood-serum. As most of these cultures were made on blood-agar the usual absence of filaments may be accounted for. When filaments are present they can often be seen to be made up of strings of bacilli. They frequently contain deeply staining bodies, sometimes round, more often like bands. Vacuoles are sometimes seen in the filaments.

Occasionally on the first day of growth, but usually a few days later, spirals with from one to twenty curves are observed, sometimes in large numbers. As a rule they stain uniformly, but often it can be seen that they too are made up of rods. The spirilla sometimes contain vacuoles similar to those seen in the spirilla in smears made from the tissues of Vincent's angina and noma. On the sodium citrate and blood-agar and human serum-agar the spirilla appear shorter and more curved than on the other media employed. Involution forms in a great variety of shapes are seen in some of the older cultures.

Sometimes for several generations fusiform bacilli only will be found in the cultures, but on changing the medium, filaments and spirilla will again be formed.

The organisms stain best with heated one per cent solution of alcoholic gentian-violet in five per cent carbolic acid solution. The spirilla appear thicker when so stained than the spirilla seen in smears from lesions which have been first fixed with heat and then stained. However, when such specimens are stained deeply by the above method, the spirilla often appear just as thick as those artificially cultivated. The spirilla may also be seen to good advantage in India ink preparations. The organisms do not stain by Gram's method.

Animal experiments have been unsuccessful. Large numbers of bacteria were injected, the growth on four and five slants being used. Injections were made subcutaneously, intraperitoneally, intravenously, and into the mucous membrane of the mouth. The animals experimented with were the dog, guinea-pig, rabbit, white rat, and pigeon.

Efforts to first lower the resistance of the animal by injections

of bichloride of mercury, lactic acid, and large numbers of killed streptococci were without any effect.

FIG. 1.—Pure culture of fusiform bacilli from noma, grown 48 hours anaerobically on goat's blood-agar. Carbol-gentian-violet. ×1,000.

FIG. 2.—Pure culture of fusiform bacilli from noma, grown anaerobically on goat's blood-agar. Carbol-gentian-violet. ×750.

FIG. 3.—Pure culture of fusiform bacilli from noma, grown 10 days anaerobically on human serum-agar. Carbol-gentian-violet. ×1,000.

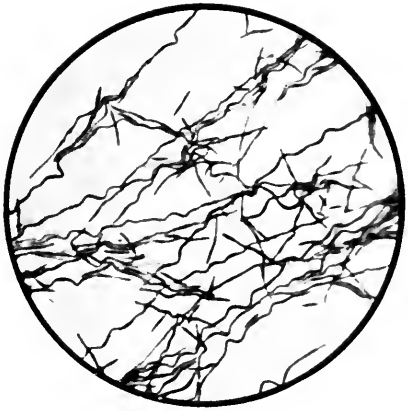

FIG. 4.—Pure culture of fusiform bacilli from the normal mouth, grown 48 hours anaerobically in 0.25 per cent glucose broth. Carbol-gentian-violet. ×1,000.

Sterile animal tissues from the dog and guinea-pig were also inoculated, but no growth of the organism occurred. It is possible that the length of time elapsing before the growth of the organisms (3–5 days) and the time required to isolate them in pure culture, generally several weeks, may account for their lack of virulency.

The case of noma from which a pure culture of fusiform bacilli and spirilla was isolated developed in a man 50 years old. The necrosis started in an ulcer on the cheek adjacent to two irregular teeth. When the patient was first seen there was great swelling and induration of the cheek, which had been perforated by the attending surgeon. There was considerable discharge from the opening in the cheek. The mucous membrane of the mouth was sloughing off and the odor was very offensive. Fusiform bacilli, filaments, and spirilla were the predominating organisms in the smears made from the gangrenous material.

The patient's opsonic index was 0.4 to a fusiform bacillus isolated from a case of gingivitis. On account of the low opsonic index, subcutaneous injections were made of fusiform bacilli, killed by heating for an hour at 60° C. These organisms were taken up quite freely by the leukocytes without the presence of serum, but not to such a degree as to interfere greatly with the estimation of the opsonic index. This strain differed in this respect from those previously described,[1] which were as readily phagocytable without as with serum, possibly because of long cultivation outside of the body before being tested.

The injections of killed fusiform bacilli and spirilla did not cause any local reaction at the site of inoculation. Following each injection there was an increase in the amount of opsonin as shown in the chart. At one time there seemed to be considerable improvement in the face, much of the swelling and induration disappearing, but it now developed that a carcinoma of the face had started, from which the patient soon died.

The cheek tissue removed during life showed carcinomatous tissue adjacent but distinct from the gangrenous tissue. This showed complete necrosis of the superficial skin with a line of demarcation separating it from the living tissue. In the superficial necrotic tissue were found cocci, fusiform bacilli, filaments, and spirilla, the last three organisms predominating. In the region of leukocytic invasion the fusiform bacilli, filaments, and spirilla alone were observed. A thick network of filaments, some being wavy, was seen outside the line of demarcation. The organisms

[1] *Jour. Infect. Dis.*, 1907, 4, p. 66.

could not be found invading the healthy tissue. The pure cultures of fusiform bacilli and spirilla isolated from this patient closely

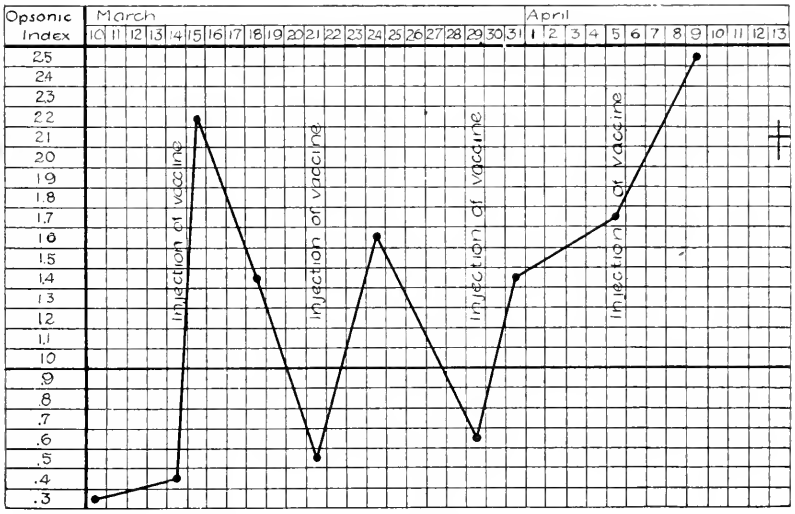

CHART 1.—Opsonic curve in case of noma injected with killed culture of fusiform bacilli and spirilla.

March 15.—Growth on one blood-agar slant injected subcutaneously.
March 21.—Growth on one and one-half blood-agar slants injected subcutaneously.
March 29.—Growth on two blood-agar slants injected subcutaneously.
April 5.—Growth on one and one-half blood-agar slants injected subcutaneously.

resembled the organisms in the tissues and in smear preparations from the necrotic tissue.

CONCLUSIONS.

The strains of fusiform bacilli isolated in pure culture from the normal mouth, ulceromembranous angina and gingivitis, and noma appear culturally and morphologically to be the same organism. The cultures show that the bacilli and the spirilla are different forms of one organism. Whether the spirilla formed from the fusiform bacilli are the same as those found in the lesions themselves cannot be decided on account of the inability to reproduce the lesions in lower animals. The injection of killed cultures of fusiform bacilli and spirilla in the case of noma caused an increase in the amount of specific opsonin and appeared to produce some beneficial effect.

TRANSMISSION OF SPOTTED FEVER BY OTHER THAN MONTANA AND IDAHO TICKS.*†

MARIA B. MAYER.

(From the Pathological Laboratory of the University of Chicago.)

Experiments having shown that the mild Idaho type of spotted fever may be transmitted by *D. venustus* of Montana and that the more virulent northern type of fever may be carried by *D. modestus* of Idaho, it became a matter of interest to determine whether ticks of other species, indigenous in widely separated areas, might also be capable of acting as agents of transfer of the spotted fever virus. In order to carry out experiments on this point arrangements were made with several entomologists for supplies of wood ticks from their localities. Ticks were received from Maine, California, Texas, Utah, and Missouri. The study of these ticks as intermediate hosts for the parasite of spotted fever was taken up according to the same plan as that used in studying the transmission of the virus by the Idaho and Montana ticks.

Two strains of the diseases were maintained in guinea-pigs at the Chicago laboratory at this time—the "Bradley" strain, which had arrived at the 179th consecutive passage from the human virus, and a so-called "natural tick strain," also in guinea-pigs in its 8th passage, produced by the bite of *D. venustus* (Montana) from nature. Both strains were used in these experiments.

1. *Transmission by Dermacentor variabilis* (Say).—Specimens of this tick have been found from Labrador to Florida. West of the Mississippi it is not common. It has been found on a great variety of small animals, more frequently on cattle and dogs, and, occasionally, on man.

During the month of July, 1909, two collections of this species were received from Dr. Ricketts, having been collected by him in the vicinity of Woods Hole, Mass. Fifty adult ticks, the females in various stages of engorgement, comprised these groups.

* Received for publication January 5, 1911.

† This work was supported by a grant from the Memorial Institute for Infectious Diseases, Chicago.

Guinea-pig 2,266, 4th passage "natural tick strain," after an incubation period of four days, was placed in the stock, with a temperature of 104° F. On the fifth day after inoculation, one male—No. 1—and one female—No. 5—tick were attached to this guinea-pig for 48 hours during the period of fever. This animal died on the eighth day of the disease, the autopsy showing specific spotted fever lesions with the hemorrhagic vulva. The impregnated female tick became much enlarged and dropped off semi-mature after two days' feeding. Any further test of this tick was impossible owing to the fact that semi-mature females rarely become reattached. The female was placed in a pill box for oviposition and a later test of her larvae for inherited infection was made.

The male tick which had fed on guinea-pig 2,266 was placed on normal guinea-pig 2,284 and remained attached for 72 hours; the temperature of this guinea-pig remained normal during a period of 18 days following attachment. The tick was found off in the sack, reattached, and removed four days later, in all a period of seven days' attachment. An immunity test given this pig with 1 c.c. of "Bradley" virus developed a characteristic case of the disease. This male tick did not transmit the disease.

Guinea-pig 1,840 of the 197th "Bradley" strain passage was taken on the third day of fever with a temperature of 105.6° F. Two female ticks—Nos. 8 and 9—attached themselves behind the ears of the pig after a short period. This guinea-pig died four days later with the engorged ticks still attached.

The female tick 8 was immediately transferred to normal guinea-pig 2,321, and remained in the same location on this pig for six days. No disease developed in this guinea-pig, and on being given 1 c.c. of "Bradley" virus it died of spotted fever in eight days. For further maturation and impregnation of female No. 8, male No. 1 of this group was placed with her. Oviposition began eight days later. Examinations of the stained specimens of these fresh eggs were made for bacilli, but no organisms were found. Examinations of eggs from normally developed ticks were also negative. Fifty fresh eggs from female No. 8 were crushed in physiological salt solution, and injected into a guinea-pig intraperitoneally, but with no production of symptoms. About 600 larvae were hatched

from the eggs. These larvae matured on a normal guinea-pig without infecting it. 26 enlarged larvae being collected from this pig. After their normal period of moulting, the nymphs were again tested on a normal pig. In no period of their development did they transfer the disease. Seven of the eleven females matured and deposited eggs, hatching large numbers of larvae.

From female 5 about 300 larvae were placed with infected guinea-pig 2,375 of the 189th "Bradley" passage; 35 larvae fed on this pig. Five infected guinea-pigs were placed in this sack and died of the disease during the feeding of these larvae. Fourteen nymphs and 19 enlarged larvae were collected. Normal guinea-pig 2,536 was infected by this batch of nymphs. The temperature of the pig ran 103°, 105.6°, 105.6°, 104.5°, and it died on the fifth day. On the third day 3 c.c. of its blood was withdrawn and inoculated into a normal and an immune guinea-pig of the natural tick strain. The immune pig did not respond to the virus. The normal died on the sixth day of fever with specific spotted fever lesions. Again blood was transferred from this guinea-pig to immune and normal pigs with the same result; the normal guinea-pig died on the eighth day, the immune showed no indication of disease.

The larvae from five females were associated with infected guinea-pigs 2,371, 2,436, and 2,482 and 2,311 of the "Bradley" strain. These pigs died of the disease, one after the other, while the larvae were feeding. A second test of these nymphs was made on normal guinea-pig 2,563. Two of the above larvae fed on this pig. On the seventh day after attachment a fever temperature began. The pig died on the eighth day. Two passages of blood were made to normal pigs and to immune pigs. The normal pigs developed spotted fever; the immune pigs were unaffected.

Two adults ticks from this group produced infection in normal guinea-pig 2,873. This pig died on the eighth day after tick attachment; transfer of blood to normal pig 2,915 produced a less severe course of fever, which conferred immunity to the "Bradley" virus.

2. *Dermacentor marginatus Utah* (Banks).—Note received with these specimens: Dallas, Sub. acc. No. 654, seed ticks hatched from eggs deposited August 14, and days following. Hatching began

September 3, adult females taken from jack rabbit, August 9, 1909, at Milford, Utah, by W. V. King.

The larvae were placed with normal and infected guinea-pigs in equal numbers. Eight nymphs matured on the normal animal. As a routine this pig was given an immunity test with "Bradley" blood. This resulted in the usual course of fever. Five infected guinea pigs were used to mature and infect the other half of these larvae. After a period of moulting four of these nymphs were allowed to feed on normal guinea-pig 2,567. On the seventh day after attachment a fever began, resulting in death on the fifth day. Passage of the heart's blood of this pig into an immune and a normal pig gave no results in the immune animal but produced typical spotted fever in the normal. A second passage was made with the same result.

3. *Amblyomma Americanum Linnaeus.*—This species is commonly known as the "lone star tick" because of the single yellow spot on the shield of the female. These specimens were received from Missouri in all stages of adult engorgement. The many attempts made to attach these ticks to guinea-pigs were unsuccessful. Nine of the number were selected for oviposition, which was evidently premature in every case. The ticks were small and comparatively few eggs were deposited. Hatching took place from the eggs of females 1, 2, 8, and 9, with about 1,200 larvae in all. Two groups were made of these larvae, about 600 in each, and placed with normal and infected guinea-pigs.

From two pigs, 2,410, 2,426, infected with the "natural tick strain" 100 larvae fed and were collected later from the débris in the sack. These enlarged larvae developed in two weeks into nymphs, passing through a moulting stage. These nymphs were associated with normal guinea-pig 2,544 for a period of two weeks and eleven attachments noted before fever temperature with the characteristic hemorrhagic scrotum developed. The pig was bled from the heart and died on the fifth day. Two passages of blood from this infected pig to normal and immune pigs were made, both resulting as before in infection of the normals and no disease in the immunes. From the 14 infected nymphs, four females and two males were attached to normal guinea-pig 2,874. Three days

later the disease was indicated in this pig by a temperature of 104°, reaching 106.8°, and the development of hemorrhagic genitalia. Death resulted after a slight decline in temperature on 13th day. The ticks were removed after the death of the pig and immediately transferred to another pig. This pig died on the tenth day, after a severe run of fever, with necrosis of the ears and scrotum. The autopsy in each case showed typical spotted fever lesions.

4 and 5.—A large number of the larvae of the species *D. albipictus* were dead when they reached the laboratory. About 50 of the living ones were placed with infected guinea-pig 2,391. Only one of these was found mature and died before further test could be made. The larvae of the *D. variabilis* (Utah) were in poor condition when received. An effort made to keep them alive on a normal pig failed.

6. *Ornithodoros megnini Duges* (California) was a very interesting specimen. Much time was given to the work of attaching these ticks to rabbits, without success. After a latent period of five months, oviposition took place, and a great number of larvae hatched out. When placed with an infected guinea-pig they immediately filled the ears and hung in bunches about the face of the pig. The pig died in this condition. The ears were cut off in the hope that these larvae would detach themselves later. This did not prove the case, and the whole mass died attached.

SUMMARY.

Ticks from six different localities were received, and of these three groups developed so that complete tests could be made, and by means of each of these three, spotted fever was transmitted from infected to normal guinea-pigs. In the case of *Dermacentor marginatus* (Utah) and *Amblyomma Americanum Linnaeus* (Missouri) the transfer was made with nymphs; no adult ticks were tested. In the case of *Dermacentor variabilis* (Mass.) transmission of the virus was effected by nymphs and later by adult ticks.

From these experiments it appears that in so far as ability of ticks from various regions to transmit the virus of spotted fever is concerned, the disease might find favorable conditions for its existence in localities other than those to which it now is limited.

TRANSMISSION OF SPOTTED FEVER BY THE TICK IN NATURE.*†

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The discovery of ticks in nature carrying the virus of spotted fever is published by Dr. Ricketts,[1] who points out the importance of this fact in connection with the theory of transmission of spotted fever by the tick.

The following experiments were made in order to throw further light on the natural infectivity of the tick.

Six hundred and fifty-six ticks were collected during the spring months of 1909: 254 of the species *D. modestus* and 402 *D. venustus*, for the purpose of studying the infectivity of the tick in nature. These ticks were found on cows, bushes, and vegetation in the Lo Lo Valley and Owl Canon, Montana, and in the vicinity of Pocatello, Idaho.

Fourteen groups were arranged, consisting of 25 male and 25 female ticks each. Each group was placed in a new tick-proof sack, with a selected guinea-pig of medium size; nine sacks of Montana ticks (*D. venustus*) and five sacks of Idaho ticks (*D. modestus*) were arranged in this way. The daily temperature of each pig was taken and the number of ticks attached noted.

In Groups IV and V the guinea-pig became infected. The ticks in both groups were gathered in the Lo Lo Valley from cows.

Experiment IV.—Normal guinea-pig 2,155 was the host of Group IV. For two weeks this pig kept up a vigorous scratching. The ticks were gathered daily and placed among the hair. Many were attached on the fourth day. On the sixth day two engorged females were found. On the 14th day the first indication of a disease was noted in a temperature of 104°, followed on the next two days by a rise to 106°, with hemorrhagic vulva; 1½ c.c. of blood was with-

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† This work was supported by a grant from the Memorial Institute for Infectious Diseases, Chicago.

[1] *Jour. Am. Med. Assn.*, 1907, 49, p. 24.

drawn from the heart, and the pig died two days later, with lesions characteristic of spotted fever.

The heart's blood taken from the pig was immediately injected into normal guinea-pig 2,203, producing a typical fever curve with extensive vulvar changes, and death on the 13th day after injection.

This pig was also bled on the fourth day of fever and the blood passed into two normal animals. Of these guinea-pigs, one died of the disease and the other survived and proved immune to 1 c.c. of the virus carried in the "Bradley" strain. It was not possible to determine how many of these ticks transmitted the infection.

Experiment V.—This group of ticks was also gathered in the Lo Lo Valley from cows. These ticks immediately attacked normal guinea-pig 2,162; 30 males and females were found attached to the abdomen inside of 72 hours. On the fourth and succeeding days the temperature range was 104.8°, 105.6°, 105.4°, 97°, death resulting on the eighth day. No external lesions were observed. The spleen and inguinal glands were greatly enlarged. Blood was transferred to normal guinea-pigs 2,181 and 2,185 and both died of spotted fever. Blood from guinea-pig 2,185 was injected into two normal pigs. One of these pigs died of spotted fever, the other survived a typical and severe attack of the disease. A subsequent immunity test with "Bradley" passage blood proved that this pig had become immune. A "passage strain," using two pigs for each passage, was started with virus drawn from this guinea-pig at the height of the disease. Fifteen pigs in the first eight passages died with lesions typical of spotted fever. A survivor of the ninth passage proved immune to a known active virus. During the following months 49 passages of this strain were made. Of the 98 guinea-pigs infected in this manner, in 49 the disease was allowed to run its course without interference; of this latter group, 8 pigs recovered from the disease, and were thereafter immune to the active virus which had been obtained originally from a patient sick with spotted fever. In more than one-half of all these pigs hemorrhagic lesions were noted.

To render this series of experiments somewhat more complete, normal Montana ticks 190, 191, 192, 193, and 195 were allowed to feed on guinea-pigs infected with this so-called "natural tick

strain." Ticks 191 and 192 became infected and transferred the disease to normal guinea-pigs 2,394 and 2,398, thus completing a tick cycle of this strain.

Five experiments were attempted with 254 ticks gathered in the vicinity of Pocatello, Idaho. Groups of 50 ticks were associated with normal guinea-pigs. In each case a short period of low temperature was noted. In experiments III and IV passage was made to normal guinea-pigs on the third day of the fever. No disease resulted and these pigs were not immune to the "Bradley" strain. In the remaining three experiments of the group no conclusive evidence of a specific infection by the Idaho ticks was obtained.

COMPLEMENT DEVIATION IN ROCKY MOUNTAIN SPOTTED FEVER.*

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The experiments here described have been made at various times in this laboratory, the original object being to provide a basis for an early diagnostic test for Rocky Mountain spotted fever.

The first experiments were carried out by Liborio Gomez at the suggestion of Dr. Ricketts and we shall give a brief outline of his unpublished results. Gomez used an ox-rabbit system, using the serum from infected guinea-pigs as antigen (drawn during second to fourth day of fever) and immune serum from guinea-pigs and rabbits as antibody. He first determined that the serum containing the antigen and the serum containing the antibody possessed no hemolytic amboceptors for ox corpuscles. His experiments then followed the method of Bordet-Gengou. A positive deviation was obtained in the case of one guinea-pig which had recovered from the disease and which had received an immunity test some ten weeks before being killed. In a recovered rabbit he also obtained a positive though weak fixation of the complement. In two guinea-pigs killed four or five months after recovery from a single attack of spotted fever Gomez found a retardation of hemolysis of from two to five hours, as compared with controls of immune serum and virus alone.

In his experiments with the immune guinea-pig serum Gomez left the tubes containing the complement-antibody-antigen mixture in the incubator for over 14 hours before adding the hemolytic system. This period of time is long and leaves some doubt as to the value of the result obtained. Gomez notes that the smaller doses of antigen-antibody recommended by Bordet and Gengou failed to give results. In his experiments with the blood of a recovered rabbit he established a binding with 0.6 c.c. of the serum. Gomez notes, however, that large doses of antibody (1–2 c.c.) will,

* Received for publication October 1, 1910.

when mixed with complement, bind or destroy its action without the presence of antigen.

With the idea of further testing this reaction, Dr. Ricketts and Mr. R. M. Wilder made several experiments, using the eggs of infected ticks as antigen. It has already been shown that the eggs of infected ticks are rich in an organism which is supposedly the cause of Rocky Mountain spotted fever and it was supposed that these eggs would therefore afford a suitable source of antigen. In these experiments a goat-rabbit hemolytic serum was used and the exact dosage of fresh, normal guinea-pig complement was fixed for each experiment.

In the first three experiments of the series eggs were used in quantities varying from 1 to 100, the antibody being varied from 0.05 to 0.1 c.c. As an example of these experiments we insert Table 1.

From these experiments it will be noted in Group A (Table 1) that there is a slight decrease in hemolysis as the number of eggs is increased. In Group B with a larger amount of antibody the same result is noted, although the total amount of hemolysis is greater in each corresponding tube than in Group A. The degree of hemolysis is not dependent on the number of eggs, since Group C shows a constant amount of hemolysis in each tube. Evidently there was some reactivation[1] of the heated serum by the unheated, and this reactivation was greater when the amount of heated complement in the antibody (guinea-pig serum) was greater. This was borne out by the results in tubes 17 and 18 in which a large amount of antibody increased the hemolytic action of 0.01 c.c. of complement. In this experiment the total amount of complement used was not quite sufficient to cause complete lysis, as shown in tube 13.

In the second experiment of Ricketts' series larger numbers of eggs were used (75–100); the results were again inconclusive. It was found that the eggs alone, without the addition of antibody, were capable of preventing lysis. It was again noted that antibody and complement, when together, were more hemolytic than com-

[1] That such an activation takes place was shown in an experiment in which we first established the minimum dosage of normal complement and then added varying amounts of antibody to a subminimal dose of complement. Doses of inactivated antibody from 0.01 c.c. up caused complete laking in the tubes, although alone the antibody had absolutely no effect.

plement alone, and it was assumed that the antibody contained hemolytic amboceptors which were activated by the complement. An effort was made in the third experiment to absorb the hemolytic amboceptor supposed to be present in the antibody, but the results

TABLE 1.

COMPLEMENT DEVIATION IN SPOTTED FEVER.

Groups	Sensitized Corpuscles	Antibody	Antigen (Eggs)	Complement	Results
Group A					
1	0.5	0.05	1	0.01	Marked hemolysis (75 per cent)
2	0.5	0.05	10	0.01	Marked hemolysis (less than tube 1)
3	0.5	0.05	20	0.01	Marked hemolysis (less than tube 1)
4	0.5	0.05	50	0.01	Moderate (less than 2 or 3)
Group B					
5	0.5	0.1	1	0.01	Complete
6	0.5	0.1	10	0.01	Complete
7	0.5	0.1	20	0.01	Complete
8	0.5	0.1	50	0.01	Almost complete
Group C					
9	0.5	0	1	0.01	Moderate
10	0.5	0	10	0.01	Moderate
11	0.5	0	20	0.01	Moderate
12	0.5	0	50	0.01	Moderate
Group D					
13	0.5	0	0	0.01	Marked hemolysis (75 per cent)
14	0.5	0	0	0	0
Group E					
15	0.5	0.05	0	0	0
16	0.5	0.1	0	0	0
Group F					
17	0.5	0.05	0	0.01	Almost complete
18	0.5	0.1	0	0.01	Complete
Group G					
19	0	0	0	0.01	0 (solution clear)
20	Unsensitized	0	0	0.01	0

The following materials were used: Sensitized erythrocytes from goat, washed, in 5 per cent suspension, 1 day old; complement—fresh, normal guinea-pig serum; antibody—inactivated serum of guinea-pig 2,537, recently recovered from spotted fever; antigen—eggs of *D. Modestus* (56 [57], 11, F. 4), 10 days old, rich in bacilli.

The goat corpuscles were sensitized by adding 0.8 c.c. of immune rabbit serum to 20 c.c. of a 5 per cent suspension, incubating 20 minutes and washing once. This was done in order to avoid the necessity of making separate measurements of corpuscles and of the hemolytic serum.

Antigen+antibody+complement was allowed to incubate 20 minutes (1 hr. in Experiment 3) in a total volume of 0.3 c.c.; 5 c.c. suspension of corpuscles was then added and the total volume raised to 2 c.c. by the addition of normal salt solution. The mixture thus prepared was incubated 2 hours and then placed on ice over night.

were negative. In a fourth experiment dried serum of an immune horse was dissolved and used as an antibody, giving a fairly strong deviation with 0.2 c.c., but it was found on further tests that 0.4 c.c. of this serum alone would bind all the complement. This was possibly due to the presence of an anti-complement in the serum of

the horse, it having been actively immunized by the injection of virulent guinea-pig blood.

We have repeated these experiments with tick eggs, crushing a given number of them (100) in sterile salt solution and then using decreasing dilutions of this emulsion. Three series of eggs were used: (1) eggs from infected ticks (showing large numbers of organisms); (2) eggs from normal ticks; and (3) eggs from ticks raised on immune pigs. The three series, however, showed complete lysis. It is true that no lysis was obtained in the 100 per cent emulsion, but it was found that tubes without antibodies would give the same result. In these experiments the antigen-antibody-complement mixture was permitted to incubate as long as $3–3\frac{1}{2}$ hours.

All the results so far obtained, while showing some deviation, were not as clear-cut as might be expected in a disease like spotted fever in which the serum of recovered animals possesses such a marked protective power.[1] We therefore undertook the testing of various tissue extracts and of sera taken from animals during fever, in the hope of finding an efficient antigen. The inactivated serum of recovered guinea-pigs served as antibody. An ox-rabbit hemolytic system was used, the corpuscles being first sensitized, and the exact dosage of complement established for each experiment. In the case of various bacterial suspensions used as antigens, a standardized loop of a 24-hour agar culture was always made up in 1 c.c. of normal salt solution.

In one experiment varying amounts of a watery extract of macerated lymph glands, spleen, and testicle of a guinea-pig which had died of spotted fever were used as antigen, with varying amounts of antibody (0.025 to 0.1 c.c.), but there was no deviation.[2] This negative result led us to investigate whether the antibody was thermostable or whether it had been destroyed by the heating at 56° C. We found that both fresh and heated immune sera protected equally well. Evidently the difficulty remained in the antigens. We therefore made separate extracts

[1] Ricketts and Gomez, *Jour. Infect. Dis.*, 1908, 5, pp. 221–44.

[2] It may be stated here that the most marked internal changes during a course of spotted fever in the guinea-pig occur in the lymph glands, testicle, and spleen. Large numbers of bodies similar in appearance to the bacillus found in the tick eggs can be seen in the spleen, lymph glands, and liver when sections are stained with Giemsa (Dr. Maria B. Maver). The organism must also be present, of course, in the blood, which is highly virulent, but it has not been seen equally well here in stained preparations.

from the macerated material of infected pigs, using serum, lungs, liver, spleen and lymph glands, kidneys, muscles, brain, and intestine. Varying quantities were used (0.1 c.c. to 0.5 c.c.), with a constant amount of immune guinea-pig serum. The organ extracts were not hemolytic. The results of the experiment were again negative. In connection with these experiments a series was made with a suspension of the culture of the "Spaniard" organism[1] as antigen and the serum of guinea-pigs recovered from infection with it and from Rocky Mountain spotted fever as antibody.

As will be seen from Table 2 complete binding of the complement was obtained with both antibodies. While this was to be ex-

[1] The "Spaniard" organism, culturally, morphologically, and immunologically, seems to be practically identical with a stock culture of *B. cholera suis* obtained from the Department of Bacteriology of the University of Chicago. It crept into the spotted fever passage virus derived from the patient "Spaniard" and gradually replaced the spotted fever virus. Whether it actually outgrew and "smothered" the virus is not known. The "Spaniard" patient was suffering from the Idaho spotted fever—a strain which on three previous occasions it had been impossible to keep going by passage through the guinea-pig—so that the adventitious infection may not have been responsible for the "dying out" of the spotted fever virus. By proper attention to dosage, the infection produced in the guinea-pig by the intraperitoneal injection of the "Spaniard" organism can be made to parallel very closely in some respects a case of spotted fever. There is the same three days' incubation period, the subsequent high fever, and death in about 10 days. Post mortem the enlarged, hemorrhagic lymph glands and swollen spleen are conspicuous. The gangrenous ears and scrotum, the cutaneous eruptions, and the hemorrhages in the region of the pampiniform plexus so common in spotted fever are wanting, while a fibrinous exudate in the peritoneal cavity, never found in the latter disease, is always present. In non-fatal cases the temperature of spotted fever pigs returns to normal in about 10 days and there remains; in non-fatal infections with the "Spaniard" organism, a low fever may persist for 20 days to a month with occasional exacerbations. Cultures from all spotted fever cases remain sterile; the specific bacilli may always be cultivated from the heart's blood, peritoneal exudate, and spleen of pigs dying from infection with the "Spaniard" organism. Finally, recovery from spotted fever does not confer immunity to the "Spaniard" organism, though a high grade of specific immunity is produced. Both cause a "hemorrhagic septicemia" but the "Spaniard" organism is much less prone to produce hemorrhages than is the spotted fever virus.

It is a striking coincidence that in three diseases produced by micro-organisms which apparently cannot be cultivated, namely yellow fever, Rocky Mountain spotted fever, and hog cholera, and the first two of which at least may be transmitted by insects, cholera-suis-like organisms should have intruded themselves. This fact, coupled with those detailed in this paper on the comparative fixation tests, suggests that there may be something more concerned here than mere coincidence. There may be an actual relationship of some kind between the various causal organisms. With this idea in mind the following preliminary experiment with precipitins is of interest:

The precipitogens consisted of filtered, 24-hour broth cultures of *B. cholera suis*, "Spaniard," *Staph. aureus*, and *B. typhosus;* the precipitins were contained in the unheated sera of spotted fever immune guinea-pigs Nos. 2,658 and 2,381 and of "Spaniard" immune guinea-pigs Nos. 2,342 and 2,669. Precipitates formed in those tubes containing the cholera suis and "Spaniard" filtrates with the spotted fever and "Spaniard" immune sera alike in a serum dilution of 1–20 and not at all in the staphylococcus and typhoid tubes. Control tubes remained clear.

SAMPLE PROTOCOLS.

Precipitogen	Precipitin	NaCl Sol.
0.5 c.c.	0.01 c.c.	1.49 c.c.
0.5 c.c.	0.1 c.c.	1.4 c.c.
0.5 c.c.	0	1.5 c.c.
0	0.1 c.c.	1.9 c.c.

pected in the "Spaniard-Spaniard" combination, the "Spaniard"-spotted fever result was rather unexpected. While these controls had been intended principally as a check on our technic by the addition of known factors, and showed that our method was correct, the result indicated either that the "Spaniard" culture was a specific antigen for Rocky Mountain spotted fever, or stood in a group relationship to the same (we knew that the first supposition was not true) or that the guinea-pigs had at some previous time been infected with the "Spaniard" bacillus and were therefore immune.

TABLE 2.

COMPLEMENT DEVIATION WITH "SPANIARD" BACILLUS AND SPOTTED FEVER AND "SPANIARD" IMMUNE SERUM.

SENSITIZED CORPUSCLES	ANTIBODIES		ANTIGEN CULTURE "SPAN." VI	COMPLEMENT	RESULTS
	Sp. Fever 2,517	"Spaniard" 2,343			
0.5	.....	0.025	0.05	0.01	No hemolysis
0.5	.....	0.025	0.1	0.01	No hemolysis
0.5	.....	0.025	0.25	0.01	No hemolysis
0.5	.....	0.025	0.5	0.01	No hemolysis
0.5	.....	0.05	0.25	0.01	No hemolysis
0.5	.....	0.1	0.25	0.01	No hemolysis
0.5	.....	0.0	0.5	0.01	Complete
0.5	.....	0.1	0	0.01	Complete
0.5	0.025	.....	0.05	0.01	No hemolysis
0.5	0.025	.....	0.1	0.01	No hemolysis
0.5	0.025	.....	0.25	0.01	No hemolysis
0.5	0.025	.....	0.5	0.01	No hemolysis
0.5	0.05	.....	0.25	0.01	No hemolysis
0.5	0.1	.....	0.25	0.01	No hemolysis

Tubes incubated for 30" and corpuscles then added and tubes made up to 2 c.c. with normal salt solution.

Incubated 1 hr. 30" and then placed at room temperature over night.

This experiment was repeated using the same antibodies and fresh suspensions of "Spaniard," typhoid bacilli, and *Staphylococcus aureus* as antigens. The results with the typhoid bacilli and staphylococci were absolutely negative but there was deviation as in the previous experiment with the "Spaniard" organism.

The effect of large doses of antibody causing an increase of hemolysis, as noted in the Ricketts experiments already quoted, was observed.

Certain preliminary experiments indicated that the bacillus of hog cholera, when used as an antigen with Rocky Mountain spotted fever and "Spaniard" immune sera as the antibodies, would give

evidence of binding the complement. A quantitative experiment was performed in order to compare this binding with the results obtained using the "Spaniard" culture as antigen. These results are shown in Table 3. As will be seen in Series A (spotted fever-"Spaniard"), there is first a progressive decrease in the amount of

TABLE 3.

GROUPS	EXPERIMENT 12 SENSITIZED CORPUSCLES	ANTIBODIES		ANTIGENS		COMPLEMENT	RESULTS
		Sp. Fever 2,381	"Spaniard" 2,342	Span. VI	B. cholera suis		
Group A							
1	0.5	0.005	.....	.05	....	0.02	Incomplete laking
2	0.5	0.0075	.....	.05	....	0.02	Incomplete laking
3	0.5	0.01	.....	.05	....	0.02	Trace of laking
4	0.5	0.02	.....	.05	....	0.02	Trace of laking
5	0.5	0.03	.....	.05	....	0.02	Trace of laking
6	0.5	0.04	.....	.05	....	0.02	0
7	0.5	0.05	.....	.05	....	0.02	Slight laking
8	0.5	0.1	.....	.05	....	0.02	Moderate laking
Group B							
9	0.5	0.005	.....	...	0.05	0.02	Incomplete laking
10	0.5	0.0075	.....	...	0.05	0.02	Incomplete laking
11	0.5	0.01	.....	...	0.05	0.02	Incomplete laking
12	0.5	0.02	.....	...	0.05	0.02	Trace of laking
13	0.5	0.03	.....	...	0.05	0.02	Trace of laking
14	0.5	0.04	.....	...	0.05	0.02	Least laking
15	0.5	0.05	.....	...	0.05	0.02	Slightly more laking
16	0.5	0.1	.....	...	0.05	0.02	Slightly more laking
Group C							
17	0.5	0	0.005	.05	....	0.02	Incomplete laking
18	0.5	.....	0.0075	.05	....	0.02	Slight laking
19	0.5	.....	0.01	.05	....	0.02	Slight laking
20	0.5	.....	0.02	.05	....	0.02	0
21	0.5	.....	0.03	.05	....	0.02	Complete laking
22	0.5	.....	0.04	.05	....	0.02	Complete laking
23	0.5	.....	0.05	.05	....	0.02	Complete laking
24	0.5	.....	0.1	.05	....	0.02	Complete laking
Group D							
25	0.5	.....	0.005	...	0.05	0.02	Complete laking
26	0.5	.....	0.0075	...	0.05	0.02	Complete laking
27	0.5	.....	0.01	...	0.05	0.02	Complete laking
28	0.5	.....	0.02	...	0.05	0.02	Complete laking
29	0.5	.....	0.03	...	0.05	0.02	Complete laking
30	0.5	.....	0.04	...	0.05	0.02	Complete laking
31	0.5	.....	0.05	...	0.05	0.02	Complete laking
32	0.5	.....	0.1	...	0.05	0.02	Complete laking

laking reaching a maximum in tube 6 in which we find complete binding and there follows increased laking in tubes 7 and 8. Series B (spotted fever+hog cholera) shows a similar reaction, though the best tube still shows a trace of laking. In Series C ("Spaniard"+ "Spaniard") we find a marked deviation in the first four tubes (90 per cent to 100 per cent) and complete laking in the last four tubes. Series D, on the other hand ("Spaniard"-hog cholera), gave complete laking. This experiment was repeated twice, using sera from different immune pigs, and similar results were obtained with the

exception that now the "Spaniard"-hog cholera combination gave the reverse of the result in Experiment 4, in which we found a negative reaction. Further experiments have confirmed these results, and also seem to show (1) that the serum of normal guinea-pigs does not contain the specific antibody; (2) that the spotted fever immune serum causes no deviation in the presence of typhoid, anthrax, dysentery (Shiga), and Friedländer bacilli, staphylococci, and cholera germs, although it has a specific affinity for certain strains of bacteria of the hog cholera group. The spotted fever antibody, in certain proportions, will bind complement with the hog cholera and "Spaniard" bacilli, and the "Spaniard" immune serum will bind with the same bacilli. We determined, however, that pigs immune to Rocky Mountain spotted fever are not protected against the "Spaniard" organism.

We have failed to find a parallelism between the bacteriolytic power of these immune sera on hog cholera bacilli and the "Spaniard" bacillus, as determined by the Neisser-Wechsberg technic, and their power to deviate complement. That is, the bactericidal power of normal sera and of "Spaniard" immune sera was almost absolutely identical, while only the immune sera supplied the amboceptor necessary for the deviation of the complement. Apparently the presence of substances in sera which cause a fixation or deviation of complement need not imply the presence of bacteriolytic or protective antibodies. A somewhat similar phenomenon has been observed by Torrey and others.[1]

CONCLUSIONS.

Positive deviation of complement, using spotted fever antigen and antibody, has been obtained in but one instance, and the results of experiments undertaken to confirm this positive finding have been negative.

The negative results are probably to be explained by the small quantities of organisms present in any one preparation of antigen and by the admixture of various substances in the antigens which in themselves absorb or neutralize complement, this in turn pro-

[1] Torrey, *Jour. Med. Res.*, 1910, 22, p. 95.

hibiting the use of the large doses of antigen which might otherwise be effective.

The protective power of spotted fever immune serum is not destroyed by heating to 56° C. for 30 minutes.

An apparent reactivation of heated serum (antibody) was met with in many cases which, by causing hemolysis, tended to mask results. This might become a source of error in work of this kind.

An organism probably identical with the hog cholera bacillus replaced the spotted fever virus in one of the passages. The serum of guinea-pigs immune to spotted fever and of guinea-pigs immune to this organism deviated the complement alike when a suspension of the above bacterium was used as antigen. This fact, together with other considerations mentioned in this paper, suggests that there may be a definite relationship of some kind between the virus of Rocky Mountain spotted fever and the hog cholera bacillus.

Our results suggest, further, that the presence of substances in sera which cause a complement-fixation need not imply the presence of bactericidal or protective antibodies.

TIME RELATIONSHIPS OF THE WOOD-TICK IN THE TRANSMISSION OF ROCKY MOUNTAIN SPOTTED FEVER.*

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(From the Pathological Laboratory of the University of Chicago.)

The chief object of the following experiments, undertaken at the suggestion of the late Dr. Howard T. Ricketts, was to determine (1) the minimum duration of feeding by infected tick necessary to infect the guinea-pig; (2) the minimum duration required for the infection of the tick from the infected guinea-pig; and (3) the length of the incubation period in the tick, that is, the period of time required for the etiological factor to establish itself in the tick and render it infective.

Duration of feeding by infected tick necessary to infect the guinea-pig.—All the ticks used in this experiment were bred in the laboratory in tick-proof cages. They were of two species, *Dermacentor venustus* and *Dermacentor modestus*. The parents of the former were the second generation of that species raised in the laboratory, the original ancestors having been procured from the Bitter Root Valley in Montana in the spring of 1907. These had been infected in the usual manner with the Montana strain of fever and had transmitted this from generation to generation up to the present time.[1] The parents of the latter species (*Dermacentor modestus*) were collected in Idaho in the spring of 1909 and these also were infected with the Montana strain.

The method was as follows: In the group of experiments in Table 1, the tick was first permitted to feed for five or ten days on a normal guinea-pig to test its infectivity. After making sure that it was infected, the tick was removed and kept at room temperature for a variable period; then fed on a normal guinea-pig for a certain number of hours; again removed and kept at room temperature. The feedings were repeated at variable intervals on new guinea-pigs until the tick died.

* Received for publication October 1, 1910.

[1] Ricketts, H. T., *Jour. Am. Med. Assn.*, 1907, 49, p. 1278.

TABLE 1.

DURATION OF TICK-FEEDING REQUIRED FOR THE INFECTION OF THE GUINEA-PIG.

Ticks previously tested and proved infective.

Experiment	Tick Species and Number	End of Infectivity Test	Interval	Length of First Test and Date	Result	Interval	Length of Second Test and Date	Result	Interval
I.....	D. modestus F. 90	9/15	3 days	18 hr. 9/18	+	4 days	10 hr. 9/22	+	16 days
II.....	D. venustus M. 196	9/15	7 days	1 hr. 9/22	−	4 days	5 hr. 9/25	−	tick dead 9/27
III.....	D. modestus M. 100	10/5	17 days	10 hr. 10/22	−	4 days	5 hr. 10/26	−	13 days
IV.....	D. modestus F. 87	9/21	43 days	5 hr. 11/3	+	8 days	2½ hr. 11/11	−	19 days
V.....	D. modestus F. 88	9/20	44 days	5 hr. 11/3	+	8 days	1 hr. 11/11	−	19 days
VI.....	D. venustus F. 198	10/25	9 days	5 hr. 11/4	−	26 days	5 hr. 12/1	−	12 days
VII.....	D. venustus F. 199	10/25	9–10 days	5 hr. 11/4	−	18 days	5 hr. 11/22	−	20 days
VIII.....	D. venustus F. 202	11/2	10 days	5 hr. 11/12	−	20 days	5 hr. 12/2	−	tick dead 12/5
IX.....	D. venustus F. 203	11/16	14 days	5 hr. 11/30	−	14 days	15 hr. 12/14	−	tick dead 12/26
X.....	D. venustus M. 204	11/19	13 days	5 hr. 12/2	−	21 days	20 hr. 12/23	−	15 days
XI.....	D. modestus F. 103	10/18	37 days	5 hr. 12/8	−	17 days	10 hr. 12/19	+	tick dead 2/23
XII.....	D. modestus F. 108	11/28	10 days	5 hr. 12/8	−	tick dead 12/12	.....	..	.....
XIII.....	D. modestus F. 106	11/22	25 days	5 hr. 12/17	+	23 days	8 hr. 1/9	+	19 days
XIV.....	D. modestus F. 107	11/28	19 days	15 hr. 12/17	−	20 days	20 hr. discarded 1/10		16 days
XV.....	D. modestus F. 110	11/29	21 days	15 hr. 12/20	+	21 days	10(?) hr. discarded 1/11		20 days
XVI.....	D. modestus F. 105	11/22	31 days	3–5 hr. 12/23	−	15 days	5 hr. 1/7	−	23 days

TABLE 2.

DURATION OF TICK-FEEDING REQUIRED FOR THE INFECTION OF THE GUINEA-PIG.

Ticks not previously tested for infectivity.

Experiment	Tick Species and Number	Date of Moulting	Interval	Duration of First Test and Date	Result	Interval
XVII.....	D. modestus F. 128	Unknown	Unknown	5 hr. 1/17	−	18 days
XVIII.....	D. venustus M. 222	Unknown	Unknown	15 hr. 1/18	−	10 days
XIX.....	D. modestus M. 129	Unknown	Unknown	5 hr. 1/19	−	21 days
XX.....	D. modestus M. 130	Unknown	Unknown	5 hr. 1/19	−	23 days

TABLE 1.

DURATION OF TICK-FEEDING REQUIRED FOR THE INFECTION OF THE GUINEA-PIG.

Ticks previously tested and proved infective.

Length of Third Test and Date	Result	Interval	Length of Fourth Test and Date	Result	Interval	Length of Fifth Test and Date	Result	Interval	Length of Sixth Test and Date	Result	Interval	Length of Seventh Test and Date	Result
1 hr. 45 m. 10/9	+	11 days	6 days 10/19–10/25	+	tick dead 10/30	.....	..	.....	.....	..	.....	....	..
.....	..	.....	.....	..	.....	.....	..	.....	.....	..	.....	....	..
10 hr. 11/9	–	22 days	11 days 12/1–12/12	+	28 days	20 hr. 1/9	+	10 days	10 hr. 10/19	–	.....	....	..
2½ hr. 11/30	–	tick dead 12/5	.....	..	.....	.....	..	.....	.....	..	.....	....	..
2½ hr. 11/30	–	tick dead 12/5	.....	..	.....	.....	..	.....	.....	..	.....	....	..
10 hr. 12/13	–	tick dead 12/16	.....	..	.....	.....	..	.....	.....	..	.....	....	..
10 hr. 12/13	–	tick dead 12/18	.....	..	.....	.....	..	.....	.....	..	.....	....	..
.....	..	.....	.....	..	.....	.....	..	.....	.....	..	.....	....	..
.....	..	.....	.....	..	.....	.....	..	.....	.....	..	.....	....	..
5 hr. 1/7	–	20 days	20 hr. 1/27	+	11 days	10 hr. 2/7	–	14 days	10 hr. 2/21	–	.....	....	..
.....	..	.....	.....	..	.....	.....	..	.....	.....	..	.....	....	..
.....	..	.....	.....	..	.....	.....	..	.....	.....	..	.....	....	..
5 hr. 1/28	–	13 days	5 hr. 2/9	–	tick dead 2/20	.....	..	.....	.....	..	.....	....	..
20 hr. 1/27	+	11 days	10 hr. 2/7	+	tick dead 2/15	.....	..	.....	.....	..	.....	....	..
15 hr. 2/1	+	8 days	5 hr. 2/9	+	14 days	3 hr. 2/23	+	9 days	2 hr. 3/4	+	3 days	1 hr. 3/7	–
5 hr. 2/1	+	tick dead 2/10	.....	..	.....	.....	..	.....	.....	..	.....	....	..

TABLE 2.

DURATION OF TICK-FEEDING REQUIRED FOR THE INFECTION OF THE GUINEA-PIG.

Ticks not previously tested for infectivity.

Duration of Second Test and Date	Result	Interval	Duration of Third Test and Date	Result	Interval	Duration of Fourth Test and Date	Result
5 hr. 2/4	–	12 days	15 hr. 2/16	–	20 days	20 hr. 3/8	+
15 hr. 1/28	+	15 days	5 hr. 2/11	+	12 days	3 hr. 2/23	–
15 hr. 2/9	+	16 days	10 (?) hr. 2/25	–	tick lost	.....	..
20 hr. 2/11	+	tick dead	.....	..	.....	.....	..

In the second group of experiments (Table 2) the ticks were not given a preliminary test, but the first feed was regarded as a time test; in all other respects the experiments were similar.

When testing or feeding an individual tick, the guinea-pig was placed in a pillory and the tick allowed to attach itself to the ear. The time of feeding was taken from the minute the tick became so firmly attached that when the tick body was flipped back it could not be detached from the pig.

In the beginning of the work it was thought that a feeding of one hour would be sufficient to infect a guinea-pig, but it was soon found necessary to extend this period to five hours. If the tick proved infective in the five-hour test the time could be reduced in the second test, or if the infection were not transmitted to the guinea-pig in the first test, the time could be lengthened.

The results of the experiments as shown by the tabular summary of Tables 1 and 2 are not constant. However, when it is considered that the tick is a living factor, this inconstancy is partially explained. The tick is a slow feeder. The hungriest and most vigorous require from 15 to 20 minutes to attach themselves firmly by means of the palpi, and without this firm attachment feeding is impossible. In other ticks the process is much slower, and after two or three days of attachment there is little evidence of the ingestion of blood.

Table 1 shows that the minimum duration of tick feeding necessary to infect a guinea-pig was found to be one hour and forty-five minutes (Experiment I, with *Dermacentor modestus*, Female 90). In Experiment XV, *D. modestus*, Female 110, was infected in a two-hours' feed and again in a three-hours' feed. A five-hours' feed inoculated in Experiments IV, V, XIII, XV, XVI, and XVIII; an eight-hours' in Experiment XII; a ten-hours' in Experiments I, XI, and XIV; a fifteen-hours' in Experiments XV (twice), XVIII, and XIX; a twenty-hours' in Experiments III, X, XIV, XVII, and XX. Twenty-seven five-hour tests were tried, and six, or 22 per cent, infected; ten ten-hour tests, of which three, or 30 per cent, infected; eight fifteen-hour tests, in which four, or 50 per cent, were positive; six twenty-hour tests, with five, or 83 per cent, positive; nine one- to three-hour tests and three, or 33 per cent, infected. To

account for the higher percentage of infections in the one- to three-hour than in the five- and ten-hour tests we find that in the former cases two highly infected and voracious ticks caused the three inoculations, while in the latter the ticks were apparently not so heavily infected, and were only average feeders.

From the data given it is observed that the average time for infecting the guinea-pig by the wood tick is about ten hours, and that twenty hours is almost surely infective. Only once did this fail, but, on repeating (Experiment X), a positive result was obtained.

D. modestus, Female 90 and Female 110, gave the best results, infecting the animals upon which they were tested at practically each feed. In Experiment XIII the first five-hours' feed was positive, while two others repeated later at varying intervals did not cause infection. The results obtained in this experiment, in Experiment XVI, and also in Experiment XVII, where, after a negative fifteen-hours' test there followed positive twenty- and five-hour tests, may have been due to disinclination of the tick to feed vigorously. In Experiments I and III the weakened condition of the ticks necessitated long feeds to restore their vitality.

From the tabulated summary it appears that both males and females of the modestus species gave more positive results than those of the venustus. This was probably due to the fact that the former were younger and more vigorous and therefore fed more rapidly.

Duration of feeding required for the infection of the tick and length of incubation period in the tick.—The technic in this series of experiments was as follows: A number of normal ticks were allowed to feed on an infected guinea-pig for a definite number of hours; they were then removed and kept at room temperature for several days, when feeding was again permitted, this time, however, on a normal guinea-pig, for a definite period. Both species, *D. venustus*, and *D. modestus*, were tested. The infected guinea-pigs on which the ticks were fed were regular passage animals, and were used between their second and fifth days of fever.

In only two of the 27 was the spotted fever virus demonstrated in the tick, so that the results cannot be considered conclusive. It seems highly probable that a much shorter feed is required for

TABLE 3.

DURATION OF FEEDING NECESSARY TO INFECT TICK AND INCUBATION PERIOD IN THE TICK.

Exp.	Tick Species and Number	Date of Feeding	Length of Feeding	Interval	Date of Test	Length of Test	Result	Interval	Date of Second Test	Length of Test	Result
I.....	D. venustus										
	M. 204	12/5	5 hrs.	7 days	12/12	10 hrs.	−	....	.....	.....	..
	M. 205	12/5	5 hrs.	14 days	12/19	10 hrs.	−	.....	.....	.....	..
	F. 206	12/5	5 hrs.	tick died before b eing test ed				.....	.....	.....	..
	M. 207	12/5	10 hrs.	7 days	12/12	5 hrs.	−	.....	.....	.....	..
	M. 208	12/5	10 hrs.	13 days	12/18	20 hrs.	−	.....	.....	.....	..
	F. 209	12/5	10 hrs.	39 days	1/13	15 hrs.	−	.....	.....	.....	..
	M. 210	12/5–6	20 hrs.	6 days	12/12	20 hrs.	−	29 days	2/2	15 hrs.	−
	M. 211	12/5–6	20 hrs.	13 days	12/18	20 hrs.	−	.....	.....	.....	..
	F. 212	12/5–6	20 hrs.	39 days	1/13	15 hrs.	−	.....	.....	.....	..
II.....	D. venustus										
	M. 214	1/9	10 hrs.	tick died before b eing test ed				.....	.....	.....	..
	M. 215	1/9	10 hrs.	4½ days	1/14	20 hrs.	−	.....	.....	.....	..
	M. 216	1/9	10 hrs.	25 days	2/4	20 hrs.	−	.....	.....	.....	..
	F. 217	1/9–10	20 hrs.	4 days	1/14	20 hrs.	−	.....	.....	.....	..
	F. 218	1/9–10	20 hrs.	23 days	1/31	15 hrs.	−	.....	.....	.....	..
	M. 219	1/9–10	20 hrs.	41 days	2/18	15 hrs.	−	.....	.....	.....	..
	F. 220	1/9–10	25 hrs.	4 days	1/14	20 hrs.	−	40 days	2/18–3/4	14 days	+
	M. 221	1/9–10	25 hrs.	24 days	2/2	15 hrs.	−	.....	.....	.....	..
III.....	D. venustus										
	M. 225	1/24	25 hrs.	37 days	3/2	20 hrs.	−	.....	.....	.....	..
	M. 226	1/24	25 hrs.	tick died before b eing test ed				.....	.....	.....	..
IV.....	D. modestus										
	M. 168	3/1–2	25 hrs.	20 days	3/22	20 hrs.	−	.....	.....	.....	..
	M. 173	3/1–2	40 hrs.	10 days	3/12	20 hrs.	−	.....	.....	.....	..
	M. 174	3/1–2	40 hrs.	20 days	3/22	20 hrs.	−	.....	.....	.....	..
V.....	D. venustus										
	F. 232	3/24–25	25 hrs.	35 days	4/28	25 hrs.	−	.....	.....	.....	..
VI.....	D. modestus										
	F. 178	3/24–30	25 hrs.	30 days	4/28	30 hrs.	+	.....	.....	.....	..
	F. 179	3/29–30	25 hrs.	38 days	5/6	25 hrs.	−	.....	.....	.....	..

infection and that the incubation period in the tick is shorter than is indicated by these experiments. The complete protocols of the two tests in which complete results, as indicated by the transmission of the disease by the tick to the normal guinea-pig, were obtained, follow.

D. venustus, Female 220, was attached to infected guinea-pig 2,547 for 25 hours on January 9–10. No hyperemic area was observed around the bite and no feces were passed. On January 14, after an interval of four days, it attached itself to normal guinea-pig 2,713 and was removed after 20 hours. The tick did not appear to feed much, not increasing any in size, and passing no feces. The temperature of guinea-pig 2,713 was taken for two weeks but did not rise above 103.4° F. A month later, February 27, the pig was given an immunity test of 1 c.c. of blood from an infected pig. It became infected and died, the organs showing changes typical of spotted fever. This proved conclusively that the guinea-pig had not been infected by the tick, since Ricketts and Gomez[1] have shown that one attack of spotted fever renders the guinea-pig immune to a second inoculation.

On February 18, Female 220 attached itself to normal guinea-pig 2,779. It was found off March 5, having been attached for 15 days. It had increased markedly in size during the feeding. A hyperemic area about 2 cm. in diameter was observed around the bite.

[1] *Jour. Infect. Dis.*, 1908, 5, p. 221.

TEMPERATURE OF GUINEA-PIG 2,779.

February 18.....103.6° F.	February 25.....102.6° F.	March 4.....105.2° F.
February 19.....104° F.	February 26.....102.4° F.	March 5.....105° F.
February 20.....103.6° F.	February 27.....	March 6..... F.
February 21.....103.8° F.	February 28.....102.6° F.	March 7.....105.6° F.
February 22.....103.6° F.	March 1.....102.4° F.	March 8.....105.2° F.
February 23.....102.9° F.	March 2.....	March 9.....104° F.
February 24.....102.6° F.	March 3.....105° F.	March 10.....101.2° F.*
		March 11.....Dead.

* Blood taken from heart.

Autopsy.—Axillary glands enlarged and congested. Spleen twice the normal size and of a deep, bluish-red color. The heart, lungs, and intestines normal. The suprarenal glands and liver slightly enlarged and congested. The external genitals swollen and the skin slightly hemorrhagic.

Diagnosis.—Spotted Fever. Blood drawn from the heart on March 10 was inoculated into guinea-pig 2,831, which ran a typical spotted-fever temperature. The scrotum became swollen, the ears became necrotic and finally sloughed off. The guinea-pig recovered and later was given an immunity test during which its temperature remained normal. All these facts prove beyond doubt that guinea-pig 2,779 had spotted fever.

In most instances it has been found that an infected tick will produce the fever in a guinea-pig after an incubation period in the pig of five or six days. In the present case this is doubled. Two assumptions may explain the results: (1) either the tick did not feed for several days after becoming attached to guinea-pig 2,779; or (2) the amount of virus ingested by the tick in its feed upon infected guinea-pig 2,547 may have been exceedingly small, since the tick did not feed actively, as is shown by the absence of a hyperemic area around the bite, and this small quantity of virus may not have increased in the body of the tick sufficiently to infect a guinea-pig until the tick had enjoyed for some days what we may regard as a very favorable condition of attachment to a guinea-pig.

On March 29 *D. modestus*, Female 178, attached itself to the ear of infected guinea-pig 2,771, where it was allowed to remain for 25 hours. Considerable feces were passed by the tick, and a slight hemorrhagic area surrounded the bite. After an intermission of 30 days during which the tick was transferred from Chicago to Montana, it attached itself on April 28 to normal guinea-pig 3,004, being removed after 30 hours. On May 5 the temperature of the guinea-pig rose to 104° F.

TEMPERATURE OF GUINEA-PIG 3,004.

April 28.....103° F.	May 5.....104° F.
April 29.....102.6° F.	May 6.....105.1° F.
April 30.....102.8° F.	May 7.....106° F.
May 1.....103° F.	May 8.....106.2° F.
May 2.....103.2° F.	May 9.....105° F.
May 3.....103.1° F.	May 10.....104.1° F. scrotum swollen.
May 4.....103.6° F.	May 11.....103° F. scrotum hemorrhagic.
	May 12.....103.4° F. Recovered.

An immunity test was given May 16, but no fever resulted, showing that guinea-pig 3,004 had been infected the first time. The incubation period in the tick in this case was 30 days with a minimum duration for infecting the tick of 25 hours. With *D. modestus*, Female 179, which was attached the same length of time as Female 178 on guinea-pig 2,771, but was given a 25-hour test after an interval of 38 days, a negative result was obtained.

In previous experiments the duration of feeding required for the infection of the tick was shown to be less than was found here, 20 hours being the shortest period.[1] The incubation period is, perhaps, "represented only by the time required for the distribution of the virulent organisms throughout the tick's body and eventually into its salivary glands. In a number of instances the tick proved virulent immediately following its removal from the infected animal" (Ricketts).[2] In the latter cases, however, the tick was permitted to feed for several days on the infected guinea-pig and thus the incubation period may have been reduced by the tick's remaining attached to the guinea-pig, or it may be that the chances that the tick will infect the susceptible guinea-pig are greatest immediately after its proboscis has been contaminated by feeding on an infected animal.

More experiments will have to be performed before any definite statements can be made relative to the length of the incubation period in the tick. The great number of negative results may have been due to the fact that the ticks were raised under artificial conditions and did not feed as rapidly as those obtained from nature. It will probably be found that ticks taken directly from the woods will give better results. Similar experiments with fresh ticks were to be undertaken in the field during the spring of 1910, but the unfortunate death of Dr. Ricketts prevented the carrying-out of the work. It will, however, probably be completed at some future date.

SUMMARY.

The minimum duration of feeding necessary for a tick to infect a guinea-pig was found to be one hour and forty-five minutes. The average time necessary seems to be about ten hours, while twenty

[1] Ricketts, H. T., *Jour. Am. Med. Assn.*, 1907, 49, p. 24.

[2] *Med. Record*, 1909, 76, p. 843.

hours were almost constantly infective. The duration of feeding necessary to infect a tick is approximately twenty-five hours, while the minimum incubation period in the tick was not definitely determined. With ticks obtained from nature it is possible that the duration of feeding necessary to infect the tick and the incubation period in the tick will be found to be much less than is indicated here.

A TECHNIC FOR THE INOCULATION OF BACTERIA AND OTHER SUBSTANCES INTO LIVING CELLS.*

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The method here described is an outgrowth of the technic for the isolation of single micro-organisms as described in previous papers.[1] The new feature makes possible not only the segregation of one or more micro-organisms but the injection of them as well as of measured doses of fluids into the protoplasm or vacuoles of living cells. In order to accomplish this, pipettes have to be constructed of such a fineness as to minimize the injury to the cells injected and of sufficient rigidity to pierce the cell wall. Further, an injection force has to be employed sufficient to overcome cell pressure, capillarity, and any obstruction in the pipette. The first requirement was met by modifying the method of drawing pipettes, and the second by the use of the expansion of mercury as a source of power for injection.

Each phase of the method and each part of the apparatus will be described in detail, and, for the most part, in the order that one would follow in carrying out the process.

The pipette holder (*ph*, Fig. 4) is the same as that described for the isolation method. It consists essentially of an attachment to the stage of the microscope which holds the pipette, and by means of screws allows an up-and-down and a lateral movement of the pipette (*sv* and *sl*, Fig. 4). A double pipette holder, which is described below, may be used in place of the simpler form.

Any mechanical stage may be employed which allows a considerable range in both directions. The glass box is prepared as for the isolation method. Two convenient forms have been used, the smaller 60 mm. long by 25 mm. broad by 16 mm. high and a larger one 70 mm. long by 36 mm. wide by 16 mm. high. Water is kept in the bottom of the box and, further to insure moisture, the sides are lined with wet filter paper. A number 2 cover-glass

* Received for publication March 13, 1911.

[1] *Jour. Infect. Dis.*, 1908, 5, p. 380; *Kansas Univ. Sci. Bull.*, 1907, 4, p. 3.

of suitable size is sealed by means of vaseline to the top of the box. This cover may be sterilized or not depending on the sort of work to be done.

The cells to be injected are placed in water on the under side of the cover, preferably toward the inner end where there is less danger of drying. An abundance of moisture may be further insured by large hanging drops of water placed under the outer end of the cover-glass, and, if necessary, by thin cloth or paper wet and so placed as partially to close the entrance of the box.

Hanging drops of the bacteria or fluids to be injected may be placed on the cover as near to the cells as possible. The broader box and cover allows more room for cells and hanging drops.

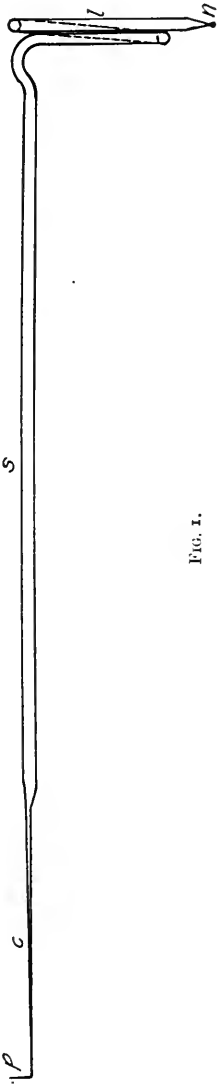

FIG. 1.

The pipette (Fig. 1) is best made from glass tubing of total diameter of about 4 mm. and wall about 0.7 mm. thick. Considerable variation in size is allowable, but one should use a somewhat thicker walled glass than is necessary for isolating pipettes. Hard or soft glass, if tough and of good quality, may be used. The advantage of the hard glass is its greater strength and the greater ease of making the fine points, while the soft glass is more easily bent and drawn out into capillaries. A piece of tubing about 35 cm. in length is bent at one end into the form shown in the illustration. The distance from the top to the bottom of the curved portion should be 4 to 5 cm. For convenience in description, the curved portion will be designated as the loop. The tip *n* is then drawn out into a moderately fine capillary and the whole tube nearly filled with mercury by

exhausting the air at the straight end, which may conveniently be done by attaching a rubber tube to this end. The point *n* is then sealed off and any air remaining above it after the sealing is worked around to the open end. The mercury should be clean and dry; it is best drawn into the tube when hot, and all precautions should be taken to insure freedom from air or water vapor. It is not essential to have the exact form of loop represented in the figure. The aim is to have the upper curves nearly on a level and to have sufficient bends to contain a considerable quantity of mercury. The greater the number of bends the more sensitive the instrument to changes in temperature, but the more unwieldy to handle. It is well to leave a point extending one or two centimeters below the lowest bend of the loop.

After sealing point *n* the straight end of the tube is drawn into a capillary about 7 cm. long and 0.3 to 0.4 mm. in diameter and with walls about 45 micromillimeters thick. The air is now expelled by vaporizing the mercury at point *s*. The capillary end is immersed in mercury, and the tube is filled with mercury as the vapor condenses. The small amount of air which enters when the tube cools to room temperature may conveniently be expelled by heating the loop in the flame. The capillary tip is now immersed in mercury and the loop cooled in ice water. The tube should be as free from air as possible, though a very small bubble of air does not prevent its successful use. If the pipettes are not to be used for some hours or days the capillary end may be sealed.

In making the fine capillary points for piercing cells the first step is the withdrawal of the mercury from the end of the capillary portion. This is done by immersing the looped end in ice water. The pipette is then held in the left hand, the tip of the capillary grasped by fine-pointed forceps held in the right hand, and the portion of the capillary left free by the contraction of the mercury held over, not in, a very small and narrow flame. A very convenient burner for producing a flame of this sort is made by bending a piece of hard glass tubing nearly at right angles, pinching one end into a very narrow slit, and attaching the other end to rubber tube connected with the gas supply and provided with a clamp to regulate the size of the flame. (See Fig. 2.) The

smallest flame that can be kept alive, if possible about 2 mm. high, is most serviceable. Natural gas for the microburner may be improved by causing it to pass through alcohol or benzine.

When the glass begins to soften over the flame one should pull with the forceps directly away from the other hand. The success

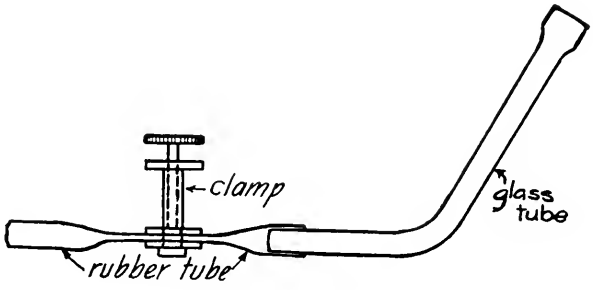

FIG. 2.

of the process depends on the timing and strength of the pull, a matter difficult to describe since much depends on the feeling. If the capillary separates with little or no feeling of resistance, the point is likely to be too long and flexible. If separation is accompanied by a snap, the point is usually too blunt. An intermediate resistance, felt as a slight tug just as the glass separates, indicates the right amount of heating and pull. After a little experience one can be sure that he has the right sort of a point by the feeling alone; but it is better to inspect the point under the low power of the microscope before proceeding farther.

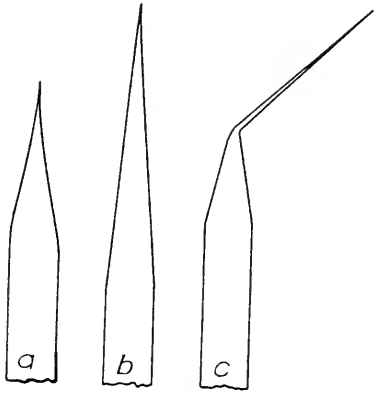

FIG. 3.

The best sort of point is shown in *a* of Fig. 3. This form combines fineness of point with rigidity and a comparatively large lumen near the tip. The end is sealed at this stage of the process. The form represented by *b* (Fig. 3) may be used, but it is more

likely to become obstructed and does not allow the regulation of the dose as well as does the form *a*. After a good point is made, the end of the capillary should be turned up at right angles as shown in Fig. 1, *p*. This is done by holding the capillary just near enough to a small flame to soften the glass while the end is lifted by the tip of the forceps. In order that the heated air in the capillary may not by expansion burst through the softened glass, a negative pressure is created in the capillary by immersing the loop in ice water just before bending the tip.

If the pipette is to be used with the mercury under considerable pressure at room temperature, the same process as described above is followed except that, just before making the point, the end of the capillary is immersed in mercury, first expelling air, if any is present, by gently heating the loop. The loop is now immersed in ice-cold water and as much mercury drawn in as possible. Then the tip of the capillary is exposed to air and the mercury is drawn from the end by immersing the loop still farther in cold water, or by cooling the portion of the tube above the loop. The pipette point is now made as described above.

The pipette is now placed in the holder, the tip brought into the center of the low-power field, and the apparatus for regulating the pressure adjusted. Two methods have been used for regulating the pressure. In the one method, the pipette is so made that the mercury in it is under considerable pressure at room temperature, as described in the preceding paragraph. In the other method, the mercury is under no pressure or a very slight negative pressure at room temperature. It will be more convenient to describe the higher-pressure method first.

The apparatus (see Fig. 4) consists of a brass tube *t* to the lower part of which is attached a brass cup *c*. This tube is held in the sleeve *s* which is attached to the arm of a simple microscope provided with a rack and pinion *r*. By means of the rack and pinion the sleeve and tube may be raised and lowered through about 5 cm. To the upper part of the sleeve a stiff wire is attached holding in a ring at its free end the bag *b* made of thin rubber tissue. All parts are adjustable. The bag may be raised, lowered, or moved laterally, the tube may be raised or lowered in the sleeve,

and a joint at *j* allows bag and cup to be swung aside together. A pan is placed beneath the cup to receive and carry away waste water.

The loop of the pipette is placed between the bag and the cup and these are so adjusted that when the sleeve is racked down to its fullest extent the upper part of the loop of the pipette will be in close contact with the bottom of the bag, and when it is racked

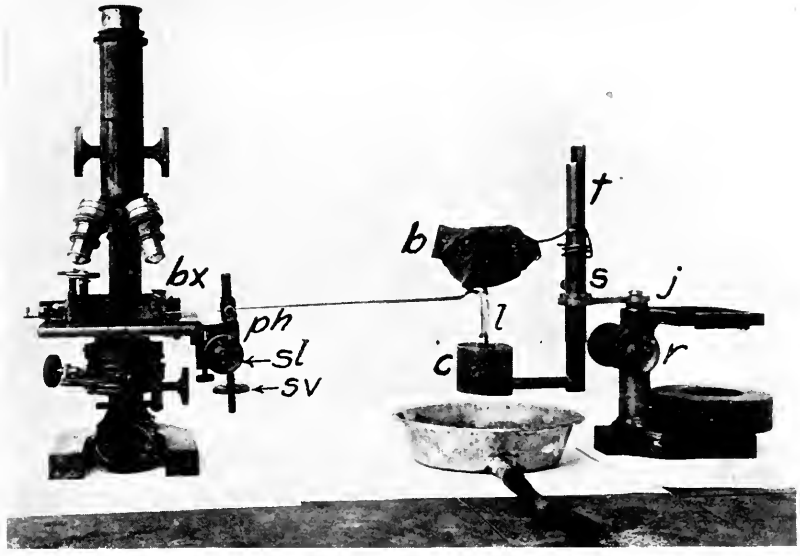

FIG. 4.

up the loop will be free from the bag and a large part of it immersed in the cup. The cup is now filled with ice-cold water and racked up so that the loop is fully immersed in it, and the point of the pipette is adjusted in the center of the high-power field. In doing this one must be careful not to break the delicate point on the cover-glass. One may avoid this by selecting under a low power a field near the margin of a hanging drop of water, and bringing the point of the pipette into this drop without touching it to the glass. The tip may then be found with the high power. If the oil immersion is to be used it is convenient to choose some conspicuous

object in a droplet of water, as a particle of foreign matter, and arrange the point of the pipette just below this object under the low power. The tip may be lowered slightly and the object found with the oil immersion. If the cover-glass has not been moved the positions of the particle and of the tip of the pipette will correspond. One has to be especially cautious in adjusting the oil immersion. A slight pressure exerted on the cover-glass in focusing may bend it down far enough to break the pipette, should the tip be left too near the glass.

When the point is found under the high power, the end, still sealed, is brought into contact with the cover-glass. Then by movement with the mechanical stage the point is scratched on the cover until the tip is broken off and the lumen of the tube opened. By varying the pressure on the cover-glass the amount broken off and the size of the opening made can be regulated. If bacteria are to be handled with the pipette a larger opening is needed than is necessary for liquids. If desired, a tube having a diameter of a micromillimeter may be made.

Next the air above the mercury must be expelled. If the mercury in the pipette is under great pressure at room temperature all that is necessary is to lower the brass cup and expose the loops to room temperature. When all of the air is driven out and the mercury appears at the tip of the pipette, pressure may be stopped by lifting the cup of ice water around the loop. If the room temperature is insufficient or too low, the rubber bag into which hot water has been poured may be racked down until its bottom is in contact with the upper part of the loop. A vessel supplied with a Bunsen burner or electric heating coil and placed somewhat above the laboratory table is a convenient source of hot water. The water may be brought to the bag through a rubber tube provided with a pinch cock.

In order to fill the pipette, the point filled with mercury to the tip is brought into a hanging drop of the liquid to be injected and the ice water racked up so as to surround the loop. The contraction of the mercury brings about a negative pressure and draws the fluid into the pipette. When a sufficient dose has been drawn in the water is partially lowered from the loop until the inflowing

ceases. The point is then lowered a short distance below the cover, and by means of the mechanical stage the cell to be injected is brought immediately over the point. The point is raised, the cell wall pierced, and the point brought to the vacuole or to any part of the cell desired. The loop is then further exposed to room temperature, and, if necessary, the bag containing hot water is lowered into contact with it. As soon as the dose is injected the cold-water cup is racked up and the pressure stopped. The pipette may be withdrawn or the action on the cell of the injected substance may be observed while the point is still in.

The second method is somewhat simpler. Adjustments are made as in the first process except that the use of warm water in the rubber bag is dispensed with, and warm water is brought into the brass cup instead. Since the mercury in the pipette is under no pressure at room temperature, warm water is brought around the loop in order to expel the air. The pipette is charged by bringing the point into the liquid or emulsion to be injected, lowering the cup, and exposing the loop to room temperature. If there is too much pressure in the pipette for convenient filling some of the mercury may be expelled by immersing the loop in warm water or the loop may be cooled by lifting a beaker of cold water around it. After being charged the point is introduced into the cell and the contents expelled by immersing the loop in warm water in the brass cup. The loop may be only partially immersed, or the water in the cup kept only slightly above room temperature if but light pressure is needed. If greater pressure is demanded, warmer water may be introduced into the cup. As a rule one should use the lowest pressure necessary for expelling the contents of the pipette.

The advantages of the second method are its simplicity and the ease with which one may obtain any desired pressure by regulating the temperature and the amount of the loop immersed. It has the disadvantage that air temperature may not be low enough to bring about a sufficiently sudden contraction of the mercury, and the injection process may not be stopped quickly enough to prevent an overdose or the injection of mercury into the cell. This disadvantage may be lessened by using the rubber bag kept

full of ice water. On the whole, the first-described process has thus far proved more convenient and reliable. It affords at least one constant, the temperature of ice water, and the room temperature is sufficiently constant for practical purposes. It is furthermore of greater importance to be able to stop the injection process quickly than to be able to apply pressure rapidly.

It is evident that an apparatus of this sort, which consists simply of a device by which heat or cold can readily be applied to a loop of glass containing mercury, may be modified in several ways. Some of these modifications have been tried, others are now being experimented on. The rubber bag may be partially filled with oil and a small electric light bulb or other electric heating device may be so arranged that the oil will be kept hot; or the bag may be dispensed with and radiated heat from some source substituted. Any electrical heating device may be arranged for keeping the water in the brass cup at any desired temperature, or for the cup a ring suitable for holding a beaker of any depth is a convenient substitute. The combination of cold water below and warm water above has thus far seemed the most feasible plan for meeting the main requirement—the gradual application of heat and the more sudden application of cold. Water may be substituted for mercury in the pipette and has the advantage of greater lightness, but the greater specific heat and conductivity of mercury makes it preferable.

In order that the dosage may be regulated and that mercury may not be driven into the cell the tip of the pipette should be unobstructed when it pierces the cell wall. Substances may successfully be injected in spite of a clogged tip, but so much force is often required to overcome the obstacle that ejection takes place with a rush and mercury is likely to follow the dose. Further, both for the regulation of the dosage and the prevention of clogging, pressure in the pipette should be nearly in equilibrium at the time of the entrance of the point within the cell. Positive pressure may not be a disadvantage if one does not care to avoid ejecting some of the contents of the pipette outside of the cell, but a too strong negative pressure causes the pipette to take up liquid surrounding the cell and increases the danger of clogging when the point enters

the cell. A clogged pipette may often be opened by gently scratching it on the surface of the cover-glass. Sometimes it is necessary to enlarge the opening somewhat.

If a volatile liquid is to be injected, or if large or repeated doses of the same substance are to be used, one may fill part of the capillary with the substance to be injected before making the injecting point, but in most cases one had better regulate the dosage by filling from the point. If very small doses are to be injected, one may keep the top of the mercury column in view after charging. The cell is pierced, pressure applied, and the rising of the mercury column to the tip shows that the dose has been injected. Focusing on the mercury column may be facilitated by piercing the cell obliquely instead of from directly below. With the use of larger doses the top of the mercury column is usually below the reach of the lens. Here one can focus as far down as possible in the pipette after its introduction into the cell and stop the pressure on the appearance of the mercury column. If pipettes of the form represented in Fig. 3, *a*, are used, much more force is required to expel the mercury than to bring it to the tip, so that one has time to stop the pressure after the appearance of the mercury. If is it desired to remove cell contents the retreat of the mercury column indicates that the contents are being drawn into the pipette.

Small doses may be measured by estimating the cubic contents of the pipette between the top of the mercury column and the tip. Larger ones may be estimated by expelling the dose on the cover-glass and measuring the droplet expelled. The liquid is then drawn into the pipette again. If a definite number of bacteria are to be inoculated, they may first be isolated in a droplet of fluid and the whole droplet inoculated.

If the substance to be inoculated forms a precipitate with the mercury, a quantity of water or of some indifferent oil, sufficient to separate the substance to be injected from the mercury, may first be drawn into the pipette.

One should have the cell to be injected well located before filling the pipette and as near to the filling place as possible, so that little time will be lost between charging and injecting. This is the

more necessary where it is advisable to keep the pressure in the pipette in equilibrium after filling. Lines may be drawn on the cover-glass to serve as guides, the cell may be located by means of the vernier on the mechanical stage, or the droplet of substance to be injected may be placed just in line with the cell so that only one movement of the mechanical stage is necessary in passing from the one to the other.

The penetration of the plant cells thus far experimented on is easy if the tip is made fine enough and if it contracts abruptly enough so that the pipette has the necessary stiffness. If points are too pliable, there may be difficulty in penetrating the cell; if too blunt there is danger of tearing the cell wall. The loss of some of the injected fluid or of cell contents on withdrawal of the pipette cannot always be avoided, but it may be minimized and in some cases entirely prevented by withdrawing the tip very slowly so that the protoplasm has time to form a plug over the opening. Further, if the tip is brought just within the cell it may be introduced with less injury to the cell and withdrawn with less loss of liquid. The smaller the tip the less injury to the cell and the greater the ease of withdrawal.

In the multinuclear cells of the fungus group Saprolegniaceae and of Nitella and Vaucheria among the algae the cell wall has been pierced, different substances injected, and the pipette withdrawn with little or no apparent injury to the cells, as judged by the movements of the protoplasm and the subsequent behavior of the cell. With some mononuclear algal cells, as Spirogyra, the cell appears to be more sensitive to injury. Few animals have thus far been experimented on with the injection apparatus. Mercury has been injected into rotifers and substances into Paramecium, but the technic for inoculating animals has thus far not been fully developed.

Any objectives from the lower powers to the oil immersion may be used. The oil immersion should have sufficient depth of focus to reach the bottom of the cell. The cells to be injected should be in contact with the cover-glass or as near to it as possible.

In making a new capillary point the pipette may be removed from the holder, the old point broken off, and a new one made

from the same capillary. When the capillary is used up a new one may be made from the end of the straight portion of the pipette, and the process continued until all is used back to the loop.

In certain kinds of work it is of advantage to use two pipettes simultaneously. This makes necessary the use of a special holder for two pipettes, a modification described in previous papers. The holder is so arranged that each pipette has its own movements, up and down, in and out, lateral and rotary with the axis at the point of attachment in the holder, and the two pipettes move together laterally besides. With this apparatus one may inject two different substances into the same cell at the same time, or by varying the pressure inject with one pipette and withdraw with the other. One pipette may be used simply as a probe or dissecting instrument, or it may be attached to a rubber tube and used as an isolating pipette, while the other is arranged for injection. The injection apparatus may be dispensed with, the tips of the pipettes left closed or made from a solid rod, and used either singly or in conjunction as needles or dissecting instruments. One or both of the pipettes may at once be converted into isolating pipettes, or through them one may treat the dissecting parts with stains, fixatives, or other reagents. An improved type of the double pipette holder is now being constructed under the direction of Dr. F. Hecker of this laboratory.

With a single point the writer has successfully divided amebae and also removed the nuclei of amebae, with little loss of protoplasm or apparent immediate injury to the organisms. The division of amebae has been accomplished with the straight point and with one bent at a considerable angle (Fig. 3, *c*). With two points spores of fungous parasites have been removed from their host, and sporangia of fungi have been dissected. In one experiment a rotifer has been held by the blunted point of one pipette while mercury was injected into the body with the other pipette. Points may be made fine almost to invisibility with sufficient stiffness for piercing the wall or even the nuclear membranes of cells.

It has seemed to the writer that this technic in its different forms may assist in the solution of various problems in the biology

of microscopical plants and animals. Experiments are now being carried on in this laboratory on the infection of cells through the injection of various micro-organisms and on the effect on cells of different chemical substances inoculated directly into the protoplasm or vacuoles. The introduction of foods, poisons, stains, and fixatives is made possible and cells may be probed or dissected under high powers, methods which may be of use in the study of the structure, chemistry, and physiology of cells. Finally, materials may be withdrawn from one cell and injected into another, and it is possible that investigations on fertilization and heredity may be extended by this technic.

NOTE.—In the process of isolation of micro-organisms the use of points such as those illustrated in Fig. 3, *a*, will be found an improvement on those described in earlier papers. The pipette is fixed in the holder and focused under the higher powers while the tip is still closed. The point is brought into a hanging drop of broth or other fluid to be used and the tip opened by gently scratching the cover-glass, as described for the inoculation pipettes. An opening of the size suited to the micro-organisms to be manipulated is made, the pipette filled from the drop, and the process carried on in the usual way. Further it will be found of advantage to clamp the pipette holder on a plate screwed on to the stage of the microscope instead of to the stage itself. (See Fig. 5.) This brings the pipettes nearer to the level of the stage and allows more working room for the mechanical stage. The longer isolation box described above will be found of advantage in allowing more room and in keeping the hanging drops under better conditions of moisture; and the broader form of box and cover will be found useful in some forms of isolation.

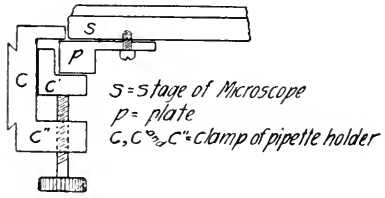

FIG. 5.

THE NUMBER OF CELLULAR ELEMENTS IN MILK.*

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The new method for determining the number of cellular elements in milk, recently devised by Prescott and Breed (1910), reveals such an inaccuracy in the older methods for determining the number of these cells that it again raises the question of how many body cells may be expected in milk under normal and pathological conditions. On this account, an investigation was undertaken to determine the number of cells present under a variety of conditions as a preliminary to experimental work to determine the significance of these cells in milk and the cause of the variations which are known to occur.

HISTORICAL SURVEY.

No attempt will be made here to criticize the older methods for determining the number of cells present in milk, as that will be done in another paper.[1] The question of the origin of these cells is in dispute at the present time, but the drift of opinion seems to be toward ascribing an epithelial, rather than a leukocytic, origin to them. On account of this dispute only noncommittal terms will be used in this paper.

A considerable amount of work has been done by the so-called "smeared-sediment" (Stokes-Stewart-Slack) method and various "volumetric" methods to determine the number of cells present in milk under a variety of conditions. However, it is generally admitted that the smeared-sediment method even in its best form does not yield sufficiently accurate results to give more than a general impression of the number of cells present, if indeed this be true. On this account, very little reference will be made in this review to work done by this method. Just how accurate the results of the various volumetric methods may be remains to be seen, but in any case they are regarded as being of sufficient importance to summarize them at this place. The earliest of these methods is the Doane-Buckley, which has recently been modified by Russell and Hoffman. This modification is the method recommended by the Committee on Standard Methods of Bacterial Milk Analysis appointed by the Ameri-

* Received for publication February 7, 1911.

[1] "The Effect of the Centrifuge and Separator on the Distribution of Cellular Elements in Milk with a Criticism of the Present Methods for Determining the Number of Such Cells in Milk" (R. S. Breed). This paper will probably be published in the *Milchwirtsch. Centralbl.* for 1911.

can Public Health Association.[1] An account of all of the methods mentioned above is given in the committee's publication. Other volumetric methods, based on a similar principle, have been devised by Savage (1906) and Hewlett, Villar, and Revis (1910). The test devised by Trommsdorff has been the most popular in European countries but the results of this test are not expressed in numbers of cells per cubic centimeter, and the method itself is open to all of the criticism that has been directed against the smeared-sediment method, so that the work done by this method is not referred to here.

a. The most important point in connection with these cells is the determination of the number of cells present and the variation in number to be expected in the milk of individual normal cows. A large amount of work has already been done by various men to determine these points. Unfortunately, the authors quoted do not always state clearly whether or not the sample examined was the mixed milk of the entire milking of the individual cow. The statements of the various authors regarding the question whether or not the cows examined were normal are unsatisfactory. No one, not even a competent veterinarian, can say what an entirely normal udder is, and the finding of an udder that is more fibrous or fleshy than usual is not proof in itself that the secretion from it will be abnormal. All figures given in this review represent the number of cells per cubic centimeter.

Doane (1905) (p. 210, Doane-Buckley test) gives the record of 25 cows from the Maryland Agricultural Experiment Station herd as follows:

December 15, 1904, 23 out of 25 cows: average 123,000, maximum 1,000,000, minimum 4,000.

January 4, 1905, 22 out of 25 cows: average 168,000, maximum 1,600,000, minimum 3,000.

March 1, 1905, 16 out of 25 cows: average 91,000, maximum 300,000, minimum 8,000.

On p. 211, he gives the record for 102 cows from "one of the best and most carefully kept herds in the United States": average 252,000, maximum 4,600,000, minimum 2,000.

Savage (1906) (p. 130, Savage test) gives the record for 40 cows where only the middle milk of the milking was used in obtaining the sample. "As a rule no selection was exercised in picking out the cow to be examined. In several instances, however, cows recently calved and just readmitted to the herd for milking purposes were chosen; in one or two instances the cowkeeper was asked to select his finest and healthiest cows." All of the cows were kept in Colchester Borough, England: 40 cows averaged 826,575, maximum 4,380,000, minimum 35,000.

Ward, Henderson, and Haring (1906) (p. 150, Doane-Buckley test) give a set of figures for 87 healthy cows from California: average 49,000, maximum 581,000, minimum 2,000. On pp. 152–54 they give figures for 71 cows, selected because of udder troubles or for other reasons which might be expected to give high counts: average 260,000, maximum 4,800,000, minimum 3,000. The tests from five other cows were not counted because the cells were badly clumped or innumerable.

Russell and Hoffman (1907*a*) (Doane-Buckley test and their modification of this test) have made the most thorough and satisfactory investigations along this line, giving, as they do, a series of examinations of animals whose clinical history was known, some being normal and others not. The results of numerous tests of 18 normal animals (p. 244) show 31.1 per cent under 50,000, 18.5 per cent between 50,000 and

[1] *Am. Jour. Pub. Hyg.*, 1910, 20, p. 315.

100,000, 36.6 per cent between 100,000 and 500,000, 10.3 per cent between 500,000 and 1,000,000, and 3.5 per cent above 1,000,000: maximum 2,191,250, minimum 1,250. A similar series of tests for 11 animals showing more or less pathological conditions but giving apparently normal milk give 12.9 per cent under 50,000, 15.2 per cent between 50,000 and 100,000, 47.6 per cent between 100,000 and 500,000, 15 per cent between 500,000 and 1,000,000, and 9.3 per cent over 1,000,000: maximum 4,952,000, minimum 7,500.

Bergey (1907) (Doane-Buckley test) gives the tests for eight cows: average 181,000, maximum 640,000, minimum 16,000. Two other cows were examined but the cells were massed to such an extent that the number of cells could not be counted.

Kendall (1907) (p. 177, smeared-sediment test) has given the tests of 168 normal cows which would have been exceedingly valuable if they had been made by a better method. He found that 138 out of the number gave tests under 400,000 and none over 2,000,000.

Stone and Sprague (1909) (pp. 240–42, Doane-Buckley test) give the record of 57 cows from the Vermont Agricultural Experiment Station herd as follows: average 403,000, maximum 8,800,000, minimum 13,000; samples from the morning milking. They also give the tests for two healthy cows (p. 243) followed throughout the entire period of lactation:

Cow No. 1, average of 546 tests, 112,203, maximum 2,110,000, minimum 10,000.

Cow No. 2, average of 621 tests, 84,341, maximum 1,444,000, minimum 6,000.

Hewlett, Villar, and Revis (1910) give several tests of individual cows using a modified form of the Savage test. Six cows (pp. 64–65) gave results ranging from 3,545,000 to 12,500, all but two of the tests being under 1,000,000. Six cows from another dairy (pp. 69–70) showed a maximum of 3,900,000 and a minimum of 9,500. All of the cows are reported to have been healthy at the time these examinations were made.

b. Another important set of results are those which deal with what may be termed market milk. These results represent samples where the milk of from several to many cows has been mixed and the milk has stood for several hours under a variety of conditions. Such samples would not be expected to show the extreme of variation that would be found in the milk of individual cows, but the sum total of the averages should be the same.

Doane (1905) (p. 209) gives the results of nine tests but does not state the source of the samples: average 950,000, maximum 3,200,000, minimum 292,000.

Savage (1906) (p. 131) examined 17 samples of mixed milk from a variety of sources: average 487,000, maximum 1,980,000, minimum 21,000.

Ward, Henderson, and Haring (1907) (p. 147) give the record of 60 tests of market milk: average 64,000, maximum 539,000, minimum 9,000.

Kendall (1907) (p. 177) gives the results of testing 87 samples of market milk in New York. Of these, 60 gave counts under 400,000 and eight were above 2,000,000.

Campbell (1909) (p. 11, Doane-Buckley test) gives the results of testing twenty samples of market milk: average 46,000, maximum 125,000, minimum 7,000.

Hewlett, Villar, and Revis (1910) report on a number of tests of herds of six or less cows. Weekly tests of the first selected herd of six for six months (p. 62) show results ranging from 11,000 to 1,528,000. Individual cows were taken out during the tests and newly calved cows substituted for them so that 13 individuals were used during the test. Similar tests of a second herd of six for nine months (p. 65)

yielded tests from 17,000 to 1,735,000. This series was begun when the cows were well along in the period of lactation and were continued until all of the cows were dry. A third herd (p. 69) gave a maximum of 4,255,000 and a minimum of 136,000 during an eight-months test. A fourth herd of six, all newly calved (p. 73), gave a maximum of 4,360,000 and a minimum of 36,000 during a two-and-a-half-months test. The fifth herd of six (p. 78) during a period of nearly seven months showed a maximum of 1,258,000 and a minimum of 67,000. Herds 3 and 5 are reported to have been entirely healthy during the period of the tests but the other herds all included individuals which developed more or less abnormal conditions.

A comparison of these figures shows a surprising variation between the figures obtained by the different workers (cf. the figures given by Ward, Henderson, and Haring and those given by Stone and Sprague, for example). This can be explained in one of two ways. Either the cattle examined showed very different conditions or else the method used does not produce consistent results in the hands of different investigators. The probabilities are in favor of the second explanation, as is indicated by the discovery of the influence of preliminary heating on a sample by Russell and Hoffman (1908) and corroborated by the work of Campbell (1909), and of the use of formalin by Hewlett, Villar, and Revis (1910), as well as by the discovery of the uncertainty of the precipitation of the cells by the centrifuge by Prescott and Breed (1910).

The average given by Prescott and Breed (1910) for 46 samples of mixed milk obtained from as many different New England farms and determined by their new method is 1,485,000, maximum 10,690,000, minimum 60,000. The average of eight tests of market milk obtained from two of the highest class dealers of Boston was 2,850,000, maximum 5,210,000, minimum 1,310,000.

c. Certain other tests have been made to determine whether the different quarters of the udder vary as a unit or independently. Doane (1905) (p. 212) gives the results of testing three different cows in this way and comes to the conclusion that "the same variation that is found between individual cows is seen in the milk from different quarters of the same udder and there is considerable variation in the same quarter from day to day." An examination of two cows with a diseased quarter (p. 219) led him to conclude that one quarter may be seriously affected without influencing the other quarters.

Russell and Hoffman (1907*a*) (p. 241): "Seventy-two examinations made from the milk of individual quarters of the udder showed wide fluctuations in the milk of the same animal. It was noteworthy where any individual quarter showed a high cell content that this condition was observable through all portions of the milking."

Hewlett, Villar, and Revis (1910) give quite a body of evidence in their paper which agrees with the conclusions of Russell and Hoffman stated above. They note also a tendency to persistence of similar counts in the various quarters from day to day.

d. More investigators have tested to see whether there was any constant difference between the fore milk, middle milk, and strippings, sometimes using the mixed milk from all four quarters and sometimes testing each quarter separately.

Thus Doane (1905) (p. 212) tried to find milk without cells but found them in the fore milk, middle milk, and strippings of each quarter of the udder of a cow giving counts of but 1,000 to 5,000. He found the cells more numerous sometimes in the fore milk and sometimes in the strippings, with a considerable variation in conditions in different cases.

Savage (1906) (p. 137) found fewer cells in the middle milk of four cows than in the fore milk or strippings, but there was little difference between the latter two.

Russell and Hoffman (1907*a*) (p. 240) state that the results of testing 18 animals show that "strippings milk contains on an average three times as many cells as the fore or middle milk. As between fore and middle milk no regularity was observed. The increase in the strippings is, however, so marked that this factor should be taken into consideration in the matter of sampling."

Campbell (1909) (p. 14, modified Doane-Buckley test) made 14 tests of nine different cows in which a majority of tests show more cells in the strippings.

Hewlett, Villar, and Revis (1910) (pp. 60–61) report on six examinations but fail to find as striking results as Russell and Hoffman. Their conclusion is as follows: "From these results it is fairly evident that the cells appear practically uniformly throughout the milking, the slight increase in the strippings being probably caused by increased manipulation of the udder by the milker in stripping."

e. A more important question is whether there is any constant variation in number due to the period of lactation. There is a general belief that there is a decidedly larger number of cells present during the early days of lactation when the colostrum corpuscles are present than later, and that the increased number again makes its appearance when the animal is being dried off. These beliefs are founded on insufficient evidence, as will be seen from the following review:

Savage (1906) gives some data for 25 cows and concludes (p. 135) that there "appears to be no relation between the time interval and the number of leucocytes." One cow in the sixth day of lactation gave a count of 35,000.

Ward, Henderson, and Haring (1906) (pp. 152–54) give the following reports of examinations of fresh cows by the Doane-Buckley method:

Cow 1— 80,000, Udder indurated. Calved eight days ago.
Cow 2— 11,000, Udder indurated. Calved seven days ago.
Cow 3— 9,000, Fresh three days. Milk used.
Cow 4— 17,000, Fresh. Milk not used.
Cow 5—226,000, Fresh. Milk not used.
Cow 6—268,000, Fresh. Milk used.
Cow 7—453,000, Fresh. Milk used.
Cow 8— 17,000, Fresh.

Russell and Hoffman (1907*a*) (p. 241) make the following statements: "The effect of parturition was also studied, but examinations of colostrum milk could not be made on account of the presence of large numbers of colostral cells and of albumen. This condition persists for a period of three or four days, after which time microscopic examinations reveal no abnormal number of leucocytes." "No particular effect can be ascribed to the influence of lactation. A study of the history of the individual cows examined shows no regularity in the fluctuation as lactation advances. We have found wide variations in the number of cells from day to day and have frequently noted a high cell content in the earlier, as well as in the later, periods of lactation."

Pennington and Roberts (1908) (p. 77) make the statement that their evidence confirms the "assertion that pus makes its appearance if the cow is milked too long." In using the smeared-sediment method, they find that 75 per cent of the healthy animals show pus at the end of the milking period. However, no great weight can be given this statement because of the method used.

Stone and Sprague (1909) have made the most careful examination of two healthy

cows, twice daily, throughout their whole period of lactation. Like Doane, they find the most surprising and unexpected variations from day to day and conclude that they must be due to transient and trivial causes. One cow varied from 6,000 to 1,444,000 and the other from 10,000 to 2,100,000 cells per cubic centimeter. They state that their highest counts in the case of one cow were obtained in the first two days of lactation and that four out of eight counts between 500,000 and 1,000,000 were obtained either during the first two days or the last two days of lactation. They say nothing about the time when they obtained the high counts in the case of the other cow, probably because the same relationship does not hold true.

Campbell (1909) (pp. 12–13, Doane-Buckley test) gives the record of two cows where samples were examined on alternate days for a period of two months, but little can be gathered other than the fact noted by Doane that there is great variation from day to day. One of the cows was an animal in her first period of lactation. She gave no very high counts in the twenty tests: average 16,500, maximum 81,000, minimum 2,000. The other cow was one suffering from an inflammation of the udder especially located in one quarter. The samples tested were the mixed milk from the remaining three quarters. The numbers are greater in this case than the other, but run over 500,000 in only two instances out of 20: average 180,600, maximum 720,000, minimum 14,000.

Hewlett, Villar, and Revis (1910) (pp. 62 and 73) made two series of tests to find whether the inclusion of newly calved cows in a herd increased the cell content of the mixed milk from the herd, but failed to find any marked influence. They give the following tests of fresh cows with the statement that they had been fresh from 7–14 days (p. 73): Cow 33 339,000; Cow 34 110,000 (contained trace of blood); Cow 35 36,000; Cow 36 415,000; Cow 37 4,360,000 (nine days since calving. Details given in regard to this case which developed a case of mastitis); Cow 38 99,000. These authors also give the results of examining a herd (p. 64) where some of the cows were being dried off, but failed to find any regular increase as the cows approached the end of their period of lactation. One cow far advanced in calf gave a very high and fluctuating count while three others which were barren showed low counts.

f. Stone and Sprague (1909) tried to determine whether there was any relation between the number of cells in the milk and the number of leukocytes in the blood but discovered none in the extended series of tests made.

g. It has been suggested several times that the age of the cow might influence the number of cells in the milk but little evidence has been submitted by anyone except Russell and Hoffman (1907*a*). On p. 253 they make the following statement: "From the studies made, it appears evident that the milks of older animals are more likely to show a high cell content than the younger stock and that frequently this condition is associated with a previous udder disturbance, which in some instances may leave a more or less marked indurated condition in the udder."

h. Russell and Hoffman (1907*a*) found no relation between the cell content and the breed of the animal.

i. One of the most important points of all, because of its bearing on sanitary problems, is the question whether there is any relation between high cell content and pathological conditions. It has been generally assumed, especially by health authorities, that such is the case. The foundation for this belief has been secured largely by the use of the Stokes smeared-sediment method with its various modifications. If now we rule this evidence out, as the authors of this paper believe they are justi-

fied in doing, because of the very great inaccuracy of these methods, this belief is found to rest on the following insufficient and contradictory evidence.

Doane (1905) (p. 213) states that "it has been demonstrated that when very great numbers of leucocytes are present in the milk there is evidently inflammation present in the udder." His examination of four cows (pp. 217–19) tends to support this statement except that in the case of Cow 86 it would appear that he has concluded that the cow had a diseased quarter because she showed a high cell count and he admits (p. 220) that "some radical and unexplained variations can be seen in the results of the count with the milk of Cow No. 86 from day to day."

Savage (1906) (p. 136) reports a cow suffering from infective mammitis whose milk showed an excessively high cell content.

Ward, Henderson, and Haring (1906) give an excellent list of tests of 47 cows where the udder was indurated or suffering from some pathological condition. These tests indicate very clearly that abnormal conditions do not necessarily produce large numbers of cells in the milk. The tests of 28 cows reported to be suffering from more or less indurated udders show four tests where the cells were so badly clumped as to be uncountable, three over 500,000, while 21 are under the 500,000 limit which has been so generally used as the standard. Their final conclusion (p. 156) is that "in view of all these observations we feel that the numerical determination of leucocytes in mixed milk, for the purpose of detecting the existence of mammitis among the cows of the dairy, does not rest on a firm experimental basis."

The work of Russell and Hoffman (1907*a*) on this point is the most careful and extended that we have. In comparing a long series of tests of 18 normal healthy cows with those from 11 cows showing more or less indurated conditions, they make the following statements (p. 247): "While it is undoubtedly true that cows suffering from udder trouble often do produce milk that is rich in leucocytes, it would seem equally true that high quantitative results were found with such frequency in milks from healthy animals as to vitiate the accuracy of a test based solely on a numerical foundation"; (p. 248): "In Group I (healthy cows) there are 11 cows whose counts never exceeded 500,000 and only three where the number was ever more than 1,000,000. Of the 11 animals showing an indurated condition, all but two gave counts above 1,000,000 with only one cow whose counts never exceeded 500,000." Some of these results were obtained with the unmodified Doane-Buckley test, in others the sample was subjected to preliminary heating. The detailed tests of three cows are given (p. 249), all of whom developed garget during the course of the tests. In the series given, it is noteworthy that many of the tests made while the animals were suffering from the attack were lower than those obtained before or after the attack, and the highest test (4,132,000) was obtained, not during the period of garget, but nearly three weeks before the attack and in the cow that had the lightest attack.

The cow which Campbell (1909) studied (Doane-Buckley test) for several weeks and which had one dry quarter, atrophied and very hard at the time the first samples were taken, shows but two counts over 500,000 and only two between 200,000 and 500,000 in a total of 20 counts, and yet he makes the statement that "the majority of them showed an extremely large number of leucocytes."

Hoffman (1909) has shown that the number of these cells may be increased by the injection of such inert materials as distilled water, physiological salt solution, or a boric-acid solution into the udder. The disturbances did not last over a very extended period of time.

Hewlett, Villar, and Revis (1910) investigated several cows during periods of garget. Their conclusion (p. 82) is as follows: "We are of the opinion that the cytological examination of milk does not admit of any inference of the existence of a diseased condition of the cows supplying the milk. It may point to the desirability of veterinary inspection, but gives no 'a priori' grounds for condemnation of the milk."

j. It has been suggested that certain physiological disturbances due to pregnancy, barrenness, or excitement due to heat have an influence on the number of these cells in the milk, but there is little satisfactory evidence that even indicates that such may be the case. It has also been claimed that the udder is so sensitive to disturbances of various kinds that a change of milkers, a change in the ration of the animal, undue excitement, feeding the animal during milking, bathing the udder in cold water, and the tuberculin test all produce a marked increase in the number of cells, but there is little or no evidence to show that such may be the case and it is very questionable whether all of these factors influence the cell content of the milk.

k. Another matter upon which considerable stress has been laid is that of the presence of fibrin in its relation to high cell counts. It is especially emphasized by Doane (1905), who feels after making an extended series of examinations that the presence of fibrin is a still better proof of diseased conditions than high cell content. He states: "The presence of fibrin combined with an abnormal number of leucocytes is the only satisfactory proof that inflammation exists in the udder."

Russell and Hoffman (1907*a*) (p. 252) fail to find such a close relationship between high cell content and fibrin. Nine out of 62 tests made where the cell count was less than 500,000 showed fibrin, while 12 out of 28 tests where the cell count was over 500,000 failed to show fibrin.

l. The relation between the number of these cells and the bacterial content of the milk both quantitative and qualitative has been discussed by various authors. The principal evidence from the quantitative standpoint has been furnished by Russell and Hoffman (1907*a*) and Hastings and Hoffman (1909). The former state (p. 250): "Out of 39 examinations there appeared to be no direct relation, although in some cases a high leucocyte count was accompanied by a high germ content. Quantitatively then, no relationship between the two factors above mentioned exists." "From the qualitative standpoint, however, there apparently was some connection, for it was noted that a high leucocyte count was usually associated with the presence of organisms presumably belonging to the streptococcus type." Hastings and Hoffman report on a long series of observations on two cows whose milk continuously showed a high germ content largely streptococci. Their milk also showed a high cell content, although both animals were apparently healthy.

The relationship between the presence of streptococci and a high cell content has been much discussed by various authors, only two of whom need to be considered here. Bergey (1907, and several earlier papers) thinks that there is a relationship between the two. Savage (1906) is equally convinced that there is none. The general opinion in this country seems to be that there is such a relationship, but no evidence has yet been produced either for or against this view which will stand a critical analysis.

ORIGINAL INVESTIGATIONS.

1. MATERIAL AND METHOD.

The present investigation has not dealt directly with all of the points mentioned in the review but does throw some light on most of them. The principal object has been to determine the normal conditions as revealed by the new method. Therefore normal, healthy cows have been selected for all of the work unless otherwise noted. The first part of the work was done largely by the junior author in the Biological Laboratory of Allegheny College and the samples used were obtained through the kindness of two milkmen in the neighborhood of Meadville, Pennsylvania. The herds from which the samples were obtained were kept under excellent conditions. During the time of the investigation the cattle were kept in the stable and were fed on ensilage corn, hay, and grain. The animals were Jerseys or grade Jersey and Durham. One of the herds had been tested with tuberculin and the reacting animals removed. The other herd was tested during the time of the investigation and six reacting animals were found out of the forty. Unfortunately it is impossible to state how many of these reacting animals are included in the reports.

The second part of the work was done by the senior author in Professor W. Fleishmann's laboratory in the Landwirtschaftliches Institut der Universität Göttingen. The senior author wishes to express his appreciation here of the kindly and sympathetic interest of Professor Fleishmann, who made it possible to carry out the investigation more completely than was at first thought possible. The principal samples used in this part of the work were either market milk samples from the Central Molkerei or from individual cows kept in Engelhardt's Kindermilchanstalt, in which the animals were kept under excellent hygienic conditions. The cattle here were largely of the Harz and Swiss types.

The method used in all cases for counting the cells was the direct method devised by Prescott and Breed (1910). This method has been found to be entirely practical and very simple in operation. The junior author found little difficulty with it and made nearly one-half of the tests reported. In all of the tests given here, duplicate smears were made and 100 fields of the oil immersion

lens counted on each smear. The duplicate examinations do not show all of the agreement that could be desired, and it is possible that some means could be devised for measuring the small quantity of milk used (0.01 c.c.) more accurately. The use of larger quantities of milk makes it difficult to get even smears. The method was also found to be satisfactory for use in examining creams and skim milks as well as colostral milks, advantages not possessed by the earlier methods.

All samples were prepared for examination within a short time after milking, usually less than two hours.

2. TESTS OF COWS FROM NEAR MEADVILLE, PENNSYLVANIA.

Normal number of cells present in the milk of the average dairy cow.—Data from 37 cows, all of whom were apparently healthy and secreting milk of normal appearance. They were not subjected to a careful veterinary inspection at the time of the test and so the list possibly includes some animals where such an inspection would have revealed slight abnormalities. The list includes not more than six, and probably not more than three, animals that reacted to the tuberculin test.

Cow	Age Years	Period of Lactation Months	First Count	Second Count	Average No. of Cells per c.c.
1	3	8	800,000	640,000	720,000
2	2	5	425,000	370,000	400,000
3	6	8	410,000	410,000	410,000
4	8	8	660,000	500,000	580,000
5	12	9	510,000	480,000	495,000
6	6	10	2,800,000	2,640,000	2,720,000
7	7	10	1,030,000	1,050,000	1,040,000
8	4	5	590,000	410,000	500,000
9	3	4	470,000	440,000	455,000
10	9	7	50,000	50,000	50,000
11	2	1	540,000	710,000	625,000
12	7	9	420,000	580,000	500,000
13	4	3	1,250,000	1,050,000	1,150,000
14	2	4	1,050,000	810,000	930,000
15	2	3	790,000	820,000	805,000
16	3	8	760,000	670,000	715,000
17	6	9	960,000	860,000	910,000
18	6	9	480,000	500,000	490,000
19	7	8	5,630,000	1,730,000	3,080,000
20	6	8	1,130,000	1,070,000	1,100,000
21	8	9	670,000	500,000	585,000
22	2	5	720,000	580,000	650,000
23	7	8	5,810,000	4,870,000	5,340,000
24	6	9	1,510,000	1,700,000	1,600,000
25	2	8	1,510,000	1,100,000	1,305,000
26	6	9	1,620,000	1,060,000	1,340,000
27	8	9	400,000	300,000	350,000
28	8	8	630,000	320,000	475,000
29	7	9	450,000	290,000	370,000
30	8	7	580,000	480,000	530,000
31	..	..	1,160,000	1,395,000	1,125,000
32	..	..	220,000	240,000	230,000
33	..	..	1,280,000	1,150,000	1,215,000
34	..	..	3,695,000	3,560,000	3,630,000
35	..	..	1,785,000	1,680,000	1,730,000
36	..	..	3,850,000	3,640,000	3,745,000
37	..	..	1,135,000	1,265,000	1,200,000

This gives an average of 1,165,000 per cubic centimeter for the 37 cows, maximum 5,340,000, minimum 50,000; 30 per cent of the counts are below 500,000, 40 per cent between 500,000 and 1,000,000, and 30 per cent over 1,000,000. None of the milk was abnormal in appearance and it was used for delivery to customers.

No marked relation is shown by these tests between the number of cells and the age of the cow or between the number of cells and the period of lactation. The same remarkable variation occurs that has been noted by previous workers who have used the other methods.

The fact that the average is much higher than any noted in the review of the previous work done was to be expected from the use of the new method. This average corresponds very well with the results obtained by Prescott and Breed (1910) for 46 samples of market milk where the average was 1,485,000 per cubic centimeter.

Number of cells present in the milk of the various quarters of the udder.—The samples were obtained by milking all of the milk of each quadrant separately and then sampling.

Cow	Quadrant	First Count	Second Count	Average No. of Cells per c.c.	Notes
38.....	R. F.	715,000	600,000	660,000	Fresh. First day of lactation.
	R. H.	21,885,000	17,790,000	19,840,000	Average of all four quadrants
	L. F.	14,225,000	14,600,000	14,410,000	—9,105,000 cells per c.c.
	L. H.	1,350,000	1,680,000	1,515,000	
39.....	R. F.	3,500,000	3,450,000	3,475,000	Fresh. Third day of lactation.
	R. H.	2,380,000	3,000,000	2,690,000	Average of four quadrants—
	L. F.	2,780,000	3,510,000	3,145,000	2,485,000 per c.c.
	L. H.	745,000	510,000	630,000	
40.....	R. F.	3,390,000	3,190,000	3,290,000	Within one week of the end of
	R. H.	3,145,000	2,770,000	2,960,000	the lactation period. Aver-
	L. F.	3,215,000	3,005,000	3,110,000	age of four quadrants—
	L. H.	5,920,000	6,285,000	6,100,000	3,865,000 per c.c.
41.....	R. F.	220,000	210,000	215,000	Period of lactation—several
	R. H.	300,000	360,000	330,000	months. Average of four
	L. F.	525,000	375,000	450,000	quadrants—280,000 per c.c.
	L. H.	115,000	150,000	135,000	
42.....	R. F.	70,000	55,000	65,000	Period of lactation—several
	R. H.	35,000	55,000	45,000	months. Average of four
	L. F.	50,000	50,000	50,000	quadrants—60,000 per c.c.
	L. H.	65,000	80,000	70,000	
43.....	R. F.	675,000	575,000	625,000	Period of lactation—several
	R. H.	140,000	155,000	150,000	months. Average of four
	L. F.	400,000	365,000	380,000	quadrants—305,000 per c.c.
	L. H.	65,000	55,000	60,000	
44.....	R. F.	700,000	655,000	675,000	Period of lactation—several
	R. H.	660,000	815,000	740,000	months. Average of four
	L. F.	625,000	680,000	650,000	quadrants—905,000 per c.c.
	L. H.	1,710,000	1,410,000	1,560,000	
45.....	R. F.	245,000	160,000	200,000	Fresh. R. H. quarter injured so that it gave no milk. R. F. quarter affected and milk bloody. Cow 7 yrs. old.

This table brings out some interesting conditions. There is the widest variation between the four quadrants, indicating that each quadrant acts independently. No one quadrant gives a constantly higher or lower count than the others. The tests of the cows in the early and late stages of lactation are especially important and will be referred to later.

Number of cells present in the fore milk, middle milk, and strippings of the average dairy cow.—Apparently normal and healthy except as noted.

Cow		First Count	Second Count	Average No. of Cells per c.c.	Notes
46.....	Fore milk	490,000	500,000	495,000	Mixed milk of all four quadrants.
	Middle milk	445,000	425,000	435,000	
	Strippings	850,000	745,000	800,000	
47.....	Fore milk	12,420,000	12,635,000	12,525,000	Right hind quarter of a tubercular cow.
	Middle milk	12,295,000	12,875,000	12,585,000	
	Strippings	10,490,000	12,745,000	11,620,000	
47.....	Fore milk	2,385,000	2,150,000	2,270,000	Left hind quarter of the same cow.
	Middle milk	2,600,000	2,485,000	2,540,000	
	Strippings	17,995,000	17,845,000	17,920,000	
48.....	Fore milk	340,000	345,000	345,000	Mixed milk from all four quadrants.
	Middle milk	710,000	505,000	605,000	
	Strippings	2,800,0000	2,790,000	2,795,000	
49.....	Fore milk	220,000	175,000	195,000	Mixed milk of all four quadrants.
	Middle milk	120,000	150,000	135,000	
	Strippings	795,000	940,000	870,000	
50.....	Fore milk	590,000	655,000	620,000	Mixed milk of four quadrants. Cow 6 yrs. old, in good condition. Fresh 5 days.
	Middle milk	300,000	210,000	255,000	
	Strippings	995,000	955,000	975,000	
51.....	Fore milk	125,000	155,000	140,000	Mixed milk of all four quadrants. Cow 4 yrs. old. Fresh two days.
	Middle milk	Sample brok'n	.....	.....	
	Strippings	1,150,000	715,000	930,000	
52.....	Fore milk	.....	.....	.....	Mixed milk four quadrants. Cow 4 yrs. old, good condition. Within two weeks of end of lactation period.
	Middle milk	155,000	115,000	130,000	
	Strippings	200,000	185,000	195,000	

All of these tests, with a single exception, show decidedly more numerous cells in the strippings than in the fore or middle milk. The single exception noted (No. 47, right hind quarter) shows but a few less cells in the strippings and there is reason for believing that this may be due to experimental error (note duplicate tests) rather than to the real condition. The fact that some counts show much greater relative differences than others is probably at least partially explained by differences in the thoroughness of milking. Differences between the fore milk and the middle

milk are possibly due to differences in the thoroughness of the previous milking.

These tests give some further data regarding early and late stages of lactation. There is no reason for believing that the large counts obtained from the tubercular cow were due to that rather than to other causes.

3. TESTS OF COWS FROM GÖTTINGEN, GERMANY.

Number of cells found in the milk of two healthy cows during a period of four weeks.—Samples of milk from the mixed milk of the entire morning milking were received

Cow 54				Cow 55			
Date	First Count	Second Count	Average No. of Cells per c.c.	Date	First Count	Second Count	Average No. of Cells per c.c.
Nov. 9	510,000	470,000	490,000	Nov. 9	2,385,000	2,440,000	2,415,000
10	260,000	300,000	280,000	10	1,900,000	1,860,000	1,880,000
11	455,000	535,000	495,000	11	1,610,000	1,880,000	1,745,000
12	335,000	355,000	345,000	12	1,040,000	1,020,000	1,030,000
13	.....	.....	.....	13	.....	.....	.....
14	260,000	180,000	220,000	14	3,350,000	3,580,000	3,465,000
15	435,000	465,000	450,000	15	2,500,000	2,930,000	2,715,000
16	300,000	270,000	285,000	16	1,240,000	1,250,000	1,245,000
17	290,000	225,000	255,000	17	1,985,000	1,825,000	1,905,000
18	620,000	450,000	535,000	18	1,800,000	1,470,000	1,635,000
19	280,000	230,000	255,000	19	1,765,000	1,480,000	1,625,000
20	425,000	415,000	420,000	20	2,280,000	2,460,000	2,370,000
21	1,185,000	925,000	1,055,000	21	1,785,000	2,015,000	1,900,000
22	1,000,000	810,000	905,000	22	1,480,000	1,220,000	1,350,000
23	460,000	390,000	425,000	23	1,615,000	1,905,000	1,760,000
24	205,000	225,000	215,000	24	1,780,000	1,570,000	1,675,000
25	505,000	445,000	475,000	25	1,165,000	1,305,000	1,235,000
26	320,000	350,000	335,000	26	780,000	1,110,000	945,000
27	455,000	445,000	450,000	27	3,120,000	2,960,000	3,040,000
28	270,000	240,000	255,000	28	2,090,000	2,120,000	2,105,000
29	810,000	700,000	755,000	29	1,005,000	925,000	965,000
30	700,000	660,000	680,000	30	5,920,000	6,030,000	5,975,000
Dec. 1	360,000	410,000	385,000	Dec. 1	2,200,000	2,030,000	2,110,000
2	740,000	920,000	830,000	2	990,000	780,000	885,000
3	940,000	1,080,000	1,010,000	3	3,930,000	3,500,000	3,715,000
4	615,000	685,000	650,000	4	1,940,000	1,820,000	1,880,000
5	460,000	390,000	425,000	5	1,060,000	1,100,000	1,080,000
6	810,000	1,150,000	980,000	6	2,400,000	2,310,000	2,355,000
7	930,000	960,000	945,000	7	2,990,000	3,120,000	3,055,000
8	710,000	710,000	710,000	8	1,860,000	1,980,000	1,920,000

	Butter Fat	Total Solids	Specific Gravity
Nov. 22	3.4%	12.068%	1.0309
Dec. 8	3.6%	12.283%	1.0308

Cow No. 54—Average of 29 tests = 535,000

	Butter Fat	Total Solids	Specific Gravity
Nov. 21	3.4%	11.767%	1.0297
Nov. 22	3.95%	12.653%	1.0306
Nov. 28	3.4%	11.867%	1.0301

Cow No. 55—Average of 29 tests = 2,070,000

from the Kindermilchanstalt daily. The two cows were selected as the most normal animals possible to obtain and without any reference to the cell content of their milk. Both had been under competent veterinary inspection and showed no known history

of garget or other troubles. Cow 54 was a cross between a Harz and Glaner strain, 7 years old, had calved March 6, 1910, and was giving 10½ liters per day on November 1. She had been tested with tuberculin on March 20. Better than the average cow of the herd, but not one of the best cows. Cow 55 was a Harz cow, 7 years old, had calved June 1, 1910, was tested with tuberculin June 14, and was giving 8 liters per day on November 1. She had had twin calves, both normal, and was not giving as much milk as she had in previous lactation periods. The udder was said to be smaller and more fleshy than formerly. A careful veterinary inspection of these cows on December 9 failed to disclose the slightest abnormal or pathological conditions. The fodder was hay, clover, and dry grain.

From November 30 to December 8, inclusive, the milk of Cow 55 was examined in greater detail to discover the conditions prevailing in the four quadrants of the udder. Unfortunately, just at this time there was a change of milkers and the instructions were not carried out perfectly, in that a varying amount of strippings was not included in the samples. This undoubtedly caused an equally variable error in all of these tests, the numbers being smaller than they otherwise would have been. This is also the explanation of the low fat content of the milk. By chance, the first test from the four quadrants was made on the morning when the mixed milk contained the greatest number of cells found during the 29 tests.

Date	Quadrant	First Count	Second Count	Average No. of Cells per c.c.	Butter Fat	Total Solids	Specific Gravity	
Nov. 30. .	R. H.	21,750,000	23,250,000	22,500,000	2.7%	10.650%	1.0286	
	L. H.	1,165,000	1,315,000	1,240,000	2.6%	11.283%	1.0316	
	R. F.	2,665,000	2,455,000	2,560,000	2.6%	10.932%	1.0302	
	L. F.	985,000	915,000	950,000	2.7%	11.178%	1.0307	
Average of four quadrants—6,810,000. Test from mixed milk—5,975,000.								
Dec. 1. . .	R. H.	14,250,000	14,870,000	14,560,000	The milker forgot to make samples from the regular morning milking and drew these at 10 A.M.			
	L. H.	2,610,000	2,640,000	2,625,000				
	R. F.	3,820,000	4,080,000	3,950,000				
	L. F.	1,990,000	1,980,000	1,985,000				
Average—5,780,000. Mixed milk—2,115,000. This latter was from the regular morning milking and contained all of the strippings.								
Dec. 2. . .	R. H.	1,600,000	1,520,000	1,560,000	2.0%	10.463%	1.0312	
	L. H.	710,000	630,000	670,000	3.1%	11.733%	1.0310	
	R. F.	800,000	770,000	785,000	2.6%	11.183%	1.0312	
	L. F.	330,000	350,000	340,000	2.3%	11.024%	1.0320	
Average—840,000. Mixed milk—885,000.					2.55%	11.123%	1.0312	
Dec. 3. . .	R. H.	3,265,000	3,435,000	3,350,000	2.2%	10.653%	1.0310	
	L. H.	2,430,000	2,560,000	2,495,000	2.55%	11.073%	1.0310	
	R. F.	3,125,000	3,185,000	3,155,000	2.35%	10.733%	1.0306	
	L. F.	2,860,000	2,880,000	2,870,000	2.1%	10.834%	1.0322	
Average—3,465,000. Mixed milk—3,715,000.					2.3%	10.873%	1.0314	

Dec. 4...	R. H.	3,750,000	3,500,000	3,625,000	2.3%	10.999%	1.0319	
	L. H.	1,235,000	1,225,000	1,230,000	2.4%	11.394%	1.0330	
	R. F.	2,540,000	3,130,000	2,835,000	2.5%	11.113%	1.0314	
	L. F.	1,430,000	1,260,000	1,345,000	2.3%	10.973%	1.0318	
	Average—2,260,000. Mixed milk—1,880,000.				2.3%	11.149%	1.0325	
Dec. 5...	R. H.	2,160,000	2,040,000	2,100,000	1.1%	9.584%	1.0320	
	L. H.	1,570,000	1,540,000	1,555,000	2.1%	10.909%	1.0325	
	R. F.	1,010,000	960,000	985,000	2.0%	10.413%	1.0310	
	L. F.	750,000	730,000	740,000	1.6%	10.409%	1.0329	
	Average—1,345,000. Mixed milk—1,080,000.				1.8%	10.574%	1.0326	
Dec. 6...	R. H.	6,390,000	6,420,000	6,405,000	2.0%	10.112%	1.0298	
	L. H.	1,110,000	1,100,000	1,105,000	2.15%	10.969%	1.0325	
	R. F.	650,000	740,000	695,000	2.4%	11.043%	1.0316	
	L. F.	1,650,000	1,570,000	1,610,000	2.0%	10.798%	1.0325	
	Average—2,455,000. Mixed milk—2,355,000.				2.1%	10.658%	1.0315	
Dec. 7...	R. H.	2,310,000	2,540,000	2,425,000	2.4%	10.717%	1.0303	1 liter
	L. H.	2,350,000	2,530,000	2,440,000	2.6%	11.409%	1.0321	1¼ liter
	R. F.	3,930,000	4,040,000	3,985,000	2.25%	10.663%	1.0308	1 liter
	L. F.	2,380,000	2,150,000	2,265,000	1.8%	10.549%	15.302	1¼ liter
	Average—2,780,000. Mixed milk—3,055,000.				2.3%	10.873%	1.0314	

Two days later, after the explanation of the low fat content had been discovered, a more thorough test of the morning milking was made. The milk from each quadrant was milked as usual and kept separate. The same was done for the strippings that accumulated after the first milking. The tests follow:

Date	Quadrant	First Count	Second Count	Average No. of Cells per c.c.	Butter Fat	Total Solids	Specific Gravity	
Dec. 9...	R. H.	2,390,000	2,380,000	2,385,000	2.9%	11.418%	1.0307	1165 c.c.
	L. H.	2,380,000	2,510,000	2,445,000	2.9%	11.693%	1.0318	1175 c.c.
	R. F.	1,540,000	1,485,000	1,515,000	2.7%	11.529%	1.0321	1020 c.c.
	L. F.	1,500,000	1,475,000	1,490,000	2.8%	11.599%	1.0319	960 c.c.

The tests for the strippings follow:

	R. H.	4,760,000	4,800,000	4,780,000				104 c.c.
	L. H.	5,255,000	5,395,000	5,325,000				100 c.c.
	R. F.	3,470,000	3,285,000	3,375,000				96 c.c.
	L. F.	2,685,000	2,855,000	2,770,000				110 c.c.

Test of entire milk, strippings included.

		2,385,000	2,495,000	2,440,000	3.15%	11.743%	1.0308	4731 c.c.

This last test shows what a difference there is in the cell content of the middle milk and strippings. The cell count of the entire mixed milk is very markedly increased by the inclusion of the strippings. The principal conclusions to be drawn from these tests will be referred to later. Attention is called to the test of 22,500,000 cells per cubic centimeter in the right hind quarter of Cow 55 on November 30 as well as to many other tests which would be regarded as extremely high by the men who have used the older methods of counting the cells. In this connection, it should also be remembered that the tests from November 30 to December 9 for Cow 55 are lower than they would have been if the strippings had been included. Yet there is no sufficient reason disclosed for regarding this milk as in the slightest degree abnormal. Certainly the milker and the dealer both honestly believed it to be of the highest quality and it was handled in the cleanliest manner possible and sold for feeding babies.

Number of cells in each quadrant of the udder of a single healthy cow during a period of five weeks.—Samples from the entire milk obtained from the right hind quarter were taken from the evening milking daily and from the remaining three quadrants twice weekly. This cow (No. 56) was selected from the same herd as 54 and 55 and in the same way as were these animals. She was a Glaner cow, four years old, had calved June 1, 1910, was tuberculin tested on June 14, and was giving 13¾ liters per day on November 1. One of the best cows in the herd. In order to condense the report the duplicate counts will not be given in this case.

Cow 56.

Date	R. H.	L. H.	R. F.	L. F.	Notes
Nov. 3	2,500	5,000	230,000	2,500	The milk from three quadrants proved to have the fewest cells present that have been found by this new method. Where the figure given is 5,000, it means that only one cell was seen in 100 fields of the oil immersion lens. 2,500 means that no cells were seen in 100 fields but an examination with a lower power showed that cells were present.
4	.....	.....	.....	.....	
5	2,500	.....	.....	.....	
6	2,500	.....	.....	.....	
7	15,000	.....	.....	.....	
8	15,000	20,000	530,000	10,000	
9	7,000	.....	.....	.....	
10	20,000	.....	.....	.....	
11	25,000	15,000	580,000	10,000	
12	2,500	.....	.....	.....	
13	25,000	.....	.....	.....	
14	25,000	.....	.....	.....	
15	40,000	15,000	1,375,000	70,000	Butter fat 4.4 per cent, total solids 14.068 per cent, spec. grav. 1.0341 in R. H. (Nov. 15).
16	25,000	.....	.....	.....	
17	7,500	.....	.....	.....	
18	12,500	35,000	605,000	20,000	
19	15,000	15,000	780,000	20,000	
20	80,000	20,000	390,000	10,000	Morning milking. All others from evening milking.
	20,000	.....	.....	.....	
21	15,000	.....	.....	.....	
22	35,000	40,000	1,045,000	25,000	

Cow 56.—*Continued.*

Date	R. H.	L. H.	R. F.	L. F.	Notes
Nov. 23.....	30,000	.....	.....	.....	Butter fat 4.6 per cent, total solids 14.059 per cent, spec. grav. 1.0331 in R. H. (Nov. 23).
24.....	200,000	.....	.....	.....	
25.....	65,000	30,000	650,000	5,000	
26.....	10,000	.....	.....	.....	
27.....	30,000	.....	.....	.....	
28.....	12,500	.....	.....	.....	
29.....	20,000	5,000	755,000	40,000	
30.....	125,000	.....	.....	.....	
Dec. 1.....	25,000	.....	.....	.....	
2.....	10,000	25,000	1,110,000	20,000	
3.....	10,000	.....	.....	.....	
4.....	7,500	.....	.....	.....	
5.....	20,000	.....	.....	.....	
6.....	15,000	5,000	270,000	5,000	
7.....	20,000	.....	.....	.....	
8.....	10,000	.....	.....	.....	Butter fat 3.7 per cent, total solids 13.427 per cent, spec. grav. 1.0349 in R. H. (Dec. 8).

The average for the twelve days on which all four quadrants were tested is 190,000 cells per c.c.

The two charts show these results graphically and in a way that shows their real character much better than the figures. The last part of the curve for Cow 55 has been drawn in dotted lines to call attention to the fact that the results on which it is based are not as satisfactory as the others. The random selection of three cows proved very happy, for Cows 55 and 56 stand at opposite extremes so far as numbers of cells are concerned, while Cow 54 stands in an intermediate position.

Through the courtesy of Dr. George Wiegner, of the Landwirtschaftliches Institut der Universität Göttingen, samples were obtained from Cows 54, 55, and 56 on January 13 and 16, 1911, and forwarded to the senior author of this paper.

Cow 54: This cow met with an accident a few days before the samples were secured. A neighboring cow stepped upon her udder, bruising the left forward quadrant so that it gave bloody milk. The tests of the milk drawn from this quadrant on the morning of January 16 are as follows: 17,000,000–21,000,000; average, 19,000,000 cells per cubic centimeter. Only 50 fields of the microscope were counted in this case. There were large numbers of red blood corpuscles present in the smears and a great number of cell fragments, so that there is a very large error in the count.

The mixed milk of the remaining three quarters tested as follows: 760,000–660,000, average 710,000 cells per cubic centimeter. This number is very much the same as had been found previously in the milk of this cow. The number does not seem to have been influenced greatly by the bruising of the one quadrant.

Cow 55: Whole milk from morning milking of January 13, 3,160,000–2,840,000; average 3,000,000 cells per cubic centimeter. January 16, 3,000,000–3,020,000; average 3,010,000 cells per cubic centimeter. These two tests are both much higher than the average for this cow, and yet a glance at Chart 1 will show that similar tests might have been obtained several times during the previous month if taken at similar three-day intervals.

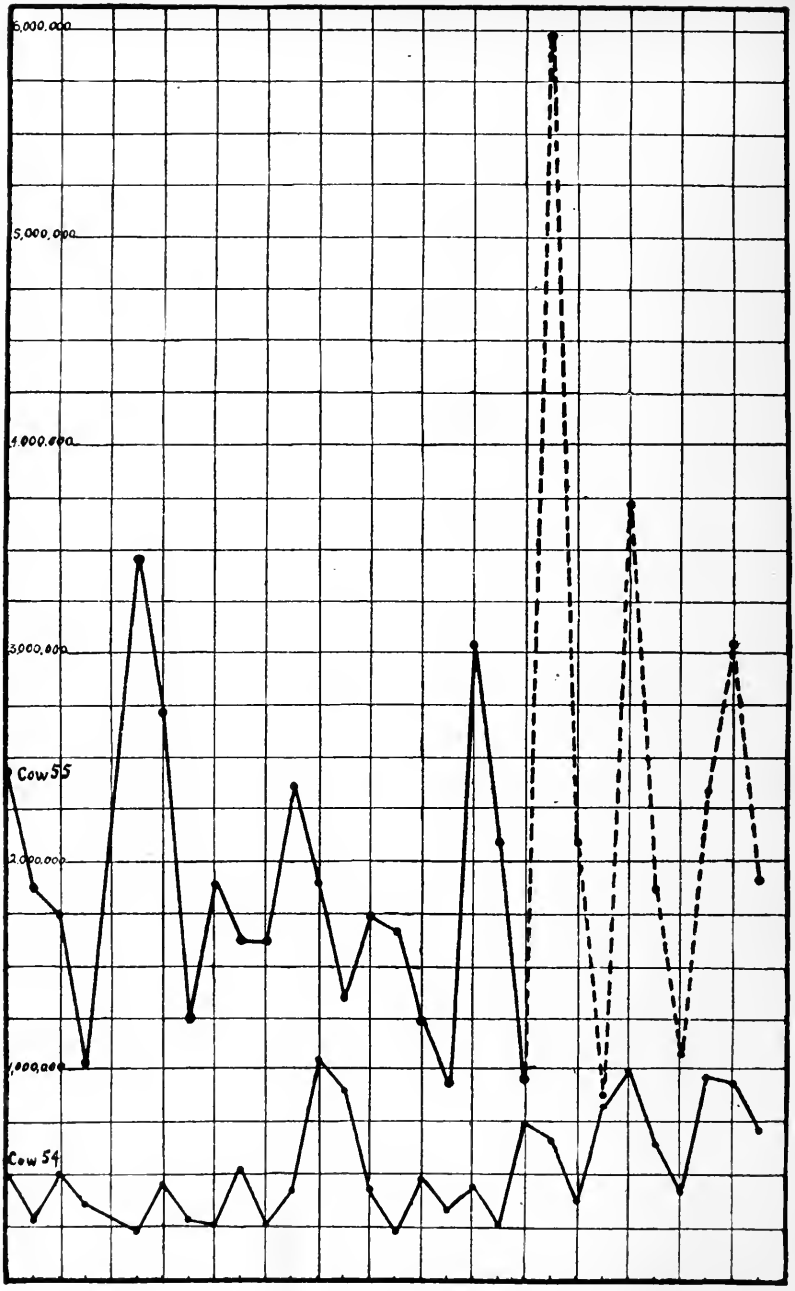

CHART 1.—Daily variation in the cellular content of the milk of Cows Nos. 54 and 55 from November 9 to December 8, 1910. Each vertical space represents 250,000 cells per cubic centimeter. Each horizontal space represents a two-day interval.

Cow 56: Separate quadrants, evening milking.

	January 13			January 16		
			Average			Average
R. H.....	40,000	40,000	40,000	90,000	65,000	75,000
R. F.....	50,000	40,000	45,000	30,000	40,000	35,000
L. H.....	3,160,000	2,810,000	2,985,000	865,000	1,025,000	945,000
L. F.....	30,000	15,000	22,000	30,000	40,000	35,000

It will be noted that all of the counts are somewhat higher than during the previous month.

Important points to notice are: first, that the cow giving the greatest quantity of milk discharged the least number of cells

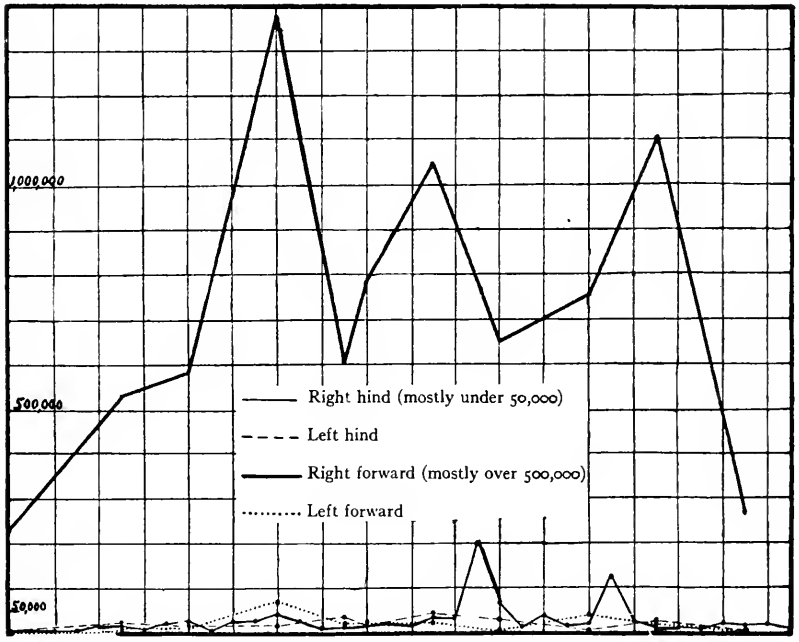

CHART 2.—Variation in the cellular content of the milk from the four quadrants of the udder of Cow No. 56 from November 3 to December 8, 1910. Each vertical space represents 100,000 cells per cubic centimeter. Each horizontal space represents a two-day interval.

both actually and relatively. So striking is this difference that if the whole number of cells discharged by the right hind quarter of Cow 56 in the 36 milkings tested had been discharged at a *single milking* and that in a liter of milk instead of in the $1\frac{3}{4}$ liters

actually secreted, the number of cells would have been approximately 2,000,000 per cubic centimeter, that is, a number which was the average number of cells found in the milk of Cow 55. In the extreme case, one quadrant of Cow 55 liberated almost as many cells at a *single milking* as the hind quarter of Cow 56 would have done in the *half of an entire lactation period* if the same low rate found in the period tested prevailed. Such facts as these are of importance in interpreting histological and physiological phenomena. The histological descriptions of the udder have been written by men who have necessarily studied but a small number of udders. No one of them has had any idea as to the number of cells which the udders studied were secreting at the time they were obtained for study. Is it any wonder that one histologist reports that cell divisions are common and another that they are rare or absent?

Second, there seems to be something of a cyclic variation in the curves of Cows 54 and 55. The time interval between maxima or between minima is almost always three days, with some two-day and some four-day intervals. The curve is ascending 20 days, descending 36 days, a ratio of almost one to two. In other words, there seems to be a tendency to jump suddenly to a maximum, usually in one day, while the descent to a minimum takes two or three days. Whether these points are of real significance or not remains to be seen.

Third, in spite of the enormous and sudden variations there seems to be a certain constancy for all three of the cows. This was sufficiently marked so that it was always possible to identify the three daily samples without recourse to the labels. This constancy held also for the separate quadrants of the udder in Cow 56.

Fourth, the greatest range of variation occurred in the cow which had the highest average number of cells in her milk, and the least range in the cow with the lowest average.

Number of cells in the market milk of Göttingen.—During the course of these investigations, market milk samples were tested seven times. All samples came from the Central Molkerei, but on different days. This milk had been purified by passing through a low speed separator which must have removed some of the cells, especially

as the milk was warmed to 40° C. before passing through the separator. It should be noted here that the samples of market milk reported on by Prescott and Breed (1910) (p. 640) had been subjected to similar treatment except that they had not been subjected to the previous warming before passing through the separator. The tests of the Göttingen milk follow:

1,540,000	2,205,000
4,185,000	1,980,000
1,885,000	1,980,000
1,645,000	Average = 2,200,000

The comparison of the average with those obtained by Prescott and Breed shows that the conditions in Boston and Göttingen are not markedly different.

Goats' milk.—Tests of the milk of two goats showed the following results:

Goat 1, Age 3 yrs., Lambed April 1, Tested November 1, 425,000 cells per c.c.

Goat 2, Age 5 yrs., Lambed April 1, Tested November 1, 2,245,000 cells per c.c.

Both reported to be normal and healthy.

Fresh cows.—The scattered evidence presented in these pages concerning colostral milk may be presented in brief form here.

Cow 38—	9,105,000.	First day. Number given is the average of the tests of the individual quadrants as follows: R. F.—660,000, R. H.—19,840,000, L. F.—14,410,000, L. H.—1,515,000.
Cow 39—	2,485,000.	Third day. Ditto: R. F.—3,475,000, R. H.—2,690,000, L. F.—3,145,000, L. H.—630,000.
Cow 45—	200,000.	In R. F. quadrant. Fresh. This cow had been injured in the R. H. quadrant so that this quarter gave no milk and the quarter tested gave milk that was somewhat bloody. Cow 7 yrs. old.
Cow 50—	620,000.	Fore milk. Cow fresh five days. 6 yrs. old.
	255,000.	Middle milk.
	975,000.	Strippings.
Cow 51—	140,000.	Fore milk. Fresh two days. 4 yrs. old.
	930,000.	Strippings.
Cow 57—	1,535,000.	Second milking after calving. Full blood Holstein, 3 yrs. old. Udder indurated but not seriously. Reported by Breed (1910).

It will be seen that not one of these tests shows numbers of cells in excess of numbers reported elsewhere for apparently normal cows in later stages of lactation. Even Cow 38 does not reach the numbers given for Cow 55.

End of lactation period.—Only two tests bear directly on this point.

Cow 40—	3,865,000.	Within one week of the end of the lactation period. The number given is the average of tests of the individual quadrants as follows: R. F., 3,290,000, R. H., 2,960,000, L. F., 3,110,000, L. H., 6,100,000.
Cow 52—	150,000.	Middle milk. Within two weeks of the end of the lactation.
	195,000.	Strippings. Cow 4 yrs. old.

Neither of these tests is exceptionally different from apparently normal conditions.

Pathological conditions.—No effort was made to examine such material. Cow 47 and the two fresh cows (45 and 57) reported just above are the only animals which were examined which showed pathological conditions. The tests of these animals do not indicate much, but they do show that further investigation must be made before the commonly accepted conclusions on this point can be regarded as proved.

CONCLUSIONS.

In view of the fact that these investigations seem to show that nearly all of our beliefs regarding the presence of cellular elements in milk will need to be revised, a conservative statement of our knowledge concerning their number and significance has been deemed of value.

1. The number of cellular elements in cows' milk varies from numbers so few as to be almost negligible (less than 5,000 per cubic centimeter) to 20,000,000 and more per cubic centimeter in milk which we have good reason for calling normal. According to the investigations thus far available, nearly one-third of normal healthy cows show a cell content in their milk less than 500,000 per cubic centimeter, somewhat more than one-third show a cell content between 500,000 and 1,000,000 per cubic centimeter, while nearly one-third give milk containing more than 1,000,000 cells per cubic centimeter. There is no evident reason for regarding the discharge of these cells in the numbers stated as indicating pathological conditions. In certain other skin glands, notably the sebaceous glands, the secretion is very largely or entirely cellular and it is not surprising to find similar conditions in glands which are possibly homologous structures.

2. Competent evidence to support the common statements that colostral milk and milk from animals nearly dry contain larger numbers of cells than milk obtained during other periods of lactation is lacking. Striking variations in the number of cells occur from day to day, and there is some evidence to show that this variation may be cyclic in nature. Individual cows maintain a certain constancy of numbers for several weeks at a time, at least. It is possible that the number of cells may be correlated to some degree with the amount of milk secreted, udders which give large amounts discharging fewer cells than where less milk is secreted.

3. The range of variation between individual quarters of a single udder is apparently as great as the range of variation between different cows. Whether or not they vary absolutely independently of each other is not clear, but the relationship does not seem to be close.

4. There is good reason for believing that there is always an increase of the number of cells in the strippings where the milking is done thoroughly. No constant relation between the fore milk and the middle milk is apparent.

5. The cause of the normal variations is not clear from the evidence at hand and probably will not be found until the nature of the cells is clear. Conclusive evidence to show whether such suggested causes as age, period of lactation, race of cow, barrenness, pregnancy, heat, the ration used, undue excitement, etc., have or have not an influence on the number of these cells is not at hand.

6. The numerical relation between these cells and pathological conditions is not at all clear. The statement that large numbers of these cells in the milk indicate a pathological condition is as unjustified by the evidence as is the converse that all pathological conditions produce an increased number of cells. Such evidence as there is indicates that very evident pathological conditions do not necessarily produce milk which has even an average number of cells for normal milk. Yet it is undoubtedly true that some pathological conditions do produce excessively large numbers of cells in the milk.

7. The evidence that there is a relation between the bacterial content of the udder, especially streptococcic, and the number of these cells is very inconclusive when subjected to a critical analysis. It may be that such a relationship holds true, but a fair verdict must be the old Scotch one of "not proven."

8. Inasmuch as it is evident from these investigations that where market milk is the mixed milk of any large number of cows the number of cells is bound to be considerably in excess of 1,000,000 per cubic centimeter, it is evident that all of the present numerical standards must be discarded. As there is at present no good evidence to show that the number of these cells has more

than minor hygienic significance, if any at all, there is no reason for suggesting other standards.

It may be said that these conclusions do not give sufficient weight to the previous investigations on this subject. Yet it seems to the authors of this paper that greater weight cannot be given in view of the evidence presented here and elsewhere.

BIBLIOGRAPHY.

BERGEY, D. H. 1907. "The Leucocyte and Streptococcus Content of Cows' Milk," *Univ. Penn. Med. Bull.*, 20, pp. 106–9.

BREED, R. S. 1910. "Cellular Elements in Cows' Milk," *Proc. 8th Internat. Zoöl. Cong., Graz, Austria*, August, 1910.

CAMPBELL, H. C. 1909. "Leucocytes in Milk: Methods of Determination and the Effect of Heat upon Their Number," *U.S. Dept. Agri., Bur. Animal Ind., Bull. 117*, 19 pp.

Committee on Standard Methods of Bacterial Milk Analysis. 1910. Final Report. *Am. Jour. Pub. Hyg.*, 20, pp. 315–45.

DOANE, C. F. 1905. "Leucocytes in Milk and Their Significance," *Md. Agri. Exper. Sta., Bull. 102*, pp. 205–23.

HASTINGS, E. G., AND HOFFMAN, C. 1909. "Bacterial Content of the Milk of Individual Animals," *Univ. Wis. Agri. Exper. Stat., Research Bull. 6*, pp. 189–96; and *Centralbl. f. Bakt.*, Abt. 2, 25, pp. 465–70.

HEWLETT, R. T., VILLAR, S., AND REVIS, C. 1910. "On the Nature of the Cellular Elements Present in Milk. Part II, Quantitative and Qualitative Results," *Jour. Hyg.*, 10, pp. 56–92.

HOFFMAN, C. 1909. "Experimental Leucocytosis in the Cow's Udder," *Univ. Wis. Agri. Exper. Sta., Research Bull. 6*, pp. 207–18.

KENDALL, A. I. 1907. "The Significance and Microscopical Determination of the Cellular Contents of Milk," *Coll. Stud. Research Lab. Dept. Health City of New York*, 3, pp. 169–81.

PENNINGTON, M. E., AND ROBERTS, E. L. 1908. "The Significance of Leucocytes and Streptococci in the Production of a High-Grade Milk," *Jour. Infect. Dis.*, 5, pp. 72–84.

PRESCOTT, S. C., AND BREED, R. S. 1910. "The Determination of the Number of Body Cells in Milk by a Direct Method," *Jour. Infect. Dis.*, 7, pp. 632–40. Abst. in *Am. Jour. Hyg.*, 20, pp. 663–64; also in *Rev. Gên. du Lait*, 8, pp. 404–5.

RUSSELL, H. L., AND HOFFMAN, C. 1907. "Leucocyte Standards and the Leucocyte Content of Milk from Apparently Healthy Cows," *Jour. Infect. Dis.*, Supp. 3, pp. 63–76.

1907*a*. "Distribution of Cell Elements in Milk and Their Relation to Sanitary Standard," *Univ. Wis. Agri. Exper. Stat., 24th Ann. Rep.*, pp. 231–53.

1908. "Effect of Heating upon the Determination of Leucocytes in Milk," *Am. Jour. Pub. Hyg.*, 18, pp. 285–91.

SAVAGE, W. G. 1906. "Streptococci and Leucocytes in Milk," *Jour. Hyg.*, 6, pp. 123–38.

STONE, B. H., AND SPRAGUE, L. P. 1909. "Some Studies of the Physiological Leucocyte Content of Cows' Milk," *Jour. Med. Res.*, 20 (N.S. 15), pp. 235–43.

WARD, A. R., HENDERSON, M., AND HARING, C. M. 1906. "The Numerical Determination of Leucocytes in Milk," *State Bd. Health Calif., 19th Biennial Rep.*, pp. 142–56.

NOTE.

ERRATA IN ARTICLE BY PRESCOTT AND BREED, *Jour. Infect. Dis.*, 1910, 7, pp. 632 ff.:

P. 634, l. 14: "0.005" should read "0.0002 or 1/5000."

P. 638, l. 6: "eight half-pint" should read "cans containing 16 ½ pints."

ON INSTITUTIONAL DYSENTERY.

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(From the Memorial Institute for Infectious Diseases.)

A STUDY OF THE DYSENTERY OCCURRING AT THE COOK COUNTY INSTITUTIONS AT DUNNING, ILL., IN 1910.*

Dysentery has been endemic in the Cook County Institutions at Dunning for a number of years. The report of this investigation, which was suggested by Dr. Hektoen, concerns etiology, diagnosis, and therapy.

I. ETIOLOGY.

The etiology of asylum dysentery has been investigated in many instances in this and in other countries. In most cases the dysentery was found to be of the bacillary type. The cases reported by Vedder and Duval[1] were mostly due to the Flexner type of bacilli, only a few Shiga bacillus cases being found. Fisher, in an epidemic at the Middletown Hospital for the Insane,[2] found only bacilli of the Flexner type. In an epidemic at the Danvers State Hospital, Danvers, Massachusetts,[3] bacilli of the Shiga type predominated. Only a few cases due to the Flexner bacillus were found.

The dysentery bacilli are classified, according to their properties of sugar fermentation, into mannite fermenters and non-mannite fermenters. The Shiga bacillus represents the non-fermenters, and the bacilli which ferment mannite are divided into (1) the Flexner-Harris type, which ferments maltose but not saccharose, (2) the Hiss Y type, fermenting neither maltose nor saccharose, (3) the Strong type, which ferments maltose but not saccharose. These types may also be distinguished from each other by agglutination tests.

* Received for publication January 12, 1911. This work was done by aid of a grant from the Fellowship Fund of the Alumni Association of Rush Medical College.

[1] Vedder and Duval, *Jour. Exper. Med.*, 1902, 6, p. 181.

[2] MacConkey and Hill, *Thompson Yates Laboratory Reports*, 1901, 4, p. 160.

[3] E. T. F. Richards, *Boston Med. and Surg. Jour.*, 1909, 161, p. 679.

A search was made in a number of the cases of diarrhea occurring in the institution as follows:

The stools were transferred to sterile test-tubes and taken to the laboratory. They were plated within a few hours after passage. Particles of mucus were selected for examination and washed in two changes of sterile salt solution or broth. The mucus was then shaken in a small quantity of broth until thoroughly disintegrated, and, by means of a platinum spatula, different dilutions of this mixture were smeared

TABLE 1.

Case Number	Motility	Gram Stain	Lactose Acid	Glucose Gas	Mannite Acid	Whey 24 hrs. Acid	Whey 48 hrs. Acid	Milk Coagulation	Cultural Characters of
1	−	−	−	−	−	−	+	−	Shiga bacillus
2*a*	−	−	−	−	+	+	Alk.	−	Flexner "
2*b*	−	−	−	−	−	−	−	−	Shiga "
3	Negative								
4	−	−	−	−	−	+	+	−	Shiga "
5	Negative								
6	Negative								
7	−	−	−	−	−	?	+	−	Shiga "
8	−	−	−	−	+	+	Alk.	..	Flexner "
9	−	−	−	−	−	+	+	−	Shiga "
10	Negative								
11*a*	+	−	−	+	+	+	+	−	?
11*b*	−	−	−	−	−	−	−	−	Shiga "
12	−	−	−	−	−	−	−	−	Shiga "
13	−	−	−	−	+	−	−	−	Flexner "
13	?	−	−	−	−	−	+	−	Shiga "
14	−	−	−	−	−	−	−	−	Shiga "
15	−	−	−	−	−	−	−	−	Shiga "
16	−	−	−	−	−	−	+	−	Shiga "
17	−	−	−	−	−	+	+	−	Shiga "
18	−	−	−	−	−	−	+	−	Shiga "
19	−	−	−	−	+	+	Alk.	−	Flexner "
20	−	−	−	−	+	+	Alk.	−	Flexner "
21	−	−	−	−	−	−	−	−	Shiga "
22	−	−	−	−	−	−	−	−	Shiga "
23	−	−	−	−	−	+	+	−	Shiga "
24	−	−	−	−	+	+	Alk.	−	Flexner "
25	−	−	−	−	−	−	−	−	Shiga "
26	Pyocyaneus								
27	−	−	−	−	+	+	Alk.	−	Flexner "
28	−	−	−	−	−	−	−	−	Shiga "
29	−	−	−	−	−	+	+	−	Shiga "
30	Negative								
31	−	−	−	−	−	+	+	−	Shiga "
32	−	−	−	−	+	+	Alk.	−	Flexner "
33	−	−	−	−	−	+	+	−	Shiga "
34	−	−	−	−	−	−	−	−	Shiga "
35–36	Negative								

over the surface of dextrose free litmus lactose agar plates. At the end of from 18 to 48 hours, subcultures were made from colonies which had not produced acid upon the medium. Usually about a dozen such plates were made. The sub-cultures were examined as to morphology and retention of Gram's stain. Those organisms which were like the dysentery bacillus in shape—that is, small, plump bacilli resembling colon bacilli and Gram-negative—were grown on various media as follows. Shake cultures in glucose agar were made for the purpose of testing for gas formation. Growth on litmus lactose agar was observed in slant tubes for control of the growth on the plates. Tests for acid formation were made in tubes of litmus mannite agar and litmus whey. Milk tubes were inoculated to test for the coagulating power. In a

few cases indol formation was tested for after a week's growth on Dunham's peptone solution. Because of lack of diagnostic significance the test was discontinued. The examination for motility was made from 18-hour growths in broth and agar slants. Mannite fermenters were tested for the fermentation of maltose and saccharose and proved to be of the Flexner-Harris type exclusively. The organisms which corresponded to the cultural characteristics of dysentery bacilli are given in Table 1. It will be seen that in 22 cases bacilli of the Shiga type were found, in nine cases bacilli of the Flexner type, and in seven cases no organisms of the dysentery type were recovered. In cases 2 and 13 both Shiga and Flexner organisms were found.

In case 9 bacilli of the cultural characteristics of paratyphoid A bacilli were found but were not identified by agglutination. In case 26 *B. pyocyaneus* was isolated. The report of an epidemic of dysentery due to *B. pyocyaneus*[1] lends interest to this finding.

The examination for amebae in the stools proved negative.

In order to confirm the diagnosis of the bacilli isolated from the stools, agglutination tests were made with serum of animals immunized to known strains of dysentery bacilli obtained from Dr. N. McL. Harris of the University of Chicago. A goat was immunized

TABLE 2.

Case No.	1–20	1–40	1–80	1–160	Control in Salt Sol.
1	+	±	−	−	−
2*b*	+	−	−	−	−
4	+	+	−	−	−
7	+	+	−	−	−
9*b*	+	±	−	−	−
11*b*	+	−	−	−	−
13*b*	+	−	−	−	−
14	+	+	−	−	−
16	−	−	−	−	−
17	+	−	−	−	−
18	+	+	+	−	−
21	+	+	+	−	−
22	+	−	−	−	−
23	Culture lost				
25	+	+	−	−	−
28	+	−	−	−	−
29	+	−	−	−	−
31	+	+	−	−	−
32	+	+	+	−	−
34	+	+	±	−	−
*C_1	+	+	±	−	−
†C_2	−	−	−	−	−

* C_1 = Stock Shiga bacillus.
† C_2 = Control made with colon bacilli.

to Shiga bacilli and tests were made by the macroscopic method. The tubes were incubated at 37° C. and observation was made at the end of four hours. The results are represented by Table 2.

An agglutination test was made in a similar way with Flexner

[1] Lartigau, *Jour. Exper. Med.*, 1898, 3, p. 595.

bacilli with the serum of a rabbit immunized to known Flexner bacilli (Table 3).

TABLE 3.

Case No.	1–20	1–40	1–80	1–160	Control in Salt Sol.
20.....	+	+	+	–	–
24.....	+	–	–	–	–
32.....	+	–	–	–	–
*C_1.....	+	+	+	–	–
†C_2.....	–	–	–	–	–

* C_1=Stock Flexner bacillus.
† C_2=Colon bacillus.

Unfortunately some of the strains of Flexner bacilli were lost and agglutination tests were possible only with part of the strains isolated.

Except in one instance—case 16—the agglutination test corresponded to the cultural diagnosis. The results, then, are in confirmation of the work of other investigators.

With the hope of ascertaining the way in which the dysentery bacilli are perpetuated in the institution, a number of bacteriological examinations were made of the stools of patients subject to recurring attacks of dysentery. These patients were free from dysentery at the time of the examination. Of seven such patients only one was found to harbor bacilli corresponding culturally to dysentery bacilli. The organism isolated from this case corresponded to the Shiga bacillus but was not agglutinated by the serum of the goat which was immunized to Shiga bacilli. The patient's serum was low in opsonins for this bacillus and did not agglutinate it in dilution of 1–20. Its identity therefore was not established. The failure to find dysentery bacilli in the stools of these cases of recurring dysentery is not to be taken as a point against the supposition that such patients are bacillus carriers. It is often extremely difficult to isolate dysentery bacilli from patients with dysentery at the time of examination, and the difficulties of isolating them from normal stools are much increased.

No attempt was made to determine the means of transmission from patient to patient in the institution, as the investigation of Dr. Pollock, resident physician in the summer of 1909, is fairly conclusive on this point. By using extreme care as to screening

and other means of avoiding flies and the protection of the food of the patients from flies, he was able to reduce greatly the number of cases of dysentery in one ward as compared with control wards where ordinary precautions were used. The time of development of most of the attacks of dysentery corresponds, moreover, to the fly season—July, August, and early September.

II. DIAGNOSIS.

Perhaps the greatest difficulty in the therapy of bacillary dysentery is the lack of an adequate means of diagnosis. On account of the rarity of occurrence of dysentery bacilli in normal stools, their recovery in diarrheal stools might be considered as diagnostic. This method however is not only uncertain but difficult and time-consuming. Deaths occur in many acute cases before the isolation and diagnosis of the bacilli can be accomplished.

Agglutinins develop too late to be of satisfactory use in diagnosis. The work of Lucas, Fitzgerald, and Schorer[1] gives a good idea of the deficiencies of the agglutination test and also a comparison with conglutination and complement fixation tests. They give the following percentages of positive findings in cases in which bacilli were isolated.

Agglutination: Flexner bacilli, 57.8 per cent; Shiga bacilli, 26.3 per cent.
Complement fixation: Flexner bacilli, 43.2 per cent; Shiga bacilli, 47.2 per cent.
Conglutination: Flexner bacilli, 60 per cent; Shiga bacilli, 21 per cent.

With the hope that the opsonic index might be of value in diagnosis, a number of cases in which bacilli were found were examined with respect to the opsonic index and agglutination reaction. The agglutination tests were carried out as in the diagnosis of the bacilli with immune serum. The opsonic index estimations were carried out with the ordinary Wright technic. The results are given in Table 4.

It will be seen from this table that the three Flexner bacillus cases gave positive agglutination reactions. One agglutinated both Flexner and Shiga bacilli; of the Shiga cases only three agglutinated the autogenous organism and two the stock bacillus. Four

[1] Lucas, Fitzgerald, and Schorer, *Jour. Am. Med. Assn.*, 1910, 44, p. 441.

of the Shiga cases agglutinated Flexner bacilli. This peculiarity has been noted before by Thomas[1] and in the Danvers report.[2]

TABLE 4.

Case Number	Type of Infection	Agglutination of Autogenous Organisms	Agglutination of Stock Shiga Bacilli	Agglutination of Stock Flexner Bacilli	Opsonic Index to Autogenous Bacilli	Opsonic Index to Shiga Bacilli	Opsonic Index to Flexner Bacilli
9	Shiga	—	—	1–20	0.45	0.66	1.0
11	Shiga	—	—	—	0.6	1.36	†
12	Shiga	1–20	1–10	—	2.	1.	0.9
13*a*	Flexner	1–20	1–20	1–20	1.4	1.6	0.7
13*b*	Shiga	1–20	1–20	1–40	1.5	...	....
14	Shiga	—	—	1–20	1.5	1.	1.1
15	Shiga	—	—	—	0.75	0.7	0.8
16	Shiga	—	—	1–40	1.5	*	1.8
18	Shiga	1–40	—	—	4.	1.6	1.4
19	Flexner	1–40	—	1–40	0.7	1.7	0.75
21	Shiga	—	—	—	0.8	0.7	0.8
32	Flexner	1–80	—	1–80	0.9	0.7	0.8

* Index high, but no accurate reading possible on account of bacteriolysis.

† No index estimated.

Excepting one case, all the cases examined showed a considerable variation from normal in the opsonic index to the stock bacilli and all showed a variation from normal in the index to the autogenous organism. If the contention advanced by the Wright school, that such variations from normal have a diagnostic value, is accepted, then we must conclude that the opsonic index is a valuable aid in diagnosis. The advantage of the opsonic index as a means of diagnosis lies in the fact that a specific change in opsonic concentration can be demonstrated in the negative phase of the process of immunity. Here we have a decrease in opsonic concentration due to specific absorption.

In the case of agglutinins we must wait until the positive phase before a specific change is demonstrable. The difference in opsonic index to autogenous organisms and stock bacilli is probably due to a difference in the virulence of the organisms. This difference in phagocytability of organisms is of interest in that it probably has a bearing on the way in which an organism is enabled to exist in the body of an individual possessing a considerable degree of immunity to the organism (bacillus carriers).

[1] Thomas, *Klin. Jahrb.*, 1909, 22, p. 29.

[2] *Loc. cit.*

The ease with which such a change in phagocytability is brought about *in vitro* is illustrated by the following experiment.

A laboratory strain of Shiga bacilli was grown on broth to which was added a small quantity of goat serum, which was bactericidal to the same strain in dilutions of 1–50. The organisms grew of course when a small enough quantity of serum was added. Now by increasing this quantity gradually, it was possible to grow the organism on increasing concentrations of bactericidal serum until it was possible to grow it on undiluted bactericidal serum.

Such an organism exhibits a high degree of spontaneous agglutination.

The readiness with which this immune organism was taken up by leukocytes was compared with the phagocytability of the strain from which it originated. For this purpose equal quantities of bacillus emulsion, serum dilution, and leukocytic suspension were used. The immune organisms had to be shaken for some time before a homogeneous suspension was obtained. The results are shown by the following figures. Immune goat serum was used.

Dilution of Serum	A	B
1–4	4	$\frac{1}{2}$
1–16	1	0
1–128	1	0

A represents the average number of non-immunized organisms taken up per leukocyte; B, the number of immunized bacilli.

A 24-hour broth culture of the immunized strain was filtered through a porcelain filter. A 24-hour agar culture of non-immunized bacilli was treated with this filtrate for a half-hour at room temperature and the bacilli were washed twice by centrifugation with salt solution. The bacilli so treated were compared, as to phagocytability, with untreated bacilli. As before, immune goat serum was used. The results are given in the following figures:

Dilution of Serum	Untreated Bacilli	Treated Bacilli
1–4	10	1
1–64	2	$\frac{1}{2}$
Salt solution	1	$\frac{1}{2}$

It will be seen that the substance on which the immunity to phagocytosis depends is transmitted to the culture medium, and the absorption of this substance by phagocytable organisms renders them less susceptible to phagocytosis. It will be seen that this substance bears the same relation to the "Virulin" of Rosenow[1] as a true soluble toxin does to an endotoxin. Rosenow obtains virulin only by autolysis.

III. THERAPY.

A. Prophylactic.—The results of preventive inoculations against dysentery have generally been reported as unsatisfactory. Lucksch[2]

[1] E. C. Rosenow, *Jour. Infect. Dis.*, 1907, 4, p. 285.

[2] F. Lucksch, *Centralbl. f. Bakt.*, 1908, Orig., 45, p. 365.

is the only one who reports a satisfactory result of vaccination. Shiga[1] in a vaccination of 10,000 persons reports a diminution in mortality but not in the number of cases developing. Most observers report a dangerously severe reaction, both local and general, following inoculation. Dopter[2] concludes that the only way to avoid a dangerous reaction and negative phase is by inoculation of bacilli sensitized by immune serum.

In view of the success attending inoculation against typhoid, it was thought advisable to repeat the experimental inoculation, and, in order to avoid the excessive reaction, resort was had to a preliminary very small dose, followed later by a larger one.

To study the negative phase and development of immune bodies, rabbits were inoculated with small quantities of Shiga bacilli, and the immune bodies were estimated from day to day. The results are given in Charts 1 and 2.

These curves show (1) that the negative phase must have lasted only a matter of hours, for the first estimation was made on the first day following the injection; (2) that the length of time that an increase of immune bodies is found present in the blood is not much different after three injections than after one.

It must not be inferred that immunity only lasts until the immune bodies fall to normal. In the case of the second rabbit an injection on the 36th day of two agar cultures (a dose which by control was quickly fatal) was without effect on the immune rabbit. This immunity may be explained by a changed reaction capability (allergy) or by the presence of so-called sessile receptors.

The vaccine used for preventive inoculation was prepared as follows: A mixture was made of suspensions in salt solution of 24-hour growths of the different strains of Shiga and Flexner organisms represented in Table 1. This mixture was standardized according to Wright's method and a suspension of 10 million bacilli per cubic centimeter was used for the first dose and one of 50 million bacilli per cubic centimeter for the second dose. Enough trikresol to make 0.2 per cent was added and the vaccine was sterilized 1½ hours at 60° C. The vaccine was then made into doses of 10 million and 50 million bacilli in antitoxin syringes.

For inoculation patients were selected who, so far as was possible to ascertain, had not had dysentery. The patients on one side of

[1] Shiga, *Deutsch. med. Wchnschr.*, 1903, 18, p. 327.

[2] Dopter, *Ann. Inst. Past.*, 1909, 23, p. 677.

each of three wards of the insane hospital, 62 in number, were inoculated subcutaneously with 10 million bacilli and one week later with 50 million bacilli. The patients on the other side of the

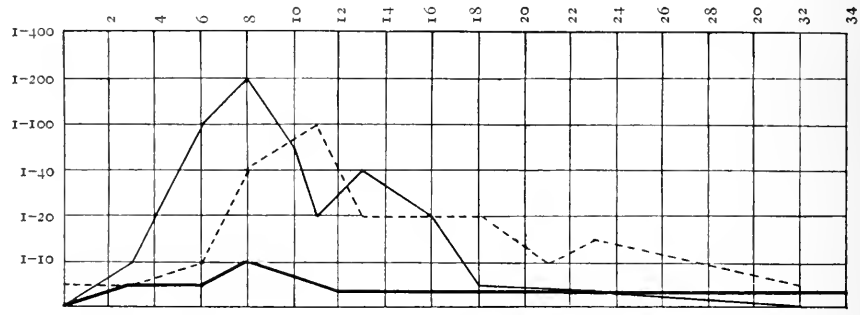

CHART 1.—Rabbit weighing 1,000 gms. inoculated with half of a 24-hour culture. The fine line indicates agglutinins, the heavy line bacteriocidins, the dotted line opsonins. The opsonins are estimated by the dilution method. The ordinates indicate the point of dilution at which opsonic, bacteriocidal, and agglutinating action disappears. The abscissae indicate the days following the injection.

In estimating opsonins and bacteriocidins, serum heated at 56° C. for a half-hour and reactivated with normal rabbit serum was used.

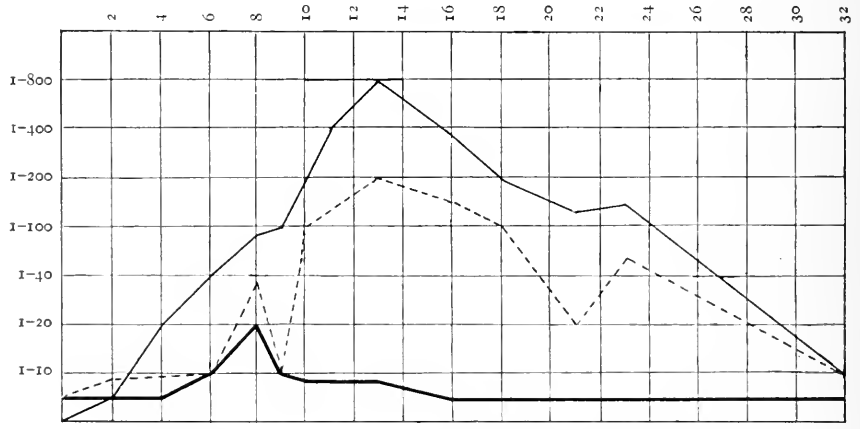

CHART 2.—This represents the result of injecting a 1,100-gm. rabbit with (1) two-tenths of a 24-hour culture of Shiga bacilli; (2) following this on the third day by an injection of half a 24-hour culture; (3) on the eighth day an injection of a 24-hour agar culture was given.

three wards, constituting about the same number, were used as controls.

Of the control patients four developed dysentery. In three of these cases Shiga bacilli were isolated, and in one, the Flexner

bacillus. Of the vaccinated patients, one developed a Shiga bacillus infection the second day after the first inoculation. The course of this case was abortive in type, lasting only 48 hours. Three of the vaccinated cases developed rather severe local reactions with some swelling and induration. Two cases had a slight rise in temperature with loss of appetite, nausea, and vomiting on the day following. These reactions occurred after the second injection.

The development of immune opsonins and agglutinins after the vaccination was observed in two cases given in Table 5.

TABLE 5.

EFFECT OF VACCINATION ON OPSONIC INDEX AND AGGLUTINATION.

CASE NUMBER	SERUM TAKEN BEFORE FIRST VACCINATION				SERUM TAKEN 9 DAYS AFTER SECOND VACCINATION			
	Agglutination		Opsonic Index		Agglutination		Opsonic Index	
	Shiga Bacillus	Flexner Bacillus	Shiga Bacillus	Flexner Bacillus	Shiga Bacillus	Flexner Bacillus	Shiga Bacillus	Flexner Bacillus
1.....	—	—	1.3	1.1	—	$\frac{1}{40}$	6	11.2
2.....	—	—	1.2	0.1	—	$\frac{1}{80}$	8	12.8

Owing to the fact that only a few of the control cases developed dysentery, a second series of vaccinations was made by Dr. Pollock. In this series half the patients in a ward of 70 were inoculated. The ward was selected for the reason that it was particularly hard to rid of flies, and dysentery cases were frequent. In view of the fact that rather severe reactions followed the injection of the second dose of 50 million bacilli in the first series, only one dose of 15 million was given in the second series. In this series there were three cases of dysentery in the controls and none in the vaccinated cases.

The conclusion would seem justified that the prophylactic inoculations were successful in immunization during the period of observation. In the first series this was about four months; in the second series, about one month. Observation was continued until November, when the development of dysentery became very infrequent.

B. Curative inoculations.—A number of cases were injected subcutaneously with small doses of killed bacilli. For this purpose

autogenous organisms were prepared in a way similar to that described for the preparation of vaccine for preventive inoculation. The treatment was carried out by the physicians in charge of the cases and reports were made as to the results. The numbers refer to the cases of the same number in Table 1.

Case 4. Diagnosis, pellagra. Dysentery about one week. Received five million bacilli and a second dose of 10 million three days later. The dysentery patient gradually improved and recovered in the course of about a month. No beneficial effect of the vaccine could be inferred.

Case 7. Diagnosis, dementia praecox. Dysentery about two weeks. An initial dose was followed the next day by marked improvement in the diarrhea, and in a few days the stools became normal. A second injection was made on the third day, after the first. Ten million bacilli were given in the second dose.

Case 9. Patient with general paresis and a terminal dysentery. A dose of five million bacilli was given in the hope that the patient would live long enough to observe the effect. Death however occurred in about 18 hours.

Case 11. Diagnosis, general paresis. Patient had had dysentery for three weeks. An initial dose of five million was followed in 24 hours by so marked an improvement that no further injection was thought necessary. One week later a relapse took place. The patient again received an injection of 15 million. There was again a marked improvement the following day and the diarrhea cleared up. No further relapse occurred.

Case 13. Diagnosis, dementia paralytica and pellagra. Complete perineal tear with infection. The patient was in very bad condition. Dysentery of some weeks' duration. An injection of five million bacilli was given and three days later one of 10 million. After the second injection there was an improvement in the diarrhea, but the patient died a few days later.

Case 16. Diagnosis, pellagra. Dysentery severe for a week. Injections of five, 10, and 20 million bacilli given together with a second organism recovered from the stools. The third injection was followed by marked improvement, both in the diarrhea and in general condition. A fourth injection of 20 million was given after the stools became normal, about the seventh day. Some weeks later the patient again developed a diarrhea and death occurred in about 24 hours.

Case 17. Diagnosis, pellagra. An initial dose of 10 million was followed by a marked improvement in about 24 hours and the diarrhea had cleared on the second day following the injection.

Case 18. Diagnosis, pregnancy, exhaustion psychosis, pulmonary tuberculosis. Dysentery of five days' duration. Dose of 10 million bacilli. This was followed by an improvement the next day. After a few days, however, the patient died of pulmonary hemorrhage.

Case 20. Diagnosis, psychosis. Dysentery for two weeks. Given 10 million bacilli. Improvement in 24 hours. Recovery.

Case 21. Chronic dysentery of a duration of several months. Initial dose of 10 million bacilli followed by increasing doses of 15, 20, 50, and 100 million with gradual improvement. The doses were given at intervals of four days. Recovery in about three weeks.

Case 28. Chronic dysentery; patient extremely emaciated as a result of a dysentery of several months' duration. Improvement after three injections at intervals of three days: first, 10 million; second, 20 million; third, 40 million. Recovery.

Case 29. Diagnosis, dementia praecox. Dysentery five days. Vaccination with 10 million bacilli. Improvement in 24 hours. Recovery.

Case 31. Chronic dysentery with a duration of over six months. The patient was given doses twice a week beginning with 10 million and increasing by double the dose each injection until 80 million were given. Then injections of 100 million for three weeks at intervals of one week. Recovery after about six weeks.

Case 32. General paresis. Dysentery of two weeks' duration. A dose of 10 million bacilli was followed in 24 hours by improvement and recovery in a few days.

From these case reports the conclusion might be drawn that the vaccine treatment is much more valuable than is really the case.

It will be noted that all of the cases treated were of some days' duration. Many of the cases were of the fulminating type and death occurred before autogenous vaccine could be prepared. As a result this vaccine treatment was tried only on cases in which the chance of spontaneous recovery was good. This fact, taken with the extreme variation in the course of the disease, renders conclusions somewhat difficult. The regularity of the period after which improvement began after vaccination (24 to 48 hours) would indicate that in these less acute cases the treatment had a beneficial effect. That this improvement was observed in case 11, both after the primary attack and after relapse, favors this conclusion.

A preparation of stock vaccine similar to that used in prophylactic vaccination was used in treatment of a few of the cases, but was without effect.

An examination of the serum of case 21 before and after recovery shows an increase of opsonic index from 0.8 to the autogenous Shiga bacillus to 2.5 and from 0.7 to stock Shiga bacillus to 2.1.

CONCLUSIONS.

The dysentery in the Cook County Institutions at Dunning is of the bacillary type. Shiga bacilli predominate.

The transmission and spread of infection is probably by means of indirect contact and fly transmission.

The perpetuation of infection may be by means of bacillus carriers.

The value of the opsonic index as a means of diagnosis in dysentery of the bacillary type deserves further investigation.

The preventive inoculation of killed dysentery bacilli is a valuable aid to prophylaxis. The curative treatment by means of vaccine deserves further investigation.

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ETIOLOGY OF CANINE DISTEMPER.*

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HISTORY OF THE DISEASE.

Canine distemper is an acute infectious disease peculiar to dogs. It has been variously termed canine distemper, canine plague, wheel plague, canine glanders, catarrhal fever, etc. It is called by the Germans "Hundestaupe," "Staupe der Hunde," or "Hundekrankheit"; by the French, "maladie des chiens" or "maladie du jeune âge"; and by the Italians "cimurro" or "moccio canino."

Distemper is caused by a specific poison, the etiological factor, probably a micro-organism, which finds its way into the system through the respiratory tract. Young animals are most susceptible to the disease, which is found running its course, according to the textbooks, as a catarrhal fever, affecting at one time or another all the mucous membranes of the body, and often accompanied with certain nervous symptoms and skin eruptions.

As with measles and whooping cough of the human race, very few of the young escape, and, usually, one attack confers immunity. It exists in all countries and may be found at any season of the year, although the spring and summer months are the most favorable

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for its production in the cities. It is said to have been known in the time of Aristotle. Laosson considers that the canine epizootic which raged in Bohemia during the year 1028 was true distemper.

It is the general opinion, however, that it was imported to Europe from America about the middle of the eighteenth century. It was first carried to Spain, whence it rapidly spread to the other European countries. One of the first to write intelligently at any length on the subject was Jenner, who in 1809, while recognizing the true infectious nature of the disease, was the first to differentiate between distemper and rabies, and also the first to show that it was not communicable to man. Later he was disputed by many of the best writers.

D'Youville must have had some conception of its contagious nature, for soon after the middle of the eighteenth century, not long after its introduction into Europe, he caused the rooms and stables where the sick dogs had been housed to be cleansed and disinfected as thoroughly as possible, so that the disease would not be conveyed to other dogs.

Between the years 1818 and 1852 many articles were written concerning the contagious nature of the disease and its communicability to man. Among those who believed that it could be transmitted to man were Waldinger, von Gemmern and Mecke, Delabère-Blain, Funke and Veith. Among those who carried out experiments on dogs to prove it was infectious, and who believed from the results of their experiments that it could not be communicated to man, were Renner and Karle.

The etiology of the disease was early brought into the discussion by Rawitsch, Krause, Zippelius, and Zündel. Chill, catching cold, imperfect nutrition, increased susceptibility and inanition due to lack of sodium salts were some of the most prominent conditions given as the cause.

Whether or not the disease was contagious and the nature of the contagium occupied the minds of most of the observers from this time on. Röll and Hertwig contended that it was not contagious while Transbot, Venuta, Semmer, Trastowo, and Krajewski believed that the disease was brought about by a certain substance or contagium and that this was specific.

Many likened distemper to diseases of man, emphasizing especially the respiratory system and the skin lesion associated with the abdominal symptoms.

Reuter with others contended that equine distemper and canine distemper were of the same etiology, and that the disease could be transmitted from one to the other.

On account of the skin lesion often found in distemper many compared it to cowpox and also to smallpox. Vaccinating, however, with heifer lymph was attended with no success.

For many years the rôle played by the skin lesions was the cause of considerable controversy, Gray, with a small following, contending that the skin lesion could be found in all cases of distemper.

During the past few years many have been actively engaged in attempting to discover the primary cause of the disease, but from the number of different organisms found, and the fact that the results cannot be confirmed, the question of etiology is still an open one, as may be seen from the summary review of this work which now follows.

PREVIOUS STUDIES OF MICROBIOLOGY OF CANINE DISTEMPER.

Semmer (1875) found an exceedingly slender and small bacillus in the blood of affected dogs a few hours after death.

Krajewski (1881) found a micrococcus.

Rabe (1883) found uniform globules of minute size, sometimes lying together in heaps or connected in twos and fours, or in rows of four or five, stained dark blue with methylene violet.

Mathis (1887) found a diplococcus in the fluid of the tissues, sputum, and pustules, which he cultivated in neutral or slightly alkaline broth.

Marcone and Meloni (1888) found cocci similar to staphylococcus.

Jacquot and Legrain (1890) found in pustules motile micrococci.

Millais (1890) found a long bacillus which liquefies gelatin, descending as a flaky mass in the almost clear fluid, which is covered by a whitish scum. Finally, when the whole of the gelatin is liquefied, the flakes gather at the base, and, if stirred, appear to be of a ropy consistency. He also found a micrococcus which he thought was the cause of the lung lesions. The two combined cause the pneumo-distemper.

Schantye (1892) divided distemper into three diseases produced by three distinct organisms.

Jensen (1896) found the pneumonia of distemper to be caused by streptococci.

Valerio (1896) found a bacillus ($0.3 \times 1.2 - 2.5\ \mu$) on agar plates from mucus of respiratory tract; often dumb-bell shaped; motile; stains by Gram; causes gas bubbles in depth of gelatin stab; does not coagulate milk; clouds broth; isolated from lungs, brain, spinal cord, and pus in frontal sinus.

Babes and Barzanesco (1897) isolated a bacillus in two cases.

Petropawlowsky (1897) found a bacillus similar to the colon bacillus.

Taty and Jacquin (1898) found a diplococcus in the central nervous system which they regarded as the cause of the nervous form of distemper.

Copeman (1900) found a cocco-bacillus in smears from broth, not infrequently in chains, sometimes of considerable length; does not stain by Gram; grows readily on agar at 36° C.; colonies grayish, glistening, semi-translucent; circular, occasionally, and especially at first, the edge is irregular. Grows well in broth, which first is turbid, later a deposit falls, liquid clears somewhat. Slow growth on serum; milk not coagulated; moist, yellowish growth on potato occasionally; gelatin liquefied.

Lignières and Phisalix (1900–1901) found a long bacillus; Gram-negative; non-motile; assumes cocco-bacillary form in guinea-pigs.

Cadiot and Breton (1901) described the infective bronchopneumonia as an independent disease.

Carré (1905) found a filterable virus in the serous discharges. The serous discharges taken at the commencement of the complication are powerfully pathogenic and the virulence is due to an organism small enough to pass through certain filters.

Hewer (1906) found short, small, non-motile bacillus; Gram negative; fine, white growth on agar; broth slightly clouded with granular sediment and stringy masses; gelatin not liquefied.

PERSONAL OBSERVATIONS—BACILLUS BRONCHICANIS.

Various micro-organisms isolated.—From the beginning of this work, many different organisms were isolated from the sick dogs, especially while my attention was confined to the discharges from the eyes, nose, and contents of the pustules. The conclusion was soon reached, however, from the infrequency and irregularity with which these different organisms were isolated, that they were simply secondary invaders and had nothing to do with the primary infection. As the distemper dog always suffers from one or more of these secondary infections, and since they often cause the most difficult symptoms to contend with, it is rather important to have some idea of them and the conditions under which they are found. It is a fact also, that the majority of investigators have considered these discharges the results of the primary infection and isolated from them the organisms they considered the real cause of the disease. From the results of my postmortem observations, in the dogs experimentally inoculated as well as in other dogs dying from the disease, I believe that the animals succumb to the secondary infections more often than to the primary disease.

The organisms most frequently found are the staphylococci, especially the albus, which are constant in all purulent secondary conditions. In the early stages of the serious eye lesions and in secondary stages of the cough, I have encountered the streptococcus, but not often. When the disease is allowed to run its course, the staphylococcus is often found in the blood, the animal probably dying from the terminal staphylococcus infection.

I have isolated in several instances a small micrococcus, probably the one mentioned by Millais and others. I have endeavored on several occasions to produce symptoms of distemper with this coccus, but have never been able to produce more than an abscess or a swelling at the site of inoculation if injected subcutaneously, while if injected in any other way it never produced symptoms other than a slight rise of temperature.

Several bacilli, resembling morphologically the bacillus I am about to describe more fully, have been isolated from the discharges as well as from the bronchi and lungs, especially in the later stages, but I have never been able to associate them with the primary infection. These are, no doubt, the organisms mentioned by Copeman, Lignières, Phisalix, and others, and from which their vaccines have been made. These organisms grow as a rule rapidly and luxuriantly, some evolving gas with glucose, some liquefying gelatin, while others coagulate milk with the formation of acid, showing that there are several organisms resembling each other morphologically but differing culturally, which may be found associated with the secondary conditions in distemper. With these organisms I have never been able to produce symptoms simulating the true disease. If inoculated subcutaneously they often produce an edematous swelling or an abscess at the site of the inoculation, and even death, and in these cases the organism may be isolated in pure culture from the blood, but that is far from proving that they are the primary cause of the disease.

In the lungs, liver, spleen, blood, etc., from animals dying with severe secondary symptoms, I have often found a large bacillus, which I believe is a terminal invader. Dogs dying when this organism is present always have a putrid, cadaverous odor several days before they die. After death the surrounding atmosphere becomes almost unbearable.

Other organisms have been found from time to time, but not often enough to consider seriously. A few times an anaerobe, resembling somewhat the bacillus of symptomatic anthrax, was isolated from the blood and organs of dogs dying from the disease. Experiments prove that they are not virulent for dogs or other animals.

While working on the eye secretions I isolated from several cases a diphtheroid bacillus resembling the *B. zerosis canis* of Graham Smith. Also, a diphtheroid bacillus was isolated from the lungs of one dog dying from typical symptoms of distemper. These organisms proved to be non-pathogenic for dogs and other animals.

On several occasions at the beginning of this work I attempted to prove the existence of a filterable virus but was never able to confirm Carré's work.

Postmortem examinations.—All autopsies were performed in an aseptic manner, eliminating as far as possible accidental contaminations. The dogs were killed by the injection of 0.5 grain of strychnine into the gluteal muscles. This is a good method, as it causes death in 30 seconds without any struggle.

Routine cultures were taken from the larynx, trachea, large and small bronchi, lungs, and blood. In obtaining cultures from the lungs and smaller bronchi the tissue was placed in a sterile dish, teased, and the juices pressed out. The juices as well as the lung tissue were placed in broth and smeared over the surface of agar.

A bacillus was found that grew slowly and with difficulty when first isolated from the body, so that extreme care was necessary when looking for the colonies. At the end of 24 hours it is often

impossible to see anything on the surface of the agar without the use of a lens. The colonies appear as pin points among the larger colonies of other bacteria and at times it is very difficult to isolate the bacillus on account of the overgrowth of other organisms.

The first pure culture of this bacillus was isolated October 23, 1908, and since then 97 strains have been isolated from dogs in all stages of the disease. Two of these cultures were isolated from dogs in the city of New York, showing that the infection is not local in Detroit. I propose to name this bacillus *B. bronchicanis*.

Blood cultures were taken as a routine measure at all autopsies, and at first nothing grew but the organisms of the secondary infections. Later, however, after the bacillus was discovered in the respiratory tract and after it was found to be so difficult to isolate when associated with other organisms on account of its slow growth, the method was changed. The heart was opened aseptically and from 1 to 5 c.c. of blood drawn off with a large pipette and planted in a flask containing 50 c.c. of broth, or distributed in several tubes of broth. After 24 hours in the incubator, plates are made or slant agar inoculated in successive tubes. Such blood cultures were taken in 63 cases. In 29 the cultures were positive, the bacillus being found in 18, in 13 of which it was uncontaminated, in three associated with staphylococcus and in two with unidentified bacteria. The remaining positive blood cultures had staphylococci only in six, staphylococcus with an unknown bacterium in one, and unidentified bacteria in four cases.

CHARACTERISTICS OF BACILLUS BRONCHICANIS.

It is a short, narrow bacillus, usually found single, but often in pairs. In liquid media it may be found at times in long chains or even filaments. In broth culture, grown directly from the dog, the organism may be larger and more oval in form. Does not take the ordinary stains as readily as most bacteria, does not stain by Gram, but stains best with Loeffler's methylene blue, with characteristic bipolar appearance. The organism is actively and progressively motile.

Plain agar stroke.—After 24 hours at 37° C. a moderate growth, filiform and slightly raised; surface moist, glistening, and smooth; growth translucent and of sticky consistency; no odor as a rule. After 72 hours growth tends to spread slightly except near bottom of tube in contact with water of condensation. As growth ages it has a tendency to become slimy, but retains its other characteristics. Stale or musty odor at times.

Plain agar stab.—After 24 hours at 37° C. a filiform growth, better near the surface. Surface growth moderate, raised and restricted; after 48 hours growth

retains form with surface spreading slightly; in 7 days surface growth tends to be restricted, although some strains spread gradually; growth is slimy and smooth.

Potato.—After 24 hours at 37° C. growth rather abundant; surface uneven, raised, moist, glistening, and contoured, and of light tan color; consistency, sticky; decided odor of stale bread; medium slightly darkened. After 48 hours growth thicker, with tendency to spread; color darker tan; medium much darker. In 72 hours the surface of medium nearly covered with growth which has become slimy.

Loeffler's blood serum.—After 24 hours at 37° C. growth is scanty, filiform, smooth, moist, glistening, and nearly flat; no color; medium not colored nor liquefied. In 48 hours the growth has increased slightly. After 30 days no special change in growth; medium browned, but not liquefied. Water of condensation decidedly alkaline.

Koch's blood serum.—After 24 hours at 37° C. growth scanty, filiform, smooth, moist, glistening, and slightly raised; of light tan color; musty odor; medium not colored nor liquefied. In 48 hours growth increased slightly but no other change; in 72 hours medium not liquefied; water of condensation decidedly alkaline. After 3 weeks no change.

Gelatin stab.—After 24 hours a filiform growth which is best on top; surface growth restricted; no liquefaction; medium not changed. In 30 days the characteristics remain the same; no liquefaction.

Nutrient broth.—In 24 hours at 37° C. no surface growth; moderate clouding which is persistent; compact sediment which is easily broken up on shaking; odor rather stale. In 72 hours cloudiness increased; sediment rather viscid; odor decidedly stale, which becomes putrefactive at times.

Growth in dextrose, mannite, maltose, saccharose, lactose, and glucose broth, in fermentation tube for seven days at 37° C., leaves fluid in open arm clouded; no visible growth in closed arm; no gas; broth alkaline.

Litmus milk.—After 24 hours at 37° C. no change. In 72 hours upper half-inch of medium has taken a deeper blue color. After 5 days color entirely disappearing from the bottom of the tube ; color at top deeper blue; brownish, slimy sediment in bottom of the tube. In 3 weeks some strains show color to have entirely disappeared from most of the medium.

Milk.—After 24 hours at 37° C. no change; in 72 hours no coagulation, no acid, sediment starting to form in bottom of tube; after 14 days color of medium light tan; slightly translucent and clouded; sediment is thick and slimy; odor stale.

No growth in Uschinsky & Cohn's solutions.

Dunham's solution.—After 7 days at 37° C. fair growth, with sediment in bottom of tube. Test for indol negative.

Colonies on agar.—After 24 hours at 37° C. colonies small, round, sometimes not much larger than a pin point; translucent and slightly raised. In 38 hours about size of a pin head; round, convex, smooth, amorphous and translucent; edge entire. In 72 hours colony has grown larger, but characteristics about the same; edge may have become undulate. After 7 days colony much thicker; grumose in center; edge undulate.

Colonies on gelatin.—Similar to those on agar; medium not liquefied.

EXPERIMENTAL DISTEMPER.

Method of caring for the dogs before and during the experiment.—In order to carry inoculation experiments to a successful termination and be sure that the dogs were not

accidentally infected, three large rooms in buildings isolated from each other were used, each room being in charge of a different attendant. The pups used were taken directly from their mother as soon as weaned. After making sure that the pups were in a healthy condition, they were at once given a bath, and then thoroughly soaked in a germicidal solution and placed in the incubation room. Here they were allowed to remain from three to six weeks under strict observation, in the charge of a man who kept away from other dogs. From the incubation room the pups were placed in another room, where they remained under observation at least three weeks longer before experiments were made. The floors and walls of these rooms were of cement, making it possible to wash and spray thoroughly with a germicidal solution as often as deemed necessary. The rooms were thoroughly disinfected and cleaned before the reception of each lot of pups and after each experiment. They were under lock and key and no one was allowed to enter except the attendant, laboratory assistants, and myself. Following these precautions and with careful feeding and handling, one could be reasonably sure that all abnormal symptoms following inoculations were due directly and solely to the treatment. In a third room, in a building far removed from the first two, were kept all convalescing, as well as all sick, dogs and others under observation and treatment. All autopsies were held in the laboratory, where cultures could be taken conveniently without fear of contamination.

A detailed account of this work has been given, because as far as I am able to learn none of the previous experimenters on distemper have mentioned taking any special care during their inoculations and after treatment.

The dogs were inoculated intratracheally or the broth culture was poured into the nostrils. Inoculating subcutaneously or intravenously did not lead to the development of any typical symptoms.

Dog No. 91.—October 28, 1908: Inoculated subcutaneously (strain No. 36).

October 30: Died. Organism seen in blood smears; no growths.

Dog No. 93.—October 31, 1908: Inoculated directly into lungs (strain No. 36). Cough, no discharge from nose or eyes.

December 3: Died. No discharge from nose or eyes. From lungs isolated *B. bronchicanis* and staphylococcus.

Dog No. 94.—November 2, 1908: Inoculated intraperitoneally (strain No. 36 carried through pig No. 13).

November 7: No symptoms.

November 13: Discharge from eyes.

November 14: Died. Discharge from eyes, and emaciation. *B. bronchicanis* not found.

Dog No. 96.—November 7, 1908: Inoculated intrapulmonarily (strain No. 36 carried through pig No. 14).

November 8: Would cry out if thorax was pressed.

November 9: Died. Consolidation of one lung and part of the other. Smears from lungs show diplococcus, large bacillus and short bacillus, which was probably the *B. bronchicanis*, but it was not isolated.

Dog No. 97.—November 7, 1908: Inoculated intrapulmonarily (strain No. 36 carried through pig No. 15).

No symptoms or signs but emaciation.

November 11: Died. Abdomen filled with fluid. Spleen, liver, and gall bladder swollen. Lungs apparently normal. *B. bronchicanis* found in cultures taken from spleen, liver, bile, and blood.

Dog No. 98.—November 11, 1908: Inoculated intraperitoneally (strain No. 96).

November 13: Watery discharge from eyes; very weak.

November 16: Much weaker; emaciation; killed with strychnine.

Cultures from peritoneum and blood gave no growth.

Dog No. 99.—November 11, 1908: Inoculated subcutaneously (strain No. 96).

November 13: Tumor at site of inoculation; watery discharge from eyes; killed with strychnine.

Culture from abscess, eyes, and blood. No growth of *B. bronchicanis*.

Dog No. 103.—February 9, 1909: Inoculated intraperitoneally (strain No. 36 carried through pig No. 16).

February 10: Very quiet, would not eat.

February 11: Found dead. Peritoneum congested and covered with thick stringy exudate.

Cultures from peritoneum, spleen, and blood sterile.

Dog No. 123.—May 19, 1909: Cultures smeared over face and nostrils (strain No. 108).

May 28: Coughing today.

June 12: Inoculated again, into trachea.

June 14: Diarrhea; slight mucous discharge from nostrils.

June 23: Has had slight cough; no discharge from nostrils or eyes; killed with strychnine.

Both lungs edematous and congested; *B. bronchicanis* found in lungs.

Dog No. 125.—May 20, 1909: Inoculated subcutaneously (strain No. 108).

May 29: No symptoms. Placed in cage just vacated by dog No. 126 which had a typical case of distemper.

June 3: Began to cough today.

June 4: Coughing badly; gave first dose of vaccine.

June 8: Vaccinated again.

July 6: Died. Very emaciated; lungs consolidated; necrotic patches in lungs; *B. bronchicanis* and staphylococcus isolated.

Dog No. 126.—May 29, 1909: Smeared over nostrils and eyes (strain No. 109).

May 26: Coughing and looks ill.

May 28: Very sick; cough; discharge from nose and eyes; vomiting.

June 3: Still coughing and sick.

June 30: Marked emaciation; discharge from nose and eyes; found dead. *B. bronchicanis* found uncontaminated in lungs. Blood sterile.

Dog No. 132.—June 3, 1909: Culture poured into nostrils (strain No. 109).

August 4: Apparently no symptoms but emaciation; killed with strychnine; *B. bronchicanis* and few staphylococci found in lungs; pure culture of *B. bronchicanis* in trachea.

Dog No. 133.—June 3, 1909: Culture poured into nose (strain No. 160).

June 9: Discharge from nose; appears very sick.

August 4: Marked emaciation; killed; *B. bronchicanis* and staphylococci found in lungs and trachea.

Dog No. 134.—June 3, 1909: Poured into nose (strain No. 109).

June 9: Discharge from nose; marked skin eruption; pustules.

June 11: Very weak and almost moribund; killed with strychnine; opacity of both corneae; discharge from eyes and nose; skin eruption; pure culture of *B. bronchicanis* found in lungs.

Dog No. 148.—August 17, 1909: Inoculated into trachea (strain No. 160).
August 19: Extremely quiet.
August 21: Slight discharge from nose; sneezes now and then.
August 26: Culture sprayed into nostrils.
August 31: Inoculated again into trachea.
September 16: No more symptoms; put into convalescing room; recovered.
Dog No. 150.—August 17: Inoculated into trachea (strain No. 160).
August 19: Quiet.
August 22: Quiet.
August 26: Sprayed culture into nostrils.
August 30: Coughed today several times; bowels very loose.
August 31: Inoculated again into trachea.
September 16: No other symptoms; recovered.
Dog No. 154.—August 26, 1909: Inoculated into trachea (strain No. 160).
August 27: Quiet.
August 28: Appears ill.
August 30: Vomited.
September 2: Vomited.
September 16: No more symptoms.
October 26: Killed with strychnine; *B. bronchicanis* found uncontaminated in lungs; in blood a larger bacillus was found.
Dog No. 155.—August 12, 1909: Inoculated into trachea (strain No. 160).
August 13: Very quiet.
August 14: Appetite poor.
August 18: Coughing today.
August 20: Still coughing.
August 22: Watery discharge from nose; does not cough much at present.
August 26: Sprayed culture into nostrils.
September 1: Very quiet.
September 16: Loss of flesh; no other sypmtoms; put into convalescing room.
October 23: Killed with strychnine; cultures from lungs show *B. bronchicanis* uncontaminated.
Dog No. 156.—August 12, 1909: Inoculated into trachea (strain No. 160).
August 13: Very quiet.
August 14: Not eating much.
August 18: Diarrhea; cough today.
August 19: Coughing a great deal.
August 22: Watery discharge from nose; does not cough so much.
August 26: Sprayed culture again into nostrils.
September 1: Very quiet.
September 16: Loss of flesh. No other symptoms or signs of distemper.
October 23: Killed with strychnine; *B. bronchicanis* uncontaminated in lungs and trachea.
Dog No. 159.—August 12, 1909: Inoculated into trachea (strain No. 160).
August 13: Very quiet.
August 14: Appetite poor.
August 16: Suspicious cough.
August 17: Much thinner.

August 18: Coughing today.
August 19: Coughing hard.
August 21: Slight discharge from nose; sneezing.
August 22: Purulent discharge from nose.
August 23: Discharge from nose very bad.
August 31: Inoculated again into trachea.
September 1: Very quiet.
September 16: Symptoms improved.
October 27: Killed with strychnine; *B. bronchicanis* uncontaminated in lungs; blood sterile.

Dog No. 163.—September 27, 1909: Inoculated into trachea (strain No. 169).
September 30: Coughing.
October 2: Very sick; coughing a great deal; vomiting; severe diarrhea.
October 3: Continues to be sick; stools watery and foul.
October 7: Still sick and coughing.
October 17: Still coughing. Put into contaminated room.
November 18: Developed a discharge from eyes and nose; very sick past week; died during the night; opacity of both corneae; complete consolidation of both lungs; smears from lungs showed several different organisms; *B. bronchicanis* not isolated.

Dog No. 164.—September 27, 1909: Inoculated into trachea (strain No. 169).
October 5: Coughing today.
October 21: Symptoms remain the same; put into contaminated room.
November 13: Been failing past week or so; discharge from eyes and nose; cough; found dead; lung consolidated in areas; cloudy fluid in peritoneal cavity; *B. bronchicanis* isolated.

Dog No. 166.—September 27, 1909: Inoculated into trachea (strain No. 169).
September 29: Coughing and sick.
September 30: Very sick and coughing.
October 1: Accidently killed; consolidation in areas in both lungs; mucopurulent exudate in trachea; smears from lungs and trachea show enormous numbers of *B. bronchicanis* which is found in pure culture in lungs and trachea and isolated with another bacillus from blood.

Dog No. 167.—September 27, 1909: Inoculated into trachea (strain No. 169).
September 29: Coughing.
October 2: Sick and coughing.
October 5: Slight purulent discharge from both eyes; still coughing.
October 21: Symptoms improved, but still coughing.
October 26: Cough has increased; killed with strychnine; lungs consolidated; cultures from lungs, trachea, and blood; *B. bronchicanis* uncontaminated from lungs and trachea.

Dog No. 184.—November 16, 1909: Inoculated *B. bronchicanis* into trachea (strains Nos. 164 and 191).
November 20: Coughing frequently.
November 21: Very quiet.
November 22: Coughing and very ill; loss of appetite.
November 25: In very bad condition.
November 26: Found dead today; from appearances died in convulsions; *B. bronchicanis* found in lungs and blood.

Dog No. 186.—November 16, 1909: Inoculated *B. bronchicanis* into trachea (strains Nos. 164 and 191).

November 20: Coughing continually.

November 21: Very quiet.

November 22: Very sick dog; appetite poor; abscess at site of inoculation.

November 23: Found dead today; much loss of flesh; *B. bronchicanis* in lungs.

CONCERNING THE PRIMARY INFECTION.

The important symptoms which may be found in typical distemper are cough, diarrhea, vomiting, purulent discharges from the eyes and nose, skin eruptions, and nervous manifestations, as convulsions and muscular twitchings or choreiform movements. The symptoms always looked for, however, are the purulent discharges from the eyes and nose. With the exception of the cough, the other symptoms are not always present.

Many veterinarians and others refuse to call a case distemper without the eye and nose symptoms, and yet, judging from the many cases I have seen from before the onset of the disease and watched till death, I feel positive that these particular symptoms are due to secondary infections and are not true manifestations of the disease. From the fact that in later stages of the disease, cultures taken from the mucous lining of the respiratory tract and also from the heart's blood show so many different organisms, and that the earlier in the disease the cultures are taken the less frequently are these organisms found, it seems to me that we have been diagnosing the disease from its secondary infections and complications. Arguing from this point, one would expect, if going back far enough in the disease, to find the causative agent unaccompanied by any other organism. And I find that in the first few days of the infection, the secondary organisms are not present at all, but *B. bronchicanis* in pure culture. Also, in many of the cases in the later stages, the smaller bronchi contain the bacillus in pure culture. The higher up in the respiratory tract, the greater variety of organisms found. In the early stages I have never failed in finding this organism in pure culture and often as high up as the trachea.

During March, 1909, I collected about 20 young normal pups for experimental purposes. They were raised in our stables and

carefully watched from birth, so as to be sure of their age and condition. As they were healthy and normal, they were all allowed to run together in one large room which was kept as clean as possible at all times.

About the first week in the following May a dog with typical distemper came in contact with the keeper of these pups. The disease was carried from the keeper to the pups, which began to show symptoms in a few days.

Considering this a rare opportunity to watch an epidemic in pups of the same age, from the very day of infection strict clinical records were kept, and the pups were killed on succeeding days, starting with the second day, so that cultures were taken at all stages of the disease.

As already mentioned, if the dogs are killed early enough in the disease, *B. bronchicanis* is present in pure culture in every case. This is shown by cases 105, 106, 107, and 108 of the epidemic of May, 1909, just mentioned and also in the dog No. 170, October 5, 1909, and 245, April 17, 1910, and others occurring sporadically. As the dogs 105, 106, 107, and 108 were from an epidemic, no one would question that they had distemper, and yet there was no discharge from the eyes or nose, no skin eruption, but simply a slight cough and convulsions.

Dog No. 245 was killed as soon as possible after the onset of the symptoms, while dog No. 170 died in a convulsion.

Dog No. 105.—May 10, 1908: Convulsions.
May 12: Slight cough.
May 15: Very ill; cough.
May 17: Killed; *B. bronchicanis* found uncontaminated from the upper trachea down.
Dog No. 106.—May 12, 1908: Convulsions; cough.
May 15: Cough increased.
May 18: Killed; *B. bronchicanis* found uncontaminated.
Dog No. 107.—May 12, 1908: Cough.
May 15: Cough increased.
May 16: Diarrhea; skin eruption.
May 18: Killed and autopsied; *B. bronchicanis* found uncontaminated.
Dog No. 108.—May 15, 1908: Cough.
May 17: Cough increased.
May 18: Killed; *B. bronchicanis* found uncontaminated through the entire respiratory tract.

Dog No. 170.—October 6, 1909: Suffering in the morning with convulsions, died in the afternoon in a prolonged convulsion; *B. bronchicanis* found uncontaminated.

Dog No. 245.—April 10, 1910: Symptoms of being ill past few days.

April 14: Prolonged convulsions in the morning; very weak; killed; *B. bronchicanis* found uncontaminated in the respiratory tract and also in the blood.

In all of these cases *B. bronchicanis* was obtained in pure culture from the respiratory tract and also in the blood of dog No. 245. The fact that in dogs Nos. 170 and 245 the culture was uncontaminated and in No. 245 in the blood as well, shows that they were typical cases of distemper taken in the very first stages and similar to Nos. 105, 106, 107, and 108. This is shown also by the fact that the culture from No. 245 injected into a healthy pup, No. 250, produced a typical case of distemper.

Dog No. 250: April 18: Injected.

April 19: Very quiet.

April 20: Coughed once or twice.

April 21: Coughed several times this morning.

April 25: Coughing most of the time; losing flesh.

April 28: Very sick; coughing continually; vomiting.

April 30: Cough; vomiting; diarrhea; no appetite; sneezing.

May 1: Slight watery discharge from nose.

May 3: Purulent discharge just starting from nose; coughing and sneezing constantly; very thin.

April 5: Purulent discharge from nose increasing.

April 7: Slight watery discharge from eyes.

April 10: Began to improve.

Furthermore, the culture from No. 245 has been agglutinated with the serum of every case of distemper tried, while the controls gave no agglutination; and the serum from No. 245 agglutinated every strain of *B. bronchicanis* tried.

The next symptom after the bronchial to make its appearance is the serous discharge from the nose and eyes, usually from the nose first as is illustrated by dog No. 109, killed a few days later in the disease.

Dog No. 109.—May 15: Cough.

May 17: Diarrhea.

May 18: Slight discharge from nose.

May 19: Killed; *B. bronchicanis* pure from bronchi.

The eyes soon become affected, while the discharge from the nose is becoming more and more purulent. First it is mucopurulent, then thick, purulent.

Dog No. 110.—May 15: Cough.
May 17: Diarrhea.
May 19: Slight discharge from nose.
May 21: Slight discharge from eyes, convulsions.
May 23: Purulent discharge from nose and beginning from eyes.
May 24: Died. The cultures here were contaminated with the staphylococcus, showing that the secondary organisms were beginning to invade the field.

Nos. 111, 116, and 117, which were allowed to go on to death, give a typical picture of true distemper.

Dog No. 111.—May 16: Cough.
May 17: Diarrhea.
May 19: Slight discharge from nose.
May 20: Slight discharge from eyes.
May 22: Purulent discharge from nose.
May 24: Purulent discharge from eyes; skin eruption.
June 6: Died.
Dog No. 116.—May 16: Cough.
May 17: Diarrhea.
May 19: Slight discharge from nose.
May 21: Slight discharge from eyes.
May 22: Purulent discharge from nose and eyes.
June 1: Died.
Dog No. 117.—May 16: Cough.
May 18: Diarrhea.
May 19: Slight discharge fron nose.
May 20: Slight discharge from eyes.
May 23: Purulent discharge from eyes and nose.
June 2: Died. Most all of the tubes were contaminated with other organisms, and many were overrun with the staphylococcus, showing that the secondary organisms are the predominating ones at this stage.

In No. 117 the staphylococcus was found in the blood as well as through the whole respiratory tract, and it was with difficulty that *B. bronchicanis* was isolated at all. Just at this stage I believe is where the previous investigators failed. They waited until the discharges made their appearance before taking their culture, and most of them obtained the supposed causative organism from these discharges.

If the diseased dogs do not succumb but are allowed to go on to recovery, the secondary symptoms will entirely disappear, leaving the dog in an apparently normal condition. Now, if the dogs are killed at this stage, and cultures are obtained from their respiratory tract, it will be found that the secondary invaders have also disap-

peared, but that the primary organisms are still present. These can usually be found in pure culture several weeks after the dogs have completely recovered. This is shown in Nos. 118, 119, and 120.

Again, if the pups which have been experimentally infected with *B. bronchicanis* are kept in clean and disinfected cages, if the fecal discharges are washed away at once and the dogs are bathed in a disinfecting solution, the secondary organisms rarely cause any trouble and the dogs will show symptoms referable only to the primary infection.

On the other hand, if the pups are placed in an unclean room along with other dogs, and allowed to run unchecked in the dirt and feces, they may be attacked with the secondary symptoms which have hitherto not made their appearance and at the same time they may be recovering from the primary infection, or the primary infection may have entirely disappeared. This is illustrated by dogs Nos. 163 and 164.

Dog No. 163.—September 27, 1909: Inoculated.
September 30: Coughing.
October 2: Very sick, coughing severely.
October 3: Stools watery and of foul odor.
September 7: Still very sick and coughing.
September 17: Still coughing. Put into convalescing room.
November 18: Developed a discharge from eyes and nose; very sick; opacity of both corneae; complete consolidation of both lungs; smears from lungs show several different organisms; *B. bronchicanis* not isolated.

Dog No. 164.—September 27: Inoculated.
October 5: Coughing.
October 21: Symptoms have not changed. Put into convalescing room.
November 13: Been failing past week; discharge from eyes and nose; cough; found dead; lung consolidated in areas; cloudy fluid in peritoneal cavity; *B. bronchicanis* isolated.

Nos. 112, 113, and 115 from the epidemic recovered from the disease and were killed, but we were unable to obtain any culture of the *B. bronchicanis* from them. These three pups were among those vaccinated, which recovered. The vaccine may have had something to do with the disappearance of the organism from the respiratory tract.

The question has naturally been raised many times, whether this organism is not a normal habitant of the dog's respiratory tract. Early in the investigation, and also several times since, I

have had occasion to kill and autopsy many, apparently normal, dogs, and in only three cases have I found this organism. It was then in pure culture. The dogs in question were some which had been associated with those suffering with the disease, but did not succumb themselves.

As far as I have been able to learn, the trachea and bronchi of healthy dogs are sterile, and it is only when the resistance is lowered or in diseased conditions that bacteria may be found growing in those situations. With this thought in mind, cultures were taken from the larger bronchi of three dogs by means of a bronchoscope. Two of the dogs were healthy and from all appearances perfectly normal; the other dog had been showing symptoms of indisposition for a few days, as loss of appetite, tendency to lie around, etc. After 24 hours all tubes from the normal dogs were sterile, while those from the sick dog showed the staphylococcus, a small micrococcus, and *B. bronchicanis*. Three days after this the sick dog was found dead and cultures from the respiratory tract as well as the blood showed the staphylococcus and *B. bronchicanis*. The lung was completely consolidated, and cultures gave a micrococcus.

It seems clear that the epidemic of May, 1909, was a typical one of distemper, that it was due to *B. bronchicanis* which was isolated in pure culture in the early stages; and that the symptoms considered typical of distemper, as the purulent discharge from the eyes and nose, were due to secondary organisms.

AGGLUTINATION, PROTECTIVE INOCULATION.

Agglutination by serum of dogs with spontaneous distemper.—The emulsions for the agglutination tests were made from 18-hour agar cultures, taken off in physiological salt solution, to which was added enough formaldehyde to make a one per cent solution. This emulsion was allowed to stand five hours at room temperature, which time was sufficient to kill all the organisms. The tubes were placed in the incubator and read at the end of 24 hours. The control animals were in most cases young and healthy dogs. In order to test as many normal dogs as possible, different controls were used for each experiment, and in all 13 dogs were used. All except one gave a negative result. In this case the animal was from the dog

pound and it was not possible to learn whether it had ever suffered from distemper. In every experiment, dogs suffering with distemper gave typical agglutinations, ranging from 1 in 40 to 1 in 800. In several cases in which the serum gave positive results, the strains isolated after death gave positive reactions with the serum of other distemper dogs, and in one case an agglutination test with positive results was made with the homologous serum obtained on the day the dog in question died.

Experiment 4.—This experiment was to test the agglutinative power of a serum from a typical case against several strains isolated at different places. Strain No. 36 was the first strain isolated, about two years old. Strains Nos. 199 and 200 were obtained in New York and were about one year old. Strain No. 232 was of recent date, isolated in Detroit.

April 8, 1910: Serum from dog No. 242, black and white, part hound, and very sick with purulent discharges from eyes and nose; severe cough.

	36	199	200	232
1–10	+++	+++	+++	+++
1–20	+++	+++	+++	+++
1–40	+++	+++	+++	+++
1–60	+++	+++	+++	+++
1–80	+++	+++	+++	+++
1–100	+			+
1–200				
1–400				
1–600				
1–800				

Experiment 2.—Test of several serums against one strain.

March 24, 1910: Serum from dogs Nos. 233, 240, 241, all obtained from pound. Controls—well dog, well pup. Emulsions were from culture No. 232.

Dog No. 232 was large, shaggy white, with cough, bloody mucous discharges from nose; quiet, but cross and snappy.

Dog No. 233 was a tan mongrel with cough, weakness; purulent discharge from eyes and nose developed later.

Dog No. 240 was black and tan with purulent discharges from eyes and nose; cough and emaciation.

Dog No. 241 was a mongrel with purulent discharge from nose; cough; very weak.

The two controls were normal, one 6 weeks, one a year old, and perfectly well.

	240	241	233	Well Dog	Well Pup
1–10	+++	+++	+++	–	–
1–20	+++	+++	+++	–	–
1–40	++	++	+	–	–
1–60	++	++	+	–	–
1–80	++	++	+	–	–
1–100	++	+	.....	–	–
1–200	++	+	.....	–	–
1–400	+	+	.....	–	–
1–600	.....	+	.....	–	–
1–800	.....	+	.....	–	–
1–1000	.....	.....	.....	–	–

Agglutination by serum of dogs experimentally infected and of immune dogs.—Young pups were immunized against *B. bronchicanis* by two methods, namely live cultures and killed cultures, both being injected intravenously and subcutaneously.

Experiment 1 was made with serum of dog No. 250, which had been experimentally inoculated with the strain from No. 245. The dog contracted a typical case of distemper, and during convalescence the serum agglutinated the bacillus in dilutions of 1 to 200. Normal serum caused no agglutination.

Experiment 2 was made with serum of dogs Nos. 248 and 249, which had been inoculated with the bacillary strain from No. 245: No. 248 intravenously, and No. 249 subcutaneously. The only marked symptom shown by these two dogs was diarrhea. The serum of No. 248 agglutinated in 1 to 600, serum of No. 249 in 1 to 100; normal serum had no effect.

Experiment 3 was made with serum of these same dogs after two had been immunized by several injections: No. 248 intravenously, and No. 249 subcutaneously. The serum of No. 248 now agglutinated in a dilution of 1–1000, serum of No. 249 in 1–200.

Experiment 4 was made with serum of No. 248, and of dogs Nos. 252 and 253; the latter two were immunized by killed cultures injected subcutaneously. The serum of a guinea-pig (No. 160) was also immunized by repeated injections of the killed cultures subcutaneously, followed by live cultures intraperitoneally, and was tested on the bacillary strain from No. 245.

The results follow:

	248	252	253	Pig 160	Control Dog	Control Pig
1–10	+++	+++	+++	++	–	–
1–20	+++	+++	+++	++	–	–
1–40	+++	+++	+++	++	–	–
1–60	+++	+++	+++	+	–	–
1–80	+++	+++	+++	+	–	–
1–100	+++	+++	+++	....	–	–
1–200	+++	+++	+	....	–	–
1–400	+++	+++	+	....	–	–
1–600	+++	++	+	....	–	–
1–800	+++	++	+	....	–	–
1–1000	+++	.....	.....	....	–	–
1–2000	+	.....	.....	....	–	–
1–4000	+	.....	.....	....	–	–

In Experiment 5 the agglutinative effect of the serum of dog No. 248 was tested on several strains. Strain No. 36 is the first one isolated (New York, October, 1908).

SERUM NO. 248. BACILLARY STRAINS.

	245	199	200	168	36	233
1–10	+++	+++	+++	+++	+++	+++
1–20	+++	+++	+++	+++	+++	+++
1–40	+++	+++	+++	+++	+++	+++
1–60	+++	+++	+++	+++	+++	+++
1–80	+++	+++	+++	+++	+++	+++
1–100	+++	+++	+++	+++	+++	+++
1–200	++	+++	+++	++	+++	+++
1–400	++	+++	+++	++	+++	+++
1–600	++	+++	+++	++	+++	+++
1–800	.....	+++	++	++	+++	+++
1–1000	.....	+++	++	++	+	+
1–2000	.....	.....	++	+	+	+
1–4000	.....	.....	.....	+	.....	+
1–6000	.....	.....	.....	.....	.....	+

We see that the distemper dogs give the same agglutination tests as the dogs experimentally inoculated with *B. bronchicanis* and also those experimentally immunized by repeated injections.

Protective inoculation.—Experiments were made to test the immunizing properties of *B. bronchicanis* against infection after exposure to the true disease. Forty dogs were used in all. Nine were immunized with live cultures and 17 with dead cultures, while 14 were saved as controls. All of these dogs were exposed to at least three dogs suffering with typical symptoms of the disease, including the respiratory, abdominal, and nervous types. Eight of the controls died while all of the immunized dogs remained well.

Experiments have also been attempted to test the curative properties of a vaccine made from emulsion of the dead organism and the results have so far been encouraging.

CONCLUSIONS.

Canine distemper is found, as a result of this research, to be an acute infectious disease primarily of the respiratory tract of the young dog, caused by a micro-organism first described by me,[1] to which I have given the name *B. bronchicanis*.

These assertions are based on the following facts:

When cultures are taken early in the disease *B. bronchicanis*

[1] Preliminary report, *Am. Vet. Rev.*, 1910, p. 499.

is found in the respiratory tract in every case, and, if taken in the first stage, is found uncontaminated.

B. bronchicanis was isolated from 97 dogs at autopsy. In 68 cases it was isolated in pure culture either from the respiratory tract or blood. Of 29 positive blood cultures it was found 18 times, in 13 of which it was unaccompanied by any other organism.

B. bronchicanis was isolated from the only case attempted during life, in the very earliest stage, by means of the bronchoscope.

The serum from cases of distemper in all stages has given positive agglutination with *B. bronchicanis* while the controls were invariably negative.

Koch's law has been fulfilled and cases of typical distemper have been produced by artificial inoculation with pure cultures, the bacillus being again isolated and grown in pure culture, all under conditions which have precluded infection from any other source.

In the above research I was materially aided by Dr. H. K. Miller and Dr. C. G. Rohrer, of the New York Canine Infirmary, wherein a part of the work was carried on, and also by Dr. T. F. Krey, whose large experience was valuable to me in the clinical portion of the work.

REFERENCES.

BABES AND BARZANESCO. *Ann. de l'Inst. de path. de Bucarest*, 1897, 5.
CADIOT AND BRETON. Quoted from Friedberger and Fröhner.
CARRÉ. *Bull. soc. de méd. vét.*, 1905, 59, p. 148.
———. *Ibid.*, 1905, 59, p. 335.
———. *Réc. de méd. vét.*, 1906, 23.
COPEMAN. *Proc. Roy. Soc.*, 1900, 57, p. 459.
FERRY. *Am. Vet. Rev.*, 1910, 37, p. 499.
FRIEDBERGER AND FRÖHNER. *Lehrbuch der spez. Pathologie und Therapie der Haustiere*, 6. Auflage, Stuttgart, 1904.
GALLI-VALERIO. *Centralbl. f. Bakt.*, 1896, 19, p. 694.
GRAY. *Vet. Rec.*, 1903, 16, p. 381.
HEWER. *Johns Hopkins Hosp. Bull.*, 1906, 17, p. 385.
KRAJEWSKI. *Rev. der Tierheilkunde und Tiersucht*, 1881, 4.
———. *Ibid.*, 1882, 5.
KRAUSE. *Compendium der spez. Pathologie und Therapie*, 1867.
LAOSSON. *Inaug. Dissertat.*, Dorpat, 1882.
LEGRAIN AND JACQUOT. *Réc. de méd. vét.*, 1890, 7, p. 149.
LIGNIÈRES. *Rév. vét.*, 1903, p. 568.
———. *Ibid.*, 1903, p. 697.
———. *Ibid.*, 1903, p. 698.
———. *Contribution à l'étude et à la classification des septicémies hémorrhagiques*, Buenos Aires, 1900.
———. *Bull. soc. de méd. vét.*, 1903, 21, p. 339.
———. *Ibid.*, 1906, 60, p. 622.

MARCONE AND MELONI. Quoted from Friedberger and Fröhner.
MATHIS. *Réc. de méd. vét.*, 1887, 41, p. 229.
MILLAIS. *Berl. Tierärztl. Wchnschr.*, 1906.
———. *Vet. Jour. and Ann. Comp. Path.*, 1890, 30, p. 313.
———. *Ibid.*, 1891, 32, p. 313.
———. *Brit. Med. Jour.*, 1890, 1, p. 856.
———. *Veterinarian*, 1896, p. 482.
PETROPAWLOWSKY. Quoted from Friedberger and Fröhner.
PHISALIX. *Rév. vét.*, 1903, p. 667.
———. *Compt. rend. Acad. d. Soc. Par.*, 1902, 134, p. 1252.
———. *Ibid.*, 1903, 135, p. 980.
———. *Ibid.*, 1903, 135, p. 1085.
———. *Progrès méd.*, 1903, 18, p. 129.
——— AND RABIEUX. *Rév. vét.*, 1903, p. 697.
RABE. *Wchnschr. für Tierheilkunde und Wiehsucht*, 1883, 21, p. 126.
RÖLL. *Lehrbuch der Pathologie und Therapie*, 3. Auflage, Vol. 2, Wien, 1867.
SCHANTYR. *Deutsch. Ztschr. f. Tiermed.*, 1892, 18, p. 1.
SEMMER. *Ibid.*, 1875, 1, p. 204.
———. *Encyklopädie der Tierheilkunde und Tiersucht von Koch*, Vol. 10, p. 261.
TATY AND JACQUIN. *Lyon méd.*, 1898, 68, p. 261.
TRASBOT. *Arch. vét.*, 1879, 4, p. 161.
———. *Rév. vét.*, 1903, pp. 569 and 649.

A CONTRIBUTION TO THE PATHOLOGICAL ANATOMY OF ROCKY MOUNTAIN SPOTTED FEVER.*†

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Six postmortem examinations were made by Dr. Ricketts on the bodies of persons dying of the disease. The cutaneous hemorrhages were general in all and varied from petechiae no more than pin-head sized to large confluent and grotesquely shaped, bluish-black hemorrhages covering all parts of the body except the palms and soles. In a few of the bodies hemorrhages were observed in the pleurae or omentum, in one instance in the substance of the lungs. The superficial and visceral lymph glands were moderately enlarged. An icterus was always present. Enlargement of the spleen is quite characteristic of the disease. It was from two to three times the normal size in all the bodies examined. In other respects it was but little altered, fairly firm, and moderately hyperemic. A large infarct was present in one instance. The large veins and right chamber of the heart were distended with blood. The liver and kidneys were enlarged and hyperemic and in both organs varying degrees of cloudy swelling, and fatty degeneration were met with. The changes in the other organs were neither marked nor constant enough to deserve mention, with the exception of the bone marrow. In two bodies where this was examined the marrow of the long bones was red to bluish red.

In monkeys and guinea-pigs the changes are like those in man with the exception of the absence of generalized cutaneous hemorrhages; in white-skinned guinea-pigs these are sometimes seen. Localized hemorrhages with necrosis of the scrotum, vulva, or prepuce are a more constant result of the disease in these animals;

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† Several years before the death of Dr. Ricketts he and I planned a joint work on Rocky Mountain spotted fever to which he was to contribute accounts of its etiology, clinical aspects, and gross morbid anatomy, and I a study of the minute changes. The plan dealt with a monographic consideration of the disease. Some of the phases of this work were to be reported by me at the last meeting of the Association of Pathologists and Bacteriologists in Washington, May, 1910, and for this purpose he sent me notes of the gross changes he had observed in human and animal bodies. No report was made, news of his death arriving during the meeting. His observations are included in the following account.

occasionally the ears become necrotic.[1] Hemorrhages are also met with in the lymph glands in guinea-pigs. In other respects the lesions of both guinea-pigs and monkeys are quite like those in man.

It will not be necessary except in a few instances to compare the microscopic changes in human tissues with those in animals; they may be considered as a whole. The animal tissues examined were from six monkeys and 32 guinea-pigs; the human tissues from the six bodies mentioned.

The changes are of two sorts, those connected with the occlusion of vessels and the more diffuse lesions affecting entire groups of organs. The diffuse changes are hyperplasia of lymphoid tissues and cloudy swelling and acute fatty changes in organs commonly the seat of such lesions in acute infectious diseases. The focal lesions are more varied in their nature since they include not only the processes leading up to the occlusion of vessels, but the results of such obstructions, necrosis in different degrees and the hemorrhages responsible for so many of the clinical and gross anatomic features of the disease as well as for the name "spotted fever." In sections of the skin, liver, kidney, spleen, and adrenal both vascular occlusion and the necroses resulting from obstruction were present. In the lung and heart the capillaries and small veins were found practically occluded with leukocytes, but there were no serious consequences of these conditions with exception of minute hemorrhages beneath the endocardium.

In the least marked lesions of this sort the vessels contain simply an excess of small and large lymphocytes and some of the latter cells have as inclusions red cells or other leukocytes. In the more obvious occlusions agglutinated red cells, fibrin, and polymorphonuclear leukocytes are also present. The agglutinated red cells are often stained a deeper red with eosin than adjacent single red cells. Fibrin is not demonstrable in all the sections of each occluded vessel, but is intermingled here and there with the other constitutents. In the human spleen, for example, and in the monkey spleen, small sinuses occur crowded to distension with polymorphonuclear leukocytes, and these obstructed vessels form the centers

[1] In the spotted fever of Idaho gangrene occurs rather frequently in the human fauces, tonsils, and palate, also in the skin of the penis and scrotum; such changes have not been met with in Montana.

for minute necroses scattered throughout the spleen so abundantly that in each field of lenses of an amplification of 100 diameters or thereabouts, at least one such lesion is present. The necroses referred to of the ears and skin of the scrotum in guinea-pigs are due apparently to anaemia from the plugging of small blood vessels. In serial sections of ears, the seat of such necrosis, and made from base to the peripheries, the entire gamut of early and late changes which terminate as necrosis are present as well as concomitant results of the altered circulation—edema, hemorrhage, and depositions of blood pigment. In the propagation of the disease the blood serum of an infected animal was sometimes injected into the fresh animal, but the lesions described have nothing to do with the use of alien or homologous serum in the manner indicated, for they are present in the human tissues and organs as well as in those of the animals infected experimentally by tick bites. A possible exception is the presence of rather compact masses of polymorphonuclear leukocytes in the perivascular sheaths of lymphoid tissue in the spleens of monkeys, for in this animal the disease was brought about by the inoculation of blood from an infected guinea-pig or monkey; the disease was not produced by tick bites in any of the monkeys.

The excess of leukocytes in the vessels, which is such a conspicuous feature in different organs, is in marked contrast to the estimation of the leukocytes made during life; in the sections they seem too numerous to be consistent with estimates of 10 to 14 thousand per cubic millimeter.[1] But we are acquainted with a similar condition in typhoid fever and in acute interstitial nephritis[2]—that is, an apparent disparity between the leukocyte content of the blood during life and the great number in the vessels of certain localities or organs. It would seem, therefore, as though the action of the toxin of this disease was manifest in certain places, and at such points, even though the vessel occlusions were not present, there was likely to be an excess of leukocytes in the blood, mainly of the mononuclear type.

[1] John A. Anderson (*Hygienic Laboratory Bulletin No. 14*, 1903, p. 22) noted an increase in the large mononuclears in the blood of two patients as the most interesting change from the normal.

[2] Mallory, "The Histology of Typhoid Fever," *Jour. Exp. Med.*, 1898, 3, p. 661; Councilman, "Acute Interstitial Nephritis," *ibid.*, p. 393; Le Count and Batty, "Purpura Hemorrhagica with Generalized Infection with B. *paratyphosus*," *Jour. Infect. Dis.*, 1907, 4, p. 175.

No extensive search for the bacillus described by Dr. Ricketts[1] was made. In the examination of the focal lesions it was difficult to dismiss the idea that the cause of the disease was in all likelihood within the field of vision. This was naturally more true of the minuter lesions. The minuteness of the bacillus, and the shrinkage which is probably greater in tissues than in cover-glass preparation, as well as its refractory staining peculiarities, are some of the obstacles to such a search.

It is reasonable to believe that the focal lesions are much more widely spread than has been demonstrated. The bone marrow of a few animals was examined without finding any focal lesions; no human marrow was obtained. Similar necessity for further study concerns the central nervous system. In the few instances in which sections were examined of the cord or brain (some of these had to do with human tissues) no local lesions were seen.

In dismissing this phase of the subject it is proper to liken the focal necroses and the preliminary vessel changes to the alterations caused by the so-called "endothelial toxins"; furthermore, to recall that some such toxins, it is believed, are liberated from the bodies of bacteria.

As for the diffuse changes, the cloudy swelling and fatty changes are not marked. In the lymph glands an hyperplasia occurs and in the spleen a similar process is in part responsible for its considerable enlargement. Hemorrhages are not met with in microscopic preparations of the lymph glands as frequently as in the gross examinations. In two instances, both guinea-pigs killed on the sixth and seventh days of the disease respectively, the microscopic preparations of the lymph glands look not very unlike liver at first glance, due to the great amount of cytoplasm visible with low powers of the microscope and its affinity for the cell-body stains. These large cells crowd and distend the sinuses and are markedly phagocytic.

One of the interesting features of the enlargement of the spleen in guinea-pigs is the appearance of large numbers of multinucleated cells and cells with nuclei aggregated into rings, cells corresponding in some respects to the giant cells of bone marrow. So far as

[1] *Jour. Am. Med. Assn.*, 1909, 52, p. 379.

indicated by these megacaryocyte-like cells, this assumption by the spleen of the characteristics of bone marrow was met with in only a few of the spleens of guinea-pigs and then early in the disease. The guinea-pig spleen normally contains such cells in small numbers, but the number present in the spleens of some of the animals is very great. In the human spleens, too, large multinucleated cells possessing some points of resemblance to megacaryocytes are occasionally found, most of them with several small nuclei in the central part of the cell, a few with ring-shaped nuclei. Cells similar to either the megacaryocytes or to multinuclear endothelial cells were absent from the spleens of monkeys. To what extent these large cells in the spleen are to be attributed to a myeloid transformation of the spleen is difficult to state. We are accustomed to attributing similar metaplasias to severe anaemias of some standing.

In some instances, as for example about the vessels of the guinea-pig's seminal vesicles, in and between tubules of the testes and epididymites of monkeys, some of which had not attained puberty, and in other structures, the accumulations of cells and evidence of their multiplication *in situ* suggest the formation of new depots for the production of leukocytes or other cells which presumably are in some way designed to play some rôle in the defensive processes. These are usually in perivascular situations and so limited to the regions of the lymph channels that it seems unreasonable to ascribe them to the focal processes of blood vessel obstruction and their sequences.

The evidences of obstruction to the outflow of bile are present in the human livers, more marked in some than in others. I have not observed them in the livers of other animals. These changes do not differ in any noteworthy way from those encountered in the livers of persons dying from typhoid fever or lobar pneumonia when an icterus develops during the course of these diseases from retrogressive processes in the liver cells and the resulting interference with the biliary current.

EXPLANATION OF PLATES.

(Figs. 10 [Plate 10], 12 [Plate 11], 15 and 16 [Plate 13] pertain to the more diffuse lesions, the remainder to localized or focal processes.)

Plate 6.

Fig. 1.—Human Rocky Mountain spotted fever. Minute necrosis in the spleen. ×400 diam.

Fig. 2.—Larger focal necrosis in the human spleen. ×200 diam.

Plate 7.

Fig. 3.—Human Rocky Mountain spotted fever. In the peripheral capillaries of the liver lobules leukocytes are very numerous, and at various points (*a*) eosin staining rounded masses occur. In some degree they resemble those met with in typhoid fever. ×120 diam.

Fig. 4.—One of the eosin-staining ball-like aggregations, with cell inclusions referred to in Fig. 3. Human. ×400 diam.

Plate 8.

Fig. 5.—Experimental Rocky Mountain spotted fever. Large subcapsular necrosis in the liver of a guinea-pig. ×200 diam.

Fig. 6.—Experimental Rocky Mountain spotted fever. Small region of necrosis in the liver of a guinea-pig. ×400 diam.

Plate 9.

Fig. 7.—Thrombosed vein in the skin. Monkey. ×250 diam.

Fig. 8.—Aggregations of leukocytes in the perivascular sheaths in the spleen of a monkey. Experimental Rocky Mountain spotted fever. ×250 diam.

Plate 10.

Fig. 9.—Illustrating the necrosis of the guinea-pig's ear. ×30 diam.

Fig. 10.—Multinucleated cells in the human spleen. ×700 diam.

Plate 11.

Fig. 11.—Illustrating the amounts of tissue necrotic in some of the livers of guinea-pigs. ×18 diam.

Fig. 12.—Numerous megacaryocytes in the guinea-pig's spleen. ×80 diam.

Plate 12.

Fig. 13.—Aggregations of polymorphonuclear leukocytes in perivascular sheaths of lymphoid tissue. Monkey. ×400 diam. Compare with Fig. 8, Plate 9.

Fig. 14.—Collections of fibrin, agglutinated red-blood corpuscles, and leukocytes in the vessels of the renal pyramid. Human. ×45 diam.

Plate 13.

Fig. 15.—Subintimal proliferation of cells. Human spleen (*a*), mitotic nucleus. ×400 diam.

Fig. 16.—Perivascular accumulations of cells in the wall of the seminal vesicle. Guinea-pig. ×200 diam.

PLATE 6.

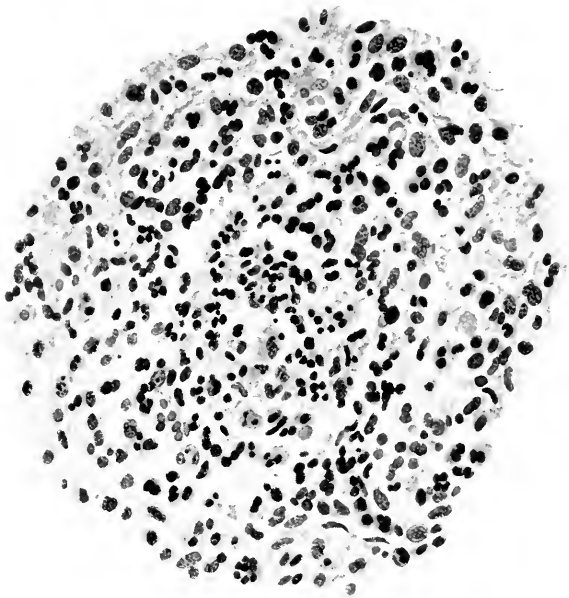

FIG. 1.

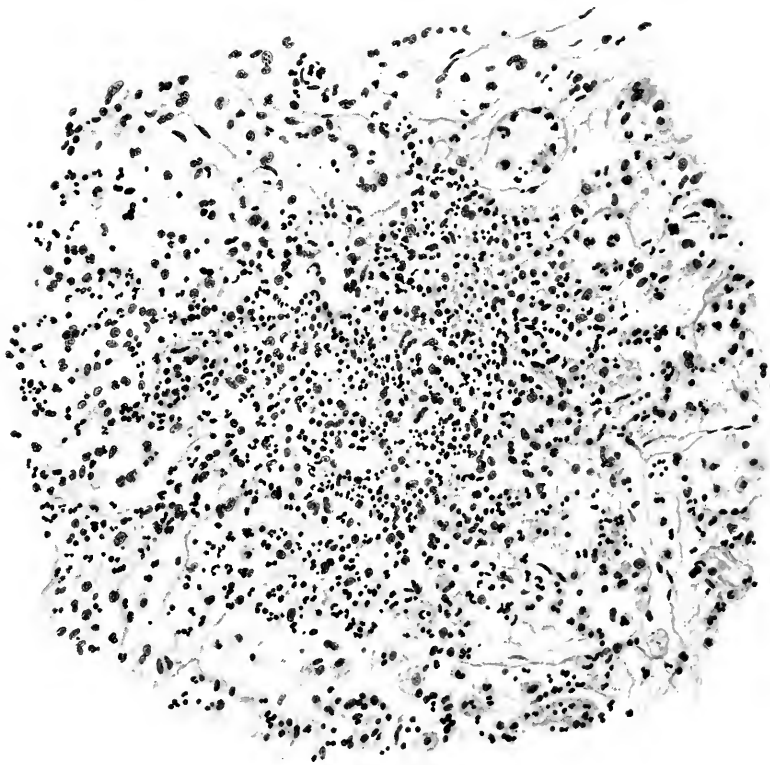

FIG. 2.

PLATE 7.

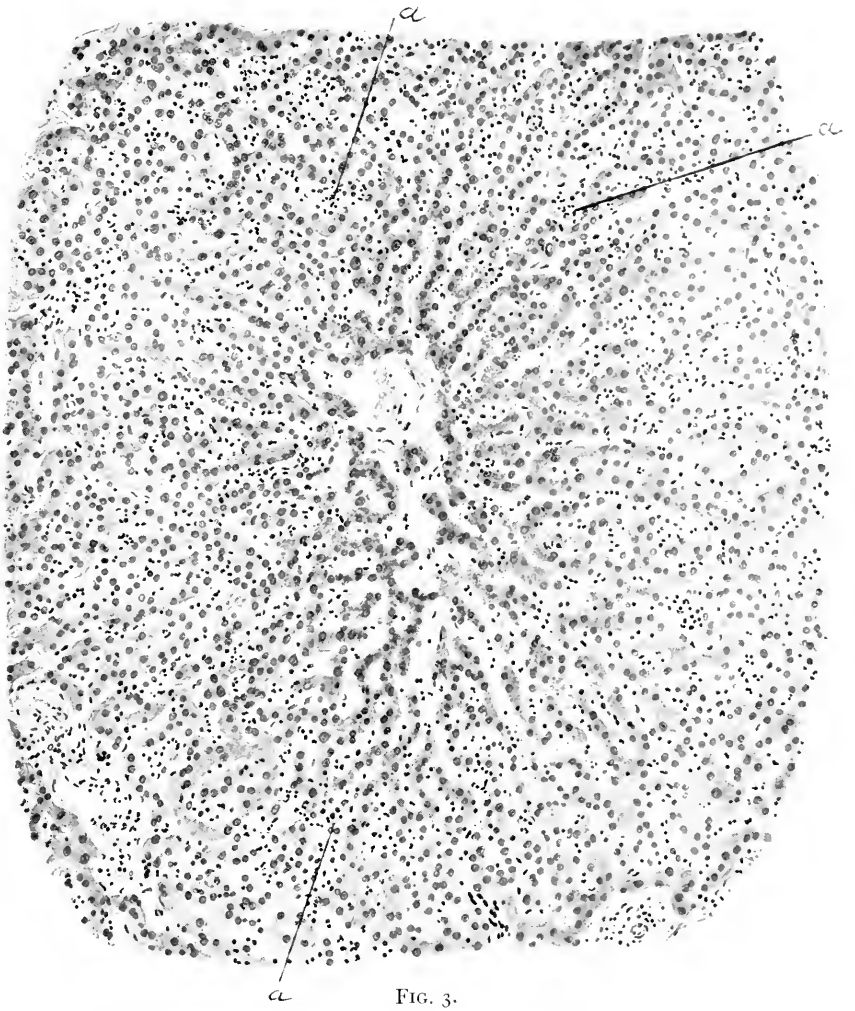

FIG. 3.

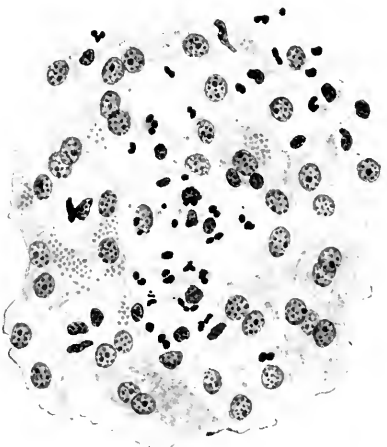

FIG. 4.

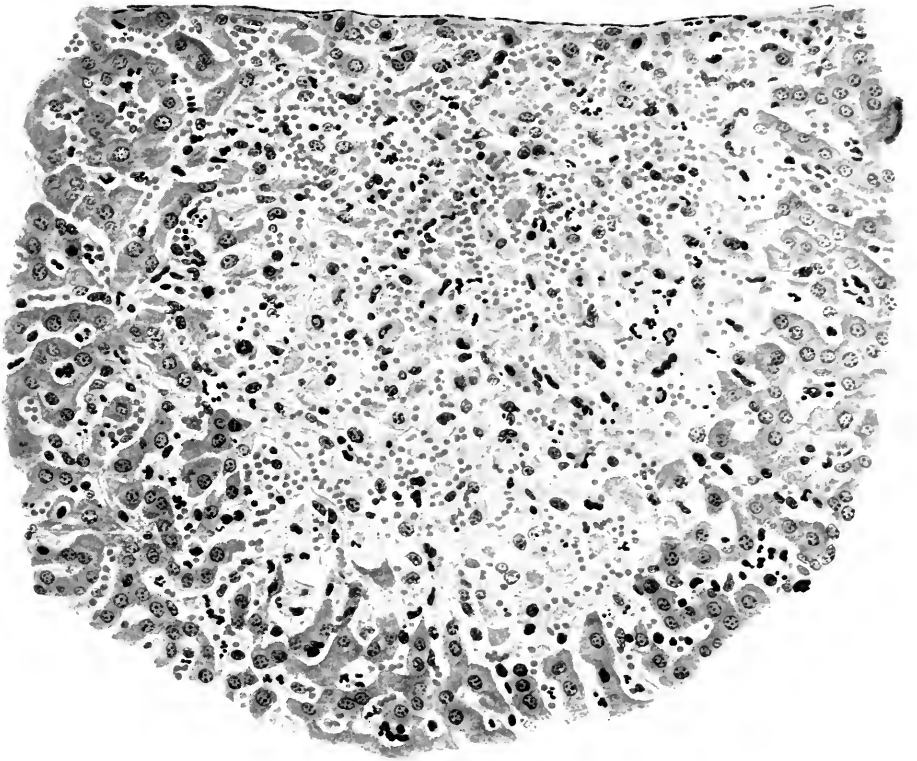

FIG. 5.

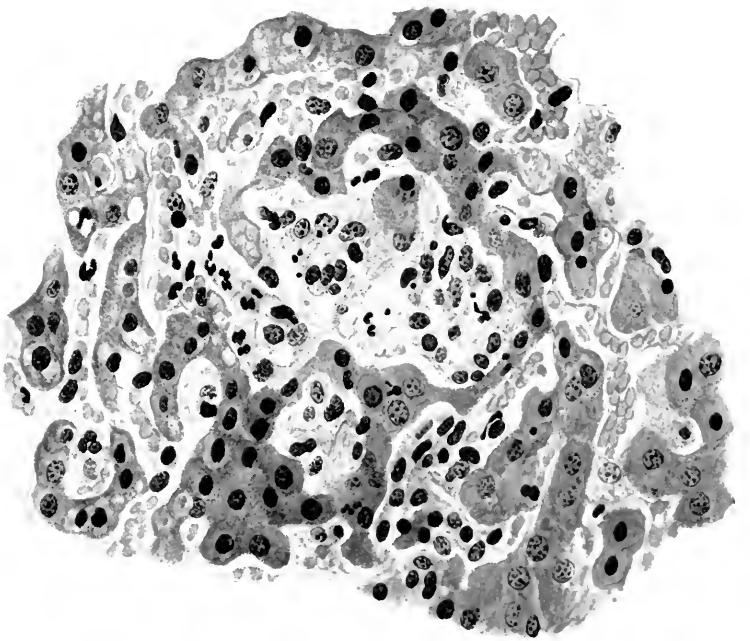

FIG. 6.

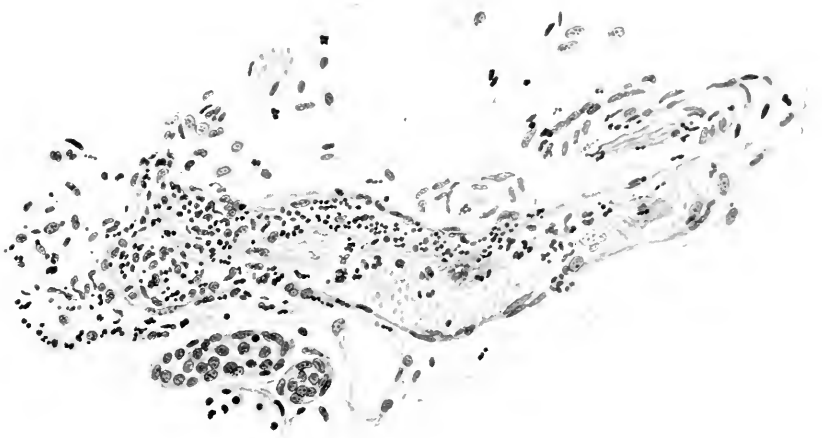

FIG. 7.

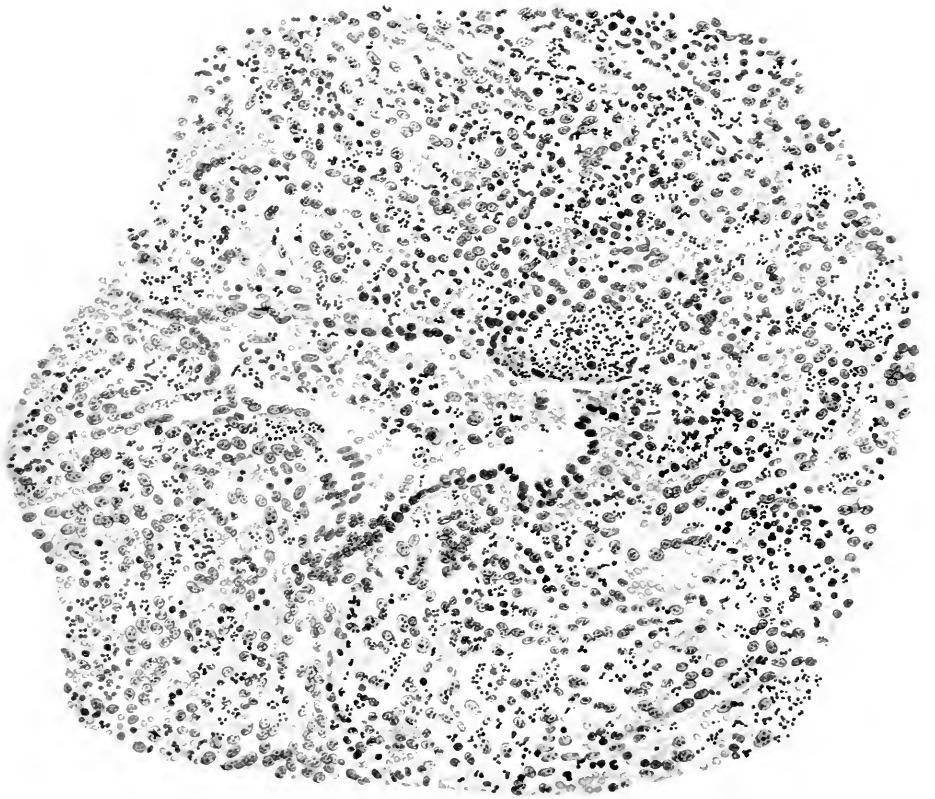

FIG. 8.

PLATE 10.

FIG. 9.

FIG. 10.

FIG. 11.

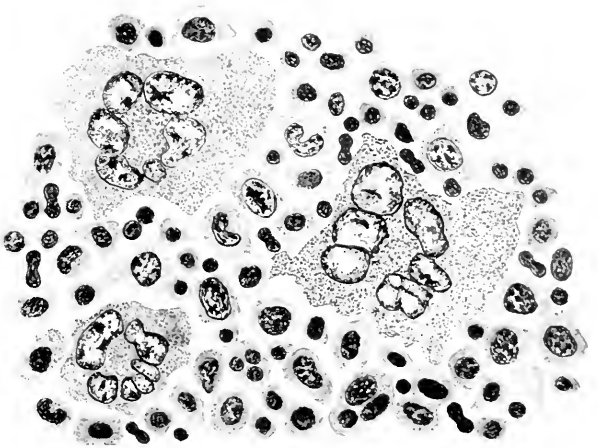

FIG. 12.

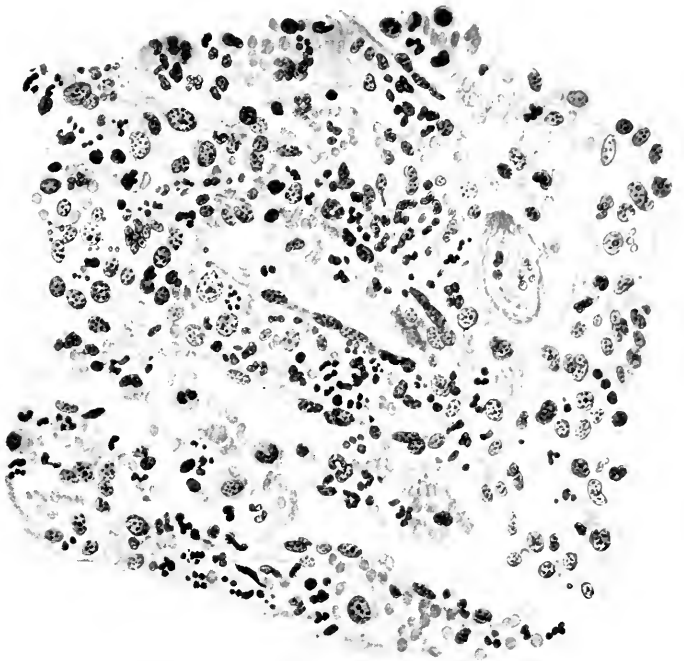

FIG. 13.

FIG. 14.

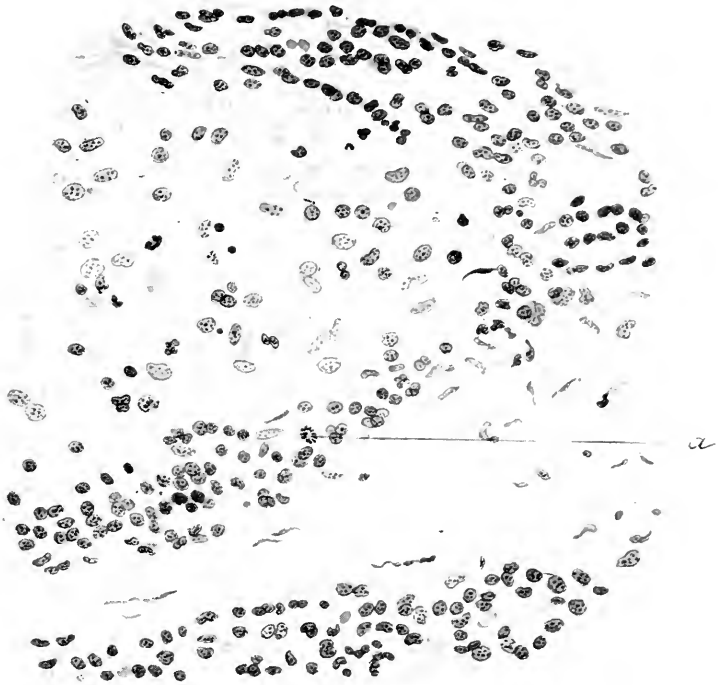

FIG. 15.

FIG. 16.

THE USE OF ACTIVE HUMAN SERUM IN THE SERUM DIAGNOSIS OF SYPHILIS.*

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Since Wassermann's original communication in 1906 of a method for the diagnosis of syphilis by means of the identification of specific bodies present in the serum of luetic individuals, many unnecessary changes and several important and valuable improvements in the methods of technic have been brought forward. Of the modifications suggested and proven valuable that of the more precise determination of the active principle of the antigen has been universally recognized. Soon after the original communications it was shown both by Wassermann himself and by others (Levaditi and Landsteiner) that extracts of the spirochete as prepared from syphilitic livers were not essential constituents, but that lipoid bodies capable of extraction, both from the liver and from other organs, preferably the human heart, were more potent. To Noguchi,[1] in particular, recognition is due for the demonstration of the rôle played by the phosphated group of lipoids in the binding of complement in the presence of the so-called syphilitic antibodies.

In addition to this standardization, as it were, of the antigenic properties of the tissue extracts, several real and important modifications of the Bordet reaction as utilized by Wassermann have been published. There is no necessity to describe, *in extenso*, the details of all the various reactions, but a review of the more important, and criticisms based on personal observation of those which appear to deserve such criticism, will be made. Excluding the use of antigens prepared in different ways, and here it may be insisted that the chief reliability of the reaction no matter how carried out depends on the specificity of the antigen, there remain two essential manners in which the performance of the reaction may differ, one of these being of the greatest possible moment, the

* Received for publication February 9, 1911.

[1] *Jour. Exp. Med.*, 1911, 13, p. 43.

other being of comparatively little importance, granted certain fundamental principles are complied with. I refer to the use of human or guinea-pig serum for complement and the employment of red-blood cells from different animals or from man. Much has been written by different authors advocating the use of some particular hemolytic series. Thus Wassermann following Bordet considers that sheep corpuscles and a specific hemolysin should be employed; still more emphatically Noguchi states that human corpuscles and a corresponding amboceptor must be used in order to get reliable results. Again others suggest the use of corpuscles from the hen and other animals.

It must be evident to all that the corpuscles used in the second part of the reaction are merely indicators and that, so long as the serum used as hemolysin can be exactly standardized and its amboceptor is capable of readily combining with complement, it is of little importance what type of red-blood cell be used, granted the accidental presence of bodies potent to increase or diminish the activity of the amboceptor can be excluded.

In general the methods for carrying out the reaction may be divided into three groups, namely (1) those in which both complement and the hemolysin are utilized from serum other than the human, usually guinea-pig serum for the former body and immunized rabbit serum for the latter; (2) those in which the normal complement content of the human serum is utilized, the hemolytic specific body being derived from rabbit serum as in the first group; and (3) those methods in which both complement and hemolysin are obtained from the serum tested.

It is apparent that one or other of these methods, if not the best under all circumstances, must be more adaptable at certain times. It will be the aim to present as clearly and fairly as possible the relative advantage of the various systems and though recommending strongly one method in particular, to appreciate the limitations and sources of error which must be considered in the use of this method. Before discussing the relative merits of the methods of Wassermann and Noguchi whose systems fall into the first group, that of Stern whose method consists in the use of normal human complement and rabbit serum hemolysin, and the methods of Hecht and Tscher-

nogubow and the modification of the latter as employed by the author, we will consider what characteristics a system of serum diagnosis should have in order to be valuable, and then note in how far the different systems comply with the essentials laid down.

In order of importance these essentials are assumed to be: (1) theoretical and experimental exactness, (2) practical correctness, (3) delicacy, (4) simplicity of procedure, (5) adaptability to use at all times and in all cases.

There being very little doubt but that methods of the third group are more simple, let us consider first the theoretical basis upon which the methods rest, discussing last the practical value and adaptability of the procedure as advocated by Tschernogubow and modified by the author.

Theoretically the Bordet-Gengou reaction depends on the fixation or binding of complement by means of an antigen and a specific antibody. That red-blood cells are employed with a specific hemolysin as an indicator is merely a matter of convenience such as the use of phenolphthalein or litmus in titration. Two things are, therefore, essential: first, since the reaction as used in the diagnosis of syphilis is a quantitative one, the complement employed must be of known quantity and, secondly, it must be susceptible to fixation. In the second place, since we know that in the presence of a larger quantity of hemolytic amboceptor a smaller quantity of complement is sufficient to produce dissolution of the blood cells, and in particular owing to the fact that in the presence of a sufficiently potent hemolysin it is possible for the fixed complement to be deviated, it is important that the hemolytic amboceptor present be also of known quantity.

In the original method as advanced by Bordet and Gengou and followed by Wassermann, guinea-pig complement was employed and measured according to the quantity of serum used, it being taken for granted that the complement content of such serum is constant. That such serum is tolerably constant with reference to its complement content we are prepared to admit; that it is not absolutely constant has been repeatedly proven both by the author and others. An article is now in preparation dealing with the variations in complement content in the serum of man and animals as

influenced by age, health, feeding, etc., and also the changes in the quantity of complement following removal from the body under different circumstances of temperature, etc. This subject will not, therefore, be taken up, *in extenso*, in this paper; suffice to say that I have found with others—Clarke,[1] Hecht,[2] König,[3] Tschernogubow,[4] and Stern and Noguchi[5]—that the complement content of serum from the guinea-pig is liable to vary at times, even in fresh serum, four to eight hours old, sometimes being double that in other sera of the same age and kept under similar conditions. Complement as present in the guinea-pig serum alters after its removal from the animal, gradually increasing in amount if kept for a short time at incubator temperature and thence in the ice chest, remaining for the first 24 to 48 hours practically constant and then, at first slowly, later more rapidly, deteriorating. The changes in complement content following removal of the blood are similar in both human and guinea-pig serum and the influence of the conditions under which the blood is kept appear to affect human complement in practically the same way as that derived from the guinea-pig. In common with Noguchi I have found that certain sera lose their complementary activity more rapidly than others. I believe, however, that as a rule accidental factors such as contamination of the serum by bacteria or protein bodies influence this variation more than any inherent property of the serum.

In 500 tests made on the complement content of human serum the quantitative constancy of this body has been proven. In all sera measured after a lapse of less than 36 hours, and this refers to over 90 per cent of those tested, in only 20 cases have demonstrable variations from the normal been detected, and even in those cases, as will be shown later, this difference is probably more apparent than real. Truly speaking, complement as such has not been estimated owing to technical difficulties not usually appreciated; what has been really tested has been the combined action of complement and the hemolysin content of human serum on guinea-pig corpuscles. For this purpose serum diluted in the proportion of 1–10 in salt solution has been added in quantities (of the diluted material) of 1 c.c., 5 c.c., 0.35 c.c., and 0.25 c.c. to 0.25 c.c. of a 5 per cent suspension of washed guinea-pig corpuscles. The mixture was placed in the incubator and read at the end of 45 minutes, at which time it has been found that the end reaction is present. The rule has been that the quantities representing 0.1 c.c., 0.05 c.c., and 0.035 c.c. of the serum uniformly cause complete lysis whereas the

[1] *Jour. Infect. Dis.*, 1910, 7, p. 476.

[2] *Wien. klin. Wchnschr.*, 1908, 21, p. 1742; *ibid.*, 1909, 22, p. 338.

[3] *Deut. med. Wchnschr.*, 1910, 36, No. 11.

[4] *Ibid.*, 1909, 35, p. 668.

[5] *Jour. Exp. Med.*, 1911, 13, p. 69.

quantity of 0.025 c.c. of serum has usually proven insufficient. In 12 cases 0.5 c.c. was insufficient and in five others 0.35 c.c. was found incapable of completing lysis. In all the former cases the serum was from syphilitic individuals. It is thought that the reason for the smaller quantity of complement was the contamination of the serum at some time in the process of handling with lipoids potent to bind some of the complementary bodies. That this error may and does occasionally take place is undoubtedly so, as I have several times noted such a false result in the results of those working in our laboratory who have employed in the transfer of the serum pipettes usually used for measuring the antigen. The importance of this liability to error will be referred to again in comparing the relative value of different methods. Contamination of the serum with protein bodies resulting in the non-specific proteotropic reaction may also result in the binding of complement, rendering it unavailable for further use. The majority of the cases in which lysis was not complete with 0.05 c.c. of serum occurred within a period lasting a couple of weeks. I noted that many of the tubes used for diluting the serum were somewhat cloudy. On washing all the test tubes used in the reaction in alcohol and ether, I found that such lack of hemolysis was prevented and since making it a routine practice to treat all tubes in this manner I have had no further false controls.

Attempts were made to test the complement content in the human serum by itself by means of a hemolytic series consisting of rabbit serum and human red-blood cells. These efforts, however, proved futile since even in the presence of 0.15 c.c. of human serum (8 hours old) absolutely no hemolysis of 1 c.c. of human corpuscle according to Noguchi's suspension (1 drop to 4 c.c.) occurred when twice the quantity of rabbit serum necessary to produce complete hemolysis when acting with 0.04 c.c. of guinea-pig serum was added. As 0.035 c.c. of this human serum was sufficient to hemolyze 0.25 c.c. of a 5 per cent suspension of guinea-pig serum, a much larger number of corpuscles, it was thought strange that such a result should be obtained, and an effort was therefore made to measure the human complement content by replacement of this body after destruction by time—e.g., one week in the ice chest, as this method has been found to weaken the amboceptor content much less than heat, while it practically allows the complete deterioration of complement. By this means it was found that in order to completely hemolyze within two hours the standard quantity of guinea-pig corpuscles the presence of 0.1 c.c. of complement was necessary. It will be noted that in this experiment three times the quantity of amboceptor necessary to cause complete lysis with 0.035 c.c. of human complement was present. In the presence of three quantities of amboceptor less than half the quantity of complement necessary should suffice, whereas in this case three times as much serum was necessary, or six times what would have been needed if human serum had been used. Repeated experiments proved that this inactivity on the part of human complement when treated with rabbit hemolysin and a similar inactivity on the part of guinea-pig complement with human amboceptors is constant. Owing, therefore, to the inactivity of the human hemolysin in the presence of guinea-pig complement and the apparent lack of activating power of human complement in the presence of rabbit hemolysin, it was considered more exact to estimate the joint action of these two bodies in the human serum, complement, and hemolysin, rather than the activating value of human complement upon immune bodies in foreign sera. It seemed unlikely also that in so large a series of reactions the coincidence of a greater complement and a smaller amboceptor (hemolytic) content would be constant.

The estimations referred to have been made in normal individuals, and in those suffering from chronic diseases, including leprosy, tuberculosis, carcinoma, syphilis, cardiorenal disease, etc., excepting those in a dying condition. In acute febrile conditions, especially in the terminal stages, complement may be diminished in quantity, but such cases are rarely the subject of syphilitic reactions and such insufficiency can hardly militate against the value of the reaction. As is well known the absence of amboceptor against foreign corpuscles in sucklings renders the use of any method of the third series useless with infants less than one and a half to two years old.

It has, therefore, been proved that in so far as the constancy of human complement is concerned it is equally as reliable as that from the guinea-pig. It has further been proved that human amboceptors, of the hemolytic order at least, are more active in the presence of human complement than that derived from the serum of the guinea-pig.

A further feature, however, theoretically renders the use of unheated human serum more valuable than that of serum heated to 56° C. as is done in the inactivation of complement. I refer to the fact that not only is the complementary body destroyed by such a temperature but at least one half of the amboceptor content is also rendered useless. Thus unheated serum will possess a larger quantity of the specific body being sought, rendering its recognition more easy and permitting a finer differentiation quantitatively.

Since no foreign serum is added at any time the possibility of a non-specific fixation, the result of the proteotropic reaction, is excluded if the antigen used is prepared in such a manner as to be free from such material. Hence no danger arises in the use of active serum in which the proteotropic bodies are not destroyed.

As Noguchi has pointed out, if a foreign serum such as that from the rabbit is to be used for its hemolysin content the titre of this serum must be as high as possible in order that a minimal amount of foreign protein substance may be added and thus obviate the possibility of the proteotropic non-specific reaction.

The possibilities for error in the original Wassermann reaction

arise from the following facts: (1) The inactivation of the tested serum results in the destruction of most of the syphilitic antibody; (2) the guinea-pig serum used for complement may vary in its complement content; (3) human serum contains a certain quantity of antisheep hemolysin, thus rendering the standardization of the hemolytic amboceptor difficult.

Noguchi's modification using fresh serum does away with the first objection, in the same way the use of a human hemolytic series excludes the third liability to error, but both of these advantages are replaced by other possibilities of error almost equally important. The use of active serum renders possible a reaction between the proteotropic bodies and the protein in either or both of the foreign sera added. Thus a positive instead of a negative reaction may occur with normal sera. This possibility of error, it is only fair to state, has not been found to be of practical importance especially if the serum from the rabbit be of a high hemolytic value.

A more influential factor is that of the antihuman hemolysin present in the guinea-pig serum used as complement. I have found that two and one-half times the quantity of serum used by Noguchi is usually sufficient to completely hemolyze the red cells used in the absence of other specific lysins. The serum from one pig was constantly found to hemolyze the entire quantity of cells in as small quantities as 0.02 c.c. This objection to the method advised by Noguchi is of importance, but in the author's experience the technical difficulties arising from the use of such small quantities of material and the difficulty of reading reactions when such a small number of red cells are employed, especially as they are agglutinated by the agglutinin present in the hemolytic serum, are even greater.

Hecht in the method usually associated with his name makes use of the normal human complement content and for hemolytic amboceptor depends on the antisheep amboceptor in human serum. This antisheep lysin is, however, present in smaller quantities than that against guinea-pig corpuscles. Thus the number of free complement units is smaller and the delicacy of the reaction interfered with to this extent. The poor activating power of human

complement of amboceptor derived from the rabbit practically prevents the use of the method advocated by Stern.

In 1909 Tschernogubow[1] recommended the employment of the natural complement in human serum as well as the natural lysin for guinea-pig corpuscles. He recommended the use of 0.1 c.c. of serum with an appropriate quantity of antigen in 1 c.c. of salt solution. To this mixture after one hour is added 0.25 c.c. of a 5 per cent suspension of guinea-pig corpuscles. Although Tschernogubow lays stress on the necessity for exact controls he does not state definitely the manner in which they are carried out. I believe then the reason why Tschernogubow's method has not been used more widely, especially in this country, is the fact that it is somewhat difficult to understand from reading his articles exactly how the reaction is performed by him. There can be no doubt but that many, if not all, of the unfavorable criticisms have been made by writers who have not conscientiously attempted to determine the value of the method but have accepted too literally the prevalent idea that the hemolytic body and complement in human serum varies. The smaller unit and consequent larger number of units of complement present in human serum when the anti-guinea-pig corpuscle lysin is employed renders the small variations in complement negligible, whereas in the Hecht method even slight variations may prove very important.

The technic employed by the author and which has been found to be very satisfactory from all points of view is as follows.

Two c.c. of blood are removed in the usual manner from a vein at the elbow. This is placed in a small test tube and kept in the incubator (37° C.) for 2 hours, and then placed in the ice chest. It has been found that such serum will remain reliable for at least 24 hours, usually for 48, and rarely for 72 hours. The most constant results are, however, obtained if sera either 4 to 6 hours or 20 to 28 hours old are employed. Thus the serum collected on two successive days can be tested at the same time.

Four-tenths c.c. of the serum is placed in a small test tube and 4 c.c. of salt solution added. Of the diluted material six test tubes are filled as follows:

	Tube 1	Tube 2	Tube 3	Tube 4	Tube 5	Tube 6
Serum	1 c.c.	1 c.c.	0.5 c.c.	0.35 c.c.	0.25 c.c.	
Antigen	1 c.c.	1 c.c.	....	....	....	1 c.c.

The antigen used is prepared according to Noguchi's acetone insoluble method, thus obviating the non-specific protcotropic reaction. The lipoids are kept dissolved

[1] *Op. cit.*

in alcohol and diluted 1–30 in salt solution for use, the dilution necessary varying, of course, with the potency of the antigen.

The mixture is placed in the incubator for one hour, at the end of which period 0.25 c.c. of a 5 per cent suspension of washed corpuscles is added to all tubes and 0.5 c.c. to tube 2. The tubes are replaced in the incubator for 30 or 45 minutes and can be read immediately or left until the following day. In the hot summer climate in New Orleans the bacterial growth is however liable to obscure the result if the reading is postponed.

Tubes 3, 4, 5, and 6 are of course controls, the last noting the absence of hemolytic action on the part of the antigen and need not be repeated with each series. The preparations containing 0.5 c.c. of the serum and that containing 0.35 c.c. will be found completely hemolyzed, whereas the one containing the smallest quantity will contain an insufficient amount of hemolysin and complement to cause complete lysis.

If the first tube shows complete inhibition of hemolysis it is evident that the Wassermann antibody is potent to fix at least five units of complement. As the units are small the reaction is delicate and the greatest reliance can be placed on this positive result. If the first tube shows hemolysis and the second tube also, a distinctly negative reaction may be reported. If the first tube is hemolyzed and the second cloudy it is known that the amount of antibody present in the suspected serum is insufficient to fix nine units, although enough is present to fix two units. Such a reaction has been called by the author weakly positive. It is extremely delicate and at the same time very easy to read. So delicate in fact, that its usefulness is more or less limited, being given by a certain number of conditions other than those frankly syphilitic. This weak reaction is, however, of the greatest value in the examination of serum used as a control to the efficacy of treatment. Following a course of treatment either with mercury or arsenobenzol the persistence of such a reaction is, in the author's experience, presumptive of the insufficiency of treatment, similarly the development of such a reaction following its disappearance as the result of treatment suggests at least the necessity for careful control of the patient by means of repeated reactions. At present the author's experience with reference to the value of the control of therapeutic measures by means of serum reactions is too limited for a definite statement of opinion. A number of cases have been followed during the past 15 months and will be reported in detail in a future publication. Suffice it to say that repeated tests are

very valuable and the importance of the weak reaction shown by this method is of the greatest possible value. The reaction, being easily read, can be reported with greater confidence than one performed by either the original Wassermann method or the Noguchi modification.

Increased delicacy in any reaction is usually considered of great value. As a rule, however, in discussing the Wassermann reaction a proper appreciation of the value of this quality is not present. Since the reaction is quantitative even more than qualitative, it is quite possible to make the identification of complement binding bodies so delicate that for practical purposes the whole reaction may be rendered useless. In general a reaction in which the complement units are individually small and collectively numerous renders it possible to estimate more exactly the antibody content of the serum examined. To obtain this quantitative reaction by the author's method it is necessary to perform two tests instead of estimating the proportionate hemolysis in a single tube. This is, however, a simple matter and the ease with which the reaction is read more than compensates. As a matter of fact 50 per cent of cases giving the weak reaction by this method are negative by the Noguchi or Wassermann system, and, as has been shown, this weak reaction is extremely valuable in estimating the efficacy of treatment.

No method which necessitates the inactivating of the tested serum can ever give as definite fixations of complement as one in which the so-called syphilitic antibody has not been depreciated by heat. Not only does the greater amount of antibody and the smaller complement unit, together with the large number of units, increase the delicacy of the reaction, but the increased activity of human amboceptors in the presence of human complement as compared with their action with guinea-pig complement results in a more complete and rapid fixation of the complementary body. The increased activity of the hemolytic amboceptor with human complement also allows the hemolytic reaction to proceed more rapidly, thus the readings can be made after the lapse of a shorter period, which in many instances is a decided advantage.

Although for practical diagnostic and even prognostic purposes

the intensity of the reaction, so long as it be sufficiently marked to be capable of binding complement to the extent of inhibiting completely hemolysis by the method described, is of comparatively little importance, such quantitative determinations may be carried out by adding to the tested serum quantities of normal serum. This can best be carried out by using a fixed quantity, say 0.1 c.c., of normal serum and adding to it varying quantities of the serum to be tested.

Naturally the real proof of the value of any reaction depends upon its practical correctness, for, unless the results obtained are valuable, no amount of theoretical reasoning as to the probable value need be considered. Not only, however, is the method recommended in this paper theoretically superior to other systems, but practically it has been found to be more correct.

Of 626 tests 381 gave positive results, 190 negative, and 55 weakly positive as shown in the following summary:

	Positive	Weakly Positive	Negative
Primary syphilis less than 3 weeks...	0	2	5
Primary syphilis over 3 weeks	17	0	0
Secondary syphilis active.....	60	0	1
Tertiary syphilis active.....	115	3	2
Distinct history, but no active syphilis	78	11	6
Treated cases (mercury)	0	15	22
General paresis and tabes.....	13	9	3
Aneurism.....	4	0	2
Congenitally syphilitic children.....	25	2	0
Mothers of syphilitic infants.....	21	1	1
Cases for diagnosis.....	30	6	64
Leprosy.....	18	3	7
No indications of syphilis.....	0	3	77
	381	55	190

It is not my intention in this paper to discuss the value of the serum reaction in syphilis as a whole. The importance of the reaction is being appreciated and it is taking its proper place in the diagnosis of the disease and in controlling the efficiency and sufficiency of treatment. The results here tabulated compare favorably with those obtained by other observers with other methods, the absence of positive findings in negative cases, in so far as the clinical diagnosis was certain, being absolute.

Among the cases classified as "Cases for Diagnosis" are included those cases in which no history or at most a very indefinite history of infection could be determined. For the most part, among the positive cases in this series the serum reaction was accepted by the clinician as probably correct. Three, however, occurred in patients in which it was supposed active destruction of brain tissue was going on as a result of tumor or hemorrhage. That the serum from a certain number of cases of brain tumor gives a positive Wassermann reaction has been shown by various authors, and the author is at present engaged in experimental work with Dr. R. Van Wart in the hope of proving the cause of this reaction.

Of undoubted syphilitic cases, other than tabes and aneurism and those who had received prolonged and energetic treatment, only three gave negative reactions. One of these, a case of late secondary lues with mucous patches from which the treponema was identified, gave an absolutely negative result. The cause in this instance may have been accidental, but as the patient could not be found in order to repeat the reaction no explanation can be offered. The remaining two cases were patients suffering from glossitis and leukoplakia, in one instance 18 and in the other 29 years after the original infection, no more marked lesions having been present at any time.

The single case in which the mother of a syphilitic child gave a negative reaction is an extremely interesting one. Twins, three months of age, were brought to the clinic of Dr. DeBuys at the Touro Infirmary: one was an undeveloped scrawny child, the other apparently healthy. Reactions performed by the Noguchi method showed the serum of the sick infant to contain complement binding bodies in the presence of the lipoids, whereas the other child's serum was normal. The result was unlooked for and the test was, therefore, repeated and the serum of both mother and father was also tested. The original result in so far as the children were concerned was again obtained. The mother's serum proved normal while the father gave a most pronounced reaction. Two weeks after the reactions were performed a typical syphilitic rash appeared on the sick twin.

The cases of tabes and general paresis examined, though not

numerous, resulted in a higher percentage of positive reactions than has been usually reported. It is the author's belief that one important reason for the number of negative reactions given by tabetic sera is that prolonged mercurial treatment, such as these patients usually undergo, results in the arrest of the syphilitic process.

In conclusion I wish to state that of about 100 cases controlled by the Noguchi system in no instance was a negative result obtained by the Tschernogubow method with serum reacting positively to the other series, whereas three undoubted syphilitics were positive by the former where by the latter method complete hemolysis occurred. In addition to laying stress on the value of Tschernogubow's original method I wish to recommend the use of the more delicate reaction obtained by doubling the number of corpuscles added, especially in cases in which the efficacy of treatment is being determined. The author's method also of controlling the complement and hemolytic content of the tested serum is simpler and more exact than that described by Tschernogubow.

It will be noted that of a total of 28 cases of leprosy examined, 21 were found to have bodies present in the serum capable of fixing complement in the presence of the Wassermann antigen. As pointed out in a previous communication by Duval and myself,[1] all of those cases failing to react with the lipoids also failed to bind complement when treated with leprosy bacilli. All these cases, moreover, were considered inactive or cured by the clinical observers. It is our belief as expressed in the paper referred to that all cases of leprosy should, and most cases do, present in their blood Wassermann antibodies. The results published by various authors have varied from 40 to 80 per cent positive. For the most part, comparatively little note has been made of the stage of the disease except that many more positive reactions have been reported in the tubercular form than in those of purely so-called anesthetic types. It is the latter class of cases in which the greatest difficulty arises in stating whether or not the disease is arrested or cured. As stated in our previous communication we believe that not only can the cure of syphilis be controlled by the serum reaction but that the same may be stated with regard to leprosy.

[1] *Arch. Int. Med.*, 1911, 7, p. 230.

The last criterion by which the value of any diagnostic reaction can be judged is its universal application or, in lieu of this, a comprehensive knowledge of the factors rendering its use untrustworthy. Two important exceptions to the general adaptability of this reaction described in this paper are, first, the uselessness of the method without some modification, in infants, and, second, the necessity for using fresh serum—less than 48 hours old—since it is found that after this time the complement content is so liable to great variations that it is unsafe to employ the serum in the active state.

Why there should be an absence of hemolysin in the serum of sucklings is not apparent, but in all probability it is due to the fact that no animal cells being included in the dietary of the individual, no receptors capable of combining with foreign-tissue cells are necessary and are, therefore, not produced. The fact, however, is well established, having been pointed out by Bauer,[1] Hecht, and others several years ago.

Since the reaction described by Tschernogubow and advocated by the author cannot be used in all cases some other method must occasionally be employed. It is possible by means of normal human serum to supply the complement and amboceptor after inactivation of the serum to be tested. This method, however, is not recommended since the possibility of a non-specific reaction is increased, and, although good results have been obtained in the 15 cases so tested, I do not consider the method theoretically good. For such cases it has been my practice to make use of the original Wassermann technic, using human corpuscles and their specific hemolysin.

The method described is not recommended for use except by those working in laboratories and in institutions where a large number of reactions are carried out. Owing to its inexpensiveness, the small amount of time necessary to make the test, and the absence of reagents which are usually prepared with more or less difficulty, it places this important diagnostic aid within the reach of the clinician in a manner that would be impossible in a similar amount of time by any other method. If the serum to be examined is procured and kept as described the results will be uniformly good, and the time taken up in the performance will be greatly reduced.

[1] *Berl. klin. Wchnschr.*, 1908, 45, p. 834.

SUMMARY.

The natural complement content of human serum can be utilized in the serum test for syphilis if the serum be properly preserved and used when fresh. In addition the natural hemolysin against guinea-pig corpuscles can be employed in the hemolytic series in infants.

Theoretically the employment of human serum ought to give more constant results than those obtained by using foreign sera for complement and amboceptor, since the human amboceptor is more active in the presence of human complement than with that from the guinea-pig. The use of active serum obviates the destruction of the greater part of the so-called syphilitic antibody, thus rendering it possible to fix a larger quantity of complement than by other methods. That the heating of the tested serum to 56° C. for the purpose of destroying the proteotropic bodies is unnecessary when protein free antigens are used, such as that prepared by Noguchi, has been proved.

Practically any method utilizing the natural complement and hemolysin present in the serum is simpler, less expensive, and requires less time than other methods in vogue. The results obtained in over 600 tests have been uniformly trustworthy and have shown fewer errors than controls performed by the original Wassermann reaction and the Noguchi modification.

Of the published methods utilizing the natural complement and hemolysin that of Tschernogubow has proved the best, both practically and theoretically. Hecht's technic using sheep corpuscles and the hemolytic amboceptor against such corpuscles is good, but since the amboceptor content for sheep erythrocytes is proportionally less in amount than that for the red cells of the guinea-pig, when a similar number of cells are used, the complement unit necessary to complete hemolysis when guinea-pig corpuscles are employed is smaller, thus rendering the reaction more delicate, since no extra complement can be readily added. Thus a quantitative estimation of the intensity of the reaction can be carried out. For whereas in the Hecht method each 0.1 c.c. of serum contains only one unit of complement, the smallest quantity of antibody results in the fixation of a sufficient quantity

of complement to inhibit hemolysis; when guinea-pig corpuscles are employed, roughly six units of complement are present, rendering the estimation of the comparative strength of the reaction easy. For practical purposes a quantitative test which has proved efficient in the author's hands is the doubling of the number of corpuscles added, thus reducing the number of excess complement units to about two.

The objection frequently raised to methods such as the one here recommended is that both complement and hemolysin vary to such an extent that the reaction is worthless. In over 500 estimations of the combined action of complement and hemolysin the author has found less than three per cent showing variations of any demonstrable importance, most of these being accounted for by accidental deterioration of complement in handling. The variations in human complement appear less than those of guinea-pig complement. Variations in the amount of complementary body present, moreover, are much more readily controlled than when guinea-pig complement is used and in practical procedure can and must be determined for every serum at the time the test is performed.

Errors liable to give a false reaction, such as those occurring as a result of contamination of the serum with lipoids capable of binding the syphilitic antibody with the normal complement giving a negative result with positive sera, are more readily noted if the complement present in the serum tested be determined at the time the reaction is performed.

The fact that but very little agglutinin is present in human serum against guinea-pig corpuscles renders the reading of the reaction easier than by methods using an artificially induced amboceptor, since in rabbit serum a sufficiently large quantity of agglutinin is produced to clump the erythrocytes, rendering the identification of a partial hemolysis very difficult.

If serum to be tested is preserved in a uniform manner, preferably two hours in the incubator and subsequently in the ice chest, it will be found to maintain its complement content usually for 48 hours and always for 24 hours. Older serum should not be used.

Since the serum of infants cannot be examined by this method and since a certain number of sera will not be properly preserved

and for other reasons unfit for the reaction, other methods must be used. For this reason and also because the mere simplicity of the technic necessarily demands a more thorough knowledge of the underlying principles, it is the author's conviction that only those working in laboratories equipped for serum diagnosis of various kinds should be recommended to employ such a reaction.

For the testing of cases not adapted to examination by this method the author prefers the Noguchi system, utilizing quantities employed by Wassermann and with inactivated serum.

AN INSTITUTIONAL OUTBREAK OF CEREBROSPINAL MENINGITIS RESTRICTED BY THE ELIMINATION OF CARRIERS.*†

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The asylum, a semi-private eleemosynary institution, is owned and conducted by the Sisters of Charity and is supported by state and private contributions. The object of the charity is the care of forsaken infants and orphan children up to the age of six years. There are accommodations for 250 children, 60 of whom are cared for at the country home.

The children are divided into three groups, the largest of which comprises those above four years of age. These are about 165 in number, but 60 of them are kept at the country home. It was among these kindergarten children at the city house that the outbreak of meningitis occurred.

This asylum has been singularly free from epidemics of all kinds and in the last eight years there had been but two cases of cerebrospinal fever, one in 1904, the other in the spring of 1905. From November, 1909, to April 28, 1910, however, four cases occurred, an interval of two months elapsing between the first and second, and of three months between the second and third. The fourth, however, occurred six days later and it was then, fearing the outbreak might assume epidemic proportions, that the Health Department was requested to make an investigation. This consisted, in part, of a careful bacteriological study of the nose and throat of each child in this group, and of every person who had been in contact with these children. All carrier cases were isolated until the treatment with antimeningitis serum cleared up the condition, quarantine being raised only after two consecutive negative cultures from both the nose and throat. There have been no more cases of menin-

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gococcic meningitis and we believe that, by this precaution, an epidemic was prevented.

The nature of the outbreak suggested that the source of infection was the presence of carriers in the asylum and careful consideration convinced us that such was the case. The long intervals between all the cases except the last two would preclude all other explanations save the one mentioned and the possibility of the disease being introduced from without. The latter assumption seemed highly improbable in view of the fact that meningitis was not prevalent in Baltimore at the time. It was, therefore, determined to make a painstaking search for carriers in the infected portion of the home. This necessitated the making of cultures from the nasal and naso-pharyngeal secretions of 94 orphans and of three other inmates who had been in contact with these children.

Although, at the time, we were cognizant of the fact that the meningococcus had been found in the nose and throat of healthy contacts, we were not aware that any similar effort had been made to control an institutional epidemic. In a review of the literature we found that up to and including 1903 the meningococcus had been isolated from the nose and naso-pharnyx of contacts only three times: once each by Kiefer, Albrecht and Ghon, and Lord. Kiefer developed a purulent rhinitis while engaged in a study of the meningococcus and was able to isolate this micro-organism from his nasal discharge. Albrecht and Ghon obtained it from the naso-pharyngeal secretion of a contact. Lord demonstrated it in the nasal secretion of a physician who had been daily in the Throat Room of the Massachusetts General Hospital, and who at the time had a severe rhinitis.

In the interim between 1903 and the present time there have been numerous cases reported, a partial review of the literature showing several hundred. Thus, Weichselbaum and Ghon isolated this organism from the nasal secretion of three persons exposed to meningitis, identifying them culturally. Goodwin and von Sholly studied the nasal mucus of 45 healthy contacts, and in 5 meningococci were found. From the nasal secretion of 2 out of 55 medical students they isolated an organism which culturally, morphologically, and tinctorially resembled the meningococcus. These strains, however, by absorption tests, were proved not to have the specific agglutinins of the meningococcus.

Von Lingelsheim obtained the meningococcus in 26 out of 289 healthy persons examined, these embracing 213 near relations, 40 close acquaintances, 30 nurses, and

6 physicians. Of the 26 carriers, 23 belonged to the first class and 3 to the second. He also found that 4 of 56 school children were carriers. In 1906 he reported upon 514 examinations of the nasal and naso-pharyngeal secretions of 471 individuals not suffering from meningitis. Three hundred and eighty-seven of the cultures were from 346 persons in more or less intimate contact with cases of epidemic cerebro-spinal meningitis. Two hundred and seventy-four were from 234 near relations, 87 from friends in infected houses, 3 from physicians, and 33 from 22 nurses. Twenty-eight of the 387 cultures contained meningococci. Nineteen relations, 4 friends, and 1 nurse proved to be infected contacts. The 87 friends included the 56 school children previously mentioned. The remaining 127 cultures were from 125 persons suffering from some disease other than meningitis, and none of these proved positive. In none of these cases was there a history of contact with meningitis.

Kolle and Wassermann examined the nasal secretion of 114 persons and isolated the meningococcus in 2 instances. One case was a girl who had had symptoms suspicious of meningitis; the other was a man whose child had probably suffered from the disease. In none of these cases did the microscopic examination of smears show intracellular diplococci which resembled the meningococcus. Ostermann isolated the meningococcus from the naso-pharyngeal mucus of 17 out of 24 members of 6 families. Kutscher in his studies found 6 carriers, one being the father of a child who had died of cerebrospinal fever. Four others were soldiers who were being treated in a garrison hospital for various catarrhal affections of the upper air passages, rheumatism, scarlet fever, and typhoid fever. In all, 56 soldiers were examined.

During the epidemic of 1905, Dieudonné examined the noses of 29 soldiers in a garrison hospital for the presence of the meningococcus, and found 4 of these were carriers. He also examined the nasal and pharyngeal secretions of 39 soldiers in a room in which several cases of epidemic cerebrospinal meningitis had occurred. From 5 of these he isolated the meningococcus. These contacts had been very closely associated with two of the cases of meningitis. Hasslauer also reports this same investigation. Bochalli found that 10 of 16, or 62 per cent of the people occupying rooms of patients were meningococcus carriers. Trautmann in his studies discovered that 100 per cent of one family were infected contacts, while 75, 50, 30, 25, and 20 per cent of other families were carriers; the average for all was 70 per cent.

Friese and Müller made 114 examinations of the naso-pharyngeal secretions of 36 sick soldiers, not, however, suffering from meningitis. Of these 79 proved positive, this representing 28 different men. In 3 cases the meningococcus was still present at end of three months. Of 60 healthy soldiers, 28 gave positive and 32, negative results. They also found 12 carriers among civilians.

Of 26 persons who had been associated with 2 cases of meningitis, Selter found 8 were carriers; while of 25 persons who had been in contact at the factory with either the father or sister of one of these cases, 7 were carriers. In another infected family he isolated the meningococcus from 6 healthy persons. He also obtained this organism from 2 out of 18 persons associated at the work shop with the father of this family, and from one other person working in a room on the opposite side of the shop. Four members of a third infected family also showed the presence of the meningococcus in the naso-pharyngeal secretion. Selter discovered that 3 of 35 patients at a nose and ear polyclinic were carriers. None of these had been in contact with meningitis.

Bethge described an epidemic in an orphan asylum in which 9 out of 187 inmates developed cerebrospinal meningitis. In April, 1909, he began a study of this out-

break and isolated the meningococcus from 60 healthy contacts. Of these 57 were isolated and all received local treatment. Following this the outbreak ceased.

Mayer made bacteriologic examinations of the troops in Munich where there had been an epidemic the year before: Two per cent of 9,111 healthy soldiers were found to be carriers. A second examination was made of 1,911 of these men, and he obtained the meningococcus from 2.46 per cent. He believes that the organism is widely distributed both during and after an epidemic.

A few of the cases reported were persons who had not, so far as could be determined, been in contact with meningitis. Thus, as already mentioned, Selter was able to isolate the meningococcus from 3 of 35 patients at a nose and ear polyclinic, not one of whom had been associated with a case of meningitis. However, it has been the experience of most observers that the meningococcus is only isolated from the nose and throat of such healthy persons as have been in contact with cerebrospinal fever. Thus, Hasslauer made cultures from the throats of 192 persons giving no history of contact with meningitis; in none of these did he find the meningococcus. Again, Davis made 200 examinations of sputum and cultured 150 throats and failed to find this organism.

We have constructed the following table which shows the percentage of contacts from whom typical meningococci were isolated:

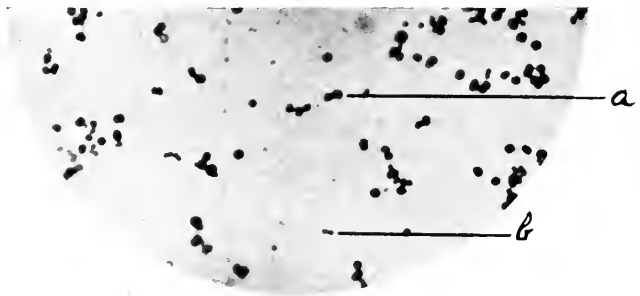
Observer	Total Examinations	Positive	Percentage
Goodwin and von Sholly	45	5	11.1
Von Lingelsheim	346	24	6.9
Ostermann	24	17	70.0
Friese and Müller	60	28	46.6
Selter	51	15	29.0
Bethge	187	60	32.0
Hachtel and Hayward	97	15	15.4
Total	810	164	20.2

ORIGINAL INVESTIGATION.

Swabs from the nose and nasal pharynx were rubbed over Loeffler's blood serum and incubated 18 hours at 37° C. Slides from these were stained according to Gram's method and counterstained with Bismarck brown. All cultures showing Gram-negative organisms morphologically like the meningococcus were selected for further study. A loop from each of these was carried through several water blanks and rubbed over four slants of Loeffler's blood serum and these cultures incubated. Typical discreet colonies were then picked and stained as above. If suspicious, the organisms were then subjected to careful cultural study, and their pathogenicity for white mice was determined. In this way we studied 194 cultures from 97 different people, and were able to demonstrate the meningococcus 18 times, this representing 15 carriers. It will thus be seen that from three of the children we

isolated it from both the nose and throat; from the other 12 it was only found in the naso-pharyngeal secretion. The percentage of carriers in this instance was, therefore, 15.4, which is between the percentage of Goodwin (11.1 per cent) and that of Bethge (32 per cent). Besides the meningococcus, we also isolated the micrococcus catarrhalis in 24 instances, and the various strains of this organism were also subjected to careful cultural study, animal inoculations, and opsonic experiments.

Morphology and staining characteristics.—In all cases the meningococcus-like organisms corresponded to the usual description, being Gram-negative, bean-shaped cocci, as a rule arranged in



a. Micrococcus catarrhalis.
b. Meningococcus.

FIG. 1.—Showing the difference in size between the meningococcus and the micrococcus catarrhalis.

pairs with the flat surfaces apposed. There was considerable variation in the size and in the staining qualities of the individual organisms, and the older cultures showed marked degenerative changes.

The micrococcus catarrhalis differed from the typical meningococcus only in being slightly, but appreciably, larger, and in being more nearly uniform in size. The difference in size is clearly shown in the accompanying photomicrograph of an artificial mixture of pure cultures of the two organisms. This was sufficient to enable us, after having examined cultures from 25 children, to separate the original cultures into two groups; the one including organisms which we considered meningococci, the other those we believed to be the micrococcus catarrhalis. In this manner we

placed 12 cultures in the first group and 22 cultures in the second, all of which were shown by cultural and opsonic tests to be the meningococcus and the micrococcus catarrhalis respectively. In only two instances out of the last 144 cultures studied did we find diplococci which we could not thus classify, these later proving to be the micrococcus catarrhalis.

Cultural properties.—All the cultures of the meningococcus were in every respect typical. Thus, on Loeffler's blood-serum, the colonies were white, viscid, and moist, having sharply defined margins, and in some cases becoming confluent. None of them liquefied this medium.

The growth was less profuse on glycerin agar than on the blood serum, and the colonies were white, translucent, and viscid, and the borders sharply defined.

Litmus milk remained unchanged in reaction, while the growth in gelatin was not profuse nor was there liquefaction in any instance. The growth in broth was scant.

We also inoculated our cultures into sheep-serum dextrose-litmus broth, and in every case there was fermentation with the formation of acid, but never of gas; never was there sufficient acid produced to cause coagulation of the sheep-serum.

Micrococcus catarrhalis.—The colonies of this organism on Loeffler's blood serum and upon glycerin agar were somewhat larger than those of the meningococcus, and after 48 hours the outlines of the colonies showed considerable irregularity. The blood serum was not liquefied. Litmus milk remained unchanged, while the growth in gelatin was not profuse and there was no liquefaction of this medium. The action of various strains of this organism on the sheep-serum dextrose-litmus broth medium was also studied, and none of them produced the slightest acidity even after standing seven days.

It would seem, therefore, that media containing dextrose should be of inestimable value in the differentiation of the meningococcus from the micrococcus catarrhalis. Thus, Arkwright, working with 36 cultures of the meningococcus isolated from cases of cerebrospinal fever, found that 33, or 91.6 per cent, caused acid fermentation of dextrose.

Symmers and Wilson studied the action on dextrose of 60 organisms isolated from cases of epidemic cerebrospinal meningitis. In nearly all instances the medium which contained litmus became red, in a few cases bleaching preceding the reddening. Of 10 strains of meningococci from sporadic cases, 80 per cent produced acid in this dextrose medium.

Pathogenicity.—Eight strains each of the meningococcus and the micrococcus catarrhalis were inoculated intraperitoneally into white mice. In all instances the mice inoculated with the meningococcus died within 18 to 48 hours of purulent peritonitis, showing many intracellular organisms, while none of the other organisms proved pathogenic. However, different strains of the meningococcus vary so greatly in their pathogenicity that animal inoculations cannot be depended upon for the differentiation of this micro-organism from the micrococcus catarrhalis.

Agglutination.—We were unable to perform any agglutination experiments with our organisms since they died out so rapidly. However, in reviewing the literature we find such great variability in the agglutination of different cultures of the meningococcus, that we do not believe that much stress should be laid upon such experiments in the separation of the micrococcus catarrhalis from the meningococcus. Both Ghon and Selter have come to the same conclusion, the latter having found that meningococci isolated from different members of the same family vary greatly in their agglutinative reaction.

Opsonic experiments.—Houston and Rankin have found that while normal serum is opsonic for the meningococcus it is much less so for this organism than for the staphylococcus. They also found the blood of cerebrospinal fever patients highly opsonic for the meningococcus, and from comparative experiments have come to the conclusion that opsonic tests are more to be depended upon than the agglutinative reaction. Davis was originally of the opinion that there was no appreciable difference between the opsonic action of normal serum and the serum of cases of meningitis. Later he made more extended studies, and from these concluded that the serum of meningitis patients is more strongly opsonic.

Since in some former work with the meningococcus we also had

found that undiluted normal serum was opsonic for this organism, but that it lost this property when used in a dilution of 1–5, we decided to employ Neufeld's method of dilution rather than Wright's technic. We employed antimeningitis serum (Flexner's) in dilutions of from 1–5 up to 1–200, using in all 15 different strengths. Equal quantities of a suspension of a 24-hour culture of the meningococcus, of washed leukocytes, and of a dilution of serum were mixed in capillary pipettes, and each of these was sealed and incubated 20 minutes. They were then opened and the contents of each spread on a slide, stained by Jenner's stain, and examined for phagocytosis. We found that what has been termed the dilution of opsonic extinction varied from 1–100 to 1–200 for our different strains.

Similar experiments were performed with our cultures of the micrococcus catarrhalis, but this serum, even at a dilution of 1–2, did not cause the leukocytes to ingest this organism. Nor was normal serum opsonic for it at a 1–2 dilution. These experiments were performed with nine strains of the meningococcus and eight of the micrococcus catarrhalis. We feel, therefore, that opsonic experiments should be of great value in the differentiation of these two organisms.

PRACTICAL METHOD FOR DETECTING CARRIERS.

In our search for the cause of this outbreak we studied most carefully the morphological, tinctorial, and biological characteristics of all the original cultures, and also of the second cultures from several of the contacts. In a purely scientific research such a procedure is necessary for the positive identification of the meningococcus. Our experience in this investigation, however, impressed us with the fact that such a method would be impracticable in practical sanitary work. We, therefore, decided to determine whether the meningococcus could be microscopically distinguished from the micrococcus catarrhalis. As previously stated we found the latter organism to be slightly larger and of more nearly uniform size than the meningococcus.

Believing that carriers are the principal source of infection in cerebro-spinal meningitis, we think that in all outbreaks such as

the one here described an attempt should be made to control the healthy contacts. As it is utterly impossible in the usually overworked municipal or state laboratory to make a complete study of the organisms in each instance, we consider, in view of our personal experience, that, for the detection of meningococcus carriers, the sanitarian is justified in depending upon the microscopic examination of the mixed cultures from the nose and naso-pharynx. Certainly this is as dependable as the microscopical method of distinguishing the diphtheria bacillus from *B. xerosis* and *B. Hoffmanni*.

Therefore, whenever a local outbreak of epidemic cerebrospinal meningitis occurs, or when carrier cases are suspected, a specimen from the nose and one from the throat should be obtained on a sterile cotton swab such as is used in the ordinary diphtheria outfit. This should be rubbed over Loeffler's blood serum and after incubating from 12 to 24 hours at 37° C. the growth should be mounted on a slide and stained by Gram's method and counterstained by Bismarck brown.

The pyogenic organisms, such as the streptococcus, staphylococci, and the pneumococcus, are eliminated by this method since they stain by Gram's method. This leaves for practical consideration only the large micrococcus catarrhalis and the somewhat smaller meningococcus. These are both negative to Gram's method and stain brown with Bismarck brown. The micrococcus catarrhalis can be distinguished from the meningococcus by being slightly larger and by its more nearly uniform size. We, therefore, propose this as a routine method for searching for carrier cases and for the determination of freedom from the meningococci for the relief of quarantine or isolation.

TREATMENT.

In reviewing the literature we find very little upon the subject of the treatment of meningococcus carriers. Ostermann used boric acid followed by the use of a one per cent solution of hydrogen peroxide for 20 minutes three times a day, but this did not prove very efficacious. Dieudonné treated his cases by having them snuff sozoiodolat; this, however, proved valueless. Selter believes that little is to be accomplished by any line of treatment. Bethge, on the

other hand, treated 60 carriers in an institution, dividing them into six groups. The first was treated with pyocyanase; the second, with hydrogen dioxide; the third, with serum (Kolle and Wassermann); the fourth, with protargol; the fifth, with one per cent salt solution; and the sixth received no treatment at all. As a result of these experiments with different methods of treatment, he concludes that hydrogen dioxide, preceded by thorough cleansing with salt solution, gives the quickest result.

The first three carriers that we discovered in the institution were given ten days' treatment with sprays of hydrogen peroxide, and also with a weak solution (1–10,000) of bichloride of mercury. This treatment, however, proved ineffectual, as cultures from these cases still showed the presence of the meningococcus, all the cultural characteristics being carefully studied. From this time on these and all other carriers were treated with sprays of antimeningitis serum, this treatment being repeated daily. As a result of this we were able to clear up the nose and throat in from 2 to 19 days, the average being 10 days. During this time, of course, the children were kept isolated, and were not released until two consecutive negative cultures were obtained from both the nose and naso-pharynx. However, we agree with Bethge in his statement that all carriers should be treated even though it may be impossible to isolate them.

CONCLUSIONS.

1. Carriers are the chief cause of the spread of cerebrospinal fever.

2. In all institutional outbreaks of epidemic meningitis, a careful and painstaking search should be made for the presence of carrier cases.

3. In practical sanitary work the identification of the meningococcus can be based on microscopic examination, this assuredly being just as reliable as the present microscopic examination of cultures for the detection of diphtheria carriers.

4. Agglutination tests are not only unnecessary for the identification of this organism, but may readily lead to erroneous conclusions since different strains of the meningococcus vary so greatly in their reaction to the same serum.

5. Opsonic experiments are of considerable value, especially when the method of dilution is employed. For the purpose of preventive medicine, however, such experiments are unnecessary.

6. Where possible all carrier cases should be isolated and treated with antimeningitis serum, the nose and throat being previously sprayed with normal salt solution. Even when isolation is impossible the cases should be treated, this subserving the double purpose of protecting the carriers and those in contact with them.

7. Release from quarantine should not be allowed until at least two consecutive cultures from both nose and throat prove negative. We do not believe, however, it is necessary to obtain the organisms in pure culture for this purpose, there being sufficient difference in size between the meningococcus and the micrococcus catarrhalis to permit a differentiation in stained specimens.

REFERENCES.

ALBRECHT AND GHON. *Wien. klin. Wchnschr.*, 1901, 14, p. 984.
ARKWRIGHT. *Jour. Hyg.*, 1909, 9, p. 104.
BETHGE. *Deut. med. Wchnschr.*, 1910, 36, p. 66.
BOCHALLI. Inaug. Diss., Breslau, 1906. Quoted from Trautmann.
DAVIS. *Jour. Infect. Dis.*, 1905, 2, p. 602.
———. *Ibid.*, 1907, 4, p. 558.
DIEUDONNÉ. *Centralbl. f. Bakt.*, 1906, 1 Abt., Orig. 41, p. 418.
FRIESE AND MÜLLER. *Klin. Jahrb.*, 1909, 20, p. 321.
GHON. *Rep. Intern. Cong. Hyg. and Demography*, 11, p. 94.
GOODWIN AND VON SHOLLY. *Ann. Rep. Dept. of Health, New York City*, 1905, 2, p. 653.
———. *Jour. Infect. Dis.*, 1906, Suppl. 2, p. 21.
HASSLAUER. *Centralbl. f. Bakt.*, 1906, 1 Abt., Orig. 41, p. 633.
———. *Ibid.*, 1906, 1 Abt., Orig. 41, p. 723.
HOUSTON AND RANKIN. *Lancet*, 1907, 1, p. 1213.
KIEFER. *Berlin klin. Wchnschr.*, 1896, 33, p. 628.
KOLLE AND WASSERMANN. *Klin. Jahrb.*, 1906, 15, p. 507.
KUTSCHER. *Deut. med. Wchnschr.*, 1906, 32, p. 1071.
LORD. *Centralbl. f. Bakt.*, 1903, 1 Abt., Orig. 34, p. 641.
MAYER, WALDMANN, FÜRST, AND GRUBER. *Münch. med. Wchnschr.*, 1910, 57, p. 1577.
OSTERMANN. *Deut. med. Wchnschr.*, 1906, 32, p. 414.
SELTER. *Klin. Jahrb.*, 1909, 20, p. 457.
SYMMERS AND WILSON. *Jour. Hyg.*, 1909, 9, p. 9.
TRAUTMANN AND FROMME. *Münch. med. Wchnschr.*, 1908, 55, p. 791.
VON LINGELSHEIM. *Deut. med. Wchnschr.*, 1905, 31, p. 1017.
———. *Klin. Jahrb.*, 1906, 15, p. 373.
WEICHSELBAUM AND GHON. *Wien. klin. Wchnschr.*, 1905, 18, p. 625.

HAND INFECTION APPARENTLY DUE TO BACILLUS FUSIFORMIS.*†

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While the actual rôle of *Bacillus fusiformis* in the production of lesions in the human body is still *sub judice*, we cannot fail to recognize its importance as a factor, if we glance at the increased number of pathologic lesions from which it has been isolated. The organism has been observed in ulcero-membranous angina, hospital gangrene, noma, appendicitis, diphtheria, foetid bronchitis, gangrenous laryngitis, pyorrhea alveolaris, brain abscess, and in the healthy mouth.

According to Jungano and Distaso,[1] Plaut first described it in 1894 in a case of ulcerous angina, while Veillon and Zuber were probably the first to isolate the bacillus in pure culture. Vincent's descriptions appeared about two years after Plaut's. The organism has also been grown in pure culture by Ellerman, Weaver,[2] Tunnicliff,[3] Lewcowitz, Leiner, Repaci, and Ghon and Mucha.

Five cases of unusual infection with fusiform bacilli and spirochaetes have been studied by me.

Case 1.—S. F., male, age 4, was admitted to the Cincinnati Hospital October 5, 1909. He had a typical lobar pneumonia with delayed resolution, which was followed by an abscess of the lung. Death ensued sixteen days after admission. Smear preparations of the pus obtained before death from the thorax, by aspiration, revealed fusiform bacilli and spirochaetes in great numbers and streptococci. The fusiform bacilli measured from 2.7 μ to 7.0 μ by 0.5 μ. Apparently two varieties of spirochaetes were observed; thick ones with irregular loose windings corresponding to the refringens type, and others composed of two or three turns and of regular amplitude. The same organisms were found in the sputum, together with a third type of spirochaete, viz., dentium.

Case 2.—P. S., a white male, age 45, was admitted to the Cincinnati Hospital October 3, 1910, with marked dyspnoea, laryngeal stridor, and an irregular pulse.

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† Photomicrographs by Dr. Chas. Goosmann, Cincinnati, Ohio.

[1] Jungano and Distaso, *Les anaérobies*, 1910, p. 155.

[2] Weaver, *Jour. Am. Med. Assn.*, 1906, 46, p. 481.

[3] Tunnicliff, *Jour. Infect. Dis.*, 1906, 3, p. 148.

He died one hour after admission. Post-mortem examination revealed a syphilitic ulceration and edema of the larynx and trachea, and other tertiary lesions. Cover-slip preparations were made from the ulcer at the base of the larynx, demonstrating fusiform bacilli resembling those described by Vincent, and spirochaetes of the refringens type.

Case 3.—F. S., the patient, had a chronic foetid bronchitis. Smear preparations of the purulent secretions showed the presence of *Bacillus fusiformis* in great numbers and an absence of spirochaetes.

These cases are mentioned briefly with a twofold purpose, viz., demonstration of the organism in question and its relative significance when associated with respiratory disorders.

Case 4.—J. K., age 38, laborer. On March 17, 1911, at Gadson, Ala., he struck a man in the teeth, injuring the index and middle fingers of the right hand. Intense swelling, edema, and a foul discharge characterized the condition. Smear preparations made from the discharge on April 9 showed the presence of fusiform bacilli and streptococci. No spirochaetes were demonstrable.

Before proceeding with the case that was studied culturally as well as microscopically, I should like to refer to the case reported by Hultgens,[1] a seven-year-old girl who showed partial gangrene of the left index finger. In smear preparations he found fusiform bacilli and spirochaetes. He does not describe the spirochaetes but calls them *Spirochaeta denticola*. His patient had carious teeth and had been in the habit of biting her finger nails. Film preparations made from her carious teeth showed the presence of these same organisms. Apparently no cultural studies were attempted. The source of infection makes this and the following case interesting especially in view of the fact that no cases are reported of direct transmission from one individual to another.

Case 5.—A. W., age 34, a bar tender by occupation. On September 6, 1910, he struck a man in the teeth, injuring the base of the little finger of his left hand. Two days later the finger was swollen and discharging a foul pus. He was admitted to the Cincinnati Hospital, with a temperature of 99.8°, pulse and respiration normal. Two free incisions were made, and the hand immersed in a continuous bichloride bath. Nine days after injury the wound was not doing well; had a chronic persistent appearance and was still discharging. Eighteen days following injury the left hand and forearm were swollen and markedly edematous, and the discoloration assumed a purplish hue. The wounds were ragged, irregular, and surrounded by cauliflower-like excrescences, with evidence of deep destruction of the tissues. The appearance of these wounds was suggestive of epithelioma. The patient was unwilling to submit to further surgical interference and was discharged. He made a slow recovery, as was learned later, and was well 54 days after the injury.

[1] Hultgens, *Jour. Am. Med. Assn.*, 1910, 55, p. 857.

Smear preparations from the wound, made September 20, showed numerous leukocytes and almost pure culture of fusiform bacilli and spirochaetes. The bacilli, for the most part, are long and regular, with pointed ends, and thicker in the middle. They lie side by side, between the cells, or end to end, and sometimes in irregular clumps. In size the bacilli vary from 2.7 μ to 8.1 μ in length by 0.6 μ in breadth. The spirochaetes are very numerous, and at least two types are visible. Most of them show three or four turns which are of irregular amplitude, corresponding to the refringens type. Their extremities are parallel to the long axis of the spirochaete with two or three thick regular turns corresponding to *Spirochaeta recta*, and another, with four or five regular turns, but which are much thinner, corresponds to *Spirochaeta tenuis*.[1] The spirochaetes measure from 9.0 μ to 16.2 μ in length by less than 0.4 μ

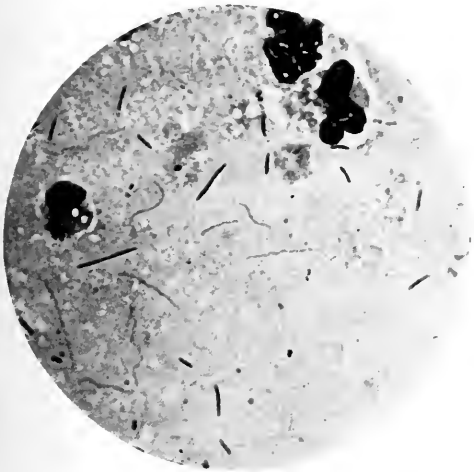

FIG. 1.—Film preparation from infected hand, Case 5, demonstrating *B. fusiformis*, ×1,000. Stained with carbol fuchsin.

FIG. 2.—Smear from infected hand, Case 5, demonstrating spirochaetes, ×1,000. Stained with polychrome methylene blue for 24 hours.

in breadth (Spencer apochromatic 2mm. Ocular No. 8). Smear preparations 46 days after the injury showed the same organisms. A mixed infection was evidenced by the presence of a limited number of cocci and small bacilli. The spirochaetes were not so abundant, appeared thinner than when first observed, and did not stain so well.

Slants of Dorset's egg medium were inoculated with the purulent secretion taken from this case of hand infection. After anaerobic incubation at 37° C. for three days, colonies of two kinds appeared, cocci and fusiform bacilli. By transplanting from the small colonies of spindle-shaped bacilli pure cultures were obtained.

Morphology and staining reactions.—In smear preparations from 24-hour cultures, the fusiform bacilli are delicate pointed rods and usually straight. As they mature, they become long, slender

[1] Gerber, *Centralbl. für Bakt.*, 1 Abt., Orig., 1910, 56, p. 508.

rods with pointed ends, and somewhat thicker in the middle. Very frequently they are slightly curved. They measure 4.5 μ to 46.8 μ in length by 0.6 μ in breadth.

In some of the cultures wavy forms may be observed. The morphologic characteristics of these are not unlike those of the bacilli. The protoplasm reacts to aniline dyes and to light in the same way; and the presence of metachromatic granules are suggestive of fusiformis. The longer bacilli most frequently contain

FIG. 3.—Pure culture of *B. fusiformis*. ×1,000. Stained with polychrome methylene blue for 48 hours.

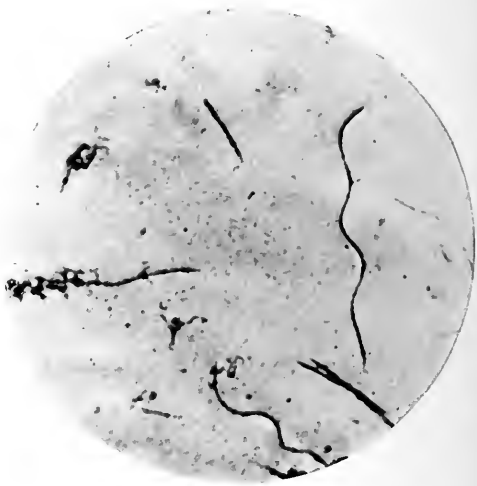

FIG. 4.—Pure culture of *B. fusiformis*, ×1,000. Stained with polychrome methylene blue for 48 hours, showing wavy forms.

four or more metachromatic granules while the shorter forms contain two. In old cultures the granules are stained less intensely or may be entirely absent. Suspended in Gram's iodine solution they do not give the starch reaction.

In making film preparations from solid cultures the bacilli often remain adherent throughout their length, forming bundles. Occasionally two bacilli may be seen lying side by side, so close together as to make one think that they divide longitudinally; however there are no indications of terminal splitting. Some appear only half as thick as others. On the other hand, there is evidence in the film preparations that transverse division occurs.

Some of the bacilli seem to be constricted, and here the protoplasm is thinner and less granular. The fusiform bacilli as well as the spirochaetes are stained by Loeffler's methylene blue, polychrome methylene blue, carbol gentian violet, carbol fuchsin, and by the Giemsa and Romanowski stains. Carbol fuchsin and polychrome methylene blue are the most satisfactory. Specimens were stained in polychrome methylene blue for 24 hours, washed with water, and mounted in balsam. The spirochaetes stain less intensely than the bacilli.

The definition of *B. fusiformis* is beautifully demonstrated by the aniline black method. Neither the bacilli nor the spirochaetes retained the stain in Gram's method, contrary to the statement of Jungano and Distaso.

Cultural properties.—The cultures were grown anaerobically by the pyrogallic acid method. The colonies, not unlike streptococcus colonies, are small and delicate with slightly raised centers about one or two mm. in diameter. The best growth was obtained at 37° C. In the hanging drop the organisms show no active or progressive motility but considerable vibratory motion especially at one end.

So far as the viability of *Bacillus fusiformis* on artificial media is concerned, Tunnicliff found them alive 55 days after inoculation. Cultures on Dorset's egg medium and Loeffler's blood serum have been found by myself, viable 20, 40, and 47 days after transplanting. The viability of the culture is conserved for some time in the refrigerator or maintained by frequent transfers. Twenty subcultures made during the past seven months have been grown successfully.

Loeffler's blood serum and Dorset's egg medium are productive of the most luxuriant growth, the colonies appearing as delicate irregular white masses with slightly raised centers. Growth is scarcely visible at the end of 24 hours, but after 48 or 72 hours' incubation the colonies measure 1 or 2 mm. in diameter. A flocculent growth is usually observed in the water of condensation. Ascites broth offers a very favorable means of cultivation. The growth is heavy, luxuriant, flocculent, and sinks to the bottom. By agitating the tubes this may be divided into small particles.

On rabbit's blood agar luxuriant growth was obtained resembling that seen on Loeffler's blood serum. When such a culture was placed under aerobic conditions the culture medium darkened and was black a week later. In Dunham's peptone solution there was a slight flocculent growth which settled on the bottom. In litmus milk limited growth occured after 72 hours at 37° but no coagulation took place. When broth with a reaction of one per cent acid to phenolphthalein and containing one per cent of dextrose, lactose, saccharose, maltose or mannite was inoculated, no growth occurred, but the addition of 0.5 c.c. of defibrinated rabbit's blood to six c.c. of these various sugar broths yielded luxuriant growths. Acid production was marked at the end of 72 hours excepting in saccharose. The litmus in dextrose and lactose broths was entirely reduced. The same luxuriant growth was obtained in litmus milk when 0.5 c.c. of defibrinated rabbit's blood was added. No growth appeared upon +1. agar, or upon one per cent glucose agar.

When the stoppers were removed from the culture tubes a foul odor was given off suggestive of skatol. The reaction for indol with potassium nitrate and sulphuric acid was negative.

Resistance.—As far as I know the resistance of the fusiform bacilli to moist heat has not been determined. Attempts were made to settle this point by heating ascites broth cultures and suspensions in 0.85 per cent sodium chloride solution. As the controls in this series often showed no growth, the following technic was adopted.

Seventy-two-hour cultures on Loeffler's blood serum were used. Anaerobic conditions were suspended and the rubber stopper and pyrogallic acid plug replaced by sterile cotton and a rubber cap. The tubes were then suspended in a water bath for 15 minutes at 50°, 55°, 60°, and 65° C. respectively. Subcultures were made from each tube and the result obtained was confirmed by an additional subculture from the first subculture. Controls were used throughout.

The bacilli are killed by exposure to moist heat for 15 minutes at 55° C. They are not affected by an exposure at 50° C. for the same length of time.

The fusiform bacillus is able to withstand the action of anti-

formin, one per cent solution, for five minutes without altering its viability. Seventy-two-hour cultures on Loeffler's blood serum were covered with the germicide for two and five minutes respectively, after which they were washed with sterile distilled water. The transplants showed luxuriant growths in 48 hours. When the colonies were covered with hydrogen peroxide, 15 per cent solution, for five minutes and washed with sterile water, no growth appeared in the subcultures. A very luxuriant growth was obtained in the transplants from colonies which had been exposed to hydrogen peroxide for one minute. Cultures were subjected to the action of hydrogen peroxide for one, five, ten, fifteen, and twenty minutes on two different occasions to verify the above results.

Inoculation experiments.—Two full-grown guinea-pigs were inoculated with ascites broth cultures. The first pig received one c.c. of a 72-hour culture intraperitoneally, and the other one c.c. subcutaneously. A white rat and a wild rat, *M. norwegicus*, were given one c.c. intraperitoneally. A large rabbit weighing 1560 gms. was inoculated with 0.75 c.c. intravenously. There was an entire absence of any local symptoms, and at the autopsy, 30 days after inoculation, no pathological conditions were visible in these animals. The fusiform bacillus was recovered from the peritoneal smear of the first guinea-pig, but there was no evidence of multiplication. Tunnicliff's results were negative in guinea-pig experiments.

In the review by Jungano and Distaso they conclude that *Bacillus fusiformis* is pathogenic for the guinea-pig and the mouse. The strain of Leiner was very virulent for the lower animals and one of Repaci's cultures was also pathogenic. Veillon and Zuber were only able to produce a very mild grade of infection with their strains.

At the present time there seems to be a difference of opinion as to the relation of the spirochaetes to *Bacillus fusiformis*. Some authors believe that they are different organisms entirely, and that the presence of the spirochaetes increases the virulence of the bacilli. Others maintain that they are two forms of one organism in its cycle of evolution. Tunnicliff claims to have observed spirilla develop from the fusiform bacilli in her cultures

after they had grown from two to five days. I was unable to note spirochaetes in any of the cultures. The terms spirilla and spirochaeta have been used indiscriminately in the literature, but I think we should adhere rigidly to the word spirochaeta because the symbiosis occurring in the mouth is one of fusiform bacilli and spirochaetes. As was mentioned above, some of the cultures contained wavy forms, but no true spirochaetes were demonstrable. Ellerman holds to the same opinion.

Future study may show that certain metabolic products of *B. fusiformis* favor the growth of the spirochaetes. Repaci isolated spirilla (spirochaetes?) from the mouth, which he claimed to be separate distinct organisms from *B. fusiformis* by reason of their chemical and biological peculiarities. Furthermore the successful cultivation of *Sp. dentium*, *Sp. refringens*, and *T. pallidum* certainly seems to separate this group of organisms from any developmental forms of the bacilli.

SUMMARY.

1. The substance of this article consists, essentially, in a preliminary study, microscopic and bacteriologic, of *Bacillus fusiformis*. Its biochemical properties with special reference to its action upon proteids will be discussed later.

2. Two cases are recorded of direct transmission from one individual to another.

3. In two cases the fusiform bacilli were not accompanied by spirochaetes.

4. The organism grows luxuriantly upon Dorset's egg medium, and in various sugar broths containing a small amount of defibrinated rabbit blood.

5. Mention is made of its resistance to moist heat, antiformin, and hydrogen peroxide.

In conclusion I wish to express my gratitude to Dr. Wm. B. Wherry for his many helpful suggestions.

AN ATTEMPT TO MODIFY THE AGGLUTINABILITY OF THE TYPHOID BACILLUS BY SELECTIVE ISOLATION OF INDIVIDUAL BACILLI.*

V. H. Moon.
(From the University of Kansas.)

All observers have noted in performing agglutination experiments, even with highly agglutinative serum in low dilutions, that some individual bacilli may resist agglutination and remain floating free in the serum. The object of this series of experiments was to determine whether this failure to clump is due to some peculiar property of the bacillus which can be transmitted to its descendants.

METHOD.

To isolate individual bacilli selectively seemed necessary as a means for this investigation. The most precise method for accomplishing this selective isolation is the technic designed and developed by Dr. M. A. Barber of the University of Kansas.[1] By using a microscopic capillary pipette supported in a mechanical holder provided with motion in three directions, one is able after considerable practice to isolate individual micro-organisms from a hanging drop. This drop is supported by a cover-glass over a moist chamber which is held and moved about under the microscope by the ordinary mechanical stage. The details of the method are fully described in the articles above referred to.

The medium used was meat broth carefully made according to the American Public Health Association standard, reaction 0.7 per cent acid to phenolphthalein. To begin with, an absolutely pure strain of *B. typhosus* was obtained by isolating an individual bacillus from a culture obtained from the laboratories of Johns Hopkins University. The resulting colony was transferred to broth and several agar slants were inoculated from this and labeled "Stock." A drop of the broth culture was placed on a sterile

* Received for publication April 1, 1911.

[1] *Jour. Infect. Dis.*, 1908, 5, p. 380; *Kansas University Science Bulletin*, 1907, p. 3.

cover-glass, and agglutination performed by adding an equal part of serum diluted 1:16. The serum used throughout this experiment was human serum from the same individual drawn under aseptic conditions and proved sterile by broth culture. It agglutinated typhoid bacilli at 1:96 dilution. As soon as clumping began a single definite clump was selected and isolated by means of the capillary pipette. The experiment was then allowed to stand one hour and 45 minutes at room temperature about 26° C. Then several individual bacilli which had resisted clumping were isolated and planted separately in small hanging drops. Chemically clean glass tubing was used for making all pipettes. For making each transfer of cultures, broth, or serum, and for each operation of isolation a new, freshly drawn pipette was used. The cover-glass was then transferred to a vaseline-sealed hollow-ground slide of large size and incubated 24 hours at 35° C. From this time on a double series of isolations and cultures was made. In the one series the first clumps to form were isolated, and in the other the individuals which resisted agglutination were selected and allowed to form colonies. For convenience we will refer to these as +S and −S respectively, the +S referring to that series selected on the basis of ready clumping and the −S to the series developed by selecting the resistant individuals. A number following the S indicates the number of selections made in that series.

After making five selections and cultures of the +S and four of the −S a preliminary comparative agglutination test was made with results as shown in the following table.

Dilution	1:16	1:24	1/32	1/48	1/64	1/96	Control
+S$_5$	+	+	+	+	+	?	−
−S$_4$	+	+	+	+	−	−	−

In making this test 24-hour agar cultures were used and the dilutions were examined microscopically. The writer in this case knew the identity of the cultures, but in all subsequent tests the cultures were marked in cipher by a second person and their identity revealed only after all observations had been made and recorded. One element of the personal equation was thus elimi-

nated. The following table shows the result of a test in which the original stock was compared with $+S_9$ and $-S_8$.

Dilution	1/32	1/48	1/64	1/96	1/128	1/192	1/250	Control
$+S_9$	+	+	+	+	+	+	−	−
$-S_8$	+	+	?	−	−	−	−	−
Stock	+	+	+	+	−	−	−	−

These results were verified by a second observer before the identity of the cultures was revealed. The test shows a marked *increase* in the agglutinability of the + series and a corresponding *decrease* in the agglutinability of the − series.

Following this test the routine of performing agglutinations and selections was continued until 24 selections in the +S and 13 in the −S had been made. The average time of making the + selection was 20 minutes, while the − series were exposed to the action of the serum for two hours before the selections were made. Frequent transfers were made to clean freshly sterilized cover-glasses.

The following test was made with 18-hour agar cultures using rabbit serum which agglutinated at about 1:600. In this test the macroscopic method was used and the observations were made after three hours at room temperature.

Dilution	1/64	1/96	1/128	1/192	1/256	1/384	1/512	1/768	Control
$+S_{24}$	++	++	++	++	++	++	++	++	−
$-S_{13}$	++	++	++	++	++	+	+	−	−
Stock	++	++	++	++	++	++	+	−	−

++ means agglutination visible to naked eye.
+ means agglutination visible with hand lens.

Following this test the cultures were kept at room temperature and transferred to fresh media three times in 18 days. Then a final test was made macroscopically, using both rabbit serum and the same human serum which was used in developing the two series. The cultures were marked in cipher as before.

RABBIT SERUM.

Dilution	1/192	1/250	1/384	1/500	1/768	1/1000	1/1500	1/2000	Control
$+S_{24}$	++	++	++	++	++	++	+	+	−
$-S_{13}$	++	++	++	++	++	++	+	+	−
Stock	++	++	++	++	++	++	+	−	−

HUMAN SERUM.

Dilution	1/16	1/24	1/32	1/48	1/64	1/96	1/128	Control
$+S_{24}$	++	++	++	++	+	+	−	−
$-S_{13}$	++	++	++	++	++	++	+	−
Stock	++	++	++	+	+	+	+	−

CONCLUSIONS.

The first two tests showed decided differences in the agglutinability of the two series. However in the final tests no such difference was evident. In the one the $-S_{13}$ agglutinated more readily than the $+S_{24}$, and in the other test the $-S_{13}$ agglutinated more readily than the stock. So we must conclude that, whatever differences in agglutinability were developed, they failed to become permanent or even to persist through the number of generations which would take place in an agar medium in 18 days at 26° C.

The author wishes to express his indebtedness to Dr. M. A. Barber for his patient instruction and oversight during the progress of the work.

MURRINA, A TRYPANOSOMAL DISEASE OF EQUINES IN PANAMA.*

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(From the Board of Health Laboratory, Isthmian Canal Commission.)

Murrina is a fatal type of equine trypanosomiasis met with on the Isthmus of Panama. The disease is known throughout the republic by the names "murrina" and "derrengadera," the natives believing that there are two distinct diseases, the one called murrina being a chronic disorder manifesting itself by edema and general weakness, while derrengadera is a disease which may be slow or rapid in its course, but in which there is always paresis of the hind legs and no edema.

The Commission corrals were free from any disorder of this type from 1904 until the middle of 1909, when there broke out among some mules and horses that had recently arrived from the United States and had been pastured with native horses at Gatun, the fatal trypanosomal disease which has been identified as murrina and derrengadera. The clinical features of this disease briefly stated are these: General weakness and droopiness, followed by progressive emaciation, the coat becoming rough and staring. At first there are febrile paroxysms and later an irregular or continued fever, reaching 104° or 106° F., rarely 107° or 108° F. A severe anemia develops, with pallor or a muddy yellow color, and ecchymoses of the nasal and ocular conjunctivae. Toward the end of the disease in some animals a staggering gait was noticed. Extreme emaciation, edema of sheath, lower abdominal wall, or legs were features observed in some animals permitted to die of the disease.

The Disease in Horses and Mules.

Period of incubation.—This was determined experimentally in two animals, pony mule 136 and horse 47. Each animal was inoculated with two or three drops of blood from infected guinea-pigs. The period of incubation in No. 136 was six days, and in No. 47 it was eight days. The end of the period of incubation, or the time of the appearance of trypanosomes in the peripheral blood in numbers sufficient to be detected

* Received for publication January 25, 1911.

microscopically in a fresh blood specimen, was followed within 24 hours by a sharp rise of temperature from normal (99.5°–100° F.) to 106°+ F.

Duration of disease.—This was determined experimentally in two animals. In horse 10 the period was 54 days after the date of inoculation. In pony mule 136 it was 98 days after the date of inoculation. This mule, however, was treated with arsenic late in the disease, the duration of which was probably not materially altered.

Animals have been kept under observation from the time that symptoms appeared and when trypanosomes were first discovered, until the time of death, and the periods were as follows:

Work horse 121: It is not known when this horse became infected. The duration was at least 73 days and may have been 219 days or more.

Mule 239: Duration 130 days. This animal received arsenic medication late in the course of the disease, the duration of which was probably not materially altered.

Mule 520: Duration of disease, 42 days; in mule 263, 28 days; in mule 257, 18 days; in mule 185, 55 days.

Symptomatology.—While there are several phases or types of the disease, the edematous, wasting, or paretic, they merge into one another, and one phase may follow the other in the same animal. In experimental animals the first indication of the disease is the appearance of trypanosomes in the peripheral blood; this is followed in 24 hours by a rise in temperature to 105° or 106° F. In the corrals, however, the first indication of the disease would be general weakness and inability to do the accustomed work. The coat becomes staring, and in some animals a conjunctivitis with considerable secretion of muco-serous fluid would be seen. The weakness appeared oftenest to be a general one associated with wasting of flanks and haunches. When the animals were exercised their gait was slow, and sometimes a trace of clumsiness in the co-ordination of the hind legs would be noted. A few animals lost rapidly in weight and died within a few days. Others would linger for several weeks, maintaining good appetites, but becoming thinner each day. Edema of the hocks, or legs, the sheath or dependent portions of the belly, appeared in some animals, generally to disappear and to be followed by further emaciation. Some animals toward the close of the disease, whether of long or short duration, and generally during a febrile paroxysm, would hang their heads as though drowsy. A few days before death they would either lean against the stall or would fall down and be unable to rise, though in some instances if helped up they could frequently stand for a day or two. One mule was down four days before he died. One horse died in a sitting posture, his hind legs being placed in a peculiar outstretched position.

The temperature has been recorded in most animals. It shows a marked tendency toward relapses, as in recurrent fever; though sometimes the febrile paroxysms in which the temperature was 103–105° F. were replaced by an elevated continued fever lasting from 3 to 22 days. The temperature at times was irregularly quotidian. On the whole the temperature was elevated during the period when trypanosomes were visible microscopically in the peripheral blood, but the trypanosomes appear before the temperature rises and generally disappear before the temperature reaches normal. The animal is inclined to be droopy during a febrile paroxysm and brightens after the crisis.

There is a progressive and severe anemia. The red blood cells early in the disease, even before trypanosomes appear in the peripheral blood, show a tendency to clump, not in rouloux, but in small and large masses. During the course of the disease the

red cells may be reduced to 2,500,000 per c.mm., or less. The blood clots rapidly. There is a lymphocytosis. The anemia is shown in the muddy pallor of the conjunctivae and nasal mucosae. Petechial hemorrhages are seen in the conjunctivae and nasal mucosae late in the disease and are valuable aids to diagnosis in the corrals.

Trypanosomes are no doubt always present in the peripheral blood, for susceptible animals may be infected with the blood of sick animals when trypanosomes are apparently absent by ordinary microscopic examinations.

In several cases trypanosomes would appear for one, two, three, or more days, to disappear for two to nine or more days. When present in greatest numbers there are approximately 600 per cubic millimeter. Usually there are much less than this.

In some of the animals, as the end approaches, there is a definite paresis of the hind quarters, with inability to stand or co-ordinate the movements of the hind legs. This has appeared in from one to six days before death.

Abstracts of records.—Horse 121: Arrived on the Isthmus April, 1909; pastured and worked at Gatun, and possibly infected there. His leg was fractured and later he was condemned and sent to Ancon, from Gatun, September 28, 1909. On November 23 he was inoculated with a porcelain filtrate from mule 545 that died at Gatun corral November 22 with symptoms resembling trypanosomiasis, but whose blood just before death contained no trypanosomes upon microscopic examination (Dr. Williamson). There were occasional rises of temperature and unimpaired appetite but slowly progressive loss of weight. Trypanosomes were present in the blood almost continuously until the day of death. They were present when the temperature was elevated and also when it was normal. Edema of the sheath and dependent belly-wall was noticed April 23, and at this time there was perceptible weakness of the left hind leg on walking the animal about. May 4 the horse was down on his haunches, while the swelling of the sheath and belly-wall had disappeared. The horse died during the night of May 7 in a sitting posture, leaning against the wall with his hind legs outstretched to one side.

Mule 228 had been at the Ancon corral continuously for two years. The animal's temperature had been elevated for several days when one trypanosome was found in the blood on the fourth daily examination. There was loss of appetite for three or four days at the onset of the disease. The animal was well nourished but was droopy and his coat was staring. There was also a discharge of muco-purulent fluid from the conjunctivae. On March 26 he was down but was able to rise. Two days later he fell down again and could not be raised; killed. This was an acute case in an animal whose blood contained very few trypanosomes and who died without there being any emaciation.

Mule 382: This animal had worked in the team with mule 204 that died of murrina July 11. On March 23, when he was taken out very sick, his blood contained one trypanosome to one film. His hind legs were outstanding and he was "pulling back on the halter." He became emaciated without edema and was shot.

Mule 185: This animal had been at the Ancon corral for four years, save for a short period, one year ago, when he was worked at Cristobal. On March 30 he fell while at work. His appetite was good but he was somewhat thin, and his blood on this day contained a few trypanosomes in one film. Later he became very much emaciated and died May 25.

Mule 204: This animal had worked in team with infected mule 382, and was no doubt infected from him. He was taken out March 28. Trypanosomes were detected

four days later. He became emaciated and on April 11 fell down in the stall and had a general convulsion. On April 13 he was slung in the stall, but on April 26, when removed from the sling, he fell to the floor, unable to rise, and was shot.

Mule 292: This mule had been on the Isthmus four years and seems to have shown considerable resistance to the disease. Some time before routine blood examinations were begun it had been observed at the Ancon corral that this mule was unaccountably weak. He was shipped to San Pablo but was too weak to work there. Later he was sent to Gatun, where he spent most of his time in the sick corral. Finally he was returned to Ancon, where he was put to work, but had to be taken out on account of weakness. Trypanosomes were discovered on the second daily examination, March 31. This animal fell down several times but was always able to regain his legs. Shot, May 12.

SUMMARY OF SUSCEPTIBLE ANIMALS.

Animal	Infected	Result
Work horse	Yes	Died
Mule	Yes	Died
Saddle horse (experimental infection)	Yes	Died
Calf	No	Refractory
Pig	Yes	Recovered
Dog	Yes	Died
Raccoon	Yes	Died
Cat	Yes	Recovered
Kitten	Yes	Died
Monkey *Cebus* and *Nyctipithecus*	Yes	Died
Rabbit	Yes	Died
Guinea-pig	Yes	Died
Rat, *M. rattus*	Yes	Died. Two recovered
Rat, *M. norvegicus*	Yes	Died
Rat, *M. alexandrinus*	Yes	Died. One refractory
Rat, albino	Yes	Died
Mouse, *M. musculus*	Yes	Died
Mouse, albino	Yes	Died
Opossum	Yes	Died
Agouti	Yes	Recovered
Coati	Yes	Died

Work horses and mules were the only equines found infected naturally. Saddle horses were not infected naturally.

Dogs are very susceptible and the disease runs a fairly rapid and fatal course.

Dog 121—2: April 7, large dog, weight 50 pounds, inoculated subcutaneously with two drops of blood in citrated saline solution from guinea-pig 121—1. The period of incubation was less than nine days. On May 12 the anterior chamber of each eye became very turbid, and the lids nearly closed on account of photophobia. He died on May 15, 38 days after inoculation.

Cats are susceptible but recover. On April 7 an adult cat was inoculated subcutaneously with two drops of infected blood in citrated saline solution from guinea-pig 121—1. Trypanosomes were always found in the peripheral blood at intervals up to June 10. On this date the animal had improved in appearance and condition, but had a dermatitis of both ears. The cat's blood has been negative for trypanosomes since June 10.

A very young kitten proved very susceptible. On April 7 the kitten was inoculated from guinea-pig 121—1. On April 15 a few trypanosomes were detected, the period of incubation being 8 days. Trypanosomes were very numerous April 19. The next day the kitten was sick and droopy and died April 21, 14 days after inoculation.

Monkeys are very susceptible. Cebus, 121—1, inoculated subcutaneously with 4 c.c. of blood direct from horse 121 when his blood contained two trypanosomes to one cover-slip. The period of incubation was less than 12 days. Trypanosomes appeared at intervals, becoming very numerous toward the end, when the blood was thin and pale. The animal became very pale and feeble and died 43 days after inoculation.

Nyctipithecus was inoculated from a guinea-pig. The period of incubation was five days. The animal became very feeble and dull. There was diarrhea. A week before death photophobia was noticed and a few days later the anterior chamber of each eye contained flakes of grayish exudation. The animal died 32 days after inoculation.

Rabbits 121—1, A and B (bred in the United States; on Isthmus 3 or 4 mos.): The period of incubation was 12 days or less. Trypanosomes were detected but once, yet each animal developed a severe dermatitis of the muzzle and ears, with blepharitis and conjunctivitis. Later there was loss of hair from the muzzle. One rabbit died 106 days, the other 163 days after inoculation.

In guinea-pigs the period of incubation was about 7 days. Trypanosomes appeared at intervals in numbers up to 10 or 20 to one immersion field. The red blood cells were clumped in the characteristic manner. Swelling of ears with dermatitis, edema and ulceration of scrotum, and edema of vulva were frequently seen. The duration of the disease was 27, 50, 75, 99, 119, 189, and 336 days after inoculation.

Agouti (Conejo): Inoculated with richly infected blood from guinea-pig 121—1. Trypanosomes appeared after 10 days. They then disappeared and were not detected on 11 subsequent examinations, and the animal has remained perfectly normal.

Opossum: Trypanosomes were detected when the animal was very sick. The animal died 34 days after inoculation.

Raccoon 10—2: His blood was examined once, when two trypanosomes were found to one immersion field. Died 52 days after inoculation.

Coati 382—2: Period of incubation six days. Died 24 days after inoculation.

Wild rats, *M. rattus*, are susceptible. Two were inoculated, whose blood contained *T. lewisi*. After several days *T. hippicum* could be detected along side of *T. lewisi* in stained films, but subsequently *T. hippicum* disappeared from the animal's blood, while *T. lewisi* remained present. Both animals recovered.

Two other specimens of *M. rattus* were inoculated, from guinea-pig 10. Trypanosomes appeared in their blood in enormous numbers, and nine days after inoculation they were dying and were killed. It is interesting to note that in neither animal could *T. lewisi* be demonstrated. This suggests the possibility of a vicarious immunization by *T. lewisi*.

M. alexandrinus: Two specimens have been inoculated. In each case their blood was negative for *T. lewisi*. One died 11 days after inoculation. The other never became infected.

M. norvegicus: One specimen inoculated September 22 from white mouse 10—2. Trypanosomes were present for several days, were absent on October 10, reappeared October 13, and the rat died November 1, 40 days after inoculation.

Rat, albino: These animals are somewhat more susceptible than wild rats. In white rat 382—2 enormous numbers of trypanosomes appeared and the animal died 20 days after inoculation.

The wild mouse, *M. musculus*, was very susceptible. Trypanosomes appeared

five days after inoculation in numbers of from 1 to 25 fields up to 12 to 1 field. They remained constantly in the blood in increasing numbers until they were more numerous than the red blood cells. The animals then died, 8, 9, 12, and 15 days after inoculation.

White mice were very susceptible. The period of incubation was from three to five days, some of the animals dying on the eighth day.

Pig: This animal is susceptible but recovers. Pig 382—2 was inoculated April 13 with blood from guinea-pig 382—1. Trypanosomes were detected on one occasion, April 28. They then disappeared and were never found again. The red blood cells were never clumped. The animal increased rapidly in weight and was perfectly well when killed June 29.

A calf was refractory. Calf 121—2 inoculated April 3 with 10 minims richly infected blood from Cebus 121—1. Trypanosomes could never be detected in the animal's blood in 17 examinations during three subsequent months, and his temperature remained normal during this period. The calf increased in weight and his condition greatly improved. On July 6, 2 c.c. of his blood were inoculated into a young guinea-pig, but trypanosomes have never appeared in the guinea-pig's blood.

PATHOLOGICAL ANATOMY OF MURRINA.

When a horse is sacrificed before the disease has run its course there may be very few gross lesions and none characteristic of the disease other than emaciation, edema of lower belly-wall, sheath or legs, excessive fluid in some of the cavities, conjunctival ecchymoses, or some splenic enlargement. If, however, the animal is permitted to die naturally there are lesions which are characteristic and usually well marked.

The spleen is only slightly enlarged, but its capsule, particularly the external surface, is dotted over with petechiae limited to the capsule. They are usually one to two mm. in diameter. The recent ones are bright red, while those somewhat older are reddish-brown in color. The pulp is only slightly increased and the reticulum is unchanged.

The kidneys contain punctate petechiae, one mm. in diameter, distributed chiefly in the cortex, just beneath the capsule.

The endocardium and epicardium usually contain large and small hemorrhages. Favorite sites are the papillary muscle, mitral leaflets, and the epicardial fat along the coronary arteries, oftenest near the base of the heart.

The pleurae and peritoneum in some cases have been the seat of numerous petechiae.

The renal lymph nodes are usually enlarged, and one or two of the set are frequently the seat of extensive hemorrhages.

There are usually hemorrhages into the conjunctival or nasal mucosae seen at some time during the disease.

Sometimes there is an excess of clear, serous fluid in the peritoneum, pleural, or pericardial cavities, but this is not constant, nor is the edema of the sheath, legs, hocks, or lower belly-wall constant.

Other viscera show no changes.

Filaria and intestinal nematodes and cestodes were encountered inconstantly.

The microscopic changes are fairly characteristic and constant. In the kidneys the focal hemorrhages and the acute glomerulitis, occasionally with necroses, make a rather striking picture. The spleen always shows the same appearance—phagocytosis of red blood cells and the presence of blood pigment in small and large clumps of refractile granules. In the liver the one unvarying feature is the presence of bronze-colored blood pigment in the endothelial cells of the sinusoids. There is a localized leukocytosis here. Atrophy and fatty infiltration of the hepatic cells are inconstant and not definitely related to the disease process. Renal lymph nodes usually show great engorgement, hemorrhage, and phagocytosis of red blood cells by large mononuclear phagocytes. The hemorrhages in other viscera present nothing of special interest.

IMMUNITY.

Embryos are not infected through the placenta (guinea-pigs), nor does an infected mother confer any immunity upon her offspring *in utero* as indicated by the result of the following experiment:

A gravid guinea-pig was inoculated with trypanosomes April 11. One young pig was born April 26, 15 days after the inoculation of the mother. The blood of the young guinea-pig, on examination, contained no trypanosomes, although the mother had a severe infection and died 99 days after inoculation. To ascertain whether there was any immunity conferred on the young, she was inoculated May 10 from guinea-pig 382, and trypanosomes appeared after a latent period of eight days.

DIAGNOSIS.

The diagnosis is made by the detection of weak animals "pulling back on the halter" or those with outstanding hind legs, animals temporarily off their feed, or those that in spite of sufficient food

show steady physical deterioration. With these symptoms there may be conjunctivitis, anemia, and subconjunctival ecchymoses. As soon as such an animal shows a temperature of 101° F. or more, a blood examination will generally reveal the trypanosome; particularly if the elevated temperature is detected at the beginning of the access. During the latter part of the paroxysm trypanosomes may not be demonstrable. The inoculation of very susceptible animals, such as mice, rats, monkeys, dogs, or guinea-pigs, will help in making a diagnosis in some of the cases or on some occasions when trypanosomes are extremely sparse in the peripheral blood.

In obtaining blood from the ear for diagnosis, no time should be lost before examining fresh cover-slip preparations, for occasionally the trypanosomes lose their motility within a few minutes after the blood is drawn, and can then only be detected in stained specimens. It has occurred not infrequently that what apparently were negative fresh preparations contained trypanosomes after staining. Trypanosomes commonly remain for a time within a clump of red cells, which can be seen to be disturbed, yet it is often impossible to detect for a minute or two the hidden trypanosome. Ross's thick-film method, or a modification in which half the drop is thinned out on the slide in order to detect morphological features, is of value in detecting trypanosomes when they are very sparse.

THE PATHOGENIC AGENT.

The pathogenic agent is *Trypanosoma hippicum*[1] nv. sp.

While there is some variation in its morphology, the typical trypanosome is 16 to 18 mikrons in length, two mikrons wide, the distance from kinetonucleus to the posterior tip about 1.75 mikrons, and the distance from the posterior tip to the middle of the trophonucleus is from 7.5 to 10 mikrons. The flagellum is usually short and frequently not entirely free, for often the attenuated process of the cytoplasm extends to the extreme end of the chromatin filament; but at times there is a free flagellum reaching 4 mikrons in length. The trophonucleus is placed a little posterior to the middle, the relation being 1:2.8 to 1:2.4. The kinetonucleus is usually placed about 1.75 mikrons from the posterior

[1] *Bull. Soc. Path. Exot.*, 1910, 3, and 1911, 3.

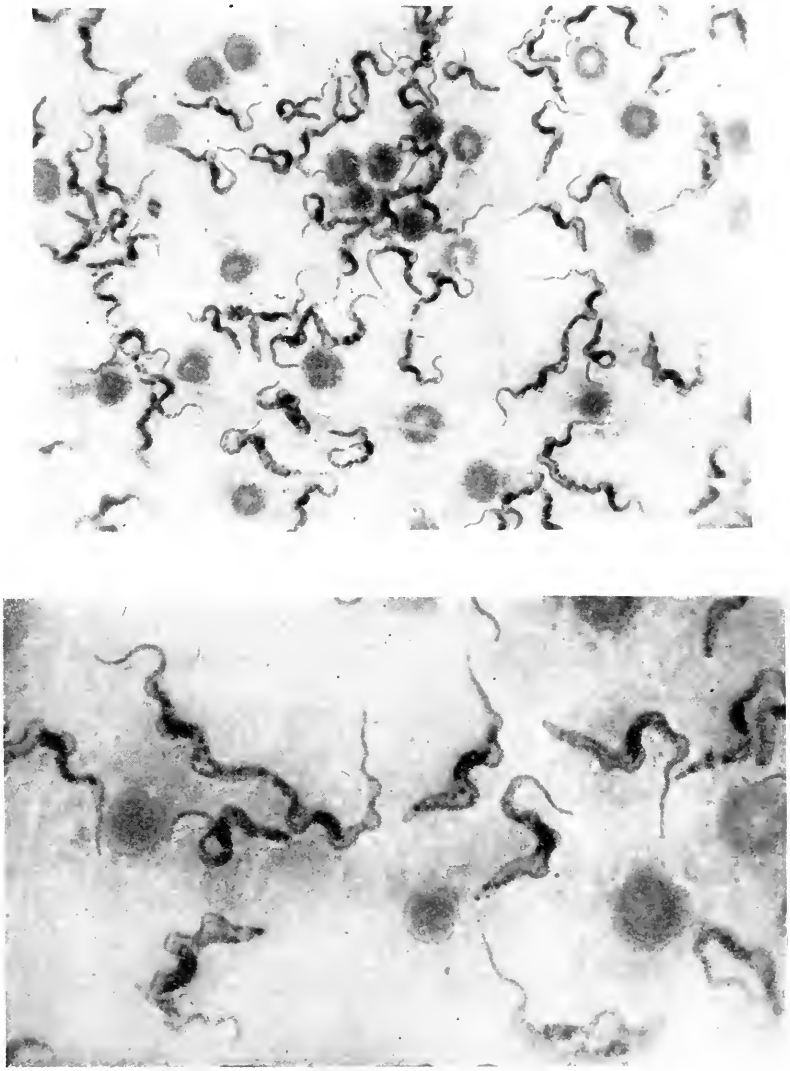

FIG. 1.—*T. hippicum* from rat.

tip; but sometimes it is quite at the tip, and occasionally may be 3 mikrons from it. The posterior tip is often rather blunt and usually obtuse, never being as attenuated as the posterior tip of *T. lewisi*. The cytoplasm usually contains numerous basophilic granules scattered throughout the cytoplasm or arranged in lines in the anterior and posterior halves. The granules are coarse and from 0.5 to 1 mikrons or more in diameter. There may be 16 to 18 in number, most of them being placed, usually, in the anterior half. The lateral margin of the trypanosome may stain more deeply than the interior. Some of the longer forms contain few or no granules. A well-developed undulating membrane is present and the chromatin filament extends from the kinetonucleus to the anterior tip of the flagellum. When rapidly proliferating in the rat or mouse some forms approach 28 mikrons in length, and become 3 to 4 mikrons in width. From this description it is quite evident that the trypanosome is different from *T. equinum* of mal de caderas, and *T. equiperdum* of dourine. Dr. Laveran, who very kindly has examined preparations of the trypanosome, has expressed his opinion that *T. hippicum* differs from the two above named trypanosomes by the presence of a centrosome, basophilic granulations, and in having a short flagellum. "Les centrosomes sont très apparents; il ne s'agit donc pas du trypanosome du mal de caderas. D'autre part le trypanosome me parait différer notablement du *Tr. équiperdum:* il est plus petit; le partie libre du flagelle est plus courte, enfin le protoplasme contient des granulations basophiles qui sont presque toujours défaut chez le *Tr. équiperdum*. Le fait que les singes et les cobayes s'enfectent facilement est aussi contraire au diagnostic de dourine."[1]

There is some variability in the presence and in the staining qualities of the basophilic granulations. Some specimens from the horse would have few or no granulations, while others had several deeply staining ones. One film from *M. alexandrinus* had many trypanosomes and none contained granules, though the cytoplasm stained deeply; from the same rat there were other specimens containing many well-stained granulations. It has appeared at times as though when the trypanosomes were multiplying very

[1] See also *Bull. Soc. Path. Exot.*, 1911, 4, p. 168.

rapidly they were longer, more attenuated, and freer from granules. The process of division appears from an examination of peripheral blood smears to take place, first, by division of the kinetonucleus, then the development of a second faint chromatin filament from the second kinetonucleus, later a division of the trophonucleus, and finally a separation of the two trypanosomes.

Trypanosomes do not die within at least 10 minutes in the drawn blood of animals to which has been added 90 per cent by volume of citrated saline solution, 0.9 per cent sodium chloride in 1.5 per cent sodium citrate solution. Inoculations, therefore, can be made with the aid of this fluid.

MODE OF TRANSMISSION.

A study of this phase of the disease impresses one very strongly that it is carried mechanically by flies. The pasture at Gatun, where the infection was supposed to have been introduced, was visited on several occasions, but tabanid flies were not found there. This pasture is not near the woods, and questions put to the corral men elicited the information that tabanids have not been seen in that locality. While they are present on the Isthmus, they do not seem to be thickly nor widely distributed. In the bush and mountains near streams they are said to be common in some places. A few were taken in August, 1909, at the new Ancon corral, and a few have been caught at the isolation corral near by, where the infected animals have been kept. None were ever taken at the old corral where the disease first appeared.

Stomoxys calcitrans naturally is found wherever horse manure is exposed. It is customary here to remove manure every 24 hours from the corrals: however, as it is not destroyed, but used for fertilizing purposes, flies are not prevented from breeding, and *S. calcitrans* has been bred out at the Laboratory from corral manure exposed on plant beds. *S. calcitrans* containing blood has been taken at Ancon corral and at the isolation corral where the infected horses were kept. Other flies taken at the stables or near by, during autopsies on mules, were representatives of Compsomyia, Hylemyia, Musca, and Pyrellia.

Tabanids cannot be held responsible for the transmission of this

disease, because the infection has spread in certain localities where apparently tabanids never visit.

While it is possible that *S. calcitrans* may have conveyed the infection by biting, it is extremely unlikely for the following reasons:

In the isolation corral at Ancon, which had been unscreened for four months and in which *S. calcitrans* was breeding, there were two animals, a horse and mule, infected with *T. hippicum*, standing alongside a pony that did not contract the disease, though the contact with these infected animals and with several others lasted altogether 10 months. At the end of this period the pony was inoculated with trypanosomes experimentally to determine his susceptibility, and he developed a severe infection in eight days (horse 47).

Similarly, at the old Ancon corral, from which several of the infected mules were taken, there were two long rows of stalls. On the outside, facing the roadway, the saddle horses were stalled, about 18 or 20 being kept there constantly. On the other side was a row of stalls occupied by work mules, from which several sick animals, suffering from murrina, were taken out and for several days segregated at one end of the row. The stalls were covered with a roof, but were not screened or protected in any other way. The mangers and hay racks came together so that only a few inches of partly open space separated the horses' and mules' heads. *S. calcitrans* was breeding in the corral at this time and several specimens were taken alive with mule's or horse's blood in their intestinal tracts. The period of exposure of the saddle horses in this way lasted at least six months, yet no saddle horse ever became infected with trypanosomes from the mules near by, though mules kept coming down with the infection during this same period.

A horse (No. 10) and a mule (No. 136) with intact skins kept in this corral with the other animals for one month showed no signs of infection, though both were susceptible, as subsequent inoculation experiments proved.

Furthermore, specimens of *S. calcitrans*, from this infected corral, were dissected and others fed from infected horses, yet no evidences of flagellates were ever detected in their intestinal tracts.

It is extremely unlikely, therefore, that *S. calcitrans* transmitted the disease by biting.

Another possible mode of transmission was that of ticks, but upon investigation it was found that the only animals harboring ticks were saddle horses that had been taken out on trails into the bush. Ticks could never be collected from corral mules, naturally, because they did not visit places infested with *Dermacentor nitens*, *Margaropus annulatus*, and *Amblyomma cajennense*, the ticks most commonly found on horses in this region. Now as none of the saddle horses ever became infected with trypanosomes, though stalled alongside infected mules in an unprotected stable, it became evident that ticks did not transmit the infection.

Before the isolation corral was screened, bats visited it and bit one of the infected horses on several occasions on both sides of the neck. Curiously enough, this was the only animal out of three or four in the stable that was bitten. While bats under such circumstances might open a channel for infection by breaking the skin of an infected animal and permitting flies to become infected, it did not seem that in this case, at any rate, they transmitted the infection: but if they had visited non-immune horses and bitten them immediately after visiting and biting an infected horse, the bats would have then possibly become a transferring medium.

None of the bats taken here have been infected with trypanosomes, though filariasis is very common.

It finally appeared from an examination of the infected animals and the nature of the work they had been doing that no matter how the infection crept into the corrals in the first place, it was subsequently transmitted mechanically by flies visiting the galls and scraper cuts of infected and non-infected animals. The infected animals were always work animals, many of them being placed on the hardest kind of work—scrapers—and were always being laid up with cuts and abrasions received in this way. The mules and horses that preserved intact skins probably never became infected. There were two positive cases of infection in mules that had been driven together in team, and it is understood that there were four or six others, but the information is not positive. It seemed right,

then, to assume that flies were the active agents in transmitting the infection, and that they conveyed it mechanically.

TREATMENT.

Prophylaxis.—The likelihood of this assumption seems to have been borne out by the result of treatment which was instituted, and which apparently has resulted in saving the work horses from further infection. Twenty-three animals, at least, had been destroyed by this disease between April, 1909, and April, 1910.

The following recommendations were made and largely carried out with the result that the epidemic has been completely wiped out:

1. Destruction of all infected animals and burial away from the corral to prevent flies becoming infected from blood at the time of shooting, if near the corral.

2. Routine daily (b. d.) temperatures for the detection of suspects.

3. The isolation in screened, vestibuled, fly-proof stables of all animals having a temperature above 100° F.

4. The detection of infected animals by blood examinations, and animal inoculations when necessary.

5. The disinfection of halters, instruments, etc., and the hands of attendants coming in contact with infected animals.

6. All wounds, sores, galls, etc., on well, infected, living, or dead animals to be protected from flies by protective dressings, containing substances such as creolin, distastful to flies.

7. Stray horses and mules are not to be impounded in or near corrals.

8. Sanitary inspectors and police are required to report all animals having this disease.

Arsenic medication.—This subject is being pursued by means of the administration of large subtoxic tertian doses of arsenious oxide (Holmes's method) to experimentally infected mules and horses, and as the work is not completed it will be made the subject of a separate report.

SPECIFIC IDENTITY OF THE DISEASE AND ITS DIFFERENTIATION FROM OTHER FORMS OF EQUINE TRYPANOSOMIASIS.

It is necessary to establish the fact that murrina is a specific disease, distinct from other forms of equine trypanosomiasis.

From the total number known to science of trypanosomes invading mammals, those of rodents, cattle, bats, etc., may at once be dismissed from consideration, as they are not pathogenic for equines, viz., *T. lewisi*, *T. Theileri*, etc.

The following trypanosomes may also be dismissed from consideration for the reason that they are either pathogenic for cattle or present well marked morphological and other points of difference from *T. hippicum: T. evansi*, *T. brucei*, *T. dimorphon*, *T. pecaudi*, and *T. cazalboui*.

T. equiperdum, the pathogenic agent of dourine (mal de coit) is differentiated by morphology, it containing no basophilic granulations, by the fact that it is extremely rare in the peripheral blood of equines and further by clinical features, mode of transmission, dourine being a disease transmitted during copulation; and further by the insusceptibility of monkeys and guinea-pigs to *T. equiperdum*.

There remains one trypanosome which must be carefully differentiated from *T. hippicum*. That one is *T. equinum*, the pathogenic agent of mal de caderas. Dr. Laveran's opinion has already been quoted. In addition to this valuable testimony we will consider what other characters may be discovered in the two diseases which may be used to differentiate them.

Mal de caderas is an epizootic trypanosomal disease of equines in South America. The trypanosome was discovered by Dr. Elmassian,[1] director of the Bacteriological Institute at Assomption, Paraguay, in 1901. Geographically, the disease is distributed in regions along the tributaries of the Rio de la Plata in Argentina, Uruguay, Paraguay, Brazil, and Bolivia, and also in the federal district of Matto Grosso, between the Madeira and Xingu rivers, tributaries of the Amazon in Brazil, and on the Island of Marajo at the mouth of the Amazon.

Certain clinical and pathological features are mentioned by

[1] Elmassian, *Rev. Soc. med. Argent.*, 1902, 10, p. 122.

Elmassian and Migone, Voges, Laveran, and Mesnil, as being more or less characteristic of mal de caderas. It should be mentioned, however, that there is much similarity among all equine trypanosomal diseases—that they, varying from one another, usually present certain characters recognizable as belonging to the type—symptom and pathological group—such as anemia, emaciation, edema, effusions, posterior paresis, hemorrhagic nephritis, and remittent pyrexia. Furthermore, if we take one disorder and observe all its vagaries, we shall see that each individual case does not necessarily conform to the specific disease type.

In mal de caderas the characteristic symptom would seem to be, as its name implies, posterior paresis, yet as Voges points out, in a series of cases unusual forms present themselves—"a number of animals may remain quietly standing up to the last moment and then fall in a heap and die at once."

The fairly constant features in which murrina differs from mal de caderas seem to be these:

Duration of disease in mules and horses.—Murrina is far more common among mules than horses. This has been undoubtedly due to the nature of the mules' work here, rendering them more liable to infection through cuts and galls. The duration of the disease is the same in mules and horses. The longest duration recorded is in a horse. According to Voges the duration of mal de caderas in horses is one to two months, occasionally four months, while in mules it is one year, if they receive good care and treatment. This is quite different from my observations of murrina.

The number of trypanosomes in the blood of mules and horses.—The blood of several mules suffering from murrina has been examined daily over periods of several weeks or months and trypansomes were easily found at intervals, particularly before and during a febrile paroxysm. Voges states, however, that in mal de caderas trypanosomes cannot be demonstrated microscopically in the blood of mules suffering from the disease for days and weeks. In one case, he states, after two months daily diligent examination, trypanosomes were found only once during an attack, and in numbers much less than in the horse. In another case, trypanosomes were only found on two days during the duration of the disease in

a mule. In murrina no difficulty has ever been experienced in detecting trypanosomes in mules if the examinations were made during a febrile paroxysm; and trypanosomes are also frequently found when the temperature is normal. Furthermore, the blood of infected horses has never been found to contain more trypanosomes than that of infected mules.

Character of fever.—A very exact idea of the type of fever in mal de caderas could not be obtained for the reason that most of the published charts are based on single daily temperature records. There appears, however, to be no very marked differences in the types of fever between murrina and mal de caderas.

Elmassian has described placques of dermatitis on the neck, shoulders, and rump of horses in mal de caderas, but these have never been observed in cases of murrina.

Keratitis and hypopyon are mentioned as being seen in horses infected with *T. equinum*, but neither of these conditions has been observed in horses or mules suffering from murrina.

Diarrhea, said to be a common symptom in mal de caderas, according to Elmassian, has not been observed in murrina, save in one horse that had received 21 gms. of arsenic.

Hemoglobinuria and hematuria have never been observed in cases of murrina. If they were at all common it would have been noticed, for all the animals have been kept under observation by the veterinarian in the corrals. These sypmtoms are said to be constant in mal de caderas.

Distinguishing pathological features of murrina.—These are very important means of differentiating the two diseases. They are as follows: splenic capsular ecchymoses; renal petechiae; epicardial and endocardial hemorrhages and ecchymoses, and less frequently pleural and peritoneal petechiae. These, with the hemorrhages into the renal lymph nodes, make up a picture quite different from mal de caderas, and one more like nagana and "equine anemia."

The spleen is not greatly enlarged in murrina but always appears to be so in mal de caderas.

Fibrinous exudation into the pleural and pericardial cavities described as constant in mal de caderas is very rarely seen in

murrina (there was a fibrinous clot in the right pleural cavity of mule 263).

SUMMARY.

There has been an epidemic of trypanosomiasis among American mules and work horses in the Canal Zone. The disease resembled other trypanosomal diseases such as nagana, surra, mal de caderas, and Senegambian horse disease; but it presented a group of symptoms and pathological features which stamp it as a distinct disease.

The disease is differentiated from mal de caderas by the morphological characters of the trypanosome, the autopsy picture, and the clinical features of the disease.

The disease also resembles in some particulars "swamp fever" or the "infectious anemia of equines," a disease of obscure etiology, prevalent in some parts of the western United States.

The disease was probably not introduced from outside sources, but represents an endemic disease of this region, known in the Republic of Panama as murrina and derrengadera.

It is distinctly a disease of mules and horses, for cattle are insusceptible.

The pathogenic agent, *T. hippicum*, has morphological and biological characters which differentiate it from other species of trypanosomes.

The animal reactions of several of the native mammals, including domestic animals, have been determined.

The chief distinguishing features of the disease are: anemia; weakness; emaciation and edema; conjunctival ecchymoses; conjunctivitis; pyrexia, and some posterior paresis. The pathological findings are constant and characteristic: splenic capsular ecchymoses; acute hemorrhagic nephritis, with acute glomerulitis and petechiae; hemorrhages in renal lymph nodes; epicardial and endocardial ecchymoses, and less constantly pleural, peritoneal, and conjunctival ecchymoses, and pleural effusion. The blood changes are those of anemia with lymphocytosis. There is much phagocytosis of blood pigment by endothelial cells of the liver and spleen.

The disease is probably transmitted mechanically by flies through the broken skin of cuts and various wounds. Saddle horses were not infected, and there are no evidences that any

animals were infected by means of stomoxys or tabanids or by ticks or bats.

The epidemic, which threatened to destroy several hundred mules and horses, has been controlled (there having been no new case in six months) by detection of infected animals, by means of daily routine temperature records throughout the corral, the microscopic examination of the blood of all animals having a temperature above 100° F., the isolation in screened stables of infected animals or suspects, and the sacrifice of all infected animals.

I wish to express my thanks to Lieutenant-Colonel C. A. Devol, U.S.A., Lieutenant W. E. Wood, U.S.A., and others in the Quartermaster's Department for various courtesies and help.

I am indebted to Dr. L. B. Bates for help with autopsies, and for keeping up the examinations of experimental animals, etc., while I was on leave, and to Colonel W. C. Gorgas, U.S.A., for permission to publish.

A PARATYPHOID-LIKE BACILLUS ISOLATED FROM A DOG.*

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During routine examination of dogs for Negri bodies the following interesting case came under observation.

History.—Three weeks ago the dog was bitten by a rabid dog. Four days ago the dog became sick, with convulsions at short intervals, flowing of saliva, and he was unable to take food. He died at six o'clock this morning, September 20, 1910.

Anatomic diagnosis.—Rabies(?); left purulent pneumonia; acute gastroenteritis; hyperemia of the organs.

A medium-sized adult dog; rigor marked; the body is free from signs of violence. Peritoneum, pericardium, and pleurae normal. Blood-stained purulent froth in the trachea. The right lung is rather dark-red in color and crepitates feebly. The left lung is pale in color; the middle lobe and the upper one-fourth of the lower lobe are gray and crepitation is absent; and a moderate quantity of gray, purulent material exudes from the bronchi and from the cut surface.

The heart appears normal. There is a small amount of bloody mucus in the stomach and the intestines, and the lining of the small intestines is redder than normal. The liver is of about normal size, distinctly mottled, and the cut surface is abnormally bloody. The gall bladder is normal. The pancreas is normal. The spleen appears slightly larger and softer than normal. Both kidneys are bluish-red in color, the capsules smooth and shining, the substance of about normal consistency; the capsules strip readily, the cut surfaces are abnormally bloody, the cortical markings and the medullary striations are prominent. The mucous membranes of the pelves appear to be normal. Both ureters and bladder are normal. The meningeal blood vessels are greatly distended with blood, giving the membranes a decidedly reddened appearance. The brain and cord also appear to contain more blood than normally. Purulent material is not found.

Microscopically the heart is normal. The gray area of the left lung is composed almost entirely of leukocytes. In many places the alveolar walls have disappeared entirely. In areas where the alveolar walls are still present the alveoli are filled with leukocytes, a small number of red blood corpuscles, desquamated epithelial cells and débris. A relatively small number of blood corpuscles are inside of the capillaries. The bronchi are completely filled with leukocytes, desquamated epithelial cells, a small number of red corpuscles and débris. The larger blood vessels are packed with blood corpuscles and a marked leukocytosis is seen there. Bacteria, both cocci and bacilli, are present in large numbers. In the right lung the alveolar walls are enormously thickened and in some places appear absent. The mass is composed almost entirely of blood cells, leukocytes being present in relatively large numbers. The bronchi are nearly filled with red and white blood cells, desquamated epithelial cells, cocci, bacilli, and granular débris. The large blood vessels are filled with blood cells, and leukocytes are present in large numbers.

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The spleen and the liver are congested. Toward the centers of the lobules the liver cells are small, granular, and frequently they are vacuolated. Occasionally a blood vessel is found in the liver which is completely filled with bacteria and débris, and the surrounding liver cells are irregular in shape, granular, the protoplasm taking a brick-red stain with hematoxylin and eosin, the nuclei being lost.

In the kidneys the glomerular tufts contain rather a large amount of blood and appear somewhat distorted, occasionally a small amount of granular, eosin-staining material is seen between the capillary tuft and the capsule. The protoplasm of the epithelial cells lining the renal tubules is granular, the cell outlines are lost but the nuclei take the hematoxylin stain well. In the medullary portion the cells are somewhat better preserved; the capillaries are moderately distended with blood.

The adrenals are normal.

In the hippocampus major and in the cerebral cortex Negri bodies were not found.

Bacteriological examination.—Microscopic examination of stained preparations of cerebrospinal fluid show no micro-organisms, and broth, agar, and serum inoculated with the fluid remain sterile.

Microscopically the blood from the heart shows a moderate number of large bacilli such as are usually found in putrefying animal matter, also a small number of small, short bacilli and some small, slender bacilli. Broth, agar-agar, and blood serum inoculated with blood yield a growth of small, short bacilli and the small, slender bacilli. The small, short bacillus proved to be the colon bacillus and the small, slender bacillus corresponds in nearly all respects to the paratyphoid bacillus.

Microscopically the purulent material from the left lung shows many cocci and bacilli. Isolated and cultivated were *Staphylococcus albus*, a streptococcus, and a bacillus closely resembling the paratyphoid bacillus.

Microscopically smears made of spleen pulp fail to show any micro-organisms. In cultures a bacillus resembling the paratyphoid bacillus was obtained.

A rabbit received a subdural injection of emulsion of the dog's brain on September 21, 1910, and died after several days' paralysis on October 17, 1910. There was great emaciation, the organs of normal appearance, the cerebrospinal fluid and the blood sterile, in the large cells of hippocampus major many typical Negri bodies.

DESCRIPTION OF THE PARATYPHOID-LIKE BACILLUS.

The surface colony on agar-agar is medium-sized, soft, moist, circular, elevated, smooth, convex surface and regular border, semi-transparent, white by reflected light and pale-brown by transmitted light. Microscopically it is brown, dark at the center, gradually fading toward the periphery; finely granular; borders regular. The deep colonies appear as small, opaque, white specks with regular border. Microscopically they are dark-brown, finely granular, lozenge-shaped.

It is a somewhat slender, actively motile bacillus; it stains well with carbol-fuchsin and with methylene blue, and loses the color when treated by Gram's method. Spores are not found.

Broth.—Uniformly clouded within a few hours after inoculation. A heavy pellicle appears after two or three days.

Agar-agar.—A fairly large, soft, moist, elevated growth, with smooth and shining surface and slightly irregular border along the line of inoculation. Semi-transparent, white by reflected light; pale-brown by transmitted light.

Glycerine agar.—Like the growth on agar-agar.

Potato.—A fairly large, light-brown growth spreading over the surface where the potato is wet.

Litmus milk.—Turned slightly red in 24 hours; after five days the milk is nearly white, and after two weeks it is of a cream yellow color and does not show any coagulation. The milk is peptonized.

Gelatine.—No liquefaction.

Indol.—None.

Gas is produced in broth which contains glucose, maltose, levulose, mannit, inosit, dextrin, or galactose. Gas is not produced from lactose or sucrose.

Agglutination.—Distinct agglutination was obtained when mixed with blood of the dog in proportion of approximately 1 to 20. Because of the small quantity of blood which was saved extensive glutination tests could not be made.

Animal inoculations.—1. A guinea-pig weighing 400 gms. received 2 c.c. of a 12-hour culture intraperitoneally on September 30, 1910. It died within 18 hours with acute exudative peritonitis. The abdominal wall edematous and studded with hemorrhages. The paratyphoid-like bacillus was obtained in pure culture from the peritoneal cavity, pleural cavity, pericardial cavity, blood from the heart, lung, spleen, and kidney. Microscopically there are extravasation of blood into the alveoli of the lungs and evidences of degeneration in the cells of the liver and renal cortex, the protoplasm being granular, the outlines indistinct, and the nuclei poorly stained.

2. On October 2, 1910, one loop-full of 24-hour agar growth of the paratyphoid-like organism was suspended in broth and injected subcutaneously in the axilla of a guinea-pig weighing 500 gms. A large swelling appeared within a few hours and the animal died about 24 hours later, with a large hemorrhagic swelling on the thoracic and abdominal walls, peritonitis, and general visceral hyperemia. The bacillus was recovered in pure culture from the site of inoculation, from the pericardial fluid, the heart's blood, pleural fluid, peritoneal fluid, spleen, and urine.

3. On October 6, 1910, a large rabbit was inoculated intraperitoneally with one loop-full of 12-hour agar culture of the bacillus. The rabbit died six hours after inoculation with an acute general fibrinous peritonitis and hyperemia of the solid viscera. The liver markedly fatty. A pure culture of the bacillus was obtained from the peritoneal fluid, from the pleural surface, from the pericardial fluid, from the heart's blood, and from the lung. There were areas of hemorrhage and leukocytic accumulation in the lungs and evidence of degenerative changes in the liver cells.

4. On October 6, 1910, one loop-full of 12-hour agar growth of the bacillus was suspended in broth and injected subcutaneously in the right axilla of a large rabbit A large swelling soon appeared at the site of the inoculation, and the rabbit died on October 9, with hemorrhage and edema of the chest and abdominal walls, fibrinous pericarditis, and pleuritis. The microscopic changes in the lungs, liver, and kidneys were like those in the preceding animals. The bacillus was recovered from the site of inoculation, from the peritoneum, the pericardium, the heart's blood, and from the pleural surface.

5. October 7, 1910, one loop-full of 18-hour agar growth of the bacillus was suspended in broth and injected into the peritoneal cavity of a medium-sized monkey. The monkey died October 11, with general peritonitis, cloudy swelling of the liver and kidneys. The bacillus was recovered from the peritoneum, the pericardium, and the heart's blood. Microscopically the liver was hyperemic and many of the liver cells

contained large and small vacuoles. In the renal cortex the changes were those of acute nephritis. The capillary tufts of the glomeruli were distorted and contained but a small amount of blood. The capsule of Bowman was normal; between the capsule and the capillary tuft granular eosin-staining material (albumen) and a few red blood corpuscles were frequently seen. The epithelial cells lining the tubules in the cortex were somewhat broken down; the protoplasm was granular and the cells were entirely without outline. Most of the nuclei took the stain well. In the medulla the lining epithelium of the tubules was fairly preserved and the capillaries contained a moderate amount of blood.

6. October 7, 1910, one loop-full of 18-hour agar growth of the bacillus was suspended in broth and injected subcutaneously in the axilla of a medium-sized monkey. A large swelling soon developed and the monkey died October 8, with hemorrhage and edema in chest and abdominal walls. There was some degeneration in the cells of the kidneys. The bacillus was recovered from the site of the inoculation, from the peritoneum, and from the heart's blood.

7. A dog fed paratyphoid bacilli for one week died October 9, 1910. The autopsy showed acute enteritis (uncinariasis) and hyperemia of the lungs, liver, spleen, and kidneys. The smaller intestine and the large intestine contained a moderate amount of bloody mucus and an enormous number of small, white worms apparently uncinaria. The mucous membrane was decidedly reddened and showed superficial ulceration. The bacillus was recovered from the heart's blood, from the lung, and from the spleen. The serum of the dog agglutinated the organism at a dilution of 1:50.

8. A healthy dog was fed cultures of the paratyphoid-like bacilli. After having been fed for about a week he became sick, with a loss of appetite and a general downcast appearance. Occasionally he would take milk when offered but would vomit soon after. After a week of sickness the appetite returned and all signs of sickness disappeared. A week after recovery the dog's serum was found to agglutinate the paratyphoid-like bacillus in dilution of 1:200.

TOXIN PRODUCTION.

A shallow layer of broth in a broad flask was inoculated with the paratyphoid-like bacillus from the dog, incubated at a temperature of 36° C. for seven days, passed through a germ-proof filter, and tested on guinea-pigs for toxicity. Four guinea-pigs, each weighing 250 gms., received subcutaneously 0.5 c.c., 1.0 c.c., 2.0 c.c., and 4.0 c.c. of the filtrate, respectively. All four guinea-pigs lived, showing that little or no toxin is produced when the bacillus is grown in broth.

THE THERMAL DEATH POINT.

55° C.—Six tubes of broth were heated in a water bath to 55° C. The temperature having reached the desired height the tubes were quickly inoculated with paratyphoid-like bacilli. At intervals of ten minutes after inoculation one tube was removed from the water bath and placed in cold water. After cooling the tubes were placed in the incubator and kept under observation for five days. If no growth became evident in five days the bacilli were regarded as killed. The result follows:

Time in Minutes at 55° C.....	10	20	30	40	50	60
Result.....	Living	Living	Dead	Dead	Dead	Dead

In the tubes of broth that were kept at 55° C. for ten and twenty minutes a good growth was obtained. All other tubes remained sterile.

60° C.—Six tubes of broth were heated in a water bath to 60° C. The temperature having reached the desired point the tubes of broth were quickly inoculated and kept at a temperature of 60° C. At intervals of ten minutes after inoculation one tube was removed from the water bath and placed in cold water. After cooling the tubes were placed in the incubator and kept under observation for five days.

Of the cultures exposed to 60° C. all were sterile. Exposure to 60° C. for ten minutes is sufficient to kill the organism.

COMPARATIVE STUDY UPON THE PARATYPHOID-LIKE BACILLUS ISOLATED FROM THE DOG, PARATYPHOID BACILLUS, TYPHOID BACILLUS, AND THE COLON BACILLUS.

In the following pages are recorded the results obtained by comparing the paratyphoid-like bacillus from the dog with the following strains of bacilli:

1. A paratyphoid bacillus (No. 10) isolated by me in 1902.[1]

2. A culture marked paratyphoid A, brought from Germany by Dr. Coca.

3. A culture marked paratyphoid B, brought from Germany by Dr. Coca.

4. A culture of paratyphoid bacillus given to me by Captain Bloomberg of the United States Army Medical Corps.

5. A culture of typhoid bacillus the source of which is unknown to me.

6. A culture of colon bacillus.

The culture media used were one per cent normal acid in reaction. Litmus milk and potato were prepared according to usual methods and incubation was done at a temperature of 36° C.

The sugar broth for the fermentation tests was Smith's sugar-free broth containing one per cent of peptone. Immediately before using one per cent of sugar was added, the broth run into graduated fermentation tubes and sterilized for fifteen minutes in the autoclave under a pressure of about ten pounds to the square inch. After sterilization the broth was inoculated in the usual manner and placed in the incubator at the temperature of 36° C. At intervals of 24 hours the level of the fluid in the closed arm was noted and recorded. It was found that gas formation was nearly complete at the end of 24 hours, but a second reading was taken and

[1] *Trans. Chicago Path. Soc.*, 1903, 5, p. 187 (Bacillus No. 10).

recorded 18 hours after inoculation. Gelatine was kept in the refrigerator at a temperature of about 10° C.

PARATYPHOID NO. 10.—Medium-sized, semi-transparent, whitish, elevated, circular, soft, moist, surface colony on agar with convex surface and regular border. Brownish by transmitted light. Microscopically they were brown, rather dark at the center and fading toward the periphery, finely granular in appearance, borders regular. The deep colonies, pin-point-sized, rather white specks with regular border, dark-brown, finely granular, lozenge-shaped with regular border on microscopical examination.

It is a slender, actively motile bacillus two to four times as long as it is thick. It stains well with carbol-fuchsin and with methylene blue, and loses the color when treated by Gram's method. Spores are not found.

Broth.—Uniformly clouded within 24 hours. A pellicle forms in three to five days.

Agar-agar.—A medium-sized, semi-transparent, soft, moist, elevated growth along the line of inoculation. The surface is smooth and shining, the border slightly irregular, and by transmitted light it appears light-brown.

Glycerine agar.—The growth corresponds to the growth on agar-agar.

Potato.—A fairly large, soft, moist, light-brown growth spreading over the surface where the potato is wet.

Litmus milk.—Turned slightly red in 24 hours but is not coagulated. Later the milk gradually turns blue and by the end of two weeks it is decidedly more alkaline than the control tube.

Gelatine.—No liquefaction.

Indol.—Negative.

Gas production in sugar broth.—

Glucose—	2.3 c.c. gas in 24 hours;	2.5 c.c. gas in 48 hours.
Maltose—	0.8 c.c. " " " "	1.6 c.c. " " " "
Lactose—	No " " " "	No " " " "
Levulose—	1.5 c.c. " " " "	1.6 c.c. " " " "
Sucrose—	No " " " "	No " " " "
Mannit—	1.7 c.c. " " " "	1.7 c.c. " " " "
Inosit—	No " " " "	No " " " "
Dextrin—	0.6 c.c. " " " "	0.6 c.c. " " " "
Galactose—	0.9 c.c. " " " "	1.4 c.c. " " " "

PARATYPHOID A.—A medium-sized, semi-transparent, whitish, elevated, circular, soft, moist colony with smooth, convex surface and regular border. Light-brown by transmitted light. Microscopically, brown, rather dark at the center, gradually fading toward the periphery, finely granular, border regular. The deep colonies are pin-point-sized, white specks with regular border, dark-brown, finely granular, lozenge-shaped, with border regular. Microscopically it is a slender, actively motile bacillus, two to four times as long as it is thick. It stains well with carbol-fuchsin and methylene blue and loses the color when treated by Gram's method. Spores are not found.

Broth.—Uniformly clouded in 24 hours. A pellicle forms after 3 days.

Agar-agar.—A medium-sized, soft, moist, elevated growth along the line of inoculation. The surface is smooth, convex, and shining; the border is regular, white, semi-transparent by reflected light and pale-brown by transmitted light.

Glycerine agar.—Like the growth on agar-agar.

Potato.—Fairly large, soft, moist, light-brown growth spreading over the surface where the potato is wet.

Litmus milk.—Turned slightly red in 24 hours; the color gradually disappears and at the end of three days the milk is almost pure white. At the end of two weeks it is yellowish and semi-transparent, apparently peptonized. No coagulation.

Gelatine.—No liquefaction.

Indol.—Negative.

Gas formation in sugar broth.—

Glucose—	1.2 c.c. gas in 24 hours;	1.9 c.c. gas in 48 hours.
Maltose—	1.2 c.c. " " " "	2.1 c.c. " " " "
Lactose—	No " " " "	No " " " "
Levulose—	1.5 c.c. " " " "	1.6 c.c. " " " "
Sucrose—	No " " " "	No " " " "
Mannit—	2.6 c.c. " " " "	2.6 c.c. " " " "
Inosit—	0.1 c.c. " " " "	0.2 c.c. " " " "
Dextrin—	0.4 c.c. " " " "	0.5 c.c. " " " "
Galactose—	2.3 c.c. " " " "	2.3 c.c. " " " "

PARATYPHOID B.—The surface colony on agar is medium-sized, elevated, circular, soft, moist, with smooth, convex surface and regular border, white, semi-transparent by reflected light and pale-brown by transmitted light; microscopically it is brown, rather dark at the center, fading gradually toward the periphery, finely granular, regular border.

The deep colony is a pin-point-sized, white, opaque speck with regular border; microscopically it is dark-brown, finely granular, lozenge-shaped; border regular.

It is a slender, actively motile bacillus two to four times as long as thick. It stains well with carbol-fuchsin and with methylene blue and loses the color when treated by Gram's method. Spores are not found.

Broth.—Uniformly clouded in 24 hours; a pellicle forms after three days.

Agar-agar.—A medium-sized, soft, moist, elevated growth along the line of inoculation. Surface smooth, shining, and convex; the border is slightly irregular; white, semi-transparent by reflected light, pale-brown by transmitted light.

Glycerine agar.—The growth on glycerine agar corresponds to the description of the growth on agar-agar.

Potato.—Fairly large, light-brown growth spreading over the surface where the potato is wet.

Litmus milk.—Turned slightly red in 24 hours; after three days it is almost pure white, and at the end of two weeks it is yellowish, semi-transparent, peptonized.

Gelatine.—No liquefaction.

Indol.—Negative.

Gas production in sugar broth.—

Glucose—	2.5 c.c. gas in 24 hours;	2.6 c.c. gas in 48 hours.
Maltose—	0.8 c.c. " " " "	1.7 c.c. " " " "
Lactose—	No " " " "	No " " " "
Levulose—	1.2 c.c. " " " "	1.3 c.c. " " " "
Sucrose—	No " " " "	No " " " "
Mannit—	2.3 c.c. " " " "	2.4 c.c. " " " "
Inosit—	0.1 c.c. " " " "	0.2 c.c. " " " "
Dextrin—	0.3 c.c. " " " "	0.4 c.c. " " " "
Galactose—	1.6 c.c. " " " "	1 7 c.c. " " " "

BACILLUS TYPHOSUS.—The surface colony is a small, soft, moist, elevated, circular colony with smooth, convex surface and regular border. Whitish, translucent by reflected light and pale-brown by transmitted light. Microscopically it is brown, rather dark at the center and fading gradually toward the periphery, finely granular,

border regular. The deep colony is a small, opaque, white speck with regular border; microscopically it is dark-brown, finely granular, lozenge-shaped, with regular border.

It is a small, slender, actively motile bacillus, two to four times as long as thick. It stains well with carbol-fuchsin and with methylene blue and loses the color when treated by Gram's method. Spores are not found.

Broth.—Uniformly clouded in 24 hours. A pellicle forms in five days.

Agar-agar.—A medium-sized, soft, moist, elevated, translucent growth with smooth, shining, convex surface and slightly irregular border. Whitish by reflected light and pale-brown by transmitted light.

Glycerine agar.—The growth on glycerine agar corresponds to the description of the growth on agar-agar.

Potato.—A fairly large, pale-brown growth along the line of inoculation, spreading over the surface where the potato is wet.

Litmus milk.—Turned slightly red in 24 hours; no coagulation. The slightly red color persists.

Gelatine.—No liquefaction.

Indol.—Negative.

No gas produced after 24 or 48 hours in glucose, maltose, lactose, levulose, sucrose, mannit, dextrin, inosit, and galactose broths.

BACILLUS COLI COMMUNIS.—The surface colony on agar is a medium-sized, circular, elevated, soft, moist colony with smooth, convex surface and regular border, semi-transparent, white by reflected light, light-brown by transmitted light. Microscopically it is brown, rather dark at the center and gradually fading toward the periphery. Finely granular, border regular. The deep colony is a small, opaque, white speck, with regular border; microscopically it is dark-brown, finely granular, lozenge-shaped, border regular.

It is a short, thick, motile bacillus, coccoid forms to two to three times as long as thick. As a whole these organisms are shorter and thicker than the typhoid or paratyphoid bacilli.

Broth.—Uniformly clouded in 24 hours. A pellicle forms in three days.

Agar-agar.—Fairly large, soft, moist, elevated growth with smooth, shining, convex surface and slightly irregular border along the line of inoculation. Semi-transparent, white by reflected light and light-brown by transmitted light.

Glycerine agar.—The growth on glycerine agar corresponds to the description of that on agar-agar.

Potato.—Fairly large, light-brown growth spreading over the surface where the potato is wet.

Litmus milk.—Turned decidedly red in 24 hours. Red and firmly coagulated in 48 hours.

Gelatine.—No liquefaction.

Indol.—Positive.

Gas production in sugar broth.—

Glucose—	1.0 c.c. gas in 24 hours;	1.2 c.c. gas in 48 hours.
Maltose—	1.7 c.c. " " " "	2.0 c.c. " " " "
Lactose—	1.5 c.c. " " " "	1.7 c.c. " " " "
Levulose—	1.4 c.c. " " " "	1.4 c.c. " " " "
Sucrose—	0.9 c.c. " " " "	1.0 c.c. " " " "
Mannit—	3.4 c.c. " " " "	3.5 c.c. " " " "
Inosit—	No " " " "	No " " " "
Dextrin—	0.3 c.c. " " " "	0.4 c.c. " " " "
Galactose—	1.7 c.c. " " " "	1.8 c.c. " " " "

PARATYPHOID-LIKE BACILLUS FROM DOG.—The surface colony on agar is a medium-sized, soft, moist, circular, elevated colony with smooth, shining, convex surface and regular border. Semi-transparent, white by reflected light and light-brown by transmitted light. Microscopically it is brown, rather dark at the center, fading toward the periphery; finely granular, regular border. The deep colony is a small, opaque, white speck with regular border; under the microscope it is dark-brown, finely granular, lozenge-shaped, with regular border.

It is a slender, actively motile bacillus, two to four times as long as it is thick. It stains well with carbol-fuchsin and with methylene blue and loses color when treated by Gram's method. Spores are not found.

Broth.—Uniformly clouded in 24 hours. A pellicle forms in three days.

Agar-agar.—A fairly large, soft, moist, elevated growth with smooth, shining, convex surface and slightly irregular border along the line of inoculation. Semi-transparent, white by reflected light, light-brown by transmitted light.

Glycerine agar.—The growth on glycerine agar corresponds to the description of the growth on agar-agar.

Potato.—A fairly large, light-brown growth spreading over the surface where the potato is wet.

Litmus milk.—Turned slightly red in 24 hours; almost pure white after three days, and peptonized after two weeks.

Gelatine.—No liquefaction.

Indol.—Negative.

Gas production in sugar broth.—

Glucose—	2.3 c.c.	gas in	24	hours;	2.4 c.c.	gas in	48	hours.	
Maltose—	1.7 c.c.	"	"	"	"	2.4 c.c.	"	"	"	"
Lactose—	No	"	"	"	"	No	"	"	"	"
Levulose—	1.2 c.c.	"	"	"	"	1.3 c.c.	"	"	"	"
Sucrose—	No	"	"	"	"	No	"	"	"	"
Mannit—	2.3 c.c.	"	"	"	"	2.4 c.c.	"	"	"	"
Inosit—	0.3 c.c.	"	"	"	"	0.5 c.c.	"	"	"	"
Dextrin—	0.5 c.c.	"	"	"	"	0.5 c.c.	"	"	"	"
Galactose	1.8 c.c.	"	"	"	"	2.0 c.c.	"	"	"	"

PARATYPHOID BACILLUS (BLOOMBERG).—The surface colony on agar is a medium-sized (about as large as that of *B. typhosus*), soft, moist, circular, elevated colony with smooth, shining, convex surface and regular border. Translucent, white by reflected light and light-brown by transmitted light. Microscopically, it is brown, rather dark at the center, gradually fading toward the periphery. Finely granular, regular border. The deep colony is a pin-point-sized, white, opaque speck with regular border; microscopically it is granular, lozenge-shaped, regular border.

It is a slender, actively motile bacillus two to four times as long as thick. It stains well with carbol-fuchsin and with methylene blue. By Gram's method it loses the color. Spores are not found.

Broth.—Uniformly clouded in 24 hours. A pellicle appears in five days.

Agar-agar.—A medium-sized, soft, moist, elevated growth with smooth, shining, convex surface and slightly irregular border along the line of inoculation. Translucent, white by reflected light and pale-brown by transmitted light.

Glycerine agar.—The growth on glycerine agar corresponds to the description of the growth on agar-agar.

Potato.—Rather small, pale-brown growth spreading over the surface where the potato is wet.

Gelatine.—No liquefaction.

Indol.—Negative.

Gas production in sugar broth.—

Glucose—	1.1 c.c. gas in 24 hours;	1.9 c.c. gas in 48 hours.
Maltose—	0.1 c.c. " " " "	0.2 c.c. " " " "
Lactose—	No " " " "	No " " " "
Levulose—	1.22 c.c " " " "	1.3 c.c. " " " "
Sucrose—	No " " " "	No " " " "
Mannit—	2.0 c.c. " " " "	2.3 c.c. " " " "
Inosit—	No " " " "	No " " " "
Dextrin—	0.33 c.c. " " " "	0.4 c.c. " " " "
Galactose—	0.7 c.c. " " " "	1.2 c.c. " " " "

ACID PRODUCTION IN SUGAR BROTH.—Broths containing one per cent of glucose, maltose, lactose, levulose, sucrose, mannit, inosit, dextrin, and galactose were inoculated with the bacilli mentioned in the following tables, incubated at a temperature of 36° C., and at intervals of 24 hours the reaction was determined by titrating with 1/20 normal solution of sodium hydrate, using phenolphthalein as indicator. The results are shown in the tables:

TABLE 1.

ACID PRODUCTION BY PARATYPHOID BACILLUS 10.

Days	1	2	3	4	5	6	7	8	9	10
Glucose	1.9	2.0	2.2	2.5	2.5	2.5	2.6	2.7	2.7	2.9
Maltose	1.8	2.0	2.1	2.2	2.3	2.5	2.5	2.5	2.5	2.5
Lactose	1.2	1.3	1.1	1.0	0.8	0.6	0.7	0.7	0.7	0.8
Levulose	2.0	2.2	2.4	2.6	2.6	2.6	2.7	2.8	2.5	2.3
Sucrose	1.2	1.2	1.0	0.7	0.7	0.7	0.6	0.5	0.7	0.8
Mannit	1.8	2.0	2.0	2.1	2.1	2.3	2.3	2.4	2.5	2.7
Inosit	1.0	1.0	0.8	0.7	0.7	0.8	0.7	0.6	0.7	0.7
Dextrin	1.4	1.5	1.5	1.5	1.5	1.5	1.3	1.2	1.2	1.3
Galactose	1.8	2.0	2.2	2.5	2.7	2.9	3.0	3.0	3.0	3.0

In glucose, maltose, levulose, mannit, dextrin, and galactose acid is produced. Lactose, sucrose, and inosit are changed to alkalinity.

TABLE 2.

ACID PRODUCTION BY PARATYPHOID BACILLUS A.

Days	1	2	3	4	5	6	7	8	9	10
Glucose	2.3	2.5	2.5	2.5	2.6	2.6	2.7	2.7	3.0	3.0
Maltose	1.6	1.8	2.0	2.4	2.5	2.5	2.5	2.5	2.5	2.5
Lactose	1.0	1.0	0.9	0.8	0.7	0.7	0.7	0.8	0.7	0.7
Levulose	2.5	2.7	2.7	2.5	2.7	2.8	2.7	2.7	2.7	2.7
Sucrose	1.0	1.0	1.0	1.0	0.8	0.7	0.5	0.5	0.5	0.5
Mannit	2.1	2.3	2.3	2.3	2.4	2.4	2.4	2.5	2.6	2.6
Inosit	2.6	2.8	2.7	2.7	2.8	2.9	2.9	2.9	3.0	3.1
Dextrin	1.5	1.5	1.5	1.5	1.2	0.7	0.8	0.9	0.9	0.8
Galactose	2.5	2.8	2.8	2.8	3.0	3.0	3.0	3.0	3.0	3.1

Acid is produced in the broth containing glucose, maltose, levulose, mannit, inosit, and galactose. Broths which contain lactose, sucrose, and dextrin become alkaline.

TABLE 3.
ACID PRODUCTION BY PARATYPHOID BACILLUS B.

Days	1	2	3	4	5	6	7	8	9	10
Glucose	2.2	2.6	2.5	2.5	2.7	2.8	2.7	2.7	2.8	2.8
Maltose	2.0	2.1	2.2	2.2	2.3	2.5	2.5	2.6	2.5	2.5
Lactose	1.0	1.0	0.7	0.5	0.6	0.6	0.7	0.8	0.6	0.5
Levulose	2.4	2.7	2.7	2.7	2.7	2.7	2.7	2.7	2.7	2.8
Sucrose	1.0	1.0	0.8	0.6	0.7	0.7	0.6	0.6	0.5	0.4
Mannit	2.0	2.7	2.5	2.3	2.4	2.5	2.5	2.5	2.5	2.5
Inosit	2.2	2.7	2.6	2.6	2.8	3.0	2.9	2.9	3.0	3.1
Dextrin	1.5	1.5	1.4	1.3	1.0	0.7	0.7	0.8	0.7	0.7
Galactose	2.4	2.8	2.6	2.6	2.4	2.7	2.8	2.9	3.0	3.1

Acid is produced in the broth which contains glucose, maltose, levulose, mannit, inosit, and galactose. Broths which contain lactose, sucrose, or dextrin are turned alkaline.

TABLE 4.
ACID PRODUCTION BY BACILLUS TYPHOSUS.

Days	1	2	3	4	5	6	7	8	9	10
Glucose	2.0	2.2	2.2	2.1	2.1	2.0	2.5	2.8	2.6	2.4
Maltose	1.8	2.0	2.0	2.1	2.2	2.3	2.3	2.3	2.3	2.3
Lactose	1.0	1.0	0.7	0.6	0.7	0.7	0.6	0.6	0.7	0.7
Levulose	2.0	2.3	2.2	2.1	2.2	2.3	2.4	2.4	2.5	2.5
Sucrose	1.1	1.0	0.8	0.6	0.7	0.7	0.7	0.7	0.8	0.8
Mannit	2.0	2.2	2.0	2.1	2.0	2.1	2.0	2.0	2.2	2.4
Inosit	0.8	0.6	0.5	0.4	0.4	0.4	0.4	0.5	0.6	0.6
Dextrin	1.0	1.0	1.0	0.9	0.8	0.8	0.7	0.6	0.7	0.8
Galactose	2.0	2.5	2.7	2.7	2.7	2.6	2.5	2.4	2.6	2.8

The typhoid bacillus produces acid in broths which contain glucose, maltose, levulose, mannit, and galactose. Alkali is produced in broths that contain lactose, sucrose, inosit, and dextrin.

TABLE 5.
ACID PRODUCTION BY BACILLUS COLI.

Days	1	2	3	4	5	6	7	8	9	10
Glucose	2.5	2.9	3.0	3.0	2.9	2.8	2.8	2.8	2.8	3.0
Maltose	2.5	2.6	2.5	2.5	2.5	2.4	2.2	2.0	2.3	2.5
Lactose	1.7	2.5	2.5	2.4	2.4	2.4	2.4	2.4	2.6	2.7
Levulose	2.6	2.8	2.8	2.7	2.8	3.0	3.0	3.1	3.1	3.2
Sucrose	2.1	2.4	2.5	2.5	2.3	2.2	2.3	2.4	2.5	2.5
Mannit	2.5	2.6	2.6	2.6	2.5	2.4	2.5	2.6	2.7	2.8
Inosit	0.8	0.6	0.7	0.8	0.8	0.8	0.7	0.7	0.7	0.6
Dextrin	1.3	1.1	1.0	1.0	0.9	0.9	0.8	0.7	0.8	0.9
Galactose	3.0	3.1	3.2	3.2	3.2	3.2	3.2	3.3	3.3	3.5

Acid is produced by the colon bacillus in broth which contains glucose, maltose, lactose, levulose, sucrose, mannit, and galactose.

The broth which contained inosit and that which contained dextrin became alkaline.

TABLE 6.

ACID PRODUCTION BY PARATYPHOID-LIKE BACILLUS.

Days	1	2	3	4	5	6	7	8	9	10
Glucose	2.3	2.5	2.5	2.7	2.8	2.9	2.8	2.8	2.9	3.2
Maltose	2.3	2.5	2.4	2.4	2.5	2.5	2.6	2.7	2.7	2.7
Lactose	1.0	1.0	0.8	0.6	0.6	0.7	0.7	0.5	0.6	0.6
Levulose	2.2	2.5	2.7	2.8	2.8	2.8	2.8	2.9	2.9	3.0
Sucrose	1.0	1.0	0.8	0.7	0.7	0.8	0.7	0.7	0.7	0.7
Mannit	2.2	2.5	2.4	2.4	2.4	2.4	2.3	2.4	2.6	2.7
Inosit	2.5	3.0	2.9	2.8	2.9	3.0	3.1	3.1	3.2	3.4
Dextrin	1.0	1.2	1.0	0.7	0.7	0.7	0.7	0.7	0.7	0.7
Galactose	2.8	3.1	3.0	3.1	3.1	3.2	3.3	3.3	3.3	3.4

The paratyphoid-like bacillus from the dog produces acid in broth which contains glucose, maltose, levulose, mannit, inosit, and galactose. Alkali is produced in broth with lactose, sucrose, and dextrin.

TABLE 7.

ACID PRODUCTION BY PARATYPHOID BACILLUS (BLOOMBERG).

Days	1	2	3	4	5	6	7	8	9	10
Glucose	2.0	2.5	2.4	2.4	2.5	2.6	2.5	2.5	2.6	2.7
Maltose	1.5	1.9	2.0	2.2	2.1	2.2	2.2	2.3	2.4	2.6
Lactose	1.0	1.1	1.0	1.0	1.0	1.1	1.0	1.0	1.0	1.0
Levulose	2.0	2.2	2.3	2.5	2.5	2.6	2.7	2.9	2.9	2.9
Sucrose	1.0	1.3	1.1	1.0	1.2	1.3	1.2	1.2	1.2	1.2
Mannit	1.8	2.1	2.0	2.1	2.2	2.3	2.4	2.4	2.5	2.6
Inosit	1.0	1.1	1.0	1.0	0.9	0.9	0.9	0.9	1.0	1.0
Dextrin	1.3	1.5	1.6	1.7	1.7	1.8	1.6	1.5	1.5	1.6
Galactose	2.0	2.4	2.4	2.5	2.7	2.9	2.8	2.8	2.9	3.1

In order to detect any change in the reaction of the broth without bacterial action a set of tubes with broths containing one per cent glucose, mannit, maltose, lactose, levulose, sucrose, inosit, dextrin, and galactose was kept at a temperature of 36° C., and at intervals of 24 hours the reaction was determined by titrating with 1/20 normal solution of sodium hydrate with phenolphthalein as indicator.

TABLE 8.

CHANGES IN REACTION OF CONTROL BROTHS.

Days	1	2	3	4	5	6	7	8	9	10
Glucose	1.0	1.0	1.0	1.0	1.0	1.0	1.1	1.1	1.2	1.2
Maltose	1.0	1.0	1.0	1.0	1.0	1.0	1.1	1.1	1.2	1.2
Lactose	1.0	1.0	1.0	1.0	1.0	1.0	1.1	1.1	1.1	1.2
Levulose	1.0	1.0	1.0	1.0	1.0	1.0	1.1	1.1	1.2	1.2
Sucrose	1.0	1.0	1.0	1.0	1.0	1.0	1.1	1.1	1.2	1.2
Mannit	1.0	1.0	1.0	1.0	1.0	1.0	1.1	1.1	1.2	1.2
Inosit	1.0	1.0	1.0	1.0	1.0	1.0	1.1	1.1	1.1	1.2
Dextrin	1.0	1.0	1.0	1.0	1.0	1.0	1.1	1.1	1.1	1.2
Galactose	1.4	1.4	1.4	1.4	1.4	1.5	1.5	1.5	1.6	1.6

Broth that contained galactose was slightly more acid than the others. In all the acidity increased slightly during the period of ten days.

INTERAGGLUTINATION OF THE VARIOUS STRAINS OF BACILLI STUDIED.—Rabbits were inoculated intraperitoneally with cultures of each of the different strains studied and the agglutinating power of the resulting serum determined with respect to each of the strains in each case. Unless otherwise stated two intraperitoneal inoculations were made one week apart. A week after the second inoculation the rabbit was bled. The blood was allowed to clot and the clear serum withdrawn.

1. The serum obtained in this way against paratyphoid bacillus No. 10 agglutinated this bacillus in a dilution of 1:400 and the Bloomberg bacillus in a dilution of 1:800; none of the other strains were agglutinated.

2. The serum against paratyphoid bacillus A agglutinated this bacillus in a dilution of 1:2,000, paratyphoid bacillus B in a dilution of 1:1,000, and the bacillus from the dog in dilution of 1:200; the other strains were not agglutinated.

3. The serum against paratyphoid bacillus B agglutinated this bacillus in a dilution of 1:4,000 and paratyphoid bacillus A in a dilution of 1:1,000; the other strains were negative.

4. The serum against the typhoid bacillus agglutinated this bacillus in a dilution of 1:400 and had no effect whatever on any of the other strains.

5. The serum against the colon bacillus agglutinated this bacillus in a dilution of 1:8,000, paratyphoid No. 10 in a dilution of 1:100, and paratyphoid Bloomberg in a dilution of 1:400; it had no effect on any other strain.

6. The serum against the paratyphoid-like bacillus from the dog agglutinated this bacillus in a dilution of 1:16,000; it had no effect on any of the other strains. In this case the rabbit received three intraperitoneal inoculations.

7. The serum against paratyphoid bacillus (Bloomberg) agglutinated this bacillus in a dilution of 1:8,000; paratyphoid No. 10 in a dilution of 1:200, paratyphoid A in a dilution of 1:50, paratyphoid B in a dilution of 1:100, and typhoid bacillus in dilution of 1:100.

CONCLUSIONS.

According to the evidence brought forth in the preceding pages the following conclusions seem to be justified.

The organism isolated from the dog and described in the foregoing belongs to the group of paratyphoid bacilli.

Because of the fact that the dog had rabies infection with the paratyphoid bacillus must be considered secondary. In all probability it was the immediate cause of death.

That infection took place before death is shown by the agglutinating power of the dog's serum.

The bacillus isolated is highly pathogenic for guinea-pigs, rabbits, and monkeys. After death of the animal the organism can be found in all organs and it has a tendency to produce pneumonia.

Apparently, soluble toxin is not produced.

Exposure to a temperature of 55° C. for 30 minutes kills it. At a temperature of 60° C. it is killed in 10 minutes.

It is doubtful whether feeding the organism to a healthy dog will produce a fatal infection.

Gas production and acid production in sugar broth varies greatly with different strains of paratyphoid bacilli.

The strains of bacilli reported on in this paper vary greatly in their behavior toward agglutinating serums.

Practically no differences were found between the culture marked paratyphoid bacillus A and that marked paratyphoid bacillus B.

A BACTERIOLOGICAL AND CELLULAR STUDY OF THE LUNG EXUDATE DURING LIFE IN LOBAR PNEUMONIA.*

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Recent investigations go to show that phagocytosis is one of the ways by which an animal combats a pneumococcus infection. But whether this method is the primary or most important reaction is still unsettled. Phagocytosis of pneumococci in the exudate of pneumonic lungs as seen after death is slight; well-preserved pneumococci within leukocytes are rarely found; pneumococci in various stages of disintegration are found more often. The following study was made to determine something as to the number of pneumococci and other bacteria in the consolidated lung throughout the disease during life; what relation, if any, this number may have to the invasion of the blood and the termination of the attack, and to analyze more closely, if possible, the mechanism by which the pneumococci are destroyed.

The following procedure was used. The skin overlying the point chosen for puncture was sterilized by means of 95 per cent carbolic acid and a sterile alcohol sponge or by means of the latter alone, in which case the superficial epithelium was rubbed off. The patient was cautioned against taking a sudden deep inspiration as the needle was passed into the lung. The upper border of the rib was used as a guide and the puncture made with an ordinary antitoxin needle. Its length being only 3.5 cm., the puncture of a good-sized artery in the lung was impossible. The flexible rubber connection between the needle and the small sterile pipette avoided tearing the lung during the exertion of ordinary breathing. The pain was trivial and no untoward results occurred. A tight-fitting metal syringe was used to produce strong suction. In this way a small amount of the exudate was aspirated into the needle or pipette, and smears and blood-agar plate cultures made immediately.

* Received for publication April 26, 1911.

The amount of material used for the cultures after making the smears was necessarily small but sufficient to estimate roughly the number of viable organisms, as shown by the results of cultures from simultaneous punctures of the same lobe; moreover, the number of cocci in the smears seemed to correspond closely to those obtained in cultures.

Twenty-seven cases[1] have been studied, of which five died. The average age of those that recovered was 32 years, of those that died, 39 years. No particular reason for the fatal termination of the latter could be found outside of an overwhelming pneumococcus infection. Five of the 22 patients who recovered and three of those who died had two or more lobes involved. Multiple blood cultures were made in five instances. Three were positive on the third, fourth, or fifth days, while two were negative, one during, the other, the day after, crisis. In each instance, when the blood contained pneumococci the material from the lung yielded a large number of organisms, and when the blood was sterile the cultures from the consolidated lung likewise proved sterile. Of 48 punctures the exudate of 26 showed viable pneumococci, while in 22 it remained sterile. The pneumococcus was present in pure culture in every instance except in one fatal case where a moderate number of colonies of *Streptococcus pyogenes* were found. Occasionally a few colonies of staphylococci and saprophytic bacilli were found, but these were looked on as accidental contaminations from the skin or air. *Bacillus mucosus* and *Streptococcus mucosus* were not found.

Positive cultures were obtained at all stages of the disease, but the percentage of positive results was much higher in the early stages (up to the fifth or sixth day) in those that recovered and throughout the course in those that died. The number of pneumococci early in the attack was equally great in the fatal and non-fatal cases. Mild cases at this time appear to have as many pneumococci as the more severe. In those with a favorable termination the number of pneumococci usually gradually diminish as a crisis is reached, although at times the numbers drop abruptly. Only six out of 20 punctures made during and after crisis yielded

[1] Only typical cases of lobar pneumonia were selected in the wards of the Cook County Hospital, during the winter of 1909. I am greatly indebted to the members of the staffs for kind assistance.

pneumococci, only a few colonies developing in each instance; while in the patients who died the number of pneumococci became progressively greater until the time of death. This result is in keeping with the results of the blood cultures in this series and with the findings which I have made in a larger series, namely, that while blood cultures are positive in the fatal and non-fatal cases early in the disease, the number of positive cultures in the cases which recover becomes less as we approach crisis. A positive blood culture late in an attack of lobar pneumonia is therefore an unfavorable sign.

Giemsa's differential stain and Gram's method with eosin or carbol-fuchsin as counterstain were used in studying the smears. The predominating cell early in the attack is the polymorphonuclear neutrophile. Eosinophiles and basophiles are rarely found. The small lymphocytes are never numerous. Early in the attack large mononuclear cells are relatively few. They are well preserved and not phagocytic for leukocytes, but later, as crisis is reached, they become more numerous and during resolution are markedly phagocytic for leukocytes. At this stage the leukocytes and large mononuclear cells become vacuolated and show other evidences of disintegration. This is most pronounced when resolution is rapid.

In lungs obtained immediately after death, the leukocytes, pneumococci, and mononuclear cells were well preserved in the parts in the early stages of consolidation but showed marked evidences of disintegration in the parts in resolution. Here, too, phagocytosis of leukocytes by mononuclear cells was marked. As many as four disintegrating leukocytes, often containing disintegrating pneumococci, could be found in a single cell. The striking difference between the microscopic picture of the lung in resolution after crisis in the patient who recovers and the lung in resolution in the patient who dies with an extension into another lobe is the almost total absence of pneumococci in the former and the presence of great numbers in the latter. Indeed cultures show that the number of viable cocci is as great in the part in resolution after death as in the part more recently consolidated.

The changes in the character and kind of cells of the exudate seem to take place irrespective of the presence or absence of living

pneumococci. This observation would seem to argue against the idea that there is produced a large amount of a pneumococcidal substance in the resolving lung and is in accord with the clinical observation that extension into another lobe is not infrequent during resolution of the lobe earlier involved.

The amount of phagocytosis of pneumococci is never very great. The smears from the punctures early in the attack show practically no phagocytosis, while those made later frequently show phagocytosis of pneumococci in various stages of disintegration. Whenever this is true a large number of disintegrating pneumococci are observed free in the exudate. This is seen best in the fatal cases, because in those which recover the number of pneumococci is usually too small after disintegration of cocci begins to study this point accurately. Certainly the amount of lysis of pneumococci in the lung seems as great in the patient that dies as in the one that recovers. Well-preserved pneumococci within leukocytes were seen in only five smears. The cocci in the exudate seem to be altered (as manifested by their loss of affinity for basic dyes) before they become susceptible to phagocytosis.

The most striking fact brought out by this study is the great difference in the number of viable pneumococci in the consolidated lung in the later stages of the disease in the cases which recover and in those which die: in the former they are relatively few, in the latter they are very numerous.

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