

THE OCCURRENCE OF PLEOMORPHISM AND MUTATION AMONG MEMBERS OF THE HÆMORRHAGIC SEPTICÆMIA GROUP OF ORGANISMS.¹

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(PLATES V.—VI.)

IN this paper we propose to discuss incidentally morphological variations among members of the hæmorrhagic septicæmia group of organisms, but more especially variations in biological activity, associated as they have been in our experience with a concomitant change in virulence. The clinical cases, which we use as illustration, have been collected by one of us (J. P. M'Gowan) in investigations into animal pathology lasting over several years. At the time the original observations were made the results were merely noted, no attempt being made to explain them. However, in the light of information obtained lately, a satisfactory explanation now seems possible.

INCREASE OF VIRULENCE OF *BACILLUS AVISEPTICUS* (*BACILLUS* OF CHICKEN CHOLERA) ASSOCIATED WITH INCREASE OF BIOLOGICAL ACTIVITY.

In November of 1914 we had occasion to undertake experiments with a view to increasing the virulence of a strain of *B. avisepticus*. This had been obtained by one of us (J. P. M'Gowan) about three years previously from one of many fowls which died, in an epidemic of chicken cholera, on a farm near Edinburgh. The strain had been regularly subcultured on agar every second month since isolation.

The protocol of one of our experiments to increase the virulence of this organism is as follows:

G.-P. 1 (November 2, 1914).—Emulsion in saline of 24-hours' agar culture of this strain injected intraperitoneally into guinea-pig (G.-P. 1) at 1 p.m. Death between 8 p.m. and following morning.

G.-P. 2 (November 4, 1914).—Emulsion in saline of 24-hours' agar culture

¹ Received March 1, 1915.

from peritoneum of G.-P. 1, a pure culture made. One c.c. of this emulsion ($=\frac{1}{6}$ agar culture) injected intraperitoneally into G.-P. 2 at 4.30 p.m. G.-P. 2 died 5th November 1914 at 12.30 p.m.

G.-P. 3 (November 5, 1914).—G.-P. 3 injected intraperitoneally with $\frac{1}{4}$ c.c. of the peritoneal fluid from G.-P. 2 at 1 p.m.; killed at 3 p.m. on 6th November 1914.

G.-P. 4 (November 6, 1914, at 4 p.m.).—G.-P. 4 injected intraperitoneally with $\frac{1}{2}$ c.c. of peritoneal fluid from G.-P. 3. This animal showed no symptoms and no peritoneal fluid for injection was obtained from it.

G.-P. 4B (November 9, 1914).—An emulsion was made in saline of a 72-hours' agar culture from the heart blood of G.-P. 3, and half of this emulsion ($\frac{1}{2}$ culture) was injected intraperitoneally into G.-P. 4B. At 11.30 a.m., on 10th November 1914, G.-P. 4B became very ill, and was killed with coal gas.

G.-P. 5 (November 10, 1914).—G.-P. 5 was injected at 4 p.m. intraperitoneally with 1 c.c. of the peritoneal fluid of G.-P. 4B. On 11th November 1914, at 12 p.m., the guinea-pig was very ill, and was killed with coal gas.

G.-P. 6A (November 11, 1914).—G.-P. 6A injected intraperitoneally with 1 c.c. peritoneal fluid of G.-P. 5 at 4.30 p.m. G.-P. 6A died during the night, but owing to circumstances another guinea-pig was not inoculated directly from it. Instead of this,

G.-P. 6B (November 13, 1914).—G.-P. 6B was inoculated at 4.45 p.m. intraperitoneally with a 24-hours' agar subculture from an agar culture of peritoneal fluid of G.-P. 5; died during the night. (It is to be noted that now for the first time the organism was found in films from the heart blood.)

G.-P. 7 (November 16, 1914).—G.-P. 7 injected intraperitoneally with an emulsion of half 48-hours' agar culture made from peritoneum of G.-P. 6B. The animal died during the night. Organism again found in the heart blood.

Subsequent to this, between 18th November 1914 and 15th December 1914, the organism from G.-P. 7 was carried by intravenous injection through a series of six rabbits, and the cultures obtained from the individual rabbits were named P.-R. 1, P.-R. 2, P.-R. 3, P.-R. 4, P.-R. 5, and P.-R. 6, according to whether their source was rabbits 1, 2, 3, etc. With reference to the condition of purity of the final organism obtained, it has to be noted that *at no time during the passage of the organism through these two series of animals was an injection made into an animal from a single colony of the culture obtained from the preceding animal.* In most cases the next animal was injected *directly* from the peritoneal fluid of the preceding animal; but where this was not done, the animal was injected from a culture, made directly from the peritoneal fluid of the previous animal, or from a subculture which had been inoculated from a platinum loop, drawn indiscriminately over the whole surface of the culture, made directly from the peritoneal fluid. It seems probable, therefore, that if any accidental contamination had gained entry during the *passage*, this contamination, short of having entirely replaced the original culture of *B. avisepticus* with which we started, ought to have persisted, along with the *B. avisepticus*, to the end of the experiment. *But as we will show, the culture at the end of the experiment (P.-R. 6) was pure, containing only one type of organism.*

As regards the nature of this organism we were first led to suspect a change in *passage*, by our noticing that the colonies on agar slopes, made during the testing of the bactericidal action of sera on the original

strain of *B. avisepticus* and on P.-R. 6 were different in size. The colonies of P.-R. 6 were much larger than those of the original strain (Plate V. Fig. 1). This led us to test the biological activity of P.-R. 6, and the original strain on various media, and subsequently to plate out and examine thoroughly all the strains that we had remaining from the various animals during the *passage*. These strains were G.-P. 7, P.-R. 3, P.-R. 4, P.-R. 5, P.-R. 6, with the result that *all five cultures were found to be pure, i.e., they consisted of colonies similar in growth, size, and appearance.* (The cultures from the other animals of this series had been accidentally laid aside and were not available at the time of this experiment, which was done on 25th December 1914.)

Individual colonies were picked off from each of them and compared as follows in various media with one another, and with the original strain :

TABLE I.—*Reaction of Original Strain and the Five Virulent Strains.*

	Original.	G.-P. 7	P.-R. 3.	P.-R. 4.	P.-R. 5.	P.-R. 6.
Lactose . .	Slight A	A + G	A + G	A + G	A + G	A + G
Glucose . .	"	A + G	A + G	A + G	A + G	A + G
Maltose . .	"	A + G	A + G	A + G	A + G	A + G
Mannite . .	A	A + G	A + G	A + G	A + G	A + G
Galactose . .	Slight A	A + G	A + G	A + G	A + G	A + G
Lactose neutral red	A	A + G	A + G	A + G	A + G	A + G
Saccharose . .	?	A + G	A + G	A + G	A + G	A + G
Raffinose . .	?	A + G	A + G	A + G	A + G	A + G
Salicine . .	0	A ?	A + G	A + G	A + G	A + G
Inulin . .	0	0	0	0	0	0
Inosite . .	0	0	A	A	A	A
Sorbite . .	0	A	A + G	A + G	A + G	A + G
Dulcite . .	Slight A	A ?	A + G	A + G	A + G	A + G
Adonite . .	?	?	A	A	A	A
Litmus milk . .	?	A ?	A + C	A + C	A + C	A + C
Peptone water . .	Indol	Marked indol	Marked indol			
Nitrate water . .	Nitrites present	Marked nitrites	Marked nitrites			
Potato . .	Slight dry granular growth	Marked dry granular growth	Marked dry granular growth	As in P.-R. 3	As in P.-R. 4	As in P.-R. 5
Broth . .	Marked turbidity	Marked turbidity	Marked turbidity			
Glucose Broth	Slight " growth	Good " growth	Very " good growth			
Maltose agar (Sabouraud)	No liquefaction	No liquefaction	No liquefaction	+	+	+
Gelatin . .	0	?	+			
Motility . .						

A + G = Acid + gas production ; A = Acid production ; A + C = acid + clot ;
? = doubtful production ; 0 = no change ; + = present.

In examining this table it will be seen that all the strains have an action on lactose, glucose, maltose, mannite, galactose, lactose neutral red, saccharose, raffinose, in the following order however,—original

< G.-P. 7, P.-R. 3, P.-R. 4, P.-R. 5, P.-R. 6; with regard to their action on salicine, inosite, sorbite, dulcitate, adonite, litmus milk, and Sabouraud's maltose agar, original is < G.-P. 7 < P.-R. 3, P.-R. 4, P.-R. 5, and P.-R. 6, and the same holds with regard to nitrite formation; the action on gelatin, broth and glucose broth is of the same nature in all. On potato the growth of original is < G.-P. 7 < P.-R. 3, P.-R. 4, P.-R. 5, and P.-R. 6; but in all the growth is of the same nature—circumscribed, dry, raised, granular, and of a tint slightly deeper than the potato itself.

It would appear, then, as if the reactions on these media differed in quantity rather than in quality, and that the culture G.-P. 7 stands somewhere intermediate between the original strain and the rabbit strains. The growth of the individual colonies of the organisms on agar lends support to this view, for there the colonies of P.-R. 6, say, though differing greatly in size from those of the original strain, are of the same naked-eye appearance (Plate V. Fig. 1). When stained films of P.-R. 6 from any of the media are compared with similarly stained films of the other strains from similar media, they are found also to have the same appearance. Especially is this the case when films from the peritoneal fluid of inoculated guinea-pigs are examined. Here the typical "bipolar" staining bacilli or "girdle-form" bacilli are found in both cases. If, however, fresh films of the original strain and P.-R. 6 are examined, P.-R. 6 is found to be motile, while the original strain is non-motile. Corresponding with this P.-R. 6 has flagella, while the original strain has none (Plate VI. Fig. 9). This modified organism gives, therefore, the reaction of a member of the coli group, but it does not correspond to any described by MacConkey (1909¹) in his work on the lactose-fermenting bacilli. The nearest approach to it in MacConkey's table is the bacillus No. "71"; *B. lactis aerogenes* and Friedländer's pneumobacillus only differ from it slightly.

Having regard to what has just been discussed, it seemed important to repeat this experiment, and this was accordingly done, starting again from the original strain of *B. avisepticus*. The following is the protocol:—

G.-P. 1 (January 8, 1915).—G.-P. 1 inoculated intraperitoneally at 10.30 a.m. with a 24-hours' agar culture of original strain; animal nearly dead at 4.30 p.m.; killed with coal gas.

G.-P. 2 (January 9, 1915).—G.-P. 2 inoculated intraperitoneally at 10.45 a.m. with culture made from peritoneum of G.-P. 1; animal very ill at 7 p.m., and killed with coal gas.

G.-P. 3 (January 11, 1915).—G.-P. 3 inoculated intraperitoneally at 10.10 a.m. with culture made from heart blood of G.-P. 2; animal very ill at 3 p.m., and therefore killed.

G.-P. 4 (January 13, 1915).—G.-P. 4 inoculated intraperitoneally with culture from heart blood of G.-P. 3 at 10.30; animal very ill at 7 p.m., and therefore killed.

G.-P. 5 (January 15, 1915).—G.-P. 5 inoculated intraperitoneally with culture from heart blood of G.-P. 4; animal very ill at 4.30, and therefore killed.

G.-P. 6 (January 18, 1915).—G.-P. 6 inoculated intraperitoneally with culture from heart blood of G.-P. 5 at 4.30 p.m.; died during the night.

G.-P. 7 (January 20, 1915).—G.-P. 7 inoculated intraperitoneally at 10.45 a.m. with one culture from heart blood of G.-P. 6; this guinea-pig killed 21st January at 4 p.m.

G.-P. 8 (January 23, 1915).—G.-P. 8 inoculated intraperitoneally at 4.30 p.m. with one heart blood culture of G.-P. 7. Animal died during the night.

G.-P. 9 (January 26, 1915).—G.-P. 9 inoculated intraperitoneally at 4.30 p.m. with one agar culture from peritoneal fluid of G.-P. 8; animal died during the night.

G.-P. 10 (January 28, 1915).—G.-P. 10 inoculated intraperitoneally at 4 p.m. with one culture from peritoneum of G.-P. 9; died during the night.

G.-P. 11 (January 26, 1915).—G.-P. 11 inoculated intraperitoneally at 5 p.m. with fluid from peritoneum of G.-P. 10; alive, but very ill next morning; killed.

G.-P. 12A (January 30, 1915).—G.-P. 12A inoculated intraperitoneally with small amount of fluid from peritoneum of G.-P. 11; this animal lived.

G.-P. 12B (February 1, 1915).—G.-P. 12B inoculated intraperitoneally with 24-hours' agar subculture from peritoneum of G.-P. 11; died during night.

G.-P. 13 (February 2, 1915).—G.-P. 13 inoculated intraperitoneally with peritoneal fluid of G.-P. 12B at 4.30 p.m.; died during night.

G.-P. 14 (February 3, 1915).—Inoculated intraperitoneally at 4.30 p.m. with peritoneal fluid of G.-P. 13; died during night.

G.-P. 15 (February 4, 1915).—Inoculated at 4.30 p.m. intraperitoneally with peritoneal fluid from G.-P. 14; died during the night.

G.-P. 16 (February 5, 1915).—G.-P. 16 inoculated intraperitoneally at 4.30 p.m. with peritoneal fluid of G.-P. 15; died during the night.

During the whole course of this experiment, cultures were made into glucose litmus peptone water from the peritoneal fluid of each individual case, and the formation of acid and gas after incubation, as indicating an increase in biological activity, was looked for. Acid alone was found in all the cases until G.-P. 14 was reached, when a small amount of gas appeared in the glucose tube. This gas formation was markedly increased in cultures made from G.-P.'s 15 and 16. The culture from the peritoneum of G.-P. 16 was then examined microscopically; it was found to be pure, and to consist of polar-staining bacilli; it was motile with flagella, when plated out it was pure, consisting of one organism only, and when tested on the various media, it gave the reactions of P.-R. 3, P.-R. 4, P.-R. 5, and P.-R. 6.

The organism was never plated out during the whole *passage*, the next injection being carried out either with the peritoneal fluid directly or with whatever grew from it; hence there was no chance of a possible contamination being picked off by mistake for the *B. avisepticus*, cultivated and used to continue the experiment. When G.-P. 10 had been arrived at, and no alteration of the properties of the bacillus had been observed, we compared our present technique with that used in the previous experiment to find out in what way it differed. We then found that whereas in this experiment we were inoculating the next guinea-pig from a culture made from the *heart blood* of the previous guinea-pig and after it had been incubated for *two days* at 37° C., in our previous experiment we had been inoculating as far as possible

directly from the peritoneum of one guinea-pig into the peritoneum of the next, without any intervening culture on artificial media. This latter method was, therefore, now adopted here, and the characters of the organism rapidly changed.

We have no data in our first experiment from which we can tell how rapidly this transformation took place, beyond that it happened in *passage* through seven guinea-pigs. Here, however, the culture of G.-P. 8 conducted itself on the various media exactly like the original strain; G.-P. 9 produced acid, but no gas; G.-P. 10, G.-P. 11, G.-P. 12A, G.-P. 12B, G.-P. 13, did the same; while G.-P. 14 began to show acid and gas. It is impossible to say exactly what happens during this change in the characters of the organisms—whether all the organisms become *pari passu* virulent; or whether there is a weeding out by the various passages of the weaker varieties possibly present in the original strain, and a concentration thus of the hardier varieties. We noticed that in the isolated colonies growing from the heart blood of G.-P. 13 there were differences in size. This might indicate a collateral existence in one culture of organisms of different vitality. In this connection, however, we plated out the original culture, subcultured four colonies selected at random, and found that there was practically no difference in the reactions on various media of these four different strains. It seems possible, however, that both the processes mentioned above may be at work in this heightening of virulence and biological activity of an organism. It was found further that, until G.-P. 14 was reached, the cultures from the heart blood of the guinea-pig when examined after incubation for eighteen hours at 37° C. contained only a few discrete colonies. It was for this reason, when we were *passing* at the beginning by means of heart blood cultures, that we had to shake the condensation water up over the surface of the agar, and incubate for another twenty-four hours to get a sufficient quantity of growth for inoculation. After G.-P. 14 was reached, however, the growth in the heart blood in eighteen hours covered the whole surface of the agar. In a similar way in our first experiment we found that, while previous to this critical point we could not detect the organism in films from the heart blood, after we had passed it we found the organism easily. It should be noted, further, that the animals died much more rapidly after this point was reached. These considerations would appear to indicate that the culture was becoming more virulent. It should be further noted that the organism only became motile in the peritoneal fluid after the crucial point just mentioned was reached.

It would seem possible, therefore, that in these two series of *passage* experiments we have succeeded, not only in increasing the virulence of the original organism, but, at the same time and parallel with this, in modifying in several ways other activities of this original strain. On all the artificial media its growth becomes more robust, and its fermenting powers more fully developed. Intermediate forms between

the original strain and the final strain have been shown by us to exist (e.g., G.-P. 7, Experiment I.). The organism has become motile, and now possesses flagella. It, however, maintains the characteristic bipolar appearance, especially in peritoneal exudates, and the character of its growth on media such as agar and potato, although more vigorous, is unchanged in quality.

THE DESCRIBED REACTIONS OF THE ORGANISMS OF THE HÆMORRHAGIC SEPTICÆMIA GROUP.

It might be well now to consider the reactions of organisms of this group, as they are described in such a general review as is found in Hutyrá's article on "Septicæmia Hæmorrhagica" (1912²), of which the following is a summary.

B. bipolaris septicus (Flügge); *B. bipolaris pluri-septicus*; *B. bipolaris pluricida* (Kitt); *Pasteurella* (Lignières); coccobacillus ovoides or girdle bacterium are its synonyms. In its typical form, as one finds it in the body juices, and especially in the blood of cases of chicken cholera, it is an organism about 1 μ long by 0.5 μ broad. It stains with watery solutions of the aniline dyes intensively at the poles, but only weakly or not at all in the middle. It does not stain by Gram's method, is non-motile, and does not form spores.

The organism, even in the same strain, varies greatly in appearance, size, and shape on artificial media. Amongst other things, one may get uniformly staining small coccid forms; more often one gets larger forms either polar staining or stained at the periphery, forming the "girdle" variety; others showed undoubted and marked polar staining and formation of long chains. As the culture gets older polar staining diminishes, and at the same time the body of the bacillus takes on the stain less well. At a later stage one finds that very few of the organisms are at all stainable. The culture now consists largely of fine granules, the remains of the broken-down bacteria (cf. Plate V. Fig. 3).

Cultural characteristics.—In acute cases of the disease the organism is usually easily obtained, but there is often difficulty in chronic cases. This is due possibly to the small number of organisms present in such cases, or it may be due to the inability of such organisms to grow on artificial media. Where culture on artificial media has failed to show its presence, one can often isolate the organism from such cases by injection of highly susceptible animals. They grow well at room temperature or body temperature, all are aerobic, very slight growth taking place under anaerobic conditions. Cultures several days old are strong and tenacious in consistence; some think this is due to capsule formation. They do not liquefy gelatin, they grow best on media with an alkaline reaction. Cultures on gelatin and agar are not characteristic. Addition of blood serum to media encourages growth, but addition of glucose or glycerin has no marked effect in this direction. There is no growth on endo-agar or malachite-green agar, they grow on Drigalski's medium without change of colour. They grow well on media containing blood; hæmolysis is not produced. The growth on solidified blood serum is much the same as on agar. They sometimes grow on potato, sometimes not, this depending on whether the potato is alkaline or acid. On this medium it forms a greyish-yellow growth. According to Bunzyl-Federn, the chicken cholera and rabbit septicæmia organisms (members of this group) grow well on untreated potato, i.e., on potato not rendered alkaline. Growth on broth is not characteristic, addition

of blood serum to broth causes growth to be larger. There is only slight growth in milk, without change of reaction and without coagulation.

Cultures inoculated with material from diseased animals can go on for a year without change of their morphological characters, provided they are subcultured every eight days. In spite of this, however, it often happens that cultures of even a few days' growth, notwithstanding that they are in apparently good cultural conditions, die out entirely without demonstrable cause. The reactions of this group of organisms on the various sugars, on formation of indol and reduction of nitrites, are treated of by Hutyra in a footnote. He states that with regard to the action on carbohydrates one may say that these bacteria produce no or, at most, very little acid. Smith and Joest (quoted by Hutyra) found slight acid production in milk, in which the swine plague organisms were grown; and Bunzyl-Federn also observed this in the case of the organisms of deer disease and swine plague. He, however, found a much stronger acid production in the case of the chicken cholera and rabbit septicæmia organisms, going the length, as it did, of clotting of the milk. As regards the amount of acid produced, litmus media with these organisms are not turned red, according to Hutyra, nor is gas formed in sugar-containing media. Indol is formed at times, and nitrates are occasionally reduced to nitrites, if good growth takes place.

Several points ought to be noted in connection with this description by Hutyra of the character of organisms of this group. Speaking broadly, there is nothing which would appear to be characteristic for them microscopically or culturally except the microscopic appearance in the body fluids. In artificial media the microscopic appearances would seem to vary so much that it is impossible to rest any part of a diagnosis on them. According to Hutyra, none of the growth reactions on artificial media would appear to be in any way characteristic. With regard to the growth on potato, which we consider of some little diagnostic importance, he mentions that growth does not take place on it unless it is rendered alkaline. Bunzyl-Federn, however, in direct opposition to this, states that he got chicken cholera and rabbit septicæmia to grow on untreated potato. The same has been our experience with chicken cholera. Our original strain grew fairly well on ordinary untreated potato, and as the organism became more virulent it grew better. We lay some stress on this growth on potato in the identification of members of the group, at least as far as the differentiation of chicken cholera is concerned. It is circumscribed, dry, granular, slightly raised, and of a colour slightly deeper than the potato.

Hutyra mentions, as characteristic, the ease with which some strains of this organism die out, apparently without any cause. When working with the *B. ovisepticus* from cases of braxy, one of us (J. P. M'Gowan) found that the medium *par excellence* for such an occurrence was blood-smear agar, due in all probability to the lytic action of the serum on the bacteria. Very little importance is attached by Hutyra to the carbohydrate reactions of organisms of this group, due in all probability to the fact that on the Continent little attention is given to these substances as a means of differentiating organisms.

Very varied statements are accordingly made, but the gist of Hutyra's view would appear to be that these organisms, as a rule, do not produce acid in any of the carbohydrate media. This is contrary to our experience, for in the organism freshly isolated from a case of the disease, if not acid and gas, at least acid production may take place in the following carbohydrates, amongst others—lactose, glucose, mannite, maltose, dulcitol, cane sugar, raffinose, dextrin, sorbitol, adonitol.

It is usually only when the organism has been isolated from a chronic case of the disease (as we found in a strain of swine plague) or after the organism has been cultured for years on artificial media (as in a strain of *B. orisepticus*) that its reactions conform to Hutyra's views. Bunzyl-Federn found the production of acid and clot in litmus milk with the chicken cholera organism. This corresponds, as will be seen, with what we found in our intensified strain of chicken cholera (Table I.), and prepares the way for consideration as to whether organisms of this intensified type do not occur in natural disease conditions to be dealt with.

WHY HAS ASSOCIATION OF INCREASED VIRULENCE WITH INCREASED BIOLOGICAL ACTIVITY NOT BEEN PREVIOUSLY OBSERVED ?

The question may now be raised as to why this association of increased virulence with raised biological activity has not been previously observed. In all likelihood this is due to the fact that when the possible methods that could be used for ensuring the purity of the culture at each stage of the *passage* of an organism of this group are examined with regard to their practical utility, and even with regard to possibility of use, they resolve themselves into microscopic examination of the peritoneal fluid for purity of the film, and examination of a culture from the peritoneal fluid, naked eye, to see if it appears pure. Neither of these would give any indication of increased biological activity, and unless some special circumstance (as in our case the comparison of the size of single colonies of the original and intensified strain) riveted the attention and caused further inquiry, the phenomenon would be missed. The sugar reactions, again, are, as has been said, not much used by those continental workers who have given most attention to the investigation of the group; and in any case where these media are employed, the production of acid and gas, other things being equal, by an organism which formerly had produced acid only or no change, would rather raise the question of a possible contamination than direct attention to an increased biological activity of the original organism.

QUESTION OF RETROGRESSION OF THE ORGANISM WITH REGARD TO THESE ACQUIRED CHARACTERISTICS.

The organism obtained by *passage* in the first experiment has been

tested several times with the object of observing if it would undergo any retrogression in its characters. The particular strain used was P.-R. 3, which was obtained from a rabbit on 24th November 1914. Since that time it has been tested five times on the various media, namely, on 6th January, 13th January, 20th January, 31st January, and 12th February. Very little change has taken place in its reactions during this time, with the exception of a possible failure now to ferment salicine with acid and gas formation. During the time mentioned the organism has been kept in a cupboard, exposed to light. The test media used at the various times were not inoculated from the original culture made on 24th November, but from a descendant of this got by subculturing this about four times a month.¹

	G.-P. 7.		P.-R. 6.	
	December 25, 1914.	February 27, 1915.	December 25, 1914.	February 27, 1915.
Galactose . .	AG	AG	AG	AG
Maltose . .	AG	A	AG	AG
Salicine . .	A	0	AG	0
Dextrin . .	AG	AG	AG	AG
Cane . . .	AG	0	AG	AG
Glucose . .	AG	AG	AG	AG
Lactose . .	AG	AG	AG	AG
Raffinose . .	AG	0	AG	AG
Mannite . .	AG	AG	AG	AG
Dulcite . .	A	0	AG	A ?
Adonite . .	0	0	A	0
Inulin . . .	0	0	0	0
Litmus milk .	A	A	AC	AC
Nitrites . .	+	+	+	+
Indol . . .	+	+	+	+
Motility . .	?	0	+	0

AG = acid + gas formation ; A = acid formation ; AC = acid + clot formation ;
? = doubtful ; + = presence of ; 0 = absence of or no change in.

¹ Since this was written the organisms from G.-P. 7 and P.-R. 6 have been tested, and the following is a comparison between their reactions now and those given by them in Table I.

It would appear, therefore, that very little alteration in the biological activities has taken place in this strain during about three months under the conditions mentioned. We have an instance of another organism of the same group which shows a similar absence of change when kept over a longer period—five and a half months. In this case the organism was the *B. bovissepticus*, obtained in pure culture from the blood of a case of typical hæmorrhagic septicæmia in cattle. The typical bipolar form was found in smears from the peritoneal fluid of an inoculated guinea-pig. At the time of isolation it fermented, with acid and gas formation, the sugars used above for *B. avisepticus*, with the exception of inuline, inosite, and adonite, and in January 1915 it gave the same reactions. Meantime it had been exposed to the light on agar slope tubes and subcultured irregularly about every month.

EFFECT OF IMMUNISING RABBITS WITH ORIGINAL AND P.-R. 6 STRAINS,
WITH RELATION TO AGGLUTINATION OF THESE STRAINS WITH THE
SERA THUS OBTAINED.

In this case rabbits were injected several times with the original strain; others were similarly injected with the P.-R. 6 strain, and at the end of about three weeks their sera were tested with regard to their agglutinating power relative to the original and P.-R. 6 strains. Normal control rabbit sera were also employed; agglutination tests were made microscopically and readings were taken after half an hour.

TABLE II.—*Agglutination Reactions.*

(A) Rabbits vaccinated with Original Strain.				
Number.	ORIGINAL STRAIN.		P.-R. 6 VIRULENT STRAIN.	
	Dilution, 1 in 2.	Dilution, 1 in 20.	Dilution, 1 in 2.	Dilution, 1 in 20.
1	C	C	?	0
2	C	$\frac{1}{2}$ C	C	0
3	C	C	0	0
(B) Rabbits vaccinated with Virulent Strain P.-R. 6.				
1	0	0	C	$\frac{1}{2}$ C
2	?	0	C	C
(C) Normal Rabbits.				
1	?	0	?	0
2	0	0	0	0
3	0	0	?	0

C = complete; ? = doubtful reaction; 0 = no agglutination.

From these results it will be seen that normal rabbit's serum has practically no agglutinating power for either strain; further, that the serum of the animals inoculated with one strain had practically no agglutinating power for the other strain and *vice versa*. It is just possible that the rabbit *versus* original serum has slightly greater agglutinating power for the virulent strain than has the rabbit *versus* virulent strain for the original strain. Table II. shows only part of the dilutions which were actually used in the experiment—thus dilutions of 1 in 40 and 1 in 80 were also used, and the preparations were examined at frequent intervals up to eighteen hours. The results, however, of these extended observations were of the nature of those just mentioned in Table II.

PROTECTION CONFERRED BY IMMUNISING ANIMALS WITH THESE STRAINS
AGAINST A SUBSEQUENT INJECTION WITH EITHER STRAIN.

In this case the animals were vaccinated by several injections of the original or virulent strains killed by heat, and after a period of three weeks of such immunisation were tested by an intravenous injection of as nearly as possible an equal dose per body weight of the original or virulent strain as the case might be. The results are given in Table III.

TABLE III.—*Injection of Immunised Animals.*

Number.	I. Normal Rabbits injected with Virulent Culture P.-R. 6.	II. Rabbits vacci- nated with Original Culture injected with Virulent.	III. Rabbits vacci- nated with Original Culture tested with Original.	IV. Normal Rabbits injected with Original Culture.	V. Rabbit vacci- nated with Virulent Culture tested with Original.	VI. Rabbit vacci- nated with Viru- lent tested with Virulent.
1	Death in 4 hours.	Death in 24 hours.	Death in 72 hours.	Death in 96 hours.	Death in 5 days.	Death in 6 days.
2	Death in 5 hours.	Death in 5 hours.	Death in 96 hours.	Death in 24 hours.
3	Death in 24 hours.	Death in 5 hours.	Recovery.	Death in 48 hours.
4	Death in 2 hours.	Recovery.	Death in 100 hours.	Death in 24 hours.
5	Death in 24 hours.
6	Death in 4 hours.

From this table it would appear that the animals died in the following order after the test injection:—

1. Normal rabbits injected with the virulent strain.
2. Original strain—immunised rabbits injected with the virulent strain.
3. Normal rabbits injected with the original strain.
4. Original strain—immunised rabbits injected with the original strain.

5. Virulent strain—immunised rabbits injected with original strain.
6. Virulent strain—immunised rabbits injected with virulent strain.

The experiments are too few from which to draw all the conclusions that are adumbrated, but one conclusion which has a bearing on the present inquiry seems justified, namely, that the strain P.-R. 6 is more virulent *qua* death of the animal than the original strain, and further, that the virulent strain immunised better, not only against a subsequent injection of itself, but also against injection of the original strain than did the original strain.

CHARACTERS OF INDIVIDUAL BACILLI.

We now deal with some characters of individual bacilli—

- (a) In the original culture.
- (b) In the same after passed once through a normal rabbit.
- (c) In the same after passed once through a rabbit immune *versus* original strain.
- (d) In the same after passed once through a rabbit immune *versus* virulent strain.

We give the reactions on the sugars of—

- (a) Four cultures obtained from single colonies after plating out original strain.
- (b) Five cultures from single colonies obtained by inoculating agar tubes with the heart-blood of a normal rabbit forty-eight hours after it had received an intravenous injection of a culture of original strain.
- (c) One culture from largest colony appearing after twenty-four hours' incubation of agar tube inoculated with blood from rabbit (immunised against original strain) three days after it had received an intravenous injection of an original strain culture.
- (d) One culture from largest colony appearing after twenty-four hours' incubation of agar tube inoculated with blood from rabbit (immunised against virulent strain) three days after it had received an intravenous injection of an original strain culture.

We had observed (p. 23) that *passage* had an effect in increasing the size of the colonies of the organism as grown on agar, concurrently with increasing the biological activity. The experiments now given were therefore undertaken to see if any difference in size of the colonies occurred under the conditions above noted, and, if so, was this difference associated with increased biological activity in other directions.

[TABLE

TABLE IV.—*Reactions of Cultures from Single Organisms of Original and once passed Cultures.*

	GROUP A.				GROUP B.										GROUP C.		GROUP D.	
	(a)	(b)	(c)	(d)	Small.		Small.		Large.		Medium.		Large.		From Rabbit versus Original.		From Rabbit versus Virulent.	
	Large.	Large.	Large.	Small.	Small.		Small.		Large.		Medium.		Large.		From Rabbit versus Original.		From Rabbit versus Virulent.	
	48 hrs.	48 hrs.	48 hrs.	48 hrs.	48 hrs.	72 hrs.	48 hrs.	72 hrs.	48 hrs.	72 hrs.	48 hrs.	72 hrs.	48 hrs.	72 hrs.	24 hrs.	48 hrs.	24 hrs.	48 hrs.
Inulin . . .	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Dulcitol . . .	a	?	0	0	0	0	0	0	0	0	0	0	0	0	0	?	0	?
Lactose . . .	a	a	a	a	0	0	0	0	0	?	0	0	0	a	0	0	0	0
Glucose . . .	a	A	A	A	A	..	A	..	A	..	A	..	A	..	A	A	A	A
Saccharose . .	0	0	0	a	0	a	0	0	0	0	0	0	0	0	0	0	0	0
Maltose . . .	a	a	a	a	0	a	?	a	a	a	0	a	0	0	a	a	a	a
Mannite . . .	A	A	A	A	0	a	A	..	0	a	A	..	A	..	a	a	a	a
Raffinose . . .	0	0	0	a	0	a	0	0	a?	a	0	a	0	0	0	0	0	0
Dextrin . . .	-	-	-	-	-	-	-	-	-	-	-	-	-	-	a	a	a	a
Salicine . . .	0	0	0	0	0	0	0	0	0	0	0	0	a?	a	0	0	0	0
Galactose . . .	a	a	a	a	0	a	a?	a	0	0	0	a	0	a	a	a	a	a
Sorbitol . . .	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Adonite . . .	a	a	a	A	0	0	0	0	0	a	0	a	0	a	0	?	0	?
Inositol . . .	0	0	0	0	0	0	0	0	0	0	0	0	0	0	-	-	-	-
Peptone water, Indol.	-	-	-	-	0	0	0	0	0	0	0	0	0	0	-	-	-	-
Litmus milk . .	?	?	?	?	?	?	?	?	?	?	?	?	?	?	-	-	-	-
Nitrate water, Nitrites.	-	-	-	-	+	..	+	..	+	+	+	+	+	+	-	-	-	-

A = large amount of acid ; a = small amount of acid ; ? = doubtful change, production, etc. ;
0 = no change, production, etc. ; + = production of ; - = test not performed.

The points to be noted in connection with Table IV. are—

1. That there is no marked increase of biological activity in one group as compared with another.

2. That in any one group the influence of the size of colony is not definite. This is possibly due to the fact that the colonies differed only slightly in size from one another, and none of them were big. There was no such marked difference as occurs say between P.-R. 6 and original strains in this respect (see Table I.).

3. That in the four groups slight differences as regards capacity for fermenting certain sugars occur in the cultures, descendants as they are from single individual bacilli. These organisms differ in their reactions from those described in text-books, and, further, although classed as the same organism they even differ from one another.

4. The process of increasing virulence with concurrently increasing biological action by *passage* would appear not to be due to the presence of virulent organisms in a minority in the original culture, followed by a weeding out of the avirulent and a consequent concentration of the virulent by *passage*, although this may play a minor part in the final result; it would appear rather to be due to a gradual increase in virility of every individual organism, a process which, however, has not advanced far by one *passage* under the conditions mentioned above.

In concluding this section we would draw attention to the fact that not only in the rabbits just mentioned, but also in nine other rabbits into which we injected intravenously the original culture, did we obtain from the blood two and three days after injection "pure" cultures of organisms resembling the culture injected, that is to say, we did not find in these cultures an organism like P.-R. 6 *suddenly* appearing. In such a case there would have been a suspicion that this latter organism did not develop from the injected culture, but came from a previous infection in the rabbit. The non-occurrence of this, however, in so many cases, taken together with what has already been mentioned regarding the gradual transformation of the original culture into the final strain, and the purity of the cultures at the beginning and end of the *passage* experiment (apart from other points brought forward and to be brought forward), go a long way in support of our view that the final and more biologically active organism is in reality evolved by the process of passage from the original less active variety.

STRAINS OF *BACILLUS BIPOLARIS SEPTICUS* RESEMBLING THOSE PRODUCED BY *PASSAGE* OCCURRING IN NATURAL CASES OF DISEASE.

Under this heading we now give instances of the recovery of organisms resembling in their biological activity the strains produced above by *passage*. All these organisms were obtained in pure culture and extensive growth from cases which presented the clinical symptoms during life, and the post-mortem appearance after death of what is known as hæmorrhagic septicæmia. In this connection attention should be here drawn to the fact that many observers quoted by Rowland (1908³), and Dieudonné and Otto (1912⁴) have described organisms with the morphology of the plague bacillus (*B. pestis*) as causal of plague-like diseases in rodents, etc. Culturally these organisms gave the reactions of members of the bacillus coli group, such as *B. enteritidis* Gaertner, *B. lactis aerogenes*, and Friedländer's pneumo-bacillus.

Chicken Cholera in Pigeons.—In an epidemic among pigeons in July 1913, massive pure cultures were obtained by one of us (J. P. M'Gowan) from the heart-blood and spleen of two pigeons (presenting as above noted the clinical symptoms and post-mortem appearances of chicken cholera), of an organism with the characteristics given in Table V., No. 1. From the trachea of one of

these cases a practically pure culture of an organism giving the reactions of No. II. in Table V. was obtained.

The growth of this organism was much slighter than that of the heart-blood and spleen organism. It would appear, therefore, as if we had to deal with a more biologically active strain in the heart-blood and spleen and a less active one in the trachea. The blood and spleen strains would appear to be slightly less active than say P.-R. 6, in that they do not ferment lactose and cane sugar, and the tracheal strain would appear to be less active than the "original" strain of the above experiments in that it does not produce acid in so many sugars. Even then its resemblance to some of the strains in Table IV. is very striking.

Hæmorrhagic Septicæmia in the Sheep (Braxy).—The reactions of two strains obtained from the heart-blood from different cases are given in III. and IV., Table V.

Hæmorrhagic Septicæmia in Cattle.—The reactions of a strain of *B. bovis-septicus* obtained in pure extensive culture from the heart-blood of a case with symptoms and post-mortem appearances typical of hæmorrhagic septicæmia are given in V., Table V.

TABLE V.

	I. Chicken Cholera Heart-blood and Spleen (pure cultures).	II. Chicken Cholera Trachea. Same case as No. I.	III. Strain 1 from Braxy case.	IV. Strain 2 from Braxy case.	V. From Hæmorrhagic Septicæmia in Cattle.
Glucose . . .	A + G	A	A	A + G	A + G
Maltose . . .	A + G	A	A	A + G	A + G
Mannite . . .	A + G	0	A	A + G	A + G
Raffinose . . .	A + G	0	A	A + G	A + G
Galactose . . .	A + G	A	A	A + G	A + G
Dulcitol . . .	A + G	0	0	0	A + G
Dextrin . . .	A + G	A	—	A + G	A + G
Sorbitol . . .	A + G	A	A	A + G	A + G
Lactose . . .	0	0	0	A + G	A + G
Cane . . .	0	0	A	0	A + G
Salicine . . .	0	0	0	0	A + G
Inulin . . .	0	0	0	0	0
Adonite . . .	0	0	0	0	0
Indol . . .	?	0	0	+	+
Nitrites . . .	+	Trace.	Trace.	+	+
Gelatin . . .	No liquefaction.	No liquefaction.	No liquefaction.	No liquefaction.	No liquefaction.
Potato . . .	Yellowish growth.	0	0	Yellowish growth.	Yellowish growth.
Litmus milk .	0	0	0	A	Acid + clot.
Morphology .	Bipolar cocco- bacillus. Present.	Bipolar cocco- bacillus. Absent.	Bipolar cocco- bacillus. Absent.	Bipolar cocco- bacillus. Present.	Bipolar cocco- bacillus. Present.
Motility . . .					

A + G = acid and gas formation ; A = acid formation ; ? = doubtful presence ;
0 = no change ; + = present in marked quantity ; — = test not performed.

In regard to this section the resemblance of some of the organisms described to members of the *B. coli* group is evident. Taking into consideration what we have shown with regard to the transformation of *B. avisepticus* and the clinical symptoms and post-mortem appearances of the diseases just described, we would hazard the opinion that the organisms isolated from these cases are in reality modified hæmorrhagic septicæmia organisms. The fact that they were obtained in pure culture from typical clinical and pathological cases of hæmorrhagic septicæmia further strengthens us in this belief.¹

PLEOMORPHISM OF ORGANISMS OF HÆMORRHAGIC SEPTICÆMIA GROUP.

We have already given evidence (p. 34) of the possibility of a reactional variability in cultures derived from different bacilli constituting what is called a single strain of *B. avisepticus*. We have further observed this variability of biological activity among different *strains*, derived from different sources, of the same organism, whether it be that of chicken cholera, sheep septicæmia, or swine plague. These variations (speaking more especially with regard to sugar reactions) may consist in a lack of power in some strains to ferment any of the sugars: on the other hand, they may act upon them in many different combinations, changing some and leaving others unchanged. Such inconstancy of action would appear, as has been mentioned, to account in some measure for the hazy and altogether indefinite description of the reactions of such organisms on the carbohydrates as is usually given in the text-books.

Not only does this group of organisms vary greatly with regard to its biological activity in the circumstances above mentioned, but it also varies greatly in its power of altering its shape. Rowland (1914⁵) has drawn attention to the great pleomorphic power of the plague bacillus, and states that probably no organism presents so marked a pleomorphism as the bacillus of plague. We propose, in order to link up more closely the *B. pestis* to the hæmorrhagic septicæmia group, to show here the pleomorphic capacities of the *B. ovisepticus* which are very marked, and of the *B. avisepticus* which are by comparison feeble. So great are they in the case of a single strain of the *B. ovisepticus* grown on two different media, say glycerin agar and salt agar, that it is often only by the previous history or by subsequent injection of the two intraperitoneally into guinea-pigs that one can be sure that the organisms on the two culture media are the same. Looked at broadly this pleomorphism is itself a biological activity and indicates a mobile protoplasm capable of being easily

¹ Since this was written, one of us (J. P. M'Gowan) has observed many more instances of these atypical organisms in cases of chicken cholera and hæmorrhagic septicæmia in the sheep.

moulded by external influences. This latter consideration has possibly a bearing on the great change produced in its activities by *passage*.

It is not proposed at this point to give a description of all the various pleomorphic changes, for this would be a rehearsal of the letterpress accompanying the plates at the end of this article illustrating these changes (Plate V. Figs. 2-5 and Plate VI. Figs. 6-8); but in regard to *B. ovisepticus*, attention may be drawn to the sharply defined and uniform character of the organism in films from the peritoneal fluid; to the long thread-like forms in the salt agar films (Plate V. Fig. 4); to the swollen globular forms on glycerin agar (Plate V. Fig. 5); to the granular detritus, remains of bacilli on blood agar (Plate V. Fig. 3); and to the general lack of uniformity of the organism in films made from sources other than the guinea-pig's peritoneum. The pleomorphism is not so great in the case of chicken cholera, but attention may be drawn to long irregular bacillary forms in some cultures and to the swollen "yeast-like" elements in films from glycerin agar cultures (Plate VI. Fig. 8).

It will be seen that the pleomorphism exhibited by the *B. ovisepticus* is possibly quite as marked as that shown by *B. pestis*. It is not proposed at this stage to go into all the resemblances between *B. pestis* and the members of the hæmorrhagic septicæmia group. The fact that they exist, should, however, be noted here, and it might be well to draw attention to the further possible resemblance between the two, perhaps in some way based on this pleomorphism, namely, that different strains of either organism have a similar erratic behaviour toward the carbohydrate media.

SUMMARY AND CONCLUSIONS.

In this paper we have shown how an organism of the hæmorrhagic septicæmia group, by an increase of virulence through *passage*, has also concurrently been endowed with an increased biological activity on artificial media, whereby it grows much faster on these media and produces acid and gas fermentation in some carbohydrate media where it only produced acid or no change previously.

It would seem to be essential for the success of this *passage* that the organism be taken directly from one animal to another without intervening growth on artificial media.

Retgression of these newly acquired biological activities would appear to be slow. With the treatment mentioned in the text there was little change in one case, even after three months. In another case the change seemed to be slightly more rapid.

The sera obtained by immunising rabbits with the original and virulent strains respectively, while agglutinating their own strains well, do not, or only very slightly, cross agglutinate. The immunity to a subsequent injection, both of the virulent and of the original

strain, appeared to be much greater after the preparatory injection of the virulent strain than after a similar injection of the original strain.

The individual bacilli which go to make up the original strain of the *B. bipolaris avisepticus*, though showing slight and well-defined differences, would appear all to be very nearly alike in their biological reactions. This holds too after the organism has been once passed through a normal rabbit, a rabbit immune *v.* original culture, or a rabbit immune *v.* virulent culture. The process, therefore, of rendering a strain virulent would appear not to consist of a weeding out of feeble elements and a concentration of the resultant strong, but rather a gradual tuning up all round of all the organisms of the original strain.

We have instanced several examples where naturally occurring diseases having all the clinical symptoms and post-mortem appearances of hæmorrhagic septicæmia were produced by organisms similar in character to the virulent variety of *B. avisepticus* just discussed. Thus we have shown this to be the case in hæmorrhagic septicæmia of the fowl, sheep, and ox.

We have drawn attention to the marked pleomorphism of members of the group, and instanced this especially by the occurrences in *B. ovisepticus bipolaris*. We have shown that it is less marked in *B. avisepticus*. We have further drawn attention to the fact that pleomorphism is possessed by the bacillus of human plague to the same marked degree as in *B. ovisepticus*.

REFERENCES.

1. MACCONKEY *Journ. Hyg.*, Cambridge, 1909, vol. ix. p. 86.
2. HUTYRA Kolle and Wassermann's "Handbuch der Path. Microorg.," Jena, 1912, Bd. vi. S. 67.
3. ROWLAND (PLAGUE ADVISORY COMMITTEE) *Journ. Hyg.*, Cambridge, 1908, vol. viii. p. 304.
4. DIEUDONNÉ AND OTTO Kolle and Wassermann's "Handbuch der Path. Microorg.," Jena, 1912, Bd. iv. S. 155 *et seq.*
5. ROWLAND *Journ. Hyg.*, Cambridge, 1914, vol. xiii. (Plague Supplement, iii.) p. 418.

DESCRIPTION OF PLATES V.-VI.

PLATE V.

FIG. 1.—48-hours' agar growths of P.-R. 6 (1) and original strain (2) showing the great difference in size of colonies of (1) as compared with (2). (Natural size.)

FIGS. 2, 3, 4, 5.—Films of *B. bipolaris ovisepticus*, fixed with heat, stained with carbol thionin. ($\times 1000$.)

FIG. 2.—24-hours' growth on blood agar.

FIG. 3.—96-hours' growth on blood agar showing granular degeneration.

FIG. 4.—24-hours' salt agar subculture of culture from which Fig. 3 was prepared. Note the long filaments.

FIG. 5.—24-hours' growth on glycerin agar: no bacillary forms; organisms broken up into granules or swollen into little round balls.

PLATE VI.

FIGS. 6, 7, 8.—Films of *B. bipolaris avisepticus*, fixed with heat, stained with carbol thionin. ($\times 1000$.)

FIG. 6.—Film from 24-hours' growth on blood agar.

FIG. 7.—Film from 24-hours' growth on salt agar: note the increase in size of the bacteria.

FIG. 8.—Film from 48-hours' growth on glycerin agar: note the great increase in size of the bacteria.

FIG. 9.—P.-R. 6, stained for flagella. The flagella are peritrichal and number about six or seven. ($\times 1000$.)

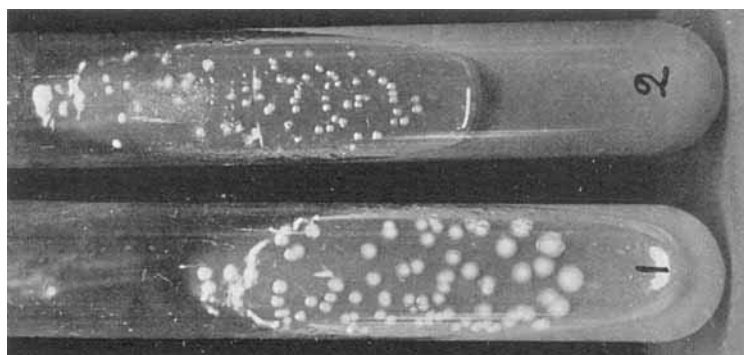


FIG. 1.

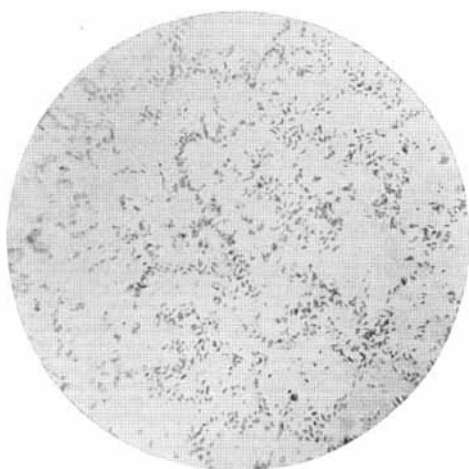


FIG. 2.

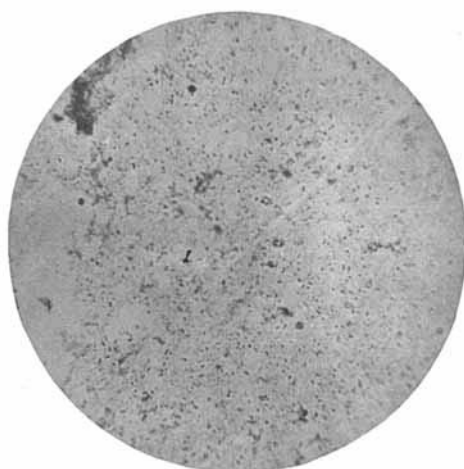


FIG. 3.

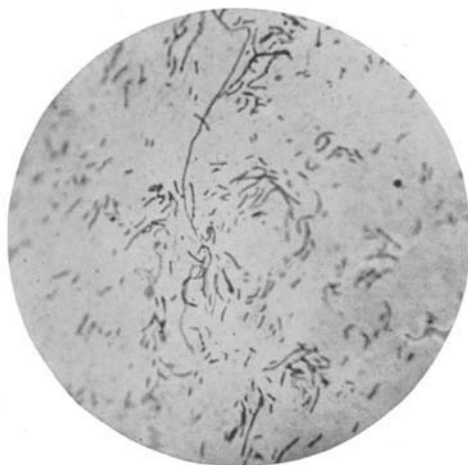


FIG. 4.

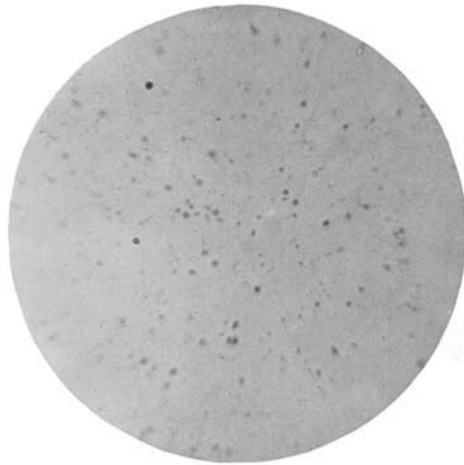


FIG. 5.

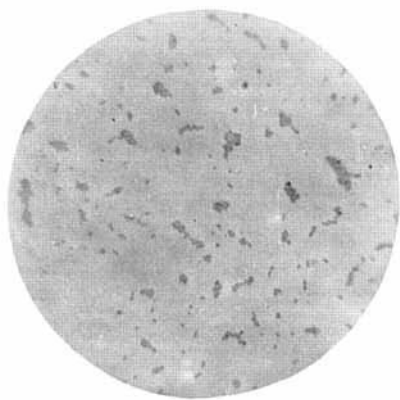


FIG. 6.

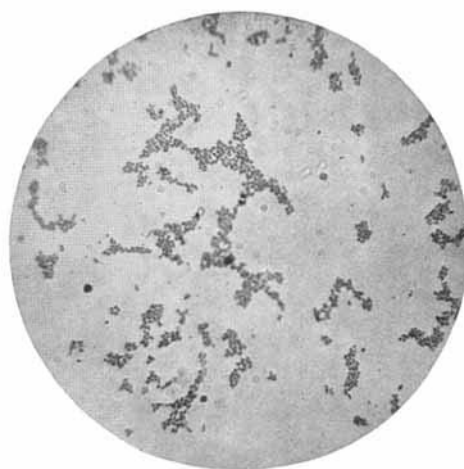


FIG. 7.

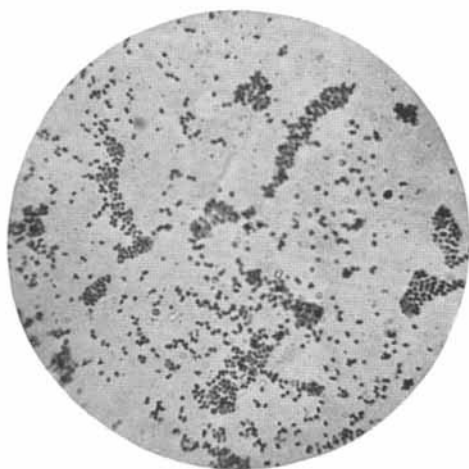


FIG. 8.

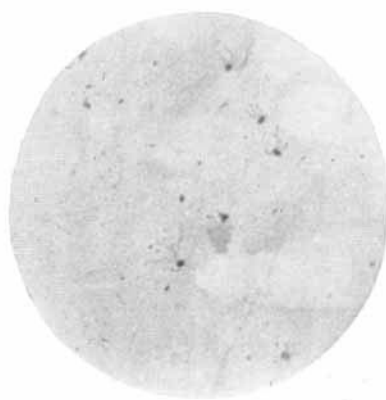


FIG. 9.