OBSERVATIONS ON THE EVOLUTION OF IMMUNITY IN DISEASE.

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CONTENTS.

I. Introduction.

The following experiments constitute an attempt to follow out in detail the stages by which immunity is established, in the course of a generalised bacterial infection (Pseudotuberculosis of rabbits). Two

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aspects of immunity are considered, firstly the presence, in the circulating fluids, of specific antibacterial substances, and secondly the power of rapidly producing such substances in answer to the specific stimulus. That is to say, attention is directed, not only to the quantity of specific antibodies present on any day of the disease, but also to the response which the animal can make to various doses of bacterial vaccine. For the immune animal is both more vigorous and more sensitive than the normal, in its reaction to a renewed dose of poison (Wassermann and Citron, 1905). Naturally the facts established with regard to one disease only, cannot be predicated at once of other diseases, in other animals. Still it is hoped that the systematic study of one disease may give some help in coordinating the large but somewhat disjointed mass of clinical observation which is already available.

It is not a new observation that, in the course of the severest bacterial infections, the early days are marked by the absence of any specific reaction on the part of the patient. The general inflammatory reaction is present but no specific antibodies are formed. After a shorter or longer period of delay, more or less immunity is developed. One may therefore distinguish two stages in the course of an infection.

(*a*) A period of inertia, as regards the formation of antibodies.

(*b*) A period of immunising reactions.

Pneumonia affords a good example of this distinction. J. G. Macdonald (1906), working with Bulloch, showed that, in this disease, the opsonic power of the blood remains low for about a week after the onset. At the end of this period, there occurs a decided rise in the amount of pneumococco-opsonins, which is followed regularly within a few hours, by the crisis. Wolf (1906) obtained similar results. In other diseases the onset of an immunising reaction may be neither so sudden nor so decidedly effective, yet the two stages may be recognised, as in the case of enteric fever. It is well known that the agglutinating power of the serum, which marks one of the specific reactions of the organism to this infection, is generally delayed in its appearance till about the tenth day. Malta fever offers another example of such delay, as in this disease the agglutination reaction is not obtained before the fifth day, and may remain absent till a much later period[1]. Reference may also be made to the streptococcal infection which seems to be a constant feature of scarlet fever. It was shown independently in America and England, by Hektoen (1907) and Banks (1907) that, in

[1] Aldridge (1898), Bert and Lamb (1908), Durham (1898).

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the first stages of this disease, the streptococco-opsonic index of the patient was generally low, but that a rise occurred in connection with defervescence, though not before the fourth day. Long continued lack of any immunising reaction has been observed by Wright (1907) in various septicaemic conditions, such as streptococcal endocarditis and Malta fever. The same paper shows how the opsonic immunity produced in response to such infections may be fluctuating and inefficient when it has at last arrived. These results in so far as they relate to Malta fever are confirmed by Bassett-Smith (1907).

In order to examine into the production of these phenomena more closely, experiments were made on the following lines:

The opsonic response following the inoculation of killed cultures into normal rabbits was investigated, and it was found that different tissues possessed different powers of response to these inoculations. Then, the effect of injecting living cultures into normal rabbits was determined, and after this the effect of inoculations of killed cultures into previously infected rabbits. The reaction of the infected rabbits towards inoculations of killed cultures was found to be profoundly altered during the course of the disease. A further examination was then made of the respective activities of living and killed cultures, in exciting a response in rabbits already infected with the disease, and recovering from this infection.

After describing these experiments, the present paper concludes with a discussion of the factors which influence the immunising reactions, which are observed in the course of the experimentally induced disease. The starting point and groundwork of these observations is provided by the work of Sir A. E. Wright and his fellow-workers, to which it is a pleasant duty to acknowledge my indebtedness.

II. GENERAL DESCRIPTION OF THE EXPERIMENTAL DISEASE.

The infective organism chosen was the *Bacillus pseudotuberculosis rodentium*, which is nearly related to that of plague (*B. pestis*). This bacillus is easily regained in pure culture from the blood of a rabbit or guinea-pig dead of the disease. Its discrete, opaque-white, colonies with crenated margins, are readily recognised; and the absence of contamination, after passage, was confirmed by the sugar reactions.

In the earlier part of the work a strain (designated P) was used which was obtained from the Pasteur Institute, early in 1907; more lately

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I have used a strain which was kindly sent me by Dr W. E. Marshall from the Lister Insitute in May 1908 (Strain M).

TABLE I.

Sugar reactions of B. pseudotuberculosis rodentium.

Sugar	Reaction	
	24 hours	48 hours
Lactose	—	—
Glucose	Acid	Acid
Maltose	Slightly acid	Acid
Mannite	Acid	Acid
Dulcite	—	—
Cane sugar	—	—
Inulin	—	—
Sorbite	—	—

The bacillus grows readily on ordinary agar, and an emulsion of bacilli washed off a 24 hours' agar slope culture, by means of broth or saline solution, formed the infective material for inoculation. A measured fraction of the emulsion obtained from one such culture was inoculated directly into the peritoneal cavity of the experimental animal. In rabbits the gravity of the infection may be determined almost at will, by varying the dose between $\frac{1}{100}$ of a culture, which dose the large majority of rabbits will survive, and $\frac{1}{5}$ which constitutes an almost certainly fatal dose. For guinea-pigs $\frac{1}{100000}$ of a culture was found to be an almost certainly fatal dose, death occurred between the 5th and 14th days. Using larger doses, up to $\frac{1}{30}$ of a culture, the fatal termination was found to arrive with considerable punctuality on the 6th or 7th day.

The intraperitoneal inoculation is followed by a general peritonitis, with a small quantity of turbid exudate. After two or three days the whole surface of the peritoneum, both visceral and parietal, is found to be studded with minute grey nodules, and the omentum is thickened and infiltrated. At this stage very few bacilli are to be found in the peritoneal fluid, but smears made from the peritoneum or omentum show large numbers of bacilli, for the most part contained within large mononuclear endothelial cells. Very soon the infection spreads, and small abscesses are formed in the spleen and liver. The small grey nodules previously noted on the peritoneal surfaces, tend to become absorbed, but some of them increase in size, and go on to the formation

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of abscesses, or caseous masses. All the abdominal glands become affected, and a large mass is often found at the root of the mesentery of the small intestine, composed of glands which have adhered to one another and undergone caseation.

In guinea-pigs the infection usually spreads to the thorax, where the mediastinal glands are found severely infected, and the lungs show numerous miliary abscesses. At death the blood always gave a pure culture of the bacillus.

The immunising reactions called forth by such an infection consist in, (1) an increase of opsonin, (2) the formation of agglutinin, (3) the formation of precipitin. No bactericidal or antitoxic substances can be demonstrated satisfactorily. It is not clear what importance attaches to the presence of agglutinin and precipitin, but it appears that an increase of opsonin is of real and vital significance in combating the disease (Part V, and Fig. 5). Consequently, in studying the fluctuations of the opsonic index, we are observing a manifestation of immunity which is of real importance in determining the fate of an infected animal. Furthermore, as will be shown below, the opsonic index gives much earlier information of an immunising response, on the part of the rabbit, than do observations of agglutination (Figs. 2 to 6, 15, 16, 18). The experience of Leishman and his colleagues of the Royal Army Medical Corps, in studying the effect of various antityphoid vaccines, also show the value of opsonic determinations in giving early information of the progress of immunising reactions[1]. And again the opsonic index has been used with success as a guide to therapeutic inoculations, so that in following its changes one was progressing along a road already partially explored. For all which reasons, in these experiments attention has been chiefly directed to the opsonic immunity.

III. Methods.

Estimations of agglutination were carried out macroscopically, in capillary tubes of about 1 mm. bore[2].

Opsonic estimations were made by Wright's method (1903) with the modifications which have been adopted in his laboratory as the result of much experience, since its first publication. I had the opportunity of working at St Mary's Hospital in the winter of 1907—1908, and became

[1] Leishman (1905, 1908), Harrison (1907).

[2] See Wright (1897), and Wright and Smith (1897).

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well acquainted with the work on which Fleming based his article on the accuracy of the method, and some of the sources of error (1908).

The corpuscular suspension used, was obtained from my own blood. About ·5 c.c. of blood was drawn from the finger into about 3 c.c. of a 1·5 per cent. solution of sodium citrate, and well shaken. This suspension was then centrifuged, and the corpuscles, after removal of the supernatant fluid, were shaken again with 3 c.c. of a ·85 or 1 per cent. solution of sodium chloride. After renewed centrifugation, and removal of the saline solution, the corpuscular sediment was thoroughly mixed. The whole of this material then afforded a uniform mixture of red corpuscles and leucocytes, which could be used to the very last drop, and sufficed, when necessary, for several dozen estimations.

Human corpuscles were used, because they were found to be more easy to work with than those of the rabbit, which are smaller and more granular, and which also have a rather awkward tendency to stick together in clumps. The human leucocytes were quite active in the presence of rabbit's serum, and gave excellent results. There was a slight tendency to phagocytosis of red blood corpuscles, but not enough to cause any inconvenience.

Guinea-pig's serum on the other hand is a very unfavourable medium for human leucocytes, and in the experiments on these animals, guinea-pig's leucocytes were used. The preparation of a satisfactory suspension of guinea-pig's leucocytes at first presented some difficulty, because the blood begins to clot very quickly. Hence if blood from the ear was allowed to drop into citrate solution many small masses of fibrin had time to form, and to these the leucocytes adhered. The masses of clot and leucocytes, thus formed, were all dragged to the end of the film in the process of spreading, and bad preparations were obtained. The method finally adopted with success was to draw blood from the heart directly into some citrate solution. This can be done quite readily as follows. Into a sterile 1 c.c. syringe are drawn up ·75 c.c. of citrate solution. Then, the guinea-pig being held for the moment fairly and squarely on its back, on a holder, the needle of the syringe is plunged directly into the heart, entering just to the left of the sternum in an intercostal space. ·25 c.c. of blood are then aspirated from the left ventricle, and the mixed blood and citrate transferred to a small tube, containing a further quantity of citrate solution. This tube is well shaken at once, and the rest of the process of washing the corpuscles is proceeded with exactly as in the case of human corpuscles. Suspensions thus prepared gave excellent results.

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The bacilli for the emulsion were removed by means of a platinum loop, from an agar slope culture, about 6 hours old, suspended in saline solution (·85 or 1 per cent.) and thoroughly mixed by the aid of a capillary pipette. This suspension was centrifuged for a minute or two, to precipitate clumps of bacilli, and the upper layers removed to a clean tube and mixed again, to insure uniformity. Emulsions were always prepared from young cultures (4 to 6 hours), as the bacilli are larger, stain better, and do not clump so readily.

The serum was obtained from the ear-vein of the rabbit to be tested. A capillary tube, drawn off to a fine point, forms the most convenient instrument with which to make a puncture. The blood was drawn into glass capsules containing about $\frac{1}{4}$ c.c.

IV. THE ACCURACY OF THE OPSONIC DETERMINATIONS.

The accuracy attainable in a series of opsonic measurements depends not only on the skill and patience of the observer and on the perfection of his methods, but on the species of the animal and microbe used. It is necessary therefore to enquire what degree of accuracy was actually attained in the experiments now before us.

1. *Accuracy of a single estimation.*

The accuracy of a single estimation is the first point to be considered. Having made one determination of the opsonic ratio between sera *A* and *B*, the question is how near this value is likely to be to the true value. Since the whole argument depends on the observation of *changes* in the opsonic indices of the experimental animals, it is of vital importance to be able to say, that the deviation of a single estimation from the true value will be *small in comparison with the changes which are to be observed.* In the absence of data for a complete mathematical statement, the following method of dealing with the problem may appear reasonable. In several series of observations the indices of all the pathological or inoculated rabbits were determined in duplicate, the counts, as also throughout the rest of these experiments, being made in ignorance of the source of every preparation. Of 210 such pairs of duplicate observations, only 2 show a divergence great enough to affect the form of the curve, of which each should define a point. Of the other 208 pairs, either member may be taken at will, or the curve may be drawn through the mean points, and those characters of the curves, from which deductions are to be drawn, remain entirely unaltered.

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Reference to Figs. 29, 30, 31, 37 to 45, and 50, 51, 52 will render this point clear.

2. *The variation of the control.*

It was found that normal rabbits showed considerable differences in the opsonic power of their sera. These differences were more or less constant, so that a rabbit which had a low opsonic power on one day, would present the same peculiarity for many weeks. This property was not, however, invariable. The normal count, on which the indices of other sera were to be calculated, was therefore obtained by taking the mean of the counts of three or four normal rabbit sera. In the experiments done at the Lister Institute three normal rabbits were used, in those done in Cambridge four. In each case these standard normal rabbits were chosen, at the beginning of a series of experiments, from amongst a considerable number of normal rabbits. The opsonic powers of the sera of the whole batch were first determined, and the normals for future use were selected as giving a fair mean sample of the batch. Under these conditions it was found that a satisfactory control was established. That is to say, no rabbit, when compared with this control, ever showed accidental variations of anything like the magnitude of those obtained after inoculation or in disease; and on the other hand, experiments could be repeated, and gave constantly the same results. The only exception to this, was in the case of inoculations of diseased animals, where the variable conditions of disease made it extremely difficult to obtain an *exact* repetition of a previous experiment.

Towards the end of these experiments one of the control animals exhibited considerable fluctuations of the index, which roused the suspicion that it had become infected accidentally. Its index then rose and was maintained at a high level for several days, confirming this suspicion (Fig. 27). The animal was therefore sacrificed, and it was found to show the changes typical of a naturally acquired infection. There were nodules in the lymphoid tissue of the vermiform appendix, and of the expanded lower end of the ileum, two small nodules in the liver, and caseating areas in the portal and mesenteric glands.

3. *The variation of the emulsion.*

It has been pointed out by Houston (1907) and Wright (1908)[1] that in the case of some microbes, especially the Meningococcus and *B. coli*,

[1] Wright, *Practitioner*, May 1908, p. 586.

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very different results may be obtained when bloods are tested with different cultures all derived from the same stock. "For in association with the attenuation which these microbes undergo upon artificial media they gradually become less and less resistant to the phagocytic attack of normal blood. A comparatively low index is now obtained where before a very high index was obtained, the result being of course simply due to an increase in the denominator in the fraction:

$$\frac{\text{phagocytic count of the patient's blood}}{\text{phagocytic count of the normal blood}} \text{ „}$$

This phenomenon is found to occur in connection with the *B. pseudotuberculosis*, as is shown in Figs. 1 to 4. For this experiment two cultures of the bacillus were used, (1) strain M, kept on artificial media, with frequent sub-culture, for seven weeks since isolation from its

Fig. 1. Rab. 10. Fig. 2. Rab. 6.

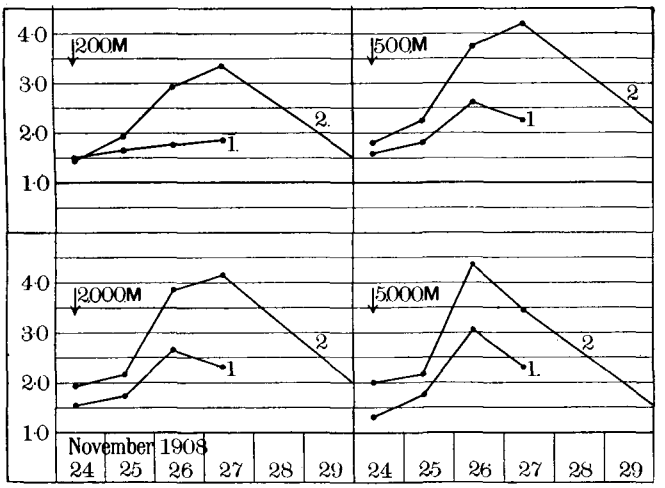

Fig. 3. Rab. 8. Fig. 4. Rab. 7.

Figs. 1 to 4. Effect of subcutaneous inoculations of vaccine, in rabbits recovering from a previous infection. Influence of attenuation of the culture used in making the opsonic determinations. Curve 1, in each figure, was obtained by using for the estimation of the index a stock which had undergone frequent subculture on agar for 7 weeks; Curve 2, in each figure, by using a stock of the same ancestry, but recently isolated from a rabbit.

Short arrows are used in all figures in this paper to mark the date of inoculations of killed cultures; long arrows denote inoculations of living cultures. The doses are given in millions of bacilli per kilogramme of body weight, or as fractions of a 24 hours' agar slope culture. For the relation between these two measures see Table II.

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last passage through a guinea-pig; (2) the same strain which during these weeks had passed through two rabbits, recently isolated from the second. Four rabbits were inoculated with a vaccine made from culture 2. Before inoculation (Nov. 24th), culture 2 gave a higher index than culture 1 in three cases, and a slightly lower index in one case. After inoculation, the indices all rose, but those obtained with culture 2 were in every case higher than those obtained with culture 1. This experiment shows that attenuation of the culture, which is used for the emulsion, may have a great effect on the value of the index obtained. But the process of modification is gradual and progresses in one direction, and so does not interfere with the recognition of changes in the opsonic index induced by inoculations, which changes are more rapid, and have phases both of increase and decrease. This phenomenon of attenuation, however, prevents us from making any trustworthy comparison between the exact heights of the opsonic rises, observed in experiments carried out at different times, or with different strains of the bacillus.

It should be added that the phagocytic counts obtained with pathological sera, giving indices below normal, are increased, during the use of attenuated cultures, in a still greater proportion than the normal counts. Consequently while the normal counts approach those of highly immune sera, low counts approach more nearly to the normal, so that there results a levelling effect. A series of indices estimated with an attenuated culture preserve the order of their relative positions, but all are nearer the normal. It will be seen that this fact provides a further test for the detection of attenuation during a continued series of daily observations, wherever the number of animals under experiment contains individuals with both high and low indices. An accidental variation in the mean of the normal counts, would cause a corresponding alteration of all indices (both high and low) in the opposite direction to this variation. Attenuation of the culture, on the other hand, lowers the high indices and raises the low indices.

The process of attenuation has been described as gradual, but one cause of sudden attenuation has come under notice during this work. When a strain of the bacillus has been grown for some time on one brew of agar, transference to a fresh brew has several times produced a marked attenuation in the first culture on this new and slightly different medium.

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4. *The kind and amount of selection exercised in presenting the results.*

In selecting the experiments, here presented, from the whole mass of experimental material collected during about 15 months' work, it was felt necessary to define the principle of what constitutes a fair method of selection, as opposed to an unfair method. If the methods of observation are liable to error, it is obviously possible to obtain, through error, a number of results which favour a preconceived opinion, whilst other results are contrary or doubtful. From such a total of data it is unfair to select those results alone, which favour the conclusion drawn. On the other hand, where all the data obtained favour one conclusion, confirming each other, it is fair to select for presentation a typical series of experiments. Following this principle, attention must here be drawn to certain observations which have been rejected, because they were of doubtful interpretation. The reader will then be able to judge whether the conclusions drawn from the published experiments are materially weakened by these doubtful results.

One curve was rejected because it contained one of the two pairs of widely divergent duplicates mentioned above, the rest of this curve was in harmony with the three given in Figs. 40, 41, and 42, which it confirms. The observations of one day (Feb. 13, 1909) were rejected because of the sudden attenuation of the culture used on this day, as shown by the fact that the indices obtained (7 high and 4 low) showed a marked tendency to approach the normal. Reference to Figs. 11, 12, 15, 16, 17, 46, 47, 48, 49, will show that the indices obtained on the 12th were distributed between 0·35 and 2·2. Those obtained on the 13th fell all between 0·64 and 1·78, ten out of eleven indices having come considerably nearer to the normal[1]. On the 14th a less altered culture of the same strain was used, and six out of seven indices showed again wider deviations from the normal, the seven being distributed between 0·26 and 2·5. It was clear, under these circumstances, that the measurements of the 13th had been made with a different scale to those of the preceding and following days, and they were omitted from the printed curves as obscuring their true form. In addition to the rejections explained above, a considerable number of experiments have been omitted as only serving to confirm points already amply demonstrated in the published figures.

[1] These indices are shown on the charts by small circles.

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V. The importance of opsonic immunity.

The general proposition that a state of well-being in the patient of a bacterial infection, is correlated with a raised opsonic index, is supported by a considerable mass of evidence obtained from observations upon man[1]. The following experiment, upon this subject, is given here, not as adding very much to the accumulated evidence of experience, but because it relates to the particular disease under consideration. I hope to be able shortly to carry out further experiments on somewhat similar lines.

Ten guinea-pigs were taken, whose opsonic indices had been estimated on two previous occasions, and found to differ widely from each other. It was thought likely that they would show corresponding differences in their powers of resisting an infection. Such wide differences, in the opsonic indices, are usually found, with regard to this bacillus, in a batch of presumably normal guinea-pigs, in which an autopsy reveals no sign of any previous infection. The disease is so fatal to guinea-pigs, that one can hardly suppose that a considerable percentage of adults have, at some time, acquired a relative immunity by way of the alimentary canal. One would be more inclined to regard these differences as natural or inborn, and comparable to the variations from the normal observed by Wells (1908) in healthy infants; but whichever view be correct matters little to the present argument. These ten guinea-pigs were inoculated on the same day, all with equal doses of the *B. pseudotuberculosis*, given intraperitoneally. The dose used was a small one, namely the one hundred thousandth part of a 24 hours' agar slope culture of the bacillus, isolated two days before from the heart's blood of a guinea-pig, dead of the disease. The animals were left untreated under the same conditions, and the course of the disease was observed. Determinations of the opsonic indices were made from time to time. Within ten days of the date of infection, seven of the guinea-pigs had died. The opsonic histories of all ten were then sorted into three groups, according to the period for which each guinea-pig had survived infection. A different opsonic curve was then seen to be characteristic of each of these groups, the opsonic curves in the same group showing a general agreement in form. A mean curve was therefore constructed for each group, and the three mean curves are presented on Fig. 5.

[1] Macdonald (1906), Wright (1907, 1908), Inman (1908), Hektoen (1907), Banks (1907), Bassett Smith (1907).

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It is seen that the three guinea-pigs which lived longest (group I.)[1] had shown high indices previous to infection, and also showed a capability to produce a relatively large increase in opsonin, in response to the stimulus of disease. The animals, on the other hand, which died earlier (groups II. and III.), were those with a past history of medium or low indices, and their indices remained low during the disease.

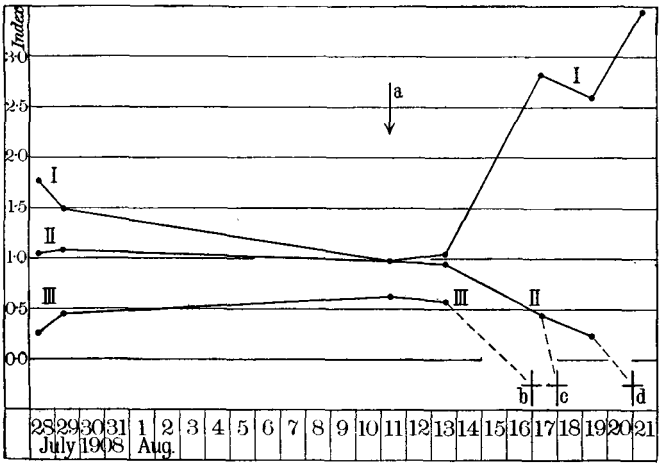

Fig. 5. Correlation between a good opsonic response, and an increased power of resisting infection.

Curve I. Mean opsonic curve for 3 guinea-pigs which survived the longest.

Curve II. Mean opsonic curve for 6 guinea-pigs which survived from 6 to 10 days.

Curve III. Opsonic index of 1 guinea-pig which only survived 5 days.

a. Date on which all the guinea-pigs were infected intraperitoneally, each with $\frac{1}{10000}$ of a culture.

b. One guinea-pig died.

c. Four died.

d. Two died.

VI. EFFECT OF INOCULATING NORMAL RABBITS WITH KILLED CULTURES OF *B. PSEUDOTUBERCULOSIS*; CONTRAST BETWEEN SUBCUTANEOUS AND INTRAPERITONEAL INOCULATION.

1. *Subcutaneous inoculation.*

The material for inoculation consisted of an emulsion of bacilli in ·85 or 1 per cent. sodium chloride solution. The bacilli were washed off the

[1] Of these three guinea-pigs two died within a fortnight of infection, one recovered, and was killed by accident after 6 months. No traces of the infection were discovered at the autopsy.

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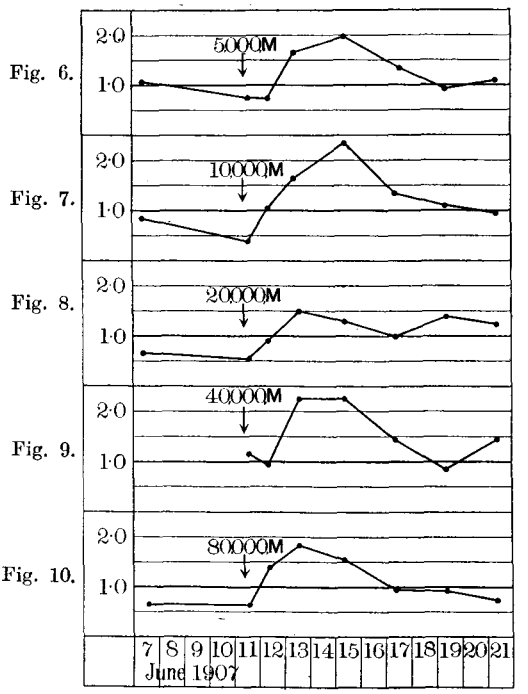
surface of a 24 hours' agar culture with the saline fluid, and, after enumeration of the bacilli against the red corpuscles of normal blood, by the method of Wright, the emulsion was heated to 60° C. for half an hour or an hour. Doses were estimated numerically, as so many millions of bacilli per kilogramme of body weight of the inoculated rabbit. For convenience this is denoted by the abreviation M. which is used, in citing doses, to mean *millions of bacilli per kilogramme of body weight.* Doses were usually made up to the volume of about 1 c.c. for subcutaneous inoculation. Some very big doses had, however, a larger volume.

A series of normal rabbits received subcutaneous inoculations ranging from 160 M. to 80,000 M. Doses from 5000 M. up to the largest given, all produced the same response, as measured by the *rapidity and duration* of the rise in the opsonic index, which followed the inoculation (Figs. 6 to 10). Reference to these figures will show that the general



Figs. 6 to 10. Response of normal rabbits to subcutaneous inoculation of large doses of vaccine.

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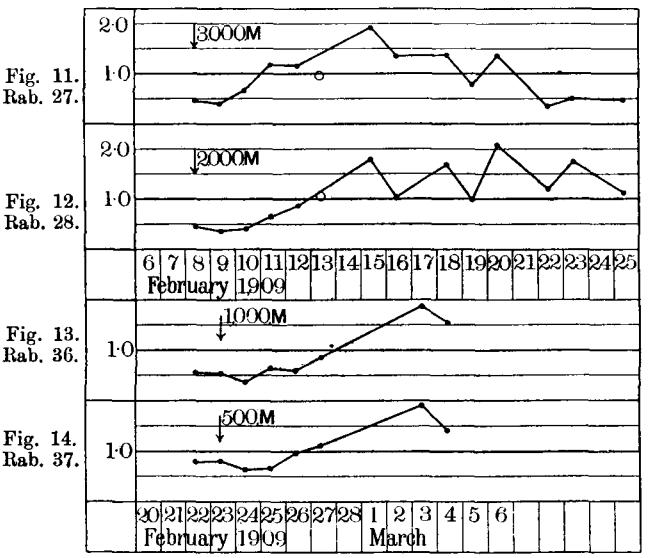
form of the curve is the same in all five cases, the differences between the curves being probably within the limits of error at this early period of the work. This form of response has been regarded as the typical form for subcutaneous inoculation, and the *minimum excitatory dose* (5000 M.), necessary to call forth this immediate reaction in a normal animal, is referred to in what follows as the M.E.D. (subcutaneous). Various doses, given in disease, or given intraperitoneally, are compared to it as to a standard. Doses smaller than 5000 M. produced a response which was delayed for some days, and did not as a rule reach so high a maximum (Figs. 11 to 14); delay was the most marked feature, characteristic of the response to small doses, in contrast to the immediate effect produced by the larger doses.



Figs. 11 to 14. Response of normal rabbits to subcutaneous inoculation of smaller doses of vaccine.

2. *Intraperitoneal inoculation of normal rabbits.*

When the vaccine is given intraperitoneally the response evoked is quite different from that obtained by subcutaneous inoculation. The most striking feature observed is a preliminary fall of the opsonic index (Figs. 15 to 18), a fall which is more marked and more prolonged the larger the dose used. The most favourable dose seems to be one of about 2000 M., for the rabbit which received this dose showed a very

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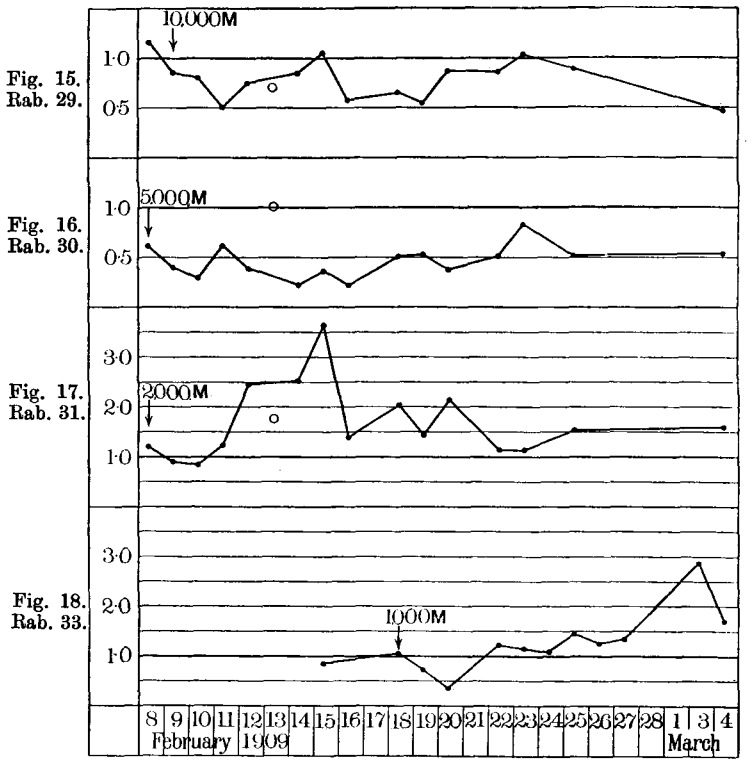
marked rise, following an initial fall of the index, which lasted for two or three days (Fig. 17). This contrast between the effects of subcutaneous and intraperitoneal inoculations, is a strong argument in favour of the local production of antibodies[1]. It appears, moreover, that the *B. pseudotuberculosis* is a microbe peculiarly well adapted for the exhibition of the differences of reaction, which are due to inoculation at different sites. This may be explained, in all



Figs. 15 to 18. Response of normal rabbits to intraperitoneal inoculation of vaccine.

probability, by supposing that the bacillus produces only a very limited amount of soluble toxic matter, so that the effects of injection of a vaccine are limited more strictly to the site of inoculation, than in the case of many other microorganisms. For it has been found to be true in most cases that subcutaneous injection of an antigen is followed

[1] Wassermann and Takaki (1898), P. Romer (1901), v. Dungern (1903), Wassermann and Citron (1905).

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in the first instance by a decrease in the quantity of the corresponding antibody circulating in the body fluids. This was first shown by Brieger and Ehrlich (1892), who estimated the antitoxin content of the milk of a goat, which was inoculated with tetanus toxin. Salomonsen and Madsen (1897) confirmed these observations, by estimations carried out on the blood and milk of a mare, inoculated with diphtheria toxin. Wright (1901, 1903) showed that similar effects followed the inoculation of antityphoid vaccine; his observations referring to the bactericidal power of the blood serum. Wright (1907) quotes similar results again, with respect to the opsonic and agglutinating powers of the blood of men and animals, inoculated with various bacterial vaccines. Further, it has been shown that, in some cases, during this period of low antitropic power, the injected antigen may be detected in the circulating blood (von Dungern 1903). Under these conditions we cannot hope to observe the pure effect of a local inoculation, as it may be masked by the diffusion of the inoculated material through the system. Conversely, in a case where inoculations in different situations produce widely different effects, we may surmise that these effects are chiefly due to a localised action. In the case of the *B. pseudotuberculosis*, its low toxicity provides another argument for the very small amount of its soluble poisonous products; large numbers of living bacilli have to be present in order to kill an infected rabbit, and extraordinarily large doses of vaccine have to be used in order to produce any effect on a normal rabbit. This is clear when one remembers that the prophylactic dose of *B. typhosus* for a man contains about a thousand million bacilli, and very few therapeutic doses as large as this have been used in respect to any of the bacterial infections of man, which have been inoculated with success. While therefore a thousand million of the bacilli, with which we are familiar, usually constitute an exceptionally large dose for a man, a normal rabbit weighing 2 kilogrammes must receive subcutaneously ten times this number of *B. pseudotuberculosis* before the typical response is produced. Parallel with this low degree of activity exists a considerable difficulty in producing a low opsonic index even in a diseased animal by subcutaneous inoculation with this bacillus (Part VIII. p. 203), which seems, therefore, to form very little nocuous material in a soluble form.

From the above it will be seen, that the effect of subcutaneous inoculation of pseudotubercle vaccine differs both from that of intraperitoneal inoculation of the same vaccine, and from that of subcutaneous inoculations of soluble toxins, or of vaccines which contain much soluble

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antigen. The most probable reason for this is, that we are here studying the effect of a purely local response of the subcutaneous tissues at the site of inoculation. On the other hand, when the vaccine is given intraperitoneally, the very rapid interchange of lymph between the peritoneal cavity and the general circulation, gives every opportunity for the absorption of the circulating opsonin, even if the vaccine material is not itself washed into the blood stream. If this be granted it will follow that the subcutaneous tissues are specially adapted to perform a protective *rôle*, against bacterial invasion. This *rôle* they fulfil by reason of their peculiar ability to react rapidly to a sufficient stimulus, and to dispose, in some way, of an excess of bacterial matter, without allowing the general stock of circulating opsonin to be diverted in order to combine with this surplus. It remains for future investigations to determine how far this speciality is confined to the subcutaneous tissues. The comparison here is only between these and the peritoneal system, by which term is denoted the peritoneal cavity and the extensive lymphatic areas, which are in easy connection with it. It is possible in view of the success which has attended the method of immunisation by way of the gut, that the mucous or submucous tissues may possess powers similar to those of the subcutaneous tissues. The experiments of Leishman (1908), however, with typhoid vaccine weigh against this surmise. It should also be pointed out that, when immunity has been established, other tissues acquire this property of rapid response, as will be shown in a later part of this paper[1].

VII. Infection with living cultures, injected into the peritoneum or into the blood stream.

If we introduce a living emulsion into the peritoneal cavity the series of events which follows this infection, is again different from either of the cases treated in the preceding part. If the rabbit is to survive for more than a day or two, the dose must not exceed $\frac{1}{5}$ of a 24 hours' agar slope culture, which quantity contains about 6,000,000,000 bacilli, of which nearly all are living (see Table II).

The injection of such a dose as $\frac{1}{5}$ of a culture is followed by a considerable fall of the opsonic index, which is maintained for several days (Figs. 19, 20, 21). It has been shown in Part VI. that such a fall may be

[1] Compare also Wassermann (1905), *Deutsch. med. Wochenschr.* p. 1101.

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produced by intraperitoneal injection of killed bacilli (Figs. 15, 16, 17, 18), but cannot be produced by subcutaneous inoculation. The living bacilli are, therefore, just as well able to abstract opsonin from the circulating fluids, as those killed by heat. On the other hand, even with their power of multiplying and spreading into the substance of the abdominal organs, they do not form any more efficient stimulus to the defensive mechanism of the animal. The production of increased quantities of opsonin is delayed just as much in the case of the living inoculation, as it was in the case of the dead vaccine. When a smaller quantity of living microbes are used for the inoculation, that is a quantity less than the minimal efficient intraperitoneal dose of bacilli killed by heat, there is an additional period of delay, while the infecting microbes have time to reach the minimal number efficient as a stimulus. This is illustrated by the experiment shown on Figs. 32 to 36 where the dose inoculated was $\frac{1}{100}$ of a culture, or about equivalent to an inoculation of 150 M.; also by Fig. 19, which represents the mean of 9 curves, obtained in experiments where the infecting doses ranged from $\frac{1}{100}$ to $\frac{1}{4}$ of a culture. From these observations it would appear that, though the bacilli have early invaded and occupied in force the omentum, liver, spleen, and abdominal lymphatic glands, and though they are even circulating in the blood stream, yet they have not, at this time, gained access to any tissue which has the power (presented by the subcutaneous tissues) of a prompt and vigorous immunising response.

TABLE II.

Number of culture tubes	Average number of microbes per tube, enumerated against red blood corpuscles	Number of living microbes by culture
4	28,000,000,000	—
1	31,000,000,000	26,000,000,000
1	31,000,000,000	31,000,000,000

After an inert period of shorter or longer duration, during which the opsonic index remains low, a reaction at length sets in, and the index rises to a high level which is only maintained, however, for a few days (Fig. 22). After this the opsonic index shows a tendency to fall gradually, though further autoinoculations occur from time to time, inducing reactions which often surpass that first produced, in intensity. An example of this was seen in the case of rabbits 19, 20, 21 and 22, whose indices rose during the first reaction to little over 2 (Figs. 32 to 36), whilst a fortnight later they were found to have attained an average value of about four (Figs. 39 to 42; Jan. 1 and 2).

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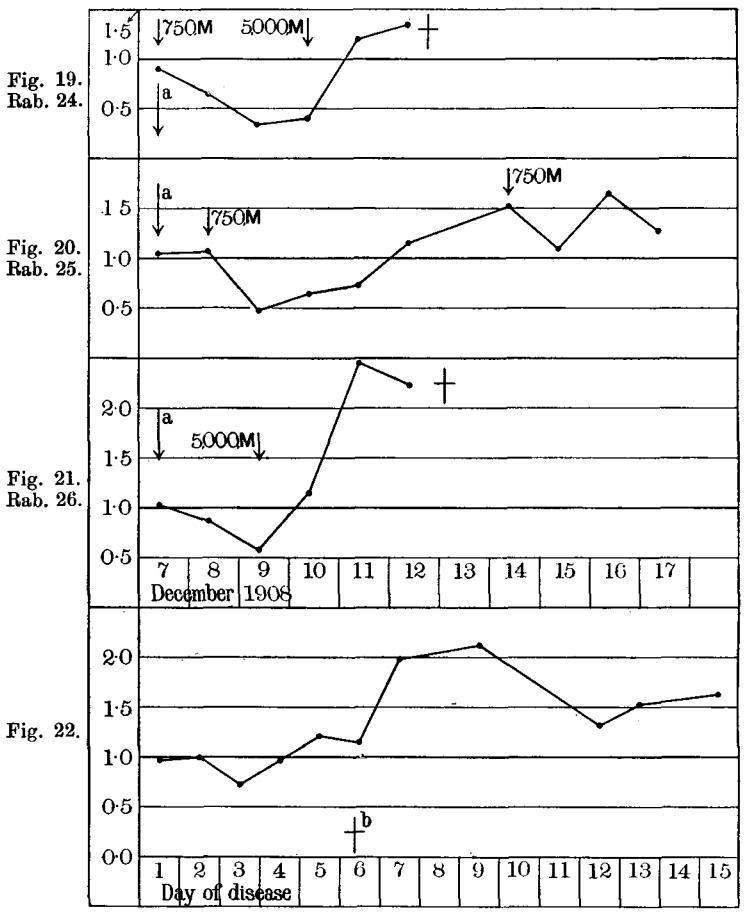
Of the rabbits infected with moderate doses (less than $\frac{1}{5}$ of a culture), four died before the first reaction set in (Figs. 22 and 36), three on the sixth day, and one on the tenth day of the disease; whilst all those which survived the inert period, and produced an immunising response, as shown by the opsonic index, went on to ultimate recovery; none died. Of those which received $\frac{1}{5}$ or $\frac{1}{4}$ of a culture, on the other



Figs. 19 to 22. Response of normal rabbits to intraperitoneal inoculation of living cultures.

Figs. 19 to 21 show also the effect of various subsequent subcutaneous inoculations.

Fig. 22. Composite curve of opsonic indices of 9 rabbits which were infected with living bacilli on day 1.

\+ signifies the death of the animal.

a. Three out of the 9 rabbits died.

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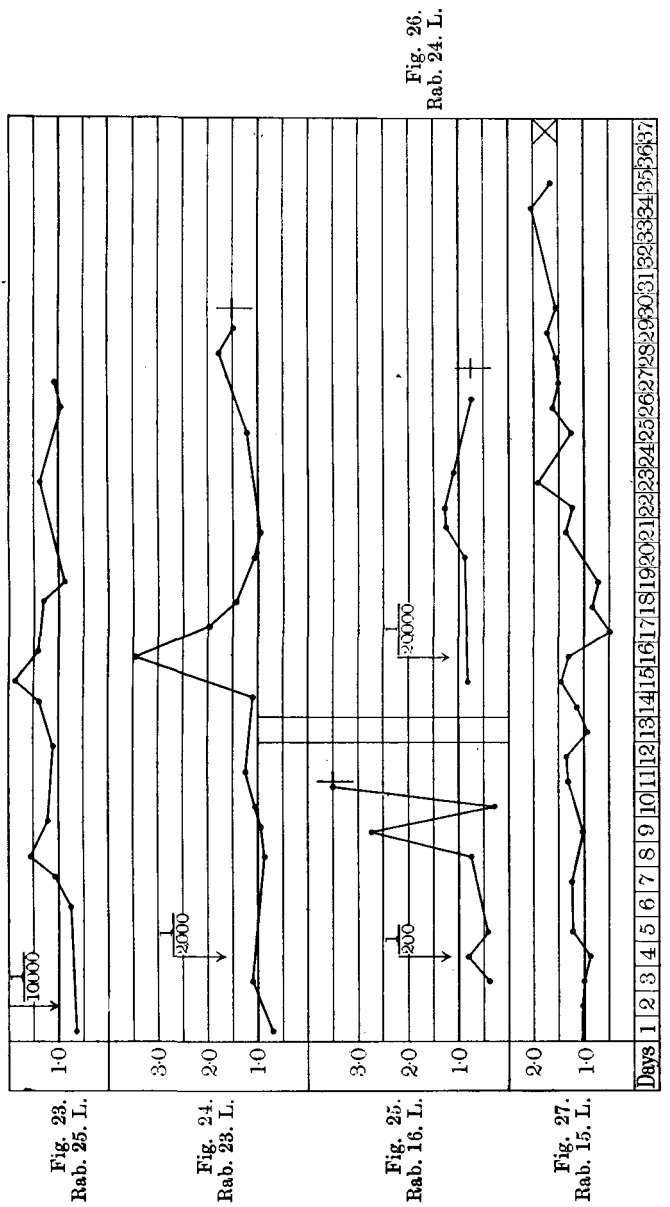
hand, three out of four died (Figs. 19, 20, 21), in spite of an immunising response, elicited in two cases by the subcutaneous injection of vaccine and in the third case supervening in the ordinary course of the disease



Figs. 23 to 26. Response of normal rabbits to intravenous inoculation of living cultures.
Fig. 27. Variations occurring in the opsonic index of a rabbit during the development of a naturally acquired infection. The exact date of the infection is not known.
× The animal was killed, and the diagnosis confirmed.

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(rabbit 25, Fig. 20, died on the 26th day of the disease). It was, of course, only to be expected that a massive infection would be able to overcome the protective mechanism of the animal, even when excited to its utmost activity.

Intravenous inoculation of living cultures produces a more fatal disease than intraperitoneal inoculation. No rabbit survived infection by way of the blood stream with more than $\frac{1}{10000}$ of a culture. Some of the rabbits which received doses between $\frac{1}{20000}$ and $\frac{1}{200}$ of a culture lived long enough to produce an immunising response. In these the inert period was at least as long as that following infection by way of the peritoneum, but it was not accompanied by any marked decrease in the quantity of circulating opsonin (Figs. 26 to 29). The bacilli therefore do not multiply in the blood stream to any great extent (as may be also shown by blood-cultures), but they become lodged in various tissues throughout the body, and are shut off from the circulation, within the necrotic foci or abscesses thus formed, so that the amount of opsonin abstracted from the circulating blood remains comparatively insignificant. The immunising reaction, when it appears, is markedly fitful and ill sustained.

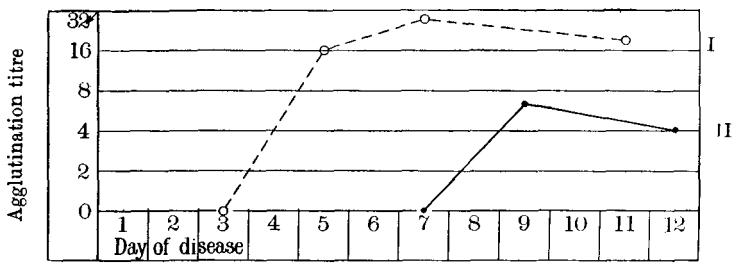

Fig. 28. Interrupted line; development of agglutinin in five rabbits which received subcutaneous inoculations of vaccine on day 1.

Continuous line; development of agglutinin in three rabbits which received intraperitoneal inoculations of living cultures on day 1. The figures at the left-hand side denote the dilutions of the serum, in which a distinct agglutinating reaction was obtained.

Fig. 27 shows the changes in the opsonic index, which occurred during the development of an infection, acquired accidentally by way of the alimentary canal.

Agglutination experiments.

The absence of any immunising reaction during the first period of the disease, which has been shown above by reference to opsonic

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observations on a series of 23 rabbits, may be equally easily demonstrated by agglutination experiments. Three infected rabbits, on which these experiments were made, only produced agglutinin after the lapse of eight days from the day of infection (Fig. 28; Curve II.). On the other hand, subcutaneous inoculation of five different normal rabbits, with various doses of killed bacilli, led to the discovery of a relatively high agglutination titre, in every case, after only four days from inoculation (Fig. 28; Curve I.).

VIII. THE RESPONSE ELICITED BY INOCULATION OF STERILISED VACCINE, IN RABBITS PREVIOUSLY INFECTED WITH THE DISEASE.

During the first stage of the disease caused by inoculating a living culture into the peritoneum, the experimental animal still reacts to a subcutaneous inoculation of vaccine in the same manner as a normal rabbit does. This fact enables us to state with more certainty the cause of the decrease in the opsonic content of the blood, which is observed during this period. This decrease was attributed solely to the abstraction of opsonin from the circulating fluids by the infecting bacilli. But there were two further possibilities, namely that the production of opsonin had been inhibited, and the supply thus cut off at the source, or, on the other hand, there might be an increased production of opsonin, which was hidden from observation because masked by the rapid absorption of this from the blood, by the bacilli. These possibilities are however disposed of by the fact mentioned above, which was established by the following experiment.

The three rabbits, 24, 25, 26, were inoculated, each with $\frac{1}{5}$ of a 12 hours' agar slope culture, on the same day. After 48 hours, rabbit 26 was obviously weaker than the other two, and appeared likely to die within the next 24 hours. It seemed that, if the presence of a severe infection can ever inhibit or mask the production of opsonins, this rabbit was in a condition to exhibit the phenomenon. It was therefore given a subcutaneous injection of 5000 M.—the M.E.D. for a normal animal. During the next two days the opsonic index rose to over 2·4 (Fig. 21). On the next day, rabbit 24, being at the time, as far as one could judge, *moribund*, received a similar subcutaneous inoculation. It lived long enough to show a rise of the opsonic index up to 1·34 (Fig. 19). It is clear that an appropriate stimulus, even in this stage of the disease, can excite the production of opsonin in increased quantity; an increase which the presence of a very grave infection is unable either to inhibit

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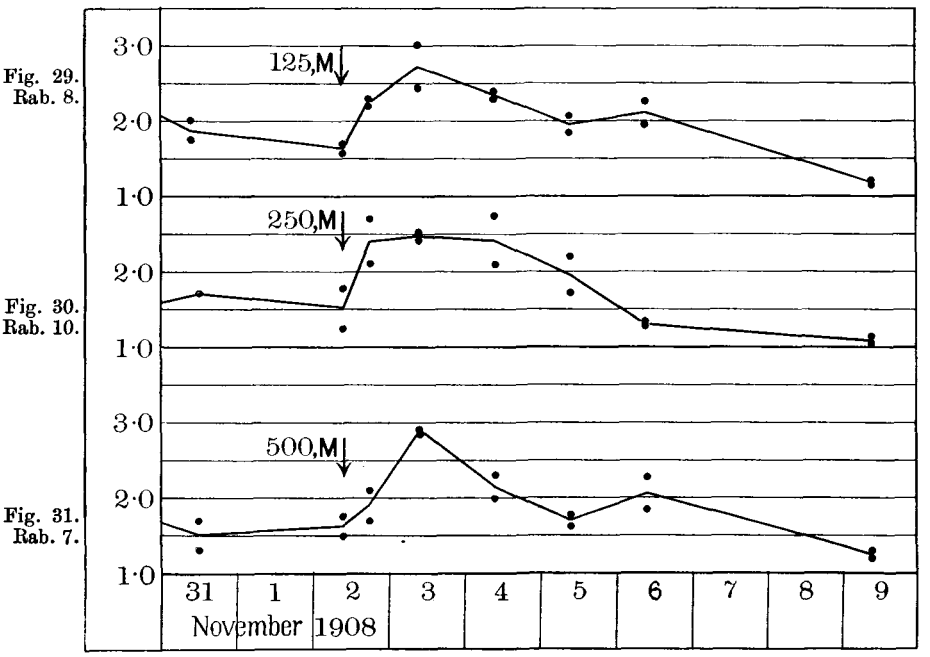
or to mask. The lowered index, which marks the beginning of this intraperitoneal infection, is therefore due only to the absorption of opsonin by the bacilli introduced; it is accompanied neither by inhibition, nor by any efficient stimulation of the immunising mechanism.



Figs. 29 to 31. Response of previously infected rabbits to small doses of vaccine, given subcutaneously.

After a certain degree of immunity has been attained in the course of the disease, the reactions of the animal towards injections of vaccine are entirely altered. In the first place it becomes possible to elicit a response with a dose one hundredfold less than the M.E.D., and secondly it becomes possible to cause a temporary lowering of the opsonic index, by subcutaneous inoculation of suitable doses of vaccine. The tissues of the (relatively) immune animal have acquired a new property, by virtue of which they react with increased vigour to the presence of microbes of the species which has induced the immunity. This tissue-immunity, which outlasts the presence of an increased quantity of antibodies in the body fluids, has been emphasised by Wassermann and Citron (1905).

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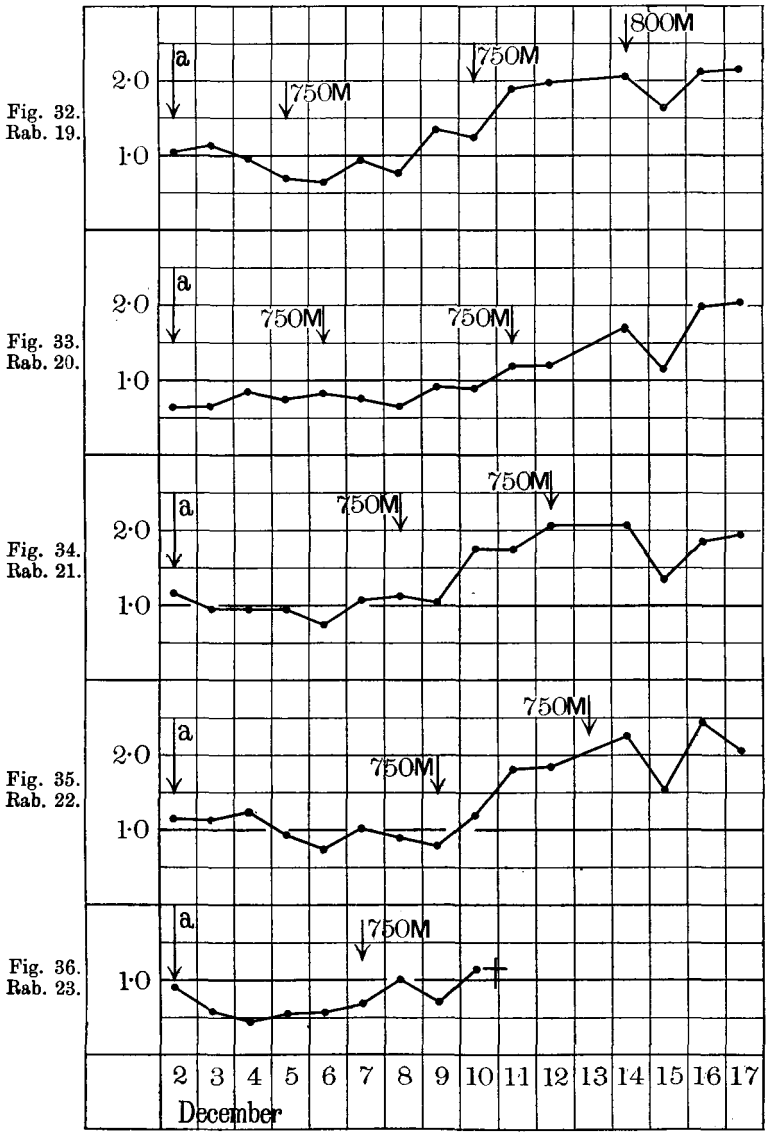
The response to small doses was first obtained in three rabbits (Figs. 29, 30, 31), one of which received the inoculation on the 15th day of the disease (rabbit 10, Fig. 30). An experiment was therefore



Figs. 32 to 36. Opsonic charts of rabbits which were infected at (*a*) with doses of $\frac{1}{100}$ of a culture. Subsequent inoculations of moderate doses of vaccine, subcute, produced no immediate response.

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instituted to determine when this property of sensibility to small doses first appeared. It was obvious that if test inoculations were made, the opsonic indices of the inoculated animals should give an indication of the effect, or absence of effect, of inoculation, within 24 hours.

For this experiment 5 rabbits were infected intraperitoneally on the same day with equal doses ($\frac{1}{100}$ of a culture) of the *B. pseudotuberculosis*. While their opsonic indices were estimated daily, they were given test doses of vaccine at intervals. The dose of vaccine chosen was 750 M. that is, 15 times the minimal effective dose for a sensitive immune rabbit, and rather less than $\frac{1}{6}$ of the M.E.D. (Figs. 32 to 36). Reference to the figures will show that certainly none of the inoculations before the eighth day were effective. Rabbit 22 (Fig. 35) gave a rise of index following immediately on an inoculation on the eighth day, but, at this time, one expected a rise in the ordinary course of the disease, so that it remains uncertain whether this dose had any effect or no. Similarly inoculations on the 9th and 12th days (rabbits 19 and 22; Figs. 32 and 35) had a doubtful effect, while doses on the 10th and 11th days (rabbits 20 and 21; Figs. 33 and 34) had no effect. In rabbit 10, however, as mentioned above, a dose of 250 M. given on the 15th day of the disease, had produced an undoubted response (Fig. 30). This rabbit had received a larger infecting dose, namely $\frac{1}{20}$ of a culture. The conclusion is, that the condition of increased sensibility to inoculation is only established some days later in the course of the disease than a high opsonic index.

The response of a diseased and sensitive animal to a small dose may have one of two forms. Either there follows immediately on the inoculation a very rapid rise of the opsonic index, which is already well begun within the first six hours, and which reaches its maximum in about 24 hours (Figs. 29, 30, 31), or there is a gradual rise, which begins slowly and goes on with increasing rapidity to a maximum after 48 to 72 hours. Such a response as this last has only been seen (in these experiments) in animals which had already received several doses of vaccine (Figs. 1, 2, 3, 4, 37, 38). The first form is that more familiar to those who have treated, by inoculation, various bacterial infections in man. Passing on to examine the effect of larger doses, it is found that the response elicited is irregular, and shows periods of decrease as well as of increase in the opsonic index (Figs. 39 to 45). The response in disease, therefore, differs from that of a normal rabbit, in the fact that a "negative phase" can be produced, which may or may not be preceded by a temporary rise of short duration, whereas in normal rabbits,

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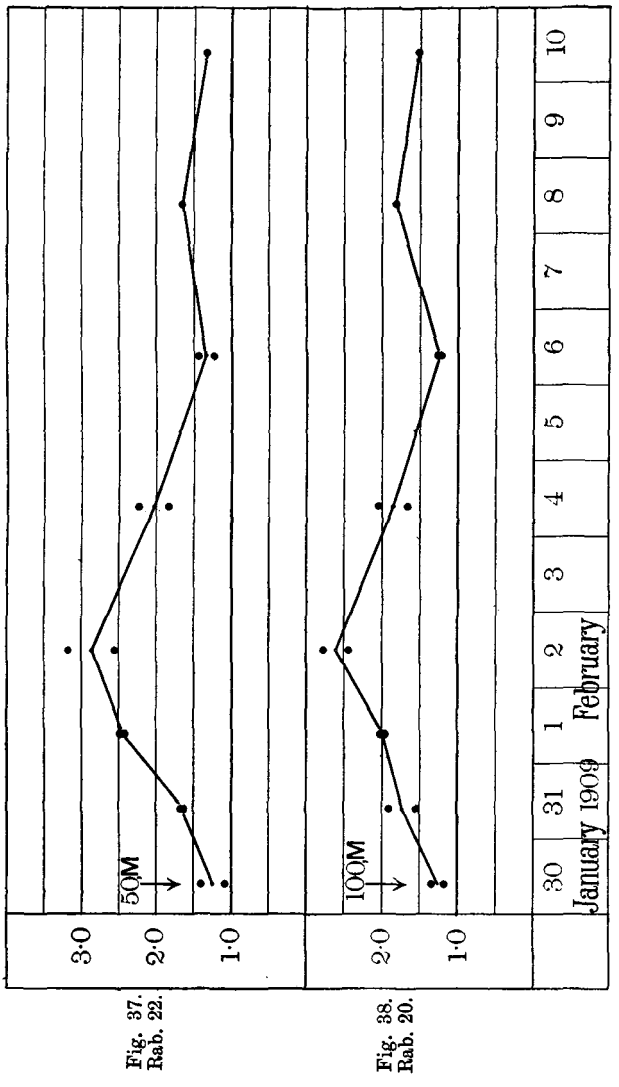
subcutaneous injections of pseudotubercle vaccine are not followed by any negative phase (Figs. 6 to 14). The dose required to produce this effect in a diseased rabbit varies in different rabbits and in different stages of the disease, and is also influenced by the height of the opsonic index at the time of inoculation, and by previous inoculations. Thus doses of 500 M. (Fig. 45) and 410 M. (Fig. 42) were both sufficient to cause a temporary fall of the index, in rabbits not previously inoculated



Figs. 37 and 38. Response of rabbits, recovering from a previous infection, to small subcutaneous doses of vaccine.

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with an effective dose, whereas the inoculations of Nov. 24th (Figs. 1 to 4), ranging from 200 M. to 5000 M., all alike acted as small doses, that is produced a pure rise of the index in rabbits which had each been inoculated on several previous occasions. It may be pointed out that Figs. 32 and 45 refer to one rabbit, the interval elapsing between the two inoculations being five weeks, during which time 2 intervening inoculations were given. Similarly Figs. 30 and 46 refer to one rabbit; there was the same time interval, of five weeks, and 3 intervening inoculations.

The inoculations shown on Figs. 39 to 42, illustrate the ease with which an already high index is lowered by inoculation, of even a relatively small dose. The four rabbits used for this experiment all had indices before inoculation of about 4. The doses given on this occasion ranged from 410 M. to 50,000 M. and the response evoked was the same in every case, the most marked feature being a very rapid fall of the index during the first 24 hours succeeding inoculation.

With regard to this experiment it should be stated that the persistence of a lowered index was partly due to the alteration of the strain from which the emulsion was prepared each day. Thus the culture used on Jan. 1st was only the third subculture since isolation of the bacillus from a rabbit's blood. But as subcultures were made once, and sometimes twice a day, that used on Jan. 6th was considerably attenuated. This attenuation being suspected on Jan. 7th, a return was made to the second subculture since isolation, and a slope was sown from this. The indices obtained, with an emulsion of this culture, were all a good deal higher than those for Jan. 6th. There is, however, no doubt that the first effect of the inoculations of Jan. 2nd, was a very pronounced fall of the opsonic index of each rabbit; and this fall was produced in one case (rabbit 22, Fig. 42) by a dose less than one tenth the M.E.D.

To sum up the foregoing section: an animal which has a raised sensibility with regard to inoculation of vaccine, reacts differently according to whether small or large doses are given. Small doses produce a pure rise in the opsonic index, large doses produce a temporary fall; but the dividing line between what constitutes a "large dose," and what a "small dose," varies considerably, and depends on the condition of the animal.

With regard to the response of a diseased animal to inoculation, the question arises whether the dose administered may not suffice to induce an inflammatory reaction at the foci of disease, just as tuberculin or

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Fig. 40. Rab. 20.

Fig. 42. Rab. 22.

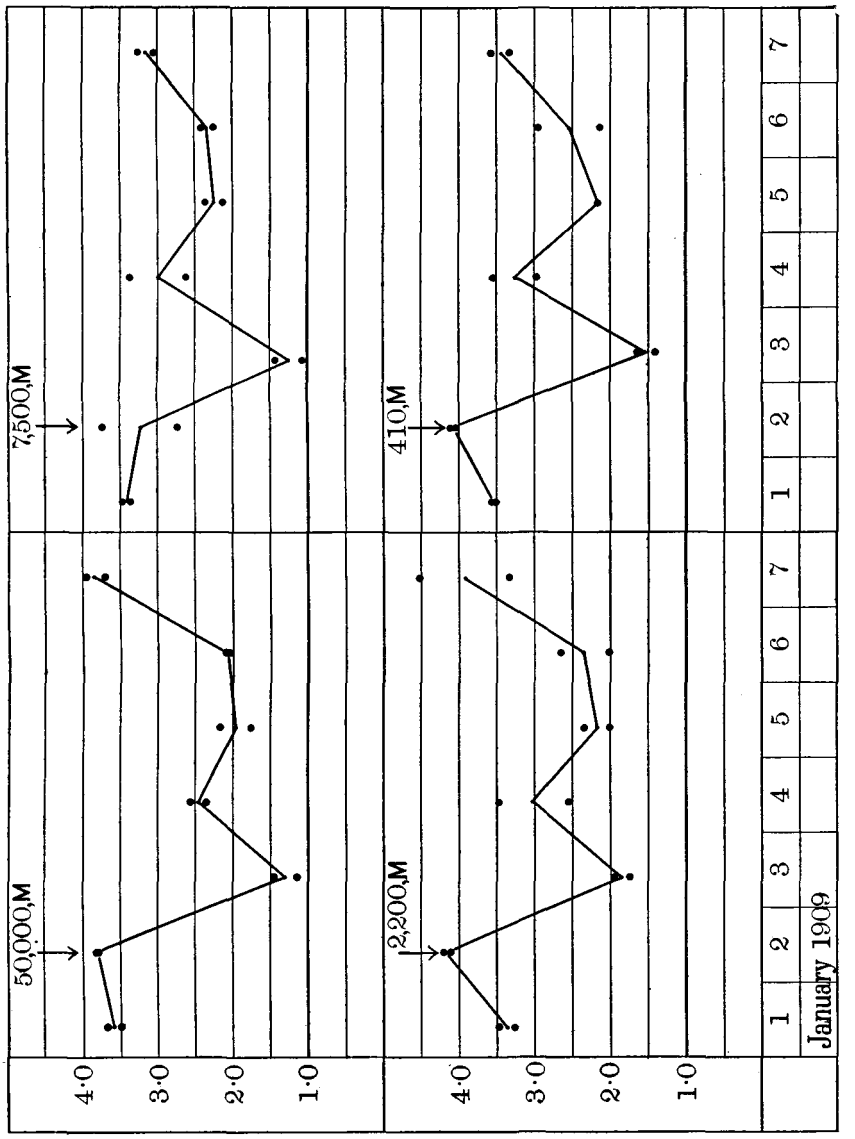

Fig. 39. Rab. 19.

Fig. 41. Rab. 21.

Figs. 39 to 42. Response of rabbits, previously infected, to large and moderate subcutaneous inoculations of vaccine. The index, which was very high in each case before inoculation, falls immediately.

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mallein may, when used in veterinary practice as a test for tuberculous disease or glanders. Such a reaction, by increasing the circulation, would tend to carry the bacterial products more widely afield, and in larger quantity, and thus a secondary autoinoculation could conceivably follow as a consequence of an inoculation from without. For the present, however, we want sufficient data for the elucidation of this complex question.

IX. Living cultures and sterilised vaccines considered as stimulus-material, and their efficiency, from this point of view, compared.

The question whether the antigen, which excites the production of opsonin, is a metabolic product of the growth of the bacillus, or, on the contrary, a product of its disintegration, is of no little interest from the point of view of vaccine therapy. If the first alternative were true, the inoculation of a living culture would provide not only a maximum quantity of ready-formed antigen, but also a source of continuous supply of fresh antigen. But if the second alternative be true, then it is possible that the process of sterilising the vaccine may increase its immediate value as stimulus-material, by breaking up the bacilli, and rendering them more readily available to the tissue cells. In this manner one could imagine a killed culture providing a more intense immediate stimulus, than a similar quantity of a living culture. To test this it was necessary to compare the immediate stimulus-value of bacilli, living, and killed by heat. In making this comparison one can only judge by the immediate results, because the later effects of inoculation would be influenced by the multiplication of the living bacilli within the body. Hence no conclusions can be drawn from the response following intraperitoneal injection, of killed and living cultures, into normal animals. In both cases the response is delayed, and multiplication of the living bacilli has had time to occur before it sets in. The effect of inoculating living bacilli into the subcutaneous tissues might be profoundly influenced by the gross changes accompanying abscess formation, so that this method of comparison between the living and dead would give untrustworthy results.

The immune animal, however, can react immediately to intraperitoneal inoculations of either living or dead cultures, so that a fair comparison could be made of their stimulus-value, by making injections into the peritoneal cavities of rabbits which had recovered from a

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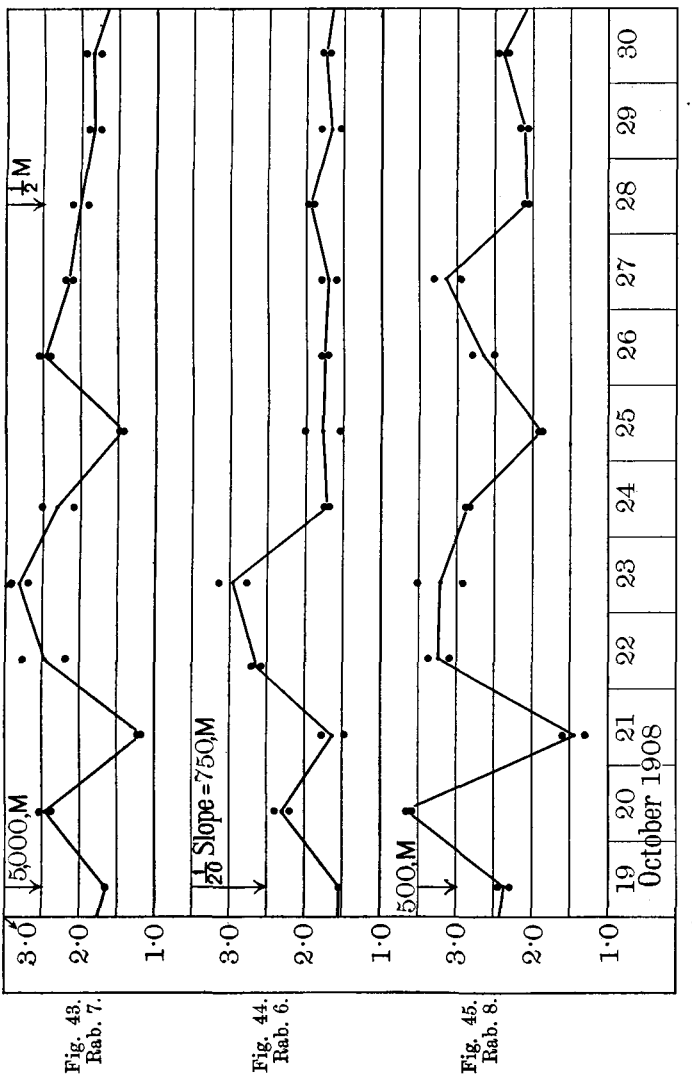
previous infection, and were consequently in a condition of immunity. A suitable quantity of living bacilli, given in this manner, was found to excite a response similar to that called forth by the injection of killed vaccine, as will be seen by comparing Fig. 44 with Figs. 43 and 45. This form of response was obtained with widely different doses of vaccine (500 M. and 5000 M.), so that the experiment did not give exact information as to what was the vaccine-equivalent of the quantity



Figs. 43 to 45. Intraperitoneal inoculation of living bacilli (Fig. 44) compared with subcutaneous inoculation of vaccine (Figs. 43 and 45). The rabbits on which these inoculations were made were recovering from a previous intraperitoneal infection with living cultures.

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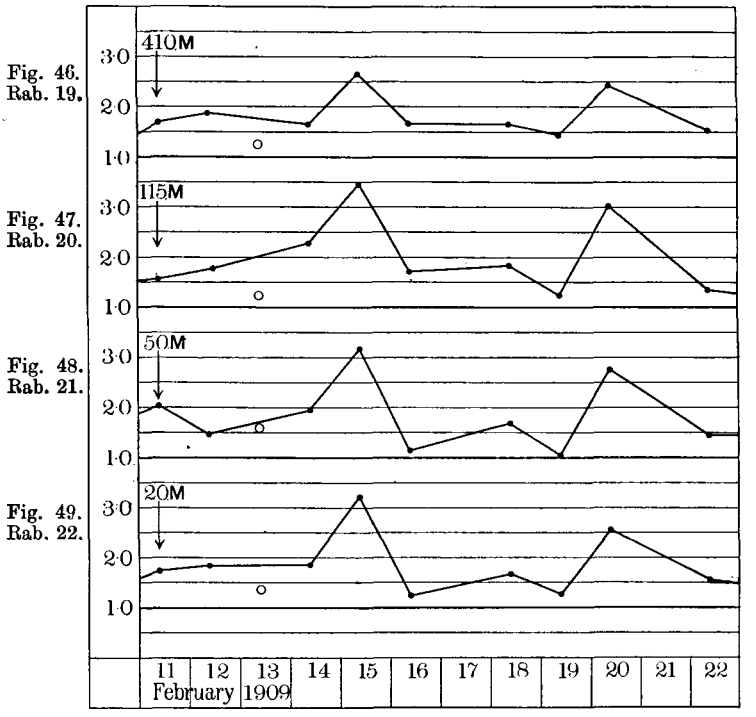
of living culture used ($\frac{1}{20}$ of a 24 hours' agar slope culture = 750 M.). A second experiment, made with smaller doses, gave more information. Four rabbits received respectively 20 M., 50 M., 115 M. and 410 M. (living culture); none of these rabbits gave an immediate response (Figs. 46 to 49), their indices remained steady for 3 days after inoculation, and only rose on the fourth day. These doses of living bacilli were, therefore, too small to excite at once an immunising response.



Figs. 46 to 49. Response of rabbits, recovering from a previous infection, to intraperitoneal inoculations of living cultures.

In contrast to this series, three rabbits were given respectively 50 M., 100 M. and 200 M. sterilised vaccine. They all gave a response, which began with a slight rise of the opsonic index on the first day, and reached its maximum on the third day after inoculation (Figs. 50 to 52). This shows that a dose of vaccine as small as 50 M. contains more immediately available antigen, than does the comparatively large dose (410 M.) of living bacilli. The living bacilli on the other hand, by the decay of successive generations, keep up a constant supply of antigen,

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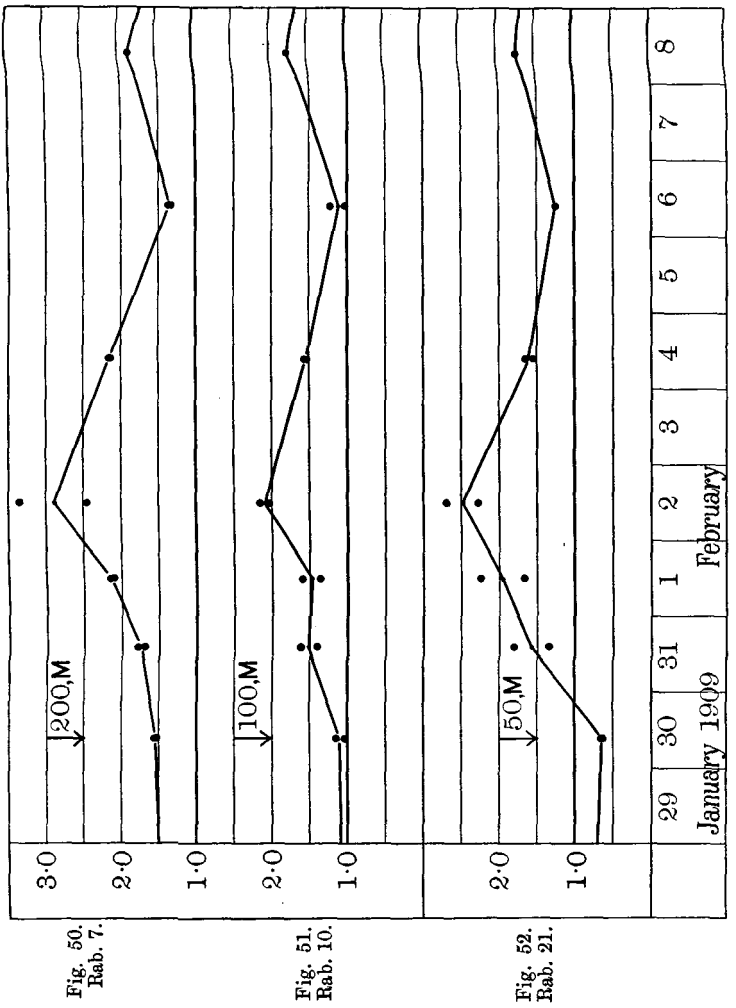
which ultimately produces a corresponding reaction on the part of the animal. It will be observed that this reaction followed the smallest dose, as well as the largest dose given.



Figs. 50 to 52. Response of rabbits, recovering from a previous infection, to intraperitoneal inoculations of vaccine.

X. CAUSES WHICH TEND TO PREVENT EFFICIENT AUTOINOCULATION IN DISEASE.

The first desideratum, for an efficient reaction, is that it should be prompt. But it has been shown that one tissue differs from another very markedly in the power of prompt response to inoculation, the

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subcutaneous tissue possessing this power in a high degree, whilst it is absent from the tissues of the peritoneal area. Hence an autoinoculation can only be considered efficient, which affects tissues gifted with this ability to react immediately; and hence also much of the antigen set free within the body will be wasted on tissues of low reactive power. The locality affected by the disease thus becomes of supreme importance, especially in the first stage, before an immunising response has been set up. After the infected animal has acquired a certain degree of immunity, other tissues have been educated to respond readily to the stimulus of inoculation, so that efficient autoinoculation is more readily attained, than it was at the beginning of the disease. But it still remains a matter of chance coincidence if the liberated antigen reaches the tissue best able to react to its presence.

The opsonic charts of rabbits infected by the blood-stream, so far as they go, entirely support this argument. Figs. 23 to 26 refer to rabbits, which suffered from severe generalised infections. In spite of the gravity of the infection, the opsonic charts (Figs. 23, 24, 26) show evidence of the occurrence of remarkably few effective autoinoculations. Rabbit 24. L. indeed, received no effective stimulus at all, in the course of its disease. Rabbit 23. L., during a disease lasting 26 days, only received two autoinoculations; and these were equivalent (judging by the results) to the quantities of vaccine treated of in Part VIII. as "small doses" (Figs. 29, 30, 31). The first, peaked elevation of the opsonic curve, culminating on May 29th, is a typical and active "small dose" response, undisturbed by any succeeding autoinoculation, so that it shows admirably the gradual fall to normal. The second elevation, immediately preceding death, was probably similar in character, but the observations for two days are lacking. Rabbit 25. L. (Fig. 23) also showed a curve indicating small autoinoculations at fairly wide intervals.

About the events of the days intervening between the elevated portions of the curves, one can only deduce that the tissues are receiving a very small amount of bacterial matter. The insignificance of this amount of constant leakage from the foci of infection, is to be argued from its lack of effect as shown on the opsonic charts. But, if it be urged against this view, that we are ignorant of the effect of a constant and diffused supply of bacterial products, bathing the tissues, as it were, in a poisonous medium, then there are two considerations to be met. First, the experiment of inoculating an animal with a large intra-peritoneal dose of bacilli, living or dead, shows that the effect of such a bath, applied to only a portion of the whole tissues of the body, is a

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prompt and marked lowering of the opsonic index (Figs. 15, 16, 19, 20, 21), and consequently such a condition, occurring in disease, should give evidence of its existence upon the opsonic chart. Secondly, if we assume that all the tissues have a constant large supply of bacterial matter to deal with, it is difficult to explain how they can respond so readily, as they certainly do, to the additional stimulus of a small dose of vaccine. It follows that the immunising mechanism of these rabbits only received small and intermittent stimuli from autoinoculation, yet the disease, which could only provide so slight a stimulus, was fatal to two of the three, which presented, post-mortem, convincing indications of a grave septicaemia.

The histological relation of the infecting microbes to the tissues is also of importance, since one condition of stimulation is a close contact between the tissue cell and the bacterial matter. Now most of the bacilli, in this infection, are either enclosed in phagocytes, or shut up in necrotic foci, which later become small abscesses. With regard to the first category, we are ignorant whether a phagocyte can produce specific antibodies, and, if it can, whether these are passed at all into the circulation, or are devoted entirely to the enclosed bacilli, which have excited their formation. With regard to the second category, those bacilli, namely, which are growing within a necrotic area, or in an abscess, consideration will show that they are, for a time, practically shut off from the living tissues of the body. When a bacterial embolus lodges, it can be seen, after a few hours, to be surrounded by a zone of dead cells. As the bacteria multiply and grow into this necrotic mass, the necrosis spreads too, so that the advancing colony is preceded in every direction by this zone of death. Except at the first lodgment of the embolus, therefore, no living tissue has contact with the invaders, only a certain amount of their soluble products diffuses out into living areas. And this, for a time, is the only material available as a stimulus. Later, when the affected area has become limited by an active abscess wall, the contents of which have been liquefied, the conditions are probably much more favourable.

It has been shown, however, by Freeman and others (1907), in the case of localised infections, that their presence may fail to call forth any notable immunising response, until the conditions are altered by massage, active or passive movement, or by induced hyperaemia. These measures insure a sudden disturbance of the infective focus, and may be supposed to bring the infecting bacteria or their products into relation with healthy, or at least active tissues, in suddenly increased

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quantity. The effect is, undoubtedly, a lively immunising response, which was absent before.

The state of the antigen itself has to be considered, as well as the tissue with which it comes into relation. When a dose of vaccine is given, the antigen is brought into contact, with the tissue elements, all at once, and in considerable concentration. In disease, on the contrary, the antigen is formed gradually by the growth and decay of bacilli spread widely through the animal body. *Pari passu,* with this growth and decay, goes on the absorption of bacteriotropic substances from the circulating fluids, so that each freshly formed bacillus, as well as the products of its growth and decay, is at once partially saturated with antibodies. Such partially saturated antigen constitutes a very much less efficient stimulus to the tissues, than unsaturated antigen, such as that contained in a sterilised vaccine. On this point the experiments of Jorgensen and Madsen (1902) are very clear. These observers found that rabbits and goats, passively immunised by the injection of anti-typhoid sera, would not react to an inoculation of killed typhoid bacilli. But as soon as the passive immunity had passed off, that is, as soon as the agglutination titre of the blood had fallen again to the normal value, the animals would react to inoculations of killed typhoid bacilli, with an energetic production of agglutinin. They argue, from these observations, that, in the case of the passively immunised animals, the inoculated material combined at once with the available antibody, and consequently its stimulating activity became neutralised.

From the arguments set forth above we may perhaps conclude that the causes, which tend to prevent efficient autoinoculation in disease, are the following:—1. The tissues chiefly affected are normally unable to make a prompt specific response to the stimulus which the presence of bacterial matter supplies, such a power being special to the sub-cutaneous tissues. 2. The *gradual* increase, by growth, of the infecting microbes and their products, which is met, at each step, by a partial saturation with antibodies supplied from the circulating fluids. 3. The temporary shutting up of every large aggregation of bacteria behind a zone of necrotic tissue. 4. (Possibly),—the inclusion of otherwise available stimulus-matter, within the various orders of phagocytes.

Conclusions.

1. Opsonic immunity is of real importance in determining recovery from an infection with *B. pseudotuberculosis.*

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2. The subcutaneous tissue of the rabbit has a special power, not possessed by the peritoneum, of reacting promptly to inoculations of killed cultures of this bacillus, with an increased production of opsonin.

3. The above observation is an instance of the local production of antibodies.

4. Intraperitoneal inoculation of *B. pseudotuberculosis*, living or dead, produces an immunising response after a considerable delay.

5. Intravenous inoculation of the living bacillus, produces an immunising response after a considerable delay.

6. Rabbits previously infected with the bacillus are profoundly altered as regards their reactions towards renewed inoculations of living or killed cultures.

7. Cultures of *B. pseudotuberculosis* killed by heat at 60° C. contain more immediately available antigen, than do equal quantities of living cultures, and hence constitute a more efficient stimulus.

8. The following causes tend to prevent the occurrence of efficient autoinoculation in disease:—The tissues affected may not be reactive; the antigen formed within the body is partially saturated with antibody *pari passu* with its formation; foci of infection are largely shut off from active tissues by necrosis; phagocytosis may prevent the ingested bacilli from functioning as a stimulus.

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