# BACTERIOLYTIC POWER OF IMMUNE SERUM AND THE THEORY OF COMPLEMENT DIVERSION.[1]

## *Part II.*

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In the first part of this article it was shown that the theory of complement diversion will not account for all of certain phenomena observed in connection with immune serum; phenomena which nevertheless appear to be due to one and the same cause. Nor apparently will a second hypothesis discussed — the formation of anti-albumens — account for the phenomena in question, and it was indicated that possibly a solution might be found in the agglutinated condition of the bacilli.

Since the anti-albumen theory, besides being inadequate theoretically, was not found to stand experimental tests, it will be disregarded, and the question will be considered at the outset as between "Complement Diversion" and "Agglutination."

It is proposed to call the phenomenon the "Pro-zone" phenomenon, and the two theories "Complement Diversion" and "Agglutination" respectively.

A. Wolff has suggested that agglutination is a protection to the bacilli, since when the bacilli are agglutinated the lysins cannot attack them so readily, but he has not given any experimental tests in support of this view.

This suggestion of Wolff's, however, coupled with the fact that the theory of complement diversion will not account for all aspects of the pro-zone phenomenon, gave rise to the following considerations:

On working with immune serums one cannot help noticing that the agglutinating power of the serum and the pro-zone phenomenon appear almost simultaneously, and when the

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agglutinating power is high the pro-zone is often broad. This does not necessarily indicate that there is any connection between the two, but it is somewhat suggestive of such a relation.

One can readily imagine that in concentrated serum agglutination of the bacilli takes place so rapidly and completely that the bacilli in the central portion of the clumps are to some extent protected from the immune bodies and complements and are therefore able to grow. As they multiply in the interior of the clumps, and some push their way out, these latter are attacked by the lysins and killed, but by degrees the complements are used up and then there is nothing to hinder the growth of the bacilli.

If the clumps are not so large and dense, as would be the case in more dilute serum, the bacilli are not sufficiently protected and the immune serum is able to kill them.

As an introduction to the subject some of the experiments already detailed may be briefly recapitulated to ascertain if they offer any indication that the agglutination theory is reasonable, and subsequently some experiments specially undertaken with the object of testing this theory will be given in detail.

It may be mentioned that in all the experiments tabulated each tube was made up to two cubic centimeters with physiological salt solution, three drops of broth and five drops of bacillary emulsion being then added. For plating out, five drops of the mixture were taken and mixed with eight cubic centimeters melted agar.

TABLE VIII. OF PART I.

| | Serum Dilution (Each Tube made up to 2 cc.) | Typhoid Bacilli, Colonies on Plates in 5 Hours. | | Serum in 24 Hours. | | |
|---|---|---|---|---|---|---|
| | | Normal Serum. | Typhoid Immune Serum. | Normal Serum. | Immune Serum. | |
| 1 | 1–1 | 0 | Very many thousands. | Clear } Killing-zone. | Growth } Pro-zone. | 1–1 |
| 2 | 1–2 | 1 | " " " | Clear } Killing-zone. | Growth } Pro-zone. | 1–2 |
| 3 | 1–5 | 2 | " " " | Clear } Killing-zone. | Growth } Pro-zone. | 1–5 |
| 4 | 1–20 | 2,500 | 4–5,000 | Clear } Killing-zone. | Clear } Killing-zone. | 1–20 |
| 5 | 1–40 | Many thousands. | 4–5,000 | Growth } Post-zone. | Clear } Killing-zone. | 1–40 |
| 6 | 1–80 | Very many thousands. | Many thousands. | Growth } Post-zone. | Clear } Killing-zone. | 1–80 |
| 7 | 1–100 | " " " | " " | Growth } Post-zone. | Growth } Post-zone. | 1–100 |

In this experiment normal and immune serums were tested side by side, under precisely similar conditions, and if the killing limits for each are compared it will at once be remarked how much more slowly the immune serum destroys the bacilli than does the normal serum. In other words, the agglutinated bacilli are destroyed less rapidly than the normal bacilli. This is not an isolated instance; it occurs constantly. There is an indication of this fact also in Table XIII., where bacilli previously agglutinated and washed appear to be less susceptible to the action of immune serum than bacilli in the normal condition, as shown by the broader pro-zone afforded by them.

ABSTRACT OF TABLE XIII., PART I.

*Paratyphoid serum and paratyphoid bacilli.*

| A. Normal Paratyphoid Bacilli. | | | B. Paratyphoid Bacilli Treated with Immune Serum and Washed. | | |
|---|---|---|---|---|---|
| | Fresh Immune Serum Dilution. | Serum in 24 Hours. | Serum in 24 Hours. | Fresh Immune Serum Dilution. | |
| 1... | 1–1 | Growth } Pro-zone. | Pro-zone { Growth. | 1–1 | ...1 |
| 2... | 1–2 | Growth } | Pro-zone { Growth. | 1–2 | ...2 |
| 3... | 1–5 | Clear } | Pro-zone { Growth. | 1–5 | ...3 |
| 4... | 1–10 | Clear } Killing-zone. | Killing-zone { Clear. | 1–10 | ...4 |
| 5... | 1–20 | Clear } | Killing-zone { Clear. | 1–20 | ...5 |
| 6... | 1–40 | Growth } Post-zone. | Post-zone { Growth. | 1–40 | ...6 |
| 7... | 1–80 | Growth } | Post-zone { Growth. | 1–80 | ...7 |

Both of these instances seem to indicate that agglutinated bacilli are better protected from the action of the serum than normal bacilli. In the case of Table XIII., B, the findings are somewhat remarkable, as one would suppose the bacilli to have become occupied by immune bodies in the course of the preliminary treatment with immune serum, and therefore more susceptible than the normal bacilli.[1]

That the bacilli treated with immune serum are less susceptible than normal bacilli might also be used as an argument in favor of complement diversion. However, it must be remarked that the results in Table XIII. given here are rather exceptional. In most of the experiments on these lines there was no apparent difference in the susceptibility of normal bacilli and those previously treated with immune serum.

## EXPERIMENTS TO TEST THE AGGLUTINATION THEORY.

It is not easy to devise experiments which will show the soundness or reverse of the agglutination theory directly, but

[1] Steinhardt remarks on the difficulty of getting typhoid bacilli to absorb intermediate bodies from normal serum.

the following should give some indications as to which is the more probable explanation of the pro-zone phenomenon, Complement Diversion or Agglutination.

I. *Series A.* — Inactivated immune serum is mixed with normal serum, and one hour later bacilli are added. This gives the complements a good chance to combine with immune bodies before the latter come into contact with the bacilli.

*Series B.* — The bacilli are added to inactivated immune serum, and one hour later the normal serum is pipetted into the mixture. The bacilli will have become thoroughly agglutinated before the normal serum is added.

If the pro-zone phenomenon is due to complement diversion, Series A should show a broader pro-zone than Series B, since in Series A there is a better opportunity for the complements to be diverted than in Series B.

If on the other hand the phenomenon is due to agglutination, then Series B should show the broader pro-zone, since the bacilli becomes agglutinated before the complement can combine with the immune bodies.[1]

Ten such experiments were made.

A showed a slightly broader pro-zone in three cases;

B showed a slightly broader pro-zone in four cases,

and the pro-zone was precisely the same in three cases.

Table I. is given as an illustration of the results obtained in one experiment with paratyphoid. Series B is given in reversed order so that the columns containing the results may be easily compared. The pro-zone is precisely the same in each instance.

[1] In this and the following experiments it is taken for granted that bacilli when added to immune serum are agglutinated. Since in dealing with lysins it is only possible to add a small number of bacilli to the serum, the fact of their actually becoming agglutinated cannot be demonstrated. But from experience with what takes place with large numbers of bacilli we may reasonably suppose that there is agglutination.

TABLE I.

*Inactivated paratyphoid serum and paratyphoid bacilli.*

| | SERIES A. Immune Serum + Normal Serum and in 1 Hour Bacilli. | | | | SERIES B. Immune Serum + Bacilli and in 1 Hour Normal Serum. | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | Immune Serum. | Normal Serum. | In 1 Hour Bacilli. | Fluid in 24 Hours. | Fluid in 24 Hours. | In 1 Hour Normal Serum. | Bacilli. | Immune Serum. | |
| | *cc.* | *cc.* | | | | *cc.* | | *cc.* | |
| 1 | 0 | .2 | 5 gtts. | Growth } Controls. | Controls { Growth | .2 | 5 gtts. | 0 | 1 |
| 2 | 1 | 0 | " | Growth } | { Growth | 0 | " | 1 | 2 |
| 3 | 1 | .2 | " | Growth } | Pro-zone { Growth | .2 | " | 1 | 3 |
| 4 | .6 | .2 | " | Growth } Pro-zone. | { Growth | .2 | " | .6 | 4 |
| 5 | .3 | .2 | " | Growth } | { Growth | .2 | " | .3 | 5 |
| 6 | .1 | .2 | " | Clear } | Killing-zone { Clear | .2 | " | .1 | 6 |
| 7 | .06 | .2 | " | Clear } Killing-zone. | { Clear | .2 | " | .06 | 7 |
| 8 | .03 | .2 | " | Clear } | { Clear | .2 | " | .03 | 8 |
| 9 | .001 | .2 | " | Growth } Post-zone. | Post-zone { Growth | .2 | " | .001 | 9 |

The results, therefore, must be considered negative. Bacilli first agglutinated by the immune serum and then treated with complement are neither more nor less resistant than those treated with immune serum and complement previously mixed, so that no data are afforded by the experiments on which to base an argument in support of either theory.

## II. *Shaking Bacilli.*

If bacilli are thoroughly shaken whilst in contact with the immune serum, the clumps should be to some extent constantly disintegrated, and the bacilli which are being shaken should, therefore, be more susceptible than bacilli at rest, provided the agglutination theory is correct.

If on the other hand the theory of complement diversion is correct, there is no reason why bacilli which are being shaken should be more susceptible than those kept under

normal conditions. We must, however, consider that the mere mechanical shaking may have some effect on the bacilli, but controls will enable us to estimate to what extent this factor is of importance.

*Shaking Experiments.*

A machine run by a small one-sixteenth horse power electric motor was devised, by which rubber-stoppered tubes containing the serum could be kept constantly whirling around at a speed of one revolution per second. The entire apparatus was put in the incubator, a parallel series of tubes, kept at rest, being made up for controls. The shaking was continuous for five hours, at the end of which time agar plates were made and the tubes then left at rest in the incubator till the next morning.

Ten experiments were made and although the results were not altogether satisfactory, there was no evidence to show that the bacilli which were being shaken were more susceptible than bacilli kept at rest. The shaking does not appear to inhibit materially the growth of the bacilli, and that the shaking itself was fairly vigorous could be judged from the foamy condition of the fluid on stopping the machine. It is doubtful, however, if the clumps are sufficiently disintegrated to obviate any protection they may afford to the bacilli in their interior, so that little importance can be attached to the experiments, but so far as they go they afford no support to the agglutination theory, since the disintegration of the clumps, to the extent it is accomplished by the shaking, does not increase the susceptibility of the bacilli to the lysins in the least.

III. Some experiments were made to determine if bacilli previously agglutinated by immune serum and then washed are more or less susceptible to normal rabbit serum than are normal bacilli. On washing bacilli free from serum by centrifugalization and suspending in salt solution, the clumps are still of large size and rapidly subside, leaving the supernatant fluid clear. It may therefore be taken for granted

that the washing does not materially affect the agglutinated condition of the bacilli.

Table II. shows that the results were negative.

TABLE II.

*Normal serum and paratyphoid bacilli.*

| | A. Normal Bacilli. | | | | B. Agglutinated and Washed Bacilli. | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | Normal Serum Dilution. | Bacilli, Normal. | Colonies in 5 Hours. | Serum in 24 Hours. | Serum in 24 Hours. | Colonies in 5 Hours. | Bacilli Agglutinated. | Normal Serum Dilution. | |
| 1 | 1–1 | 5 gtts. | Few. | Clear. | Clear. | 0 | 5 gtts. | 1–1 | 1 |
| 2 | 1–2 | " | Hundreds. | " | " | 0 | " | 1–2 | 2 |
| 3 | 1–5 | " | Thousands. | " | " | Few. | " | 1–5 | 3 |
| 4 | 1–10 | " | " | " | " | Hundreds. | " | 1–10 | 4 |
| 5 | 1–20 | " | " | " | " | Thousands. | " | 1–20 | 5 |
| 6 | 1–40 | " | Many thousands. | Growth. | Growth. | Many thousands. | " | 1–40 | 6 |
| 7 | 1–80 | " | " " | " | " | " " | " | 1–80 | 7 |
| 8 | 1–100 | " | " " | " | " | " " | " | 1–100 | 8 |

The agglutinated bacilli are destroyed somewhat more rapidly than normal bacilli, but the net effect is practically the same as seen by the condition of the serum in twenty-four hours. In each case it kills the bacilli up to 1–20 dilution, but no higher.

Since the agglutinated bacilli are certainly not less susceptible than the normal bacilli, no argument can be deduced in support of the agglutination theory, although the negative results cannot be considered as a valid argument against it, since in the normal serum there is no progressive agglutination of the bacilli as in immune serum.

Another point already noticed on page 464 here arises. Leaving the agglutination theory out of the question, the immune bodies do not appear to have attached themselves to the bacilli treated with immune serum, otherwise these should be very much more susceptible than the normal

bacilli, although as a matter of fact they are little or not at all more susceptible. This, however, will not be discussed here, since it does not bear upon the question at issue.

IV. Finally five rabbits were immunized by successive doses of typhoid or paratyphoid bacilli; their serum being tested on the seventh day after each inoculation. Every ninth day the rabbits were again inoculated, until they had received six injections. The injections were then stopped, and the serum tested every fifth day until five additional tests had been made. Rabbit 121 (Diagram III.), however, was treated somewhat differently, eight successive injections being made, but the serum not tested subsequently.

By means of these tests agglutination and pro-zone curves can be plotted and their relations to each other roughly judged.

In Diagrams I. to V. the vertical lines show the lytic power of the serum at dilutions given in the first column, the continuous black lines indicating the killing-zones and the circles the pro- and post-zones.

The dilutions for the agglutinating values are given in the second column, and the curves represent the values attained by the different serums after each inoculation. The agglutinating value was measured by the ability of the serum dilution to afford clumps visible to the eye in one hour.

Living agar cultures were used, inoculations being made into the peritoneal cavity. The figures of the lowest line indicate the amounts inoculated each time, calculated in agar cultures.

A short explanation may be given to furnish a key to the diagrams.

Taking Diagram I., the first vertical line shows the killing-zone of the normal serum extending up to dilution 1–40.

Two days after testing the normal serum the rabbit received an inoculation of one culture. Seven days later the serum was again tested, the killing-zone now running up to 1–120 dilution. After an interval of two days a second inoculation of one-half culture was given, and seven days later a pro-zone appeared, extending to about 1–3 dilution, the killing-zone

being from 1–3 to 1–20 dilution, above which is the post-zone. The agglutinating value after the first inoculation rose to about 1–1,000, and after the second inoculation to 1–2,500.

It may be remarked that the absolute height of the pro-zones and killing-zones cannot be accurately compared from experiment to experiment since it is impossible to emulsify the bacilli to more than approximately the same density each time. It is obvious that if the bacilli are thickly sown the pro- and post-zones will be broader and the killing-zone narrower than if they are more thinly sown, but the lines represent the conditions in a general way.

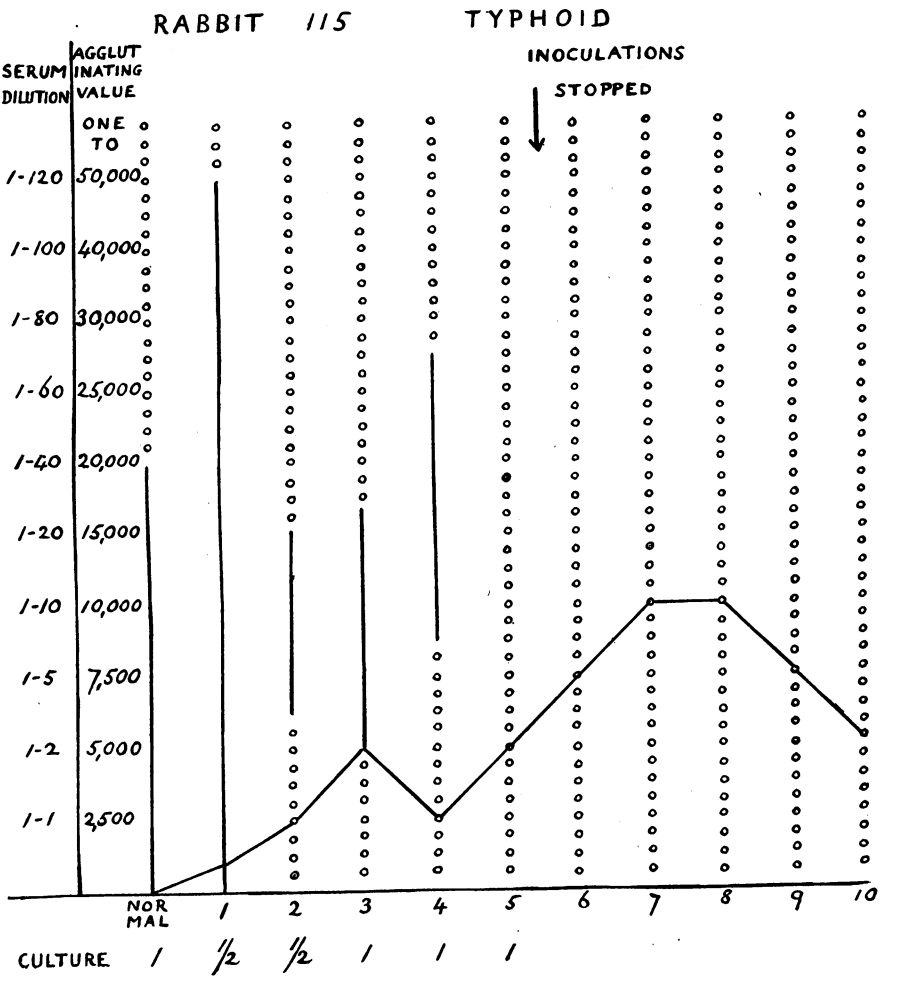
RABBIT 115
TYPHOID
INOCULATIONS STOPPED
SERUM DILUTION
AGGLUTINATING VALUE ONE TO
1-120 50,000
1-100 40,000
1-80 30,000
1-60 25,000
1-40 20,000
1-20 15,000
1-10 10,000
1-5 7,500
1-2 5,000
1-1 2,500
NOR MAL 1 2 3 4 5 6 7 8 9 10
CULTURE / ½ ½ / / /

DIAGRAM I.

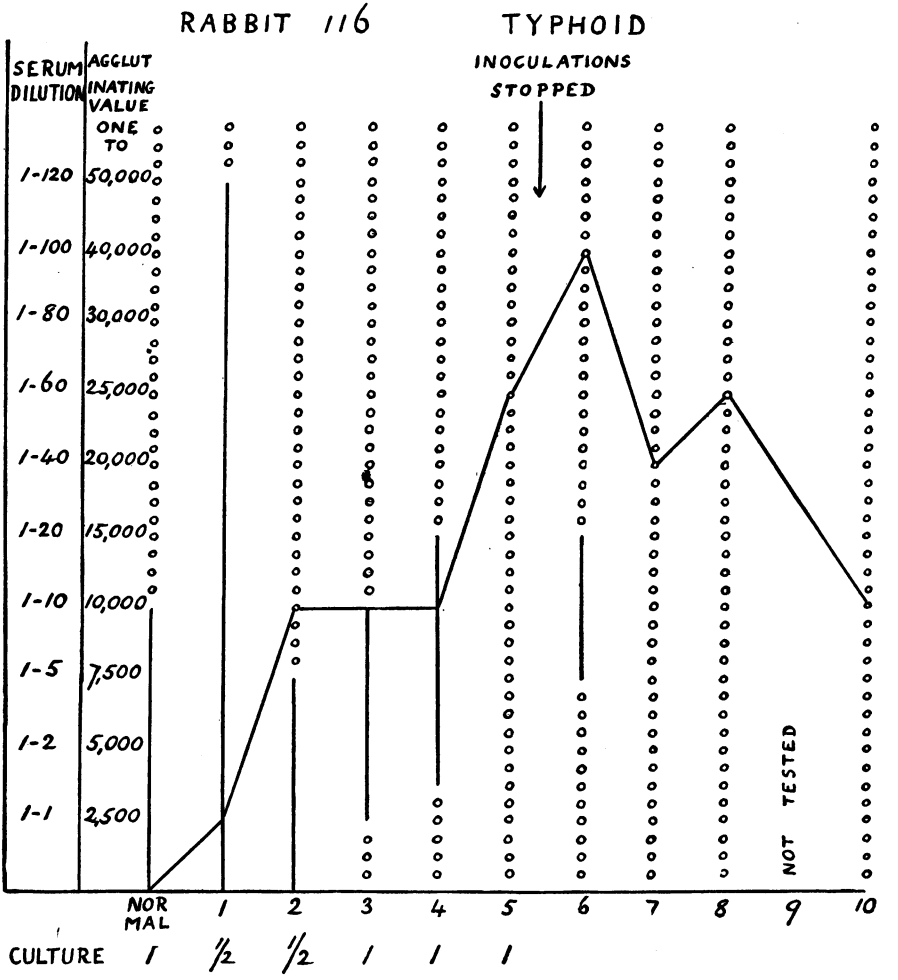
RABBIT 116
TYPHOID
INOCULATIONS STOPPED
SERUM DILUTION
AGGLUTINATING VALUE ONE TO
1-120 50,000
1-100 40,000
1-80 30,000
1-60 25,000
1-40 20,000
1-20 15,000
1-10 10,000
1-5 7,500
1-2 5,000
1-1 2,500
NOT TESTED
NOR MAL 1 2 3 4 5 6 7 8 9 10
CULTURE 1 ½ ½ 1 1 1

DIAGRAM II.

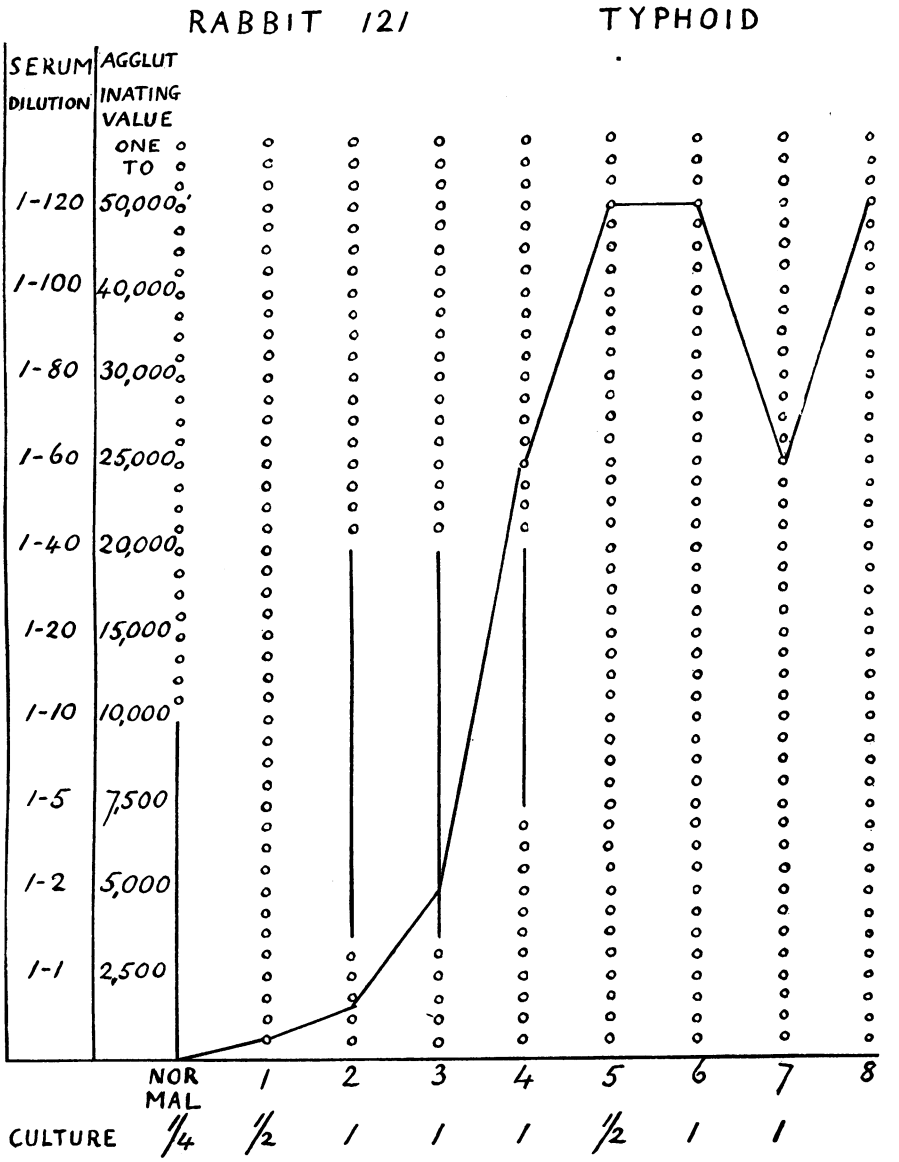
RABBIT 121
TYPHOID
SERUM DILUTION
AGGLUTINATING VALUE ONE TO
1-120
50,000
1-100
40,000
1-80
30,000
1-60
25,000
1-40
20,000
1-20
15,000
1-10
10,000
1-5
7,500
1-2
5,000
1-1
2,500
NOR MAL
1
2
3
4
5
6
7
8
CULTURE
1/4
1/2
1
1
1
1/2
1
1

DIAGRAM III.

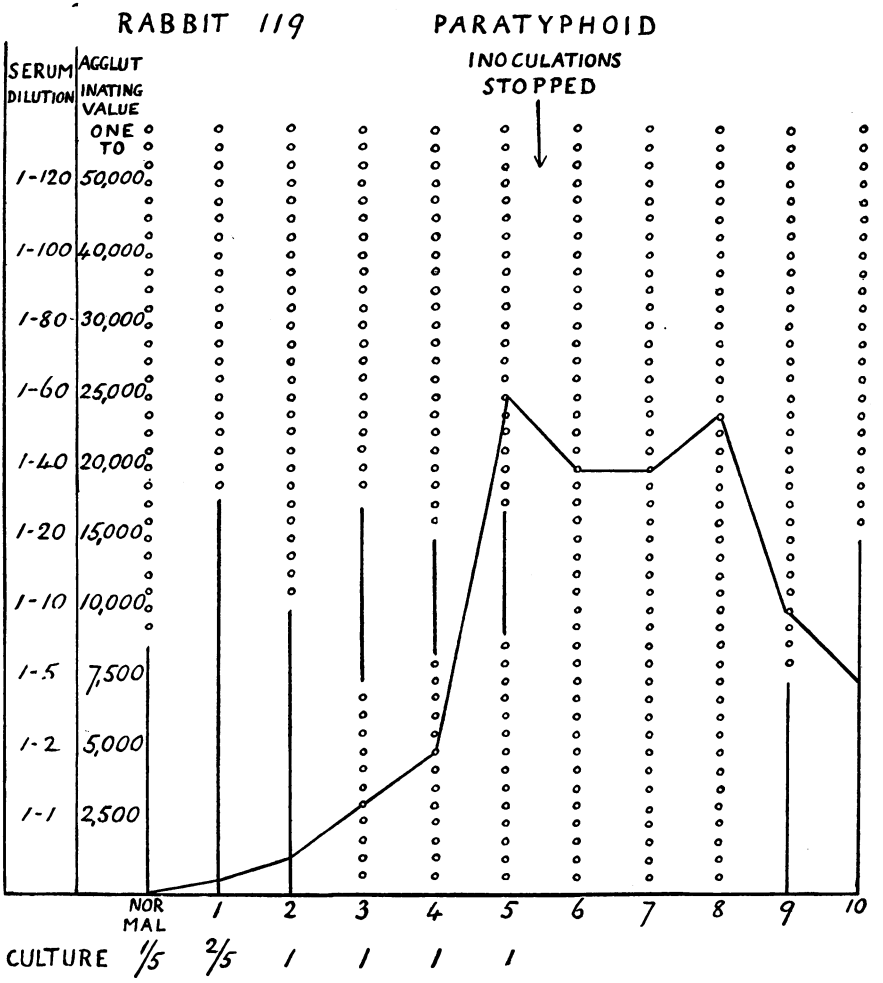
RABBIT 119
PARATYPHOID
INOCULATIONS STOPPED
SERUM DILUTION
AGGLUTINATING VALUE ONE TO
1-120 50,000
1-100 40,000
1-80 30,000
1-60 25,000
1-40 20,000
1-20 15,000
1-10 10,000
1-5 7,500
1-2 5,000
1-1 2,500
NOR MAL
1
2
3
4
5
6
7
8
9
10
CULTURE 1/5 2/5 1 1 1 1

DIAGRAM IV.

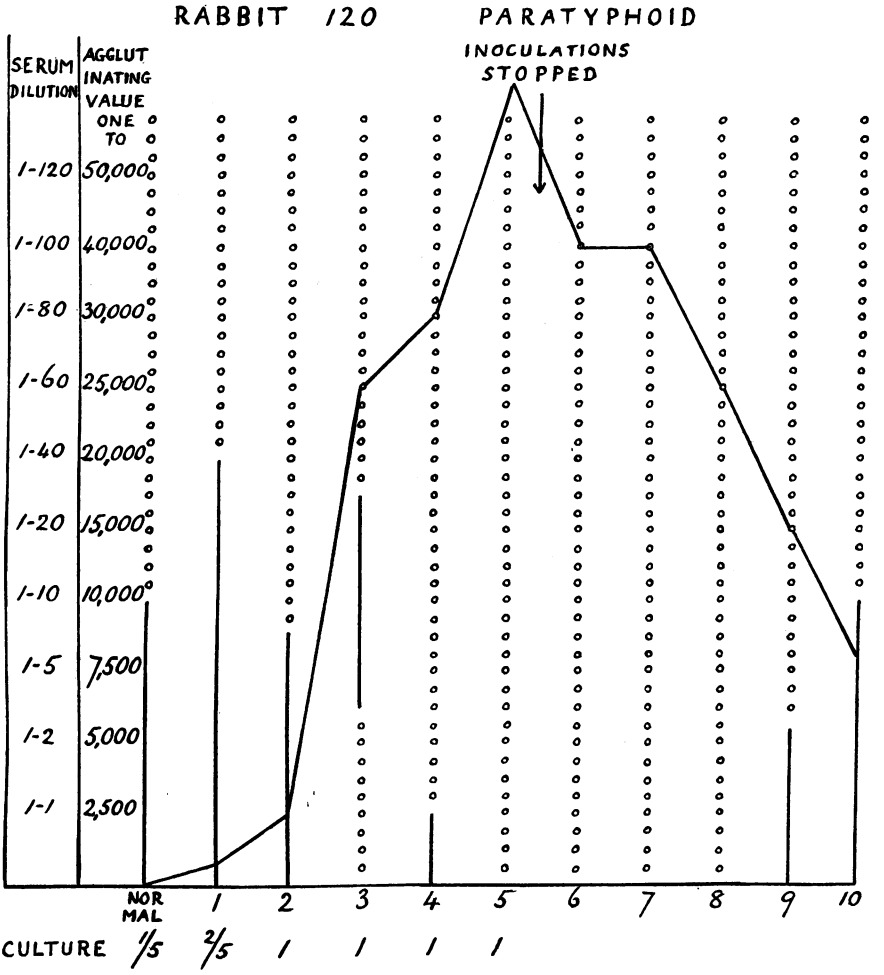
RABBIT 120
PARATYPHOID
INOCULATIONS STOPPED
SERUM DILUTION
AGGLUTINATING VALUE ONE TO
1-120 50,000
1-100 40,000
1-80 30,000
1-60 25,000
1-40 20,000
1-20 15,000
1-10 10,000
1-5 7,500
1-2 5,000
1-1 2,500
NORMAL 1 2 3 4 5 6 7 8 9 10
CULTURE 1/5 2/5 1 1 1 1

DIAGRAM V.

A study of the diagrams brings out some interesting points which may be discussed before proceeding to the main argument, *i.e.*, the relations between the agglutinating power of the serum and the pro-zone.

1. *First stage.* — After the first inoculation the killing-zone reaches a somewhat higher point than with normal serum, although the rise is not necessarily very marked. Rabbit 121 shows no killing-zone at this stage, but this was probably due to an error in manipulation.

2. *Second stage.* — Second to fourth or fifth inoculation. The upper end of the killing-zone then descends again and a pro-zone appears.

3. *Third stage.* — After about four inoculations the pro- and post-zones meet, and there is no killing-zone at all. This condition appears to persist for a considerable length of time after inoculation has been stopped. How long it persists has not yet been determined in the case of typhoid rabbits, but with the two paratyphoid rabbits there seems to be a return to normal conditions about three weeks after the last injection. On account of the serum rapidly reaching the third stage it is often difficult to demonstrate the killing-zone. I have, however, had clear evidence of a killing-zone preceded by a pro-zone in over twenty experiments with immune serums, and there is no doubt in my mind that the phenomenon occurs at certain stages of the immunization. It is important to be certain of this since its occurrence constitutes the chief argument against the theory of complement diversion (see Part I.).

4. Although the fact does not appear in the diagrams, it has been found that at this third stage, if the bacilli are sown very thinly indeed, the concentrated serum will kill them, though the killing-zone is narrow, not extending above 1–10 or 1–20 dilution. There may be some indications of a pro-zone at 1–1, but it is never clearly defined. This point will be referred to again under the general discussion.

Table III. gives an instance of a serum immune to paratyphoid sown with very few bacilli (3,000 colonies on plate). Normal serum diluted up to 1–20 will kill twenty or thirty

times this number of paratyphoid bacilli, and the serum in question did not have the slightest effect on the larger dose of bacilli given in ordinary experiments.

TABLE III.

*Paratyphoid immune serum, third stage, and paratyphoid bacilli.*

| | Fresh Immune Serum. | Broth. | Bacilli. | Colonies at Once. | Colonies in 5 Hours. | Serum in 24 Hours. |
|---|---|---|---|---|---|---|
| 1 | 1–1 | 3 gtts. | 5 gtts. | About 3,000. | 16 | Clear. |
| 2 | 1–2 | " | " | " " | 0 | " |
| 3 | 1–5 | " | " | " " | 0 | " |
| 4 | 1–10 | " | " | " " | 0 | " |
| 5 | 1–20 | " | " | " " | 16 | " |
| 6 | 1–40 | " | " | " " | 500 | Growth. |
| 7 | 1–80 | " | " | " " | 3,000 | " |
| 8 | 1–100 | " | " | " " | Many thousands. | " |
| 9 | Control. | " | " | " " | " " | " |

5. Serum in the third stage, however, contains an immense number of immune bodies, as may be seen from Table IV., conducted under the Neisser and Wechsberg conditions, with a fixed amount of complement. The serum in this case had reached the third stage, being the same as that used in the previously mentioned experiment.

With sufficient complement the serum kills large numbers of bacilli to a dilution of 1–10,000.

TABLE IV.

*Inactivated paratyphoid serum, third stage, and paratyphoid bacilli.*

| | Immune Serum. | Normal Serum. | Broth. | Bacilli. | Colonies at Once. | Colonies in 5 Hours. | Fluid in 24 Hours. | |
|---|---|---|---|---|---|---|---|---|
| | *cc.* | *cc.* | | | | | | |
| 1 | 0 | 0 | 3 gtts. | 5 gtts. | Thousands. | Thousands. | Growth | Controls. |
| 2 | 0 | .2 | " | " | " | " | Growth | |
| 3 | 1 | .2 | " | " | " | " | Growth | Pro-zone. |
| 4 | .1 | .2 | " | " | " | 0 | Clear | Killing-zone. |
| 5 | .01 | .2 | " | " | " | 0 | Clear | |
| 6 | .001 | .2 | " | " | " | 0 | Clear | |
| 7 | .0001 | .2 | " | " | " | Hundreds. | Slight growth | |
| 8 | .00001 | .2 | " | " | " | Thousands. | Growth | Post-zone. |

Experiments have not yet been sufficiently numerous to establish the five principles on a firm basis, but the findings have been remarkably constant, not only with the serums plotted on the diagrams, but also with immune serums of other rabbits less systematically tested.

Turning now to the main point of the discussion and referring again to the diagrams, we find that the agglutinating power of the serum and the pro-zone do not run together so closely as we should expect if the two were interdependent.

Rabbit 115, for instance, rapidly acquires a marked pro-zone, but its agglutinating power is comparatively low, while with Rabbit 116 the agglutinating power runs up very quickly, but the pro-zone is not at all so well defined as with Rabbit 115.

Again with paratyphoid, Rabbit 119, which shows the lower agglutinating power, acquires a pro-zone earlier than Rabbit 120, the pro-zone for the latter only showing on one occasion.

It may be remarked that Rabbits 115 and 119 stood the inoculations much better than 116 and 120. The two latter suffered a good deal, lost flesh, and had to be very carefully treated to save their lives.

But perhaps the most convincing point that can be made against the agglutination theory is the fact that towards the close of the experiments the serums of the two paratyphoid rabbits returned practically to normal so far as their bacteriolytic power was concerned, although their agglutinating value still remained comparatively high. In the case of Rabbit 119, columns three and four show a clearly defined pro-zone, though the agglutinating value only reaches 1–2,500 and 1–5,000. Columns nine and ten, on the other hand, show an agglutinating value of 1–10,000 and 1–7,500, yet there is no pro-zone at all.

This set of experiments, therefore, like those previously given, fails to indicate the probability of the agglutinated condition of the bacteria being the cause of their increased resistance. The agglutinating power of the serum may be high, while the pro-zone is narrow, and *vice versa*.

### *General Discussion.*

During the course of the experiments, detailed in the first part of this article, showing that the pro-zone phenomenon could not be satisfactorily explained by the theory of complement diversion, some incidental observations seemed to indicate that the agglutinated condition of the bacilli in the immune serum might account for the pro-zone, but experiments, undertaken with the special object of showing this to be a probable explanation, have led, as we have seen, to results not only negative but in some respects controverting such a theory.

Not one of the three theories discussed, "Complement Diversion," "Anti-albumen," or "Agglutination," will stand experimental tests. It appears, therefore, as if we must abandon the idea of regarding the anti-bodies themselves as in any way directly responsible for the phenomena observed with concentrated immune serums.

If we look a little further afield we find, apart from the bacteriolysins, that many analogous phenomena have been reported.

### 1. *Pro-agglutinoids.*

All bacteriologists are familiar with the pro-agglutinoids and pro-precipitoids of various authors, and it is not necessary to more than mention them.

There is a pro-, agglutination, and post-zone.

### 2. *Snake Venom.*

Flexner and Noguchi observed that if blood corpuscles are washed free of serum they are not dissolved by snake venom, but on addition of normal serum dissolution takes place. They concluded, therefore, that two principles are necessary for dissolution of the corpuscles — an amboceptor of the venom and a complement contained in the serum. But if an excess of snake venom is added, the washed corpuscles are not destroyed on addition of serum. Calmette attributed this to the action of an anti-hemolysin, Kyes and Sachs to diversion of complement, and Noguchi quite recently to chemical combination between the venom and the hemoglobin of the corpuscles with formation of an insoluble compound.

"Quot autores tot opiniones," but whatever the cause there is obviously a pro-, dissolving, and post-zone.

### 3. *Colloids Precipitated by Colloids.*

Bilz found that two colloids, zirconium hydroxide and colloidal gold, carrying opposite electrical charges in solution, would precipitate each other.

Girard, Mangin, and Henri observed that washed blood corpuscles are agglutinated by certain unstable colloids — ferric hydroxide, arsenic sulphide, etc. In both of these instances a zone of optimum concentration was observed, above and below which there was no precipitation of the colloids or agglutination of the blood corpuscles. In other words, there is a pro-, precipitation, and post-zone.

### 4. *Colloids Precipitated by Salts.*

Galeotti and Pauli, in experiments on precipitation of albumens by salts of the heavy metals, observed that copper, silver,

or zinc salts will cause precipitation, but only at certain optimum concentrations.

Dr. S. P. Beebe informs me that thymus nucleo-histon shows precipitation behavior with a number of salts that is quite analogous to the pro-zone phenomena under discussion.

### 5. *Colloids Precipitated by Alcohols.*

Spiro remarks that certain aromatic alcohols — carbolic acid, resorcin, etc.— precipitate albumens from solution, but if the alcohols are in excess there is no precipitation.

### 6. *Bacteria Agglutinated by Salts.*

In some experiments now being carried on, in conjunction with Dr. P. A. Shaffer, on agglutination of bacteria by means of salts of the heavy metals, we find in every case a pro-zone. $Pb(NO_3)_2$, for instance, at N/2 does not agglutinate typhoid bacilli, but shows an agglutinating zone from N/10 to N/1,000, below which again there is no agglutination. Similar results have been obtained with Fe, Al, and Cu.

Now in the last four instances there can be no question of anti-bodies and complements. For some reason or other an excess of the active agent inhibits a phenomenon which only becomes evident at certain optimum dilutions.

The following observations of Galeotti and Gengou seem to have some bearing on this question.

Galeotti, taking water, copper sulphate, and albumen, recognizes that he is here dealing with a system of two phases and three components. At certain concentrations there is precipitation of the albumen, but if any one of the components is in excess the precipitate is dissolved.

NOTE. — If then either the copper sulphate or albumen is in excess we have an example of a pro-zone, and, if the water is in excess, of a post-zone.

Galeotti finds that the proportions of the three components in the precipitate vary according to their proportions in the fluid phase with which they are in contact, and no matter

how the manipulations are carried out there is always some albumen and some copper sulphate left in the fluid phase.

He concludes, therefore, that there is no true chemical reaction between the metal ion and the albumen, but only a loose combination or adhesion of the entire $CuSO_4$ molecule to the albumen, the proportion of albumen and copper sulphate varying in both solid and fluid phases according to circumstances, and following the laws of adsorption or distribution rather than of chemical reaction.*

With the help of the phase rule he has drawn curves representing the various proportions of the components at different concentrations.

Spiro has come to similar conclusions with regard to the precipitation of casein by salts. In the fluid phase there is much water, much salt, and little albumen. In the solid phase there is much albumen, less salt, and much less water, but there is always some of each one of the three components in each phase, the proportions following certain laws of distribution.

In this connection it is worthy of note that Eisenberg and Volk found it impossible to absorb all of the agglutinin of immune serum even by repeated doses of the specific bacilli, and Arrhenius has attempted to explain this as a phenomenon of equilibrium explicable by Guldberg and Waage's law of mass action, with little success, however, according to Michaelis and Nernst, both of whom deny that the law of mass action can be applied to colloidal solutions.

We may also recall the fact that immune serum in the third stage has the power of killing a few bacilli, though only a fraction of the number that can be destroyed by normal serum. Regarding the system as immune bodies (Galeotti's $CuSO_4$), bacilli (Galeotti's albumen), and water, then if the bacilli (albumen) are comparatively few they can be acted upon even if there is a large excess of immune bodies ($CuSO_4$), as in immune serum, and if the immune bodies ($CuSO_4$) are

* There are combinations intermediate between physical mixtures and chemical compounds known under the various names of adsorption, adhesion, distribution, solid solution.

comparatively few, as in normal serum, a larger number of bacilli (albumen) can be killed.

A crude idea of the reasoning may be gained by the following considerations:

Let A = Immune bodies.
B = Bacilli.
C = Water and other inactive components of the serum.
D = Complements.
Supposing A + B = 20% or over, and C = 80% or under = Pro-zone.
" A + B = 19% to 11%, and C = 81%–89% = Killing-zone.
" A + B = 10% or under, and C = 90% or over = Post-zone.
Then if A = 16% and B 2% = 18% and the bacilli are killed.
But if A = 16% and B = 6% —
Then at serum dilution 1–1, 16 + 6 = 22% = Pro-zone.
" " " " 1–2, 8 + 6 = 14% = Killing-zone.
" " " " 1–8, 2 + 6 = 8% = Post-zone.

But it must not be forgotten that a fourth component D, which is diluted "pari passu" with A, is necessary for the reaction to take place, and if A is greatly in excess, by the time it is sufficiently diluted to reach its killing-zone D may have already reached its post-zone. This would explain why the killing-zone disappears as the serum becomes very highly immunized, but can be demonstrated if the amount of D is kept fixed as in the Neisser and Wechsberg experiments.

It is obvious that these equations have their limitations. For instance, the proportions between A and B must be considered. In the supposititious case given one could not suppose that if A = .01% and B = 18.99% = 19% that this would come within the limits of a killing-zone, but for general purposes the equations afford a clue to the situation, provided it is analogous to that investigated by Galeotti.

Gengou, discussing the Girard-Mangin-Henri experiments, points out that blood corpuscles, suspended in salt solution and treated with fine suspensions of $CaFl_2$ or $BaSO_4$, are agglutinated. But if serum is treated with such suspensions, not only is there no precipitation, but the suspended particles are dissociated and become finer than before. Yet he was able to show experimentally that in both instances there was adhesion (adsorption) of the suspended particles — to the

blood corpuscles on the one hand and to the colloidal albumens of the serum on the other, so he concludes that the process is essentially the same, although in one instance it leads to precipitation and in the other to dissociation.

Gengou suggests as an explanation

1. Blood corpuscles + $BaSO_4$. Both substances naturally tend to precipitate out, *i.e.*, are in unstable suspension, and when adsorbed by each other the tendency is heightened.

2. Serum albumen + $BaSO_4$. The albumen is in colloidal solution, *i.e.*, in stable suspension, and when adsorbed by the $BaSO_4$ particles its natural stability overcomes the instability of the latter.

There is certainly a very striking analogy between the examples of pro-zones here quoted from various authors and the pro-zone phenomenon observed with bacteriolytic immune serum, and it appears not unlikely that the laws regulating the phenomenon will have, at some future time, to be explained by the phase rule and laws of adsorption. We are probably dealing here with a purely physical phenomenon which will have to be interpreted by the physical chemist rather than by the bacteriologist

It is more difficult to conceive how the pro-zone for bacteriolysins can be explained by physical causes than the pro-zone in precipitation or agglutination phenomena, but, pending the decision of the physical chemist, we may suppose provisionally that the death of the bacteria is due to coagulation of certain albumens necessary for their existence; a coagulation which does not take place in the presence of an excess of the bacteriolytic (coagulating) agents.

In conclusion it may perhaps be mentioned that I entered into these investigations with no particular bias against current theories. On the contrary, I was inclined to believe in the probability of each one of the three theories here discussed in turn, and only abandoned them one by one on finding that they did not respond to experimental tests.

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