SOME FUNDAMENTAL EXPERIMENTS ON IMMUNITY, ILLUSTRATED.

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Repetition of Fundamental Experiments on some Phenomena accompanying Artificially Produced Immunity, as observed when one Species of Animal has been Immunised against the Erythrocytes of another Species.

It has been my experience that written descriptions of experiments on immunity cannot convey to the reader anything like so forcible an impression as a demonstration of the actual experiments themselves. On account of the long preparation necessary for the demonstration of what are but rapidly passing phenomena an opportunity of viewing them presents itself to very few. I have sought for some method of demonstrating to a wider public than the select few, having opportunity of performing similar experiments, the real value of some investigations in this field of enquiry. Since Ehrlich substituted experiments in the test-tube for experiments on living animals the advance in our knowledge has been great, so great, indeed, that we have hardly had time to properly correlate the new facts which have accumulated, and still less opportunity to impartially appreciate the value of the hypotheses which have been formulated for their explanation. If this paper contribute a little in assisting others to separate the facts from the hypotheses, and to recognise that the explanatory hypotheses and technical phraseology which form so large a part of the literature on immunity have a definite relation to undoubted facts, it will, I hope, fulfil a useful purpose. I have repeated the fundamental experiments of others, and especially those of Bordet and of Ehrlich and Morgenroth,

and I have utilised the actual experiments themselves to obtain graphic records.

The facts related in this paper are for the most part well-known, and they are not exhaustively discussed, but they are presented in a form which differs from that which is customary. The plates seem to me to be of chief importance, and to them an explanatory description is merely appended[1].

My avoidance of the terminology employed by Bordet, Ehrlich and Morgenroth, is solely due to my desire to escape the necessity of using terms, which, although applied to definite phenomena, are so applied as to involve hypothetical conceptions of their significance. I wish to fully acknowledge my indebtedness to the work of those distinguished investigators, and to the work of Max Neisser, v. Dungern, and Hans Sachs.

Methods of Experiment.

My experiments were performed *in vitro*, chiefly because it was possible to satisfy oneself that the phenomena thus observed corresponded to similar phenomena *in vivo*. For *in vitro* experiments of this nature it is essential that all test-tubes, pipettes, vessels, and saline used be sterile. The saline was always 0·86 % solution. Bullocks' blood was used to induce the immunity in rabbits[2]. For special purposes experiments were made with guinea-pigs.

In all cases, except where otherwise stated, serum was obtained by bleeding the rabbits from the marginal vein of the ear. The blood was received in a sterile porcelain capsule and *gently* defibrinated with a sterilised piece of wood. The blood was then well centrifugalised. By this method serum is more quickly obtained and in greater quantity than by allowing it to separate by coagulation of the blood. One can easily and repeatedly obtain 20 c.c. of blood from the ear vein of a rabbit weighing about 2 kilos. With careful defibrination there is rarely excess of blood

[1] The following studies formed a portion of the preliminary stages in an extensive investigation into the mechanism of antitoxine production. New duties have interfered with the satisfactory completion of this work. The subject-matter of this paper formed part of an introduction to a progress report submitted to the Scientific Assessors of the Worshipful Company of Grocers, April, 1902. For a critical discussion of the theoretical explanations of some of the facts related in this paper I would refer to the recent papers of Professor R. Muir (*Lancet*, August 15th, 1903), and Dr Ritchie (*Journal of Hygiene*, 1902), and to the literature there quoted.

[2] It is scarcely necessary to point out that immunity to blood implies immunity to all the different constituents of blood. Only some of the manifestations of immunity to the erythrocytes are considered in this paper.

pigment in the serum. It was thus also possible to utilise when necessary both corpuscles and serum. The bullocks' blood was obtained from the abattoir quite fresh and sterile for each determination. Every estimation of a reaction, and whatever was dependent on it, was carried through to a finish without interval. This was done to obtain regularity in the results and to avoid fallacies attributable either to any spontaneous weakening of action due to sera having been kept for many hours, or to increased susceptibility of the bullock's corpuscles, owing to the same cause. This made the work often very arduous, but this was necessary where great stress was being laid on the comparison of degrees of actions manifested.

In such a series of experiments each test-tube contains the same quantity of blood suspension. The serum to be tested is added in decreasing quantities to the constant quantity of blood in each tube of the series. This necessitates the employment of the serum in convenient dilutions and diminishing quantities of each dilution. Therefore to the test-tubes receiving the lesser quantities of serum, or diluted serum, amounts of normal saline must be added to equalise the volume of fluid in the tubes throughout the series.

When any phenomenon is referred to as being manifested on blood, it is to be understood, that in all cases 1 c.c. of a 5 % suspension of fresh bullock's blood in 0·86 % saline is meant. The serum had not been removed from the bullock's blood.

Throughout the experiments the dilutions of the sera of which the action was being determined were 1 : 1, 1 : 10, 1 : 100, 1 : 1000, 1 : 10,000, 1 : 100,000, etc. When in a long series of test-tubes the quantities of serum added to the standard quantity of bullock's blood were 1·0 c.c., 0·75 c.c., 0·5 c.c., 0·35 c.c., 0·25 c.c., 0·15 c.c. of each dilution respectively, a comparable series was obtained with regularly diminishing quantities of the active agent. The quantities of saline added were of course 0·0, 0·25, 0·5; 0·65, 0·75 and 0·85 respectively. The test-tubes were conveniently arranged in stands holding three rows of six each; one row for each dilution. The results in the corresponding tubes in different rows (which all had the same quantity of fluid, but different dilutions of the active agent, added to them) represented the effect of the tenth or the hundred-fold multiples of the agent added. Some such arrangement was required in order to facilitate the manifold measurements of fluid sometimes necessary, to allow of uniformity in time occupied, and of compactness in each experiment. In making colour-index comparisons, for example, those given in the graphic records which follow, it is essential that care be taken to add saline in the quantities necessary to raise the amount of fluid in each test-tube to the same quantity. So soon as the necessary mixture had been prepared all the tubes in the series were well shaken, and then placed along with the necessary control tubes for two hours at 37—38° C., after which the result was noted. The tubes were then left in the cold for 12 to 15 hours, and the results again controlled. The duration of stay in the incubator and the degree of shaking to which the tubes have been subjected are important factors in limiting the actions manifested. In experiments such as those about to be described it is important to pay as much attention to the limit where action is no longer visible, as to the limit where complete action manifests itself. The former is a very necessary control, in any single series of experiments, and in comparing the results obtained in series of experiments of different dates.

I. *Relative Indifference of Rabbit's Serum to Bullock's Erythrocytes.*

Normal rabbit serum is an almost indifferent medium for bullock's erythrocytes when the latter are suspended therein. The following experiment illustrates this statement. Each of a series of six test-tubes received 1 c.c. of blood suspension followed by varying quantities of normal rabbit's serum. Saline was added in sufficient quantity to raise the amount of fluid in each test-tube to 2 c.c. All the tubes were then shaken, and placed at rest for two hours in the incubator. At the expiration of this period the corpuscles settle down to a considerable extent and the practised eye can detect and judge if there is evidence of laking (haemolysis) of the corpuscles. This for guidance in the manipulations which follow. The tubes were then placed at 11—12° C. for 12 hours and the result recorded.

Usual Method of Recording such Experiments.

A series of test-tubes receive 1 c.c. of 5 % blood suspension, varying amounts of normal rabbit's serum, and saline q.s. to raise the total volume to 2·0 c.c. in each tube. Such an experiment is usually recorded as follows:

5 % suspension of Bullock's blood in c.c.	Amount of normal rabbit's serum in c.c.	Amount of saline added in c.c.	Result
1·0	1·0	0·	Trace of Hb diffused through lower layers of fluid
,,	0·75	0·25	Minute trace of Hb diffused through lower layers of fluid
,,	0·5	0·5	Nothing
,,	0·35	0·65	,,
,,	0·25	0·75	,,
,,	0·15	0·85	,,
,,	Control + 1 c.c. saline, result = nothing		

The above method of recording the experiments conveys no idea of the appearances to one unfamiliar with them, and does not yield very objective data for comparison with future experiments. Before alluding to what appears to me to be a better method it will be well to consider an experiment with the serum of an immune animal.

II. *Production of Immunity to Erythrocytes*[1].

If 20 c.c. of fresh bullock's blood be injected into the peritoneum of a rabbit, after an interval of a few days (8 days in the following experiment), its serum will be found to have ceased to be a relatively indifferent medium when bullock's corpuscles are suspended in it. Under the previously described experimental conditions bullock's corpuscles now undergo complete laking. To the blood there was added serum undiluted, diluted 1 : 10 and 1 : 100. The experiment is usually recorded as follows:

5% suspension of Bullock's blood in c.c.	Amount of immunised rabbit's serum in c.c.	Amount of saline added in c.c.	Result
1·0	1	0·0	Complete laking
,,	0·75	0·25	,, ,,
,,	0·5	0·5	,, ,,
,,	Undiluted 0·35	0·35	,, ,,
,,	0·25	0·75	? ,, ,,
,,	0·15	0·85	,, ,,
,,	0·1	0·0	,, ,,
,,	0·075	0·25	,, ,,
,,	Diluted 0·05	0·5	,, ,,
,,	1 : 10 0·035	0·65	,, ,,
,,	0·025	0·75	,, ,,
,,	0·015	0·85	,, ,,
,,	0·01	0·0	Trace of laking
,,	0·0075	0·35	? ,, ,,
,,	Diluted 0·005	0·5	Nothing
,,	1 : 100 0·0035	0·65	,,
,,	0·0025	0·75	,,
,,	0·0015	0·85	,,
,,	Control + 1 c.c. saline = nothing		

For a graphic record of the two preceding experiments see Plates II and III.

Graphic Method of recording the Results.

In order to obtain records of a large number of experiments on immunity I have employed a method which occurred to me seven years ago when engaged in some clinical observations with my respected teacher and friend, Sir Thomas R. Fraser. At that time we found it convenient to roughly record the amount of bile-pigment present in the

[1] See footnote No. 2 on p. 32.

serum in cases of jaundice, and the amount of haemoglobin free at different dates in the serum in a case of paroxysmal haemoglobinuria. We did this by placing drops of serum upon white blotting-paper. The records then made remain comparable at the present time. I have utilised this method to obtain, within small compass, compact records of several series of related experiments, and to obtain details of each test-tube experiment in a series.

In the one case (Method I), drops from the clear fluid from above the corpuscular sediment were placed in a series upon glazed paper. In the other case (Method II), immediately after shaking each test-tube, equal quantities of the fluid (and also therefore of suspended erythrocytes, if any were present) were transferred to white blotting-paper and allowed to diffuse. When dry both forms of record were permanent.

The technique of Method I is as follows. The tubes are gently agitated to mix the supernatant fluid as much as possible without disturbing the corpuscular sediment. Measured quantities of the fluid are now withdrawn from each test-tube by means of a marked capillary tube, and expelled in series upon the surface of a sheet of glazed paper. The drops dry on the paper and at once yield a graphic and accurate record of the actual experiment. (See Plate III, p. 68.)

Method II. Since the total volume of fluid in each of a series of test-tubes is the same, equal fractions thereof will yield comparable records. From each test-tube, immediately after shaking, a measured quantity of fluid is withdrawn by means of a fine pipette. The fluid is allowed to gradually diffuse from the tip of the pipette gently resting on white blotting-paper. By this procedure one obtains a series of circular diffusion areas which register the phenomena observed in the corresponding test-tube from which they were derived. In the test-tubes at one end of the series the erythrocytes were unaffected, they settled to the bottom, leaving a clear watery zone of fluid above. On the contrary, if haemolysis occurs, the amount of sediment is diminished, and the fluid is tinged with haemoglobin in accordance with the degree of the solution of the erythrocytes. Where complete laking takes place toward the end of the series there is no sediment, and the fluid is uniformly tinged with haemoglobin. The limits of diffusion of fluid and erythrocytes on blotting-paper do not coincide. The erythrocytes, when present in a diffusing fluid, never spread as far as the fluid, but remain localised about the centre whence diffusion has taken place. The two limits reached produce a circular zone on the blotting-paper. This circular zone is most marked where erythrocytes have not parted with

any of their haemoglobin. The zone is then colourless, and its outer limit ill-defined. On the contrary the zone is coloured if the fluid has contained free haemoglobin, and at the same time the margin reached by the fluid becomes more distinct owing to the accumulation of the blood pigment there. The marginal zone becomes less definite as the amount of free haemoglobin increases and the number of suspended erythrocytes diminishes. It disappears altogether when there has been complete haemolysis, for then a diffusion of the haemoglobin uniform with that of the fluid is possible, although a deeper shade is produced at the margin. The erythrocytes naturally diffuse uniformly, but if they have been clumped together their diffusion is proportionately irregular.

On any radius of such a circular diffusion area one has a reproduction of the appearances presented in the corresponding vertical test-tube. The erythrocytes which would have been deposited in the test-tube are represented by those occupying the central area in the diffusion on blotting-paper, and the zone of fluid in the test-tube is reproduced in the marginal zone of the diffusion area. The marginal zone on the blotting-paper also shows the character of the fluid in the test-tubes, and with the disappearance of the distinction between fluid and sediment in the test-tube the distinction between marginal zone and central area is lost in the uniformity of the appearance of the diffusion area (see Plates II and VIII).

By utilising graphic records obtained by the methods described, all the phenomena referred to in this paper have been illustrated. As regards the occurrence of these phenomena there is no room for doubt, although great diversity of opinion exists with regard to their hypothetical explanation.

III. *Experimental Modifications of the Properties of Immune Serum.*

(1) *Deprivation of acquired character.* (Effect of heat.) An immune serum differs from a normal serum in that it has acquired the power to produce laking of bullock's blood (see Experiment II). When heated in a water-bath at 56° C. for half-an-hour it becomes an even more indifferent medium than the serum of an untreated rabbit:

Heated Immune Serum Undiluted	1·0	c.c.	devoid of evident action	
	0·75	,,	,,	,,
	0·5	,,	,,	,,
	0·35	,,	,,	,,
	0·25	,,	,,	,,
	0·15	,,	,,	,,

Diluted 1 : 10	1·0	c.c.	devoid of evident action	
	0·075	,,	,,	,,
	0·05	,,	,,	,,
	0·035	,,	,,	,,
	0·025	,,	,,	,,
	0·015	,,	,,	,,
Diluted 1 : 100	0·01	,,	,,	,,
	0·0075	,,	,,	,,
	0·005	,,	,,	,,
	0·0035	,,	,,	,,
	0·0025	,,	,,	,,
	0·0015	,,	,,	,,
Control + 1 c.c. Saline			,,	,,

This corresponds to Experiment III on Plate III, and Experiment V on Plate II.

(2) *Restoration of acquired character.* Heated immune serum is placed in a series of test-tubes as above and normal rabbit serum is added thereto, in a quantity (represented by test No. 6, in Experiment I on Plate III), which of itself is devoid of all evident action. The result is as follows :

	c.c.	
Heated immune serum + fresh serum undiluted	1·0	Hb completely diffused into the fluid
	0·75	
	0·5	
	0·35	
	0·25	
	0·15	
Diluted 1 : 10	0·1	
	0·075	
	0·05	
	0·035	
	0·025	
	0·015	Diffusion of Hb diminishing
Diluted 1 : 100	0·01	
	0·0075	
	0·005	
	0·0035	
	0·0025	
	0·0015	
Diluted 1 : 1000	0·0001	? Trace of Hb diffused out
	0·00075	Nothing
	0·0005	,,
	0·00035	,,
	0·00025	,,
	0·00015	,,

Control + 1 c.c. Saline = Nothing.

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This experiment corresponds to No. IV on Plate III.

(3) *Augmentation of the acquired character.* The last experiment shows not only that heated immune serum can have its lost power of laking restored, but that the power of laking may be greatly augmented.

If to the same dilutions of the immune serum used in Experiment II (*i.e.*, unheated serum) there be added normal rabbit's serum as in the last experiment, a similar and slightly greater augmentation of the laking power is manifested. This is due to the amount of the factor which is normally present in the immune serum not having been eliminated by previous heating.

	c.c.	
Serum Undiluted	1·0	Complete laking
	0·75	
	0·5	
	0·35	
	0·25	
	0·15	
Diluted 1 : 10	0·1	Complete laking
	0·075	
	0·05	
	0·035	
	0·025	
	0·015	
Diluted 1 : 100	0·01	? Complete laking
	0·0075	Amount of laking diminishing
	0·005	
	0·0035	
	0·0025	
	0·0015	
Diluted 1 : 1000	0·001	Trace of laking
	0·00075	? ,, ,,
	0·0005	Nothing
	0·00035	,,
	0·00025	,,
	0·00015	

Control + 1 c.c. of Saline = Nothing.

This corresponds to Experiment V on Plate III.

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Difference between Normal Serum and that of an Immunised Animal.

The power of laking bullock's corpuscles has been acquired by what is practically a process of immunisation. As a concomitant consequence the serum of the rabbit has become endowed with a power previously absent. This power is specific. The acquirement of this power of itself distinguishes immune serum from the serum of a normal rabbit. The following experiment demonstrates that immune rabbit serum exerts no effect upon the erythrocytes of a normal rabbit. In this case normal rabbit's erythrocytes were freed from their own serum by centrifugalising and washing four times as a 5 % suspension in 0·86 % saline.

The rabbit's erythrocytes (1 c.c. of 5 % suspension) were then subjected to the influence of the immune serum.

Amount of immune rabbit's serum in c.c.	
1·0	In all the tubes the corpuscles settled down, leaving the fluid above them clear.
0·75	
0·5	
0·35	
0·25	
0·15	

Control + 1 c.c. Saline = Nothing.
Control for colour, Serum 0·25 + 0·25 Saline.

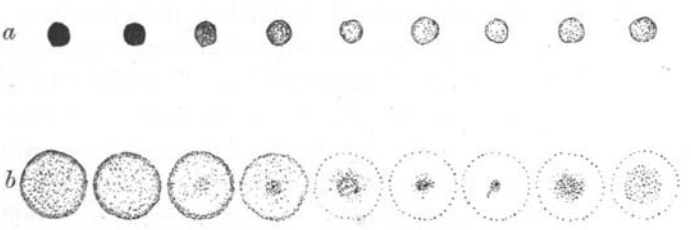
This corresponds to Experiment VI on Plate III, and illustrates the general law that the serum of the immunised animal remains innocuous for the cells of its own species. Exceptions to the specific nature of this phenomenon have indeed been reported, but as this question is still the subject of controversy I refrain from further discussion of the topic.

IV. *Agglutinating Power also acquired.*

There is often (as shown in the accompanying figure) a somewhat abrupt disappearance of the phenomena of laking, so that the range of haemolytic action does not descend so far in the series of test-tubes as would be expected.

If the tubes in which the expected haemolysis has not shown itself be shaken, the bullock's corpuscles will be found clumped

together, an occurrence which personally I have never observed to result from the action of normal rabbit's serum. The following experi-



To show how the occurrence of agglutination interferes with the lower limit of haemolysis. *a*. Fluid only on glazed paper. *b*. Fluid with suspended corpuscles, if present, diffused on blotting-paper.

ment illustrates well what is meant. In the dilution of 1 : 100 a serum was still able to induce complete laking. The laking was almost complete with 0·005 c.c., but was unexpectedly almost absent with 0·0035 c.c. as shown below. The tubes in the series containing higher dilutions are not quoted.

		Amount in c.c.	Result
Immune	(1)	0·01	Complete laking
serum	(2)	0·0075	,, ,,
1 : 100	(3)	0·005	Trace of ,,
	(4)	0·0035	Trace of laking, almost complete agglutination
	(5)	0·0025	?Trace of laking, almost complete agglutination
	(6)	0·0015	No trace of laking, almost complete agglutination
	(7)	0·001	Agglutination diminishing
	(8)	0·00075	
	(9)	0·0005	Trace of agglutination
	(10)	0·00035	Nothing
	(11)	0·00025	,,
	(12)	0·00015	,,

Control + 1 c.c. Saline = Nothing.

The phenomena exhibited in such a series are very well shown in Experiment II on Plate II.

Laking and Clumping independent phenomena.

The phenomenon of agglutination is quite independent to that of haemolysis to which, at one time, it was regarded as the necessary preliminary. By suitable experiments the entire independence of the phenomena can be demonstrated. For example, a degree of heating in

the water-bath which will abolish the power of laking will leave unimpaired the power to agglutinate. This loss of power to lake and retention of the power to agglutinate is demonstrated in Experiment III, on Plate II. On the same record a further demonstration of the independence of the two phenomena is given, for an acquired power to lake is present in the absence of agglutination. The power to lake may be abolished by heating. In the above case the heating was for $\frac{1}{4}$ hour at 56° C., but for any particular serum the degree of temperature and duration of heating has to be worked out in order to ascertain the best experimental conditions. At a definite temperature and with sufficiently prolonged exposure both the power to agglutinate and to lake are permanently abolished.

The agglutination of the red blood corpuscles is believed to be analogous to the clumping of bacteria by specific immune sera. The laking of the corpuscles or haemolysis is held to be similar to bacteriolysis.

The Serum of an Immunised Animal has acquired New Properties which do not cause, but merely accompany Immunity.

From the six fundamental experiments demonstrated on Plate III, the conclusion has been drawn that the serum of a rabbit which has been immunised against the blood of the bullock has had two new properties superadded to those naturally present. In addition to a something which is very susceptible to heat and normally contained in rabbit's serum, other bodies are present which owe their origin to the injection of the bullock's corpuscles, and which for the sake of clearness we will simply regard as concomitants of immunity. The acquired powers to agglutinate and to lake are thereby explained.

Much confusion has been introduced into these matters by the fact that the something present normally in rabbit's serum, and also in the serum of the immunised rabbit, is enabled to effect more ready liberation of the haemoglobin contents of the erythrocytes if these have been acted upon by the serum of an immunised animal. Extensive investigation has shown that the diffusion of haemoglobin is but an incidental phenomenon in the reactions here taking place. Some believe that the erythrocytes are destroyed by the actions of osmosis in a manner exactly analogous to the action of distilled water on them. Others assert that osmotic laws play only a minor part, and that the red cells are dissolved in consequence of a chemical union between them, the products due to immunity, and something normally present in serum. The reaction requires to be analysed in order that the relationship of the different factors may be made more evident.

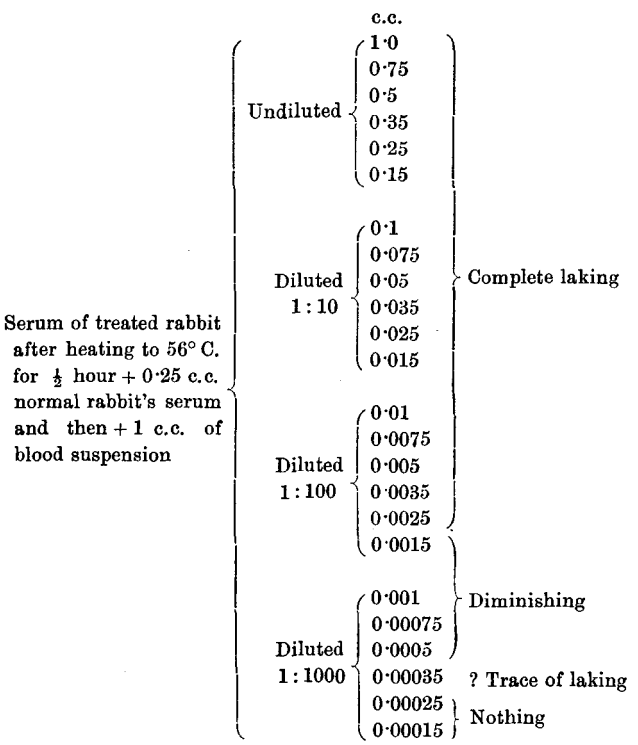
V. *Analysis of the Mechanism by which Erythrocytes are laked.*

(1) *Preliminary determination of the three constants necessary for complete haemolysis.* The following experiments were performed when an opportunity of using a serum devoid of any power to produce agglutination of bullock's erythrocytes was obtained. The experiments about to be referred to are recorded graphically on Plate II. As in previous experiments, the dose of normal rabbit serum (itself devoid of any action on bullock's erythrocytes) must first be determined.

	Amount in c.c.	Result
(1) Normal Serum	1·0	Trace
	0·75	? Nothing
	0·5	Nothing
	0·35	,,
	0·25	,,
	0·15	,,

Control + 1 c.c. Saline = nothing.
Control for colour 0·25 Serum + 0·25 Saline.

This corresponds to Experiment I on Plate IV.



Control + 1 c.c. Saline = nothing.
Control for colour 0·3 Serum + 0·3 Saline.

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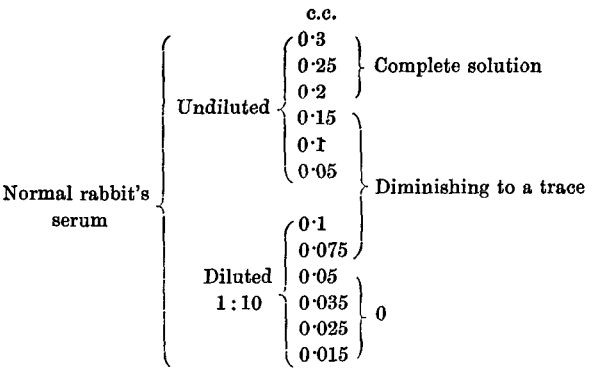
It has then to be ascertained that by heating for half-an-hour at 56° C. the serum of a treated rabbit (which had a strong power to produce laking) has been deprived of all action, also that it is possible to restore this action by the addition of normal rabbit serum, in a quantity which of itself is devoid of action. This is done as above, p. 43.

Experiments performed without the presence of normal serum show the heated serum to be quite an indifferent medium (see Experiment II and Experiment III, Plate IV). In Experiment III not 0·0025 of serum, the actual minimal laking dose, but a larger quantity, viz. 0·0035 c.c., was decided on for subsequent employment.

It had now to be determined what was the minimal quantity of normal rabbit's serum necessary to bring out the full action of 0·0035 c.c. of the heated immune serum. The employment in Experiment III (see Plate IV), of 0·25 c.c. of normal serum has been purely arbitrary, experience having, however, shown this to be a suitable quantity. The following experiment was therefore made. In each tube there was placed the above determined quantity of heated immune serum, 0·0035 c.c., and to it there was added in diminishing quantity normal rabbit's serum as follows:



Control + 1 c.c. Saline = 0.
Control for colour + 0·3 normal serum + 1 c.c. Saline.

It was decided to use 0·25 c.c. of normal serum and not 0·2 c.c. which the above experiment showed was the minimal necessary dose of normal rabbit's serum. The constant volumes of the different factors responsible for the haemolysis which are necessary for the experimental analysis of their relations to one another have now been determined.

(2) *Relations of the responsible factors to one another.* These preliminary determinations having been made, it was now possible to perform the following experiments on the behaviour of the three factors (viz. bullock's erythrocytes, the something normally present in rabbit's serum, and the new factor present in the latter after immunisation against bullock's corpuscles) to one another. We proceed in such a way that *one* of the latter two factors is placed in contact with the erythrocytes. Then, by centrifugalising the erythrocytes from the fluid, it is possible, by adding the factor previously omitted, to determine whether the factor first added has remained present in the centrifugalised fluid or if it has passed to the sediment.

The Factor called forth by Immunisation.

(1) Of the heated serum of the immunised rabbit ten times the quantity, which it had been determined was adequate to effect complete laking of the 1 c.c. of blood suspension, was taken[1]. To this was added 10 c.c. of blood suspension. The mixture was left in the incubator for half-an-hour and then centrifugalised.

(2) There was thus obtained a sediment containing the erythrocytes and a clear fluid which was decanted, and with blotting-paper its last traces removed from the sediment. The erythrocytes and the rabbit's serum have thus been again separated.

At the commencement of the experiment the conditions had been 10 c.c. of blood suspension, plus 3·5 c.c. of 1:10 dilution of heated immune rabbit serum, *i.e.* a total of 13·5 c.c., therefore 1·35 c.c. contained a solvent dose of serum, and 1 c.c. of 5 % blood suspension.

(3) If now to the centrifugalised fluid 0·05 c.c. of actual blood (not 5 % suspension) was added, these conditions were practically reestablished, provided that the sediment had not carried anything down with it before or during the process of separation from the fluid.

To the sediment there was added 13·5 c.c. of saline; then 1·35 c.c. was equivalent to 1·35 c.c. before centrifugalising. There remained the possibility that the constituents of the heated serum which had been poured off had been taken up by the erythrocytes before these were centrifugalised.

(4) Of the centrifugalised fluid the following quantities were taken, and to each was added 0·05 c.c. of blood (not 5 % suspension)

[1] In reality more than ten times the quantity determined in Experiment III, Plate IV, viz. 3·5 c.c. of 1:10 dilution.

and 0·25 c.c. of normal rabbit's serum, *i.e.* thus establishing the conditions necessary to laking of a second supply of bullock's erythrocytes, provided the fluid had remained unaltered.

c.c.	Result
2	0
1·75	0
1·5	0
1·35	0
1·25	0
1·0	0

The result as seen in Experiment V, Plate IV, was that the centrifugalised fluid had by contact with bullock's erythrocytes become an indifferent medium; for on the addition of normal rabbit's serum bullock's erythrocytes remain unchanged. The corresponding experiment with the centrifugalised sediment affords the explanation, and shows that whereas the fluid had lost the capacity to render the erythrocytes susceptible to the influence of the something present in normal rabbit's serum, the sediment had acquired this susceptibility, and in the acquisition had deprived the centrifugalised fluid of the constituent responsible for causing it. The sediment, plus the saline re-added as before described, was used in the quantity of 1·35 c.c. and normal rabbit's serum added in various quantities including also that quantity which preliminary determination of the constants had shown to be necessary to the complete laking of the 1 c.c. of blood suspension when 0·0035 c.c. of heated immune serum was present.

Sediment + saline		Normal rabbit's serum	Result
1·35 c.c.	+	0·3 c.c.	Complete laking
1·35 ,,	+	0·25 ,,	,, ,,
1·35 ,,	+	0·2 ,,	,, ,,
1·35 ,,	+	0·15 ,,	Not complete
1·35 ,,	+	0·1 ,,	Moderate
1·35 ,,	+	0·05 ,,	Slight

This corresponds to Experiment VI on Plate IV.

The quantitative conditions determined in the preliminary experiments had therefore been experimentally reestablished. The corpuscles had absorbed or otherwise used up the factor characteristic of the heated serum of an immunised rabbit in the proportion previously ascertained. By so doing they had become susceptible to the action not only of 0·25 c.c. of normal rabbit's serum, but to the actual minimal dose (viz. 0·2 c.c.) of this serum necessary to bring into effect

the action of the previously determined minimal dose of heated immune serum. The assumption of the existence of special affinities between the bullock's erythrocytes and the factor induced to appear in the serum of the rabbit by their previous injection therefore appears to be fully justified. It will now be legitimate to designate this factor as an "anti-erythrocytic body" directed against the cells (erythrocytes) responsible for calling it into existence.

The Factor normally present in Rabbit's Serum.

The something normally present in rabbit's serum is not quite indifferent[1] to bullock's corpuscles, but this relative indifference disappears when the latter have previously been subjected to the influence of the specific "anti-erythrocytic body." When the experiments just described are repeated with the normal rabbit serum the results for centrifugalised fluid and corpuscles are exactly the opposite of those obtained above. The fluid apparently loses none of its properties, and the bullock's erythrocytes which have been subjected to the action of the normal fluid, appear to have acquired no property that distinguishes their behaviour from normal bullock's erythrocytes.

(1) 10 c.c. of blood suspension and 2·5 c.c. of normal rabbit's serum gives a total of 12·5 c.c. in 1·25 c.c. of which the necessary constant dose of normal serum and 1 c.c. of 5 % blood suspension is contained.

The mixture remained half-an-hour in the incubator and was then centrifugalised.

(2) After centrifugalising the fluid from the sediment, they were tested separately.

(3) The factor omitted at the outset, viz., 0·0035 c.c. heated serum of immunised rabbit, was used to test the behaviour of the factor normally present as follows.

(4) The centrifugalised fluid was taken in the following quantities, and to each tube was added 0·35 c.c. of 1:100 dilution of the heated immune serum, and 0·05 c.c. of actual blood, so that the experimental conditions for the interaction of three factors were established.

Centrifugalised fluid		Heated immune serum		Blood (not suspension)	Result
2·0 c.c.	+	0·0035 c.c.	+	0·05 c.c.	Complete laking
0·75 ,,	+	,,	+	,,	,, ,,
1·5 ,,	+	,,	+	,,	,, ,,
1·35 ,,	+	,,	+	,,	,, ,,
1·3 ,,	+	,,	+	,,	,, ,,
1·25 ,,	+	,,	+	,,	,, ,,

[1] Cf. also Robert Muir, *loc. cit.*

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The normal serum contained in 1·25 c.c. of the centrifugalised fluid had therefore lost nothing by contact with the bullock's erythrocytes. This corresponds to Experiment VII on Plate IV.

To the sediment was added the necessary amount of saline for 1·25 c.c. to again contain 1 c.c. 5% blood suspension, and the factor previously absent, viz., the heated immune serum, was added in the quantities stated.

Sediment + saline		Heated immune serum	Result
1·25 c.c.	+	0·01 c.c.	Nothing
1·25 ,,	+	0·0075 ,,	,,
1·25 ,,	+	0·005 ,,	,,
1·25 ,,	+	0·0035 ,,	,,
1·25 ,,	+	0·0025 ,,	,,
1·25 ,,	+	0·0015 ,,	,,

The factor present normally in rabbit's serum had therefore the appearance of being quite indifferent to the bullock's corpuscles under these experimental conditions. The corpuscles had not absorbed any of it, for had they done so they must have been dissolved in the presence of the excess of heated serum of the immunised rabbit. This corresponds to Experiment VIII on Plate IV. It would lead into a very theoretical digression to discuss whether the results of the foregoing analysis are dependent upon the special experimental and quantitative relations of the factors present in normal and in immune rabbit's serum.

On the basis of the experiments which have been detailed in the preceding pages, one is justified in regarding the body present in the serum of an immunised rabbit as a body of an antitoxic nature. This is the main point.

I do not wish to discuss whether the something present in normal serum of rabbit acts *directly* on the erythrocytes, which have been subjected to what has been regarded as the mordant-like influence of the anti-erythrocytic body, nor whether the factor normally present in serum unites with the anti-erythrocytic body, the latter being regarded as an intermediate body linking on the factor of normal serum to the erythrocytes. The production of the antitoxic body and the readiness of estimation of the degree of its production are the points of interest, at present. The accident that with the assistance of a second factor normally present in serum, laking of the erythrocytes can be produced *does not affect this paper*, except in so far as normal serum has been employed as an indicator of the modification produced in erythrocytes which have been acted upon by an immune serum. It is not proposed to discuss the theoretical explanations of the mechanism of haemolysis.

VI. *The Course and Progressive Augmentation of Artificial Immunity.*

The consequences of injecting erythrocytes illustrate exceptionally well some of the features in the course and progressive augmentation of acquired immunity. As in the case of a toxine, the primary injection is followed by an interval within which immunity is being acquired, but is usually not demonstrable. Some of the phenomena of immunity soon become demonstrable, and from day to day they become more strongly developed till a maximum is reached. Thereafter, constancy is maintained for a varying period, and ultimately a gradual diminution sets in. The acquisition, rise, and constancy of the haemolytic phenomena following upon the injection of erythrocytes are well shown on Plates V and VI, on which the experimental determinations made at different dates are succinctly recorded. The comparison of the actions at different dates shows the sudden acquisition, the gradual rise, and the constancy of the haemolytic power. These plates also show that further injections of erythrocytes are followed by succeeding augmentations[1] of the haemolytic power, which resulted from the primary injection. In this way the immunisation process may be pushed in order to obtain a serum characterised by great haemolytic or antitoxic powers. The haemolytic powers of immune serum in its natural state are recorded on Plate V, and it will be noted that the curve obtained is different from that shown on Plate VI, on which the haemolytic powers of the heated immune serum are recorded when a constant quantity of normal rabbit's serum has been added to make the haemolysis effective. The difference in the two curves is important. It is due to the fact that the immune serum in its natural state has only acquired anti-erythrocytic body, and has not had any excess of the factor naturally present superadded by immunisation. This latter factor has nothing whatsoever to do with the acquisition of immunity, of which it is quite independent, and for the purposes of these experiments its addition, as in Plate VI, fulfils solely the function of making the erythrocytes effective indicators of the presence of the anti-erythrocytic body. The erythrocytes together with the factor naturally present in the serum form a kind of compound indicator of the presence of anti-erythrocytic body.

[1] Immediately following the injection there is a transitory fall in haemolytic or antitoxic power (see Bullock, *Trans. Path. Soc. London*, 1902).

How is the Progressive Augmentation Artificially Produced?

It must be borne in mind that the haemolytic or antitoxic powers which the serum of an artificially immunised animal may acquire are only some of the manifest accompaniments of immunity, and not its cause. When a certain amount of blood or a small dose of toxine is injected into a highly immunised animal, the immunity is increased although the serum of the animal would be expected to at once abolish the effect[1] of the injections.

Outside of the body 1 c.c. of the immune animal's serum may be able to account for many multiples of the amount of blood, or the toxic effects of many times the dose of toxine, and yet notwithstanding what must be the much greater potency of the total amount of serum in the body, small doses of corpuscles or of toxine are still effective, immunising agents.

It is easy to allow bullock's erythrocytes to load themselves with the anti-erythrocytic body in an immune rabbit's serum until they are unable to take up any more. If this be done before the erythrocytes are injected into an immune rabbit, one may suppose that the process of neutralisation assumed to occur when injections are practised on immune animals, has been imitated outside the body.

We will also be justified in assuming that the supposed neutralisation within the body really does occur, if erythrocytes after saturation with the anti-erythrocytic body *in vitro* give rise to an augmentation in the immunity of an already immune animal. As a matter of fact, erythrocytes which have been loaded with anti-erythrocytic body[2] produced the augmentation in immunity which is demonstrated by the records on Plates Nos. V and VI. The conclusion from these observations is obviously that augmentation of the immunity to erythrocytes occurs notwithstanding the probability that when injected into an immune rabbit the serum of the latter will neutralise them. But this neutralisation involves retention, by the resulting product, of those features which in the unneutralised erythrocyte are responsible for the production of the anti-erythrocytic body.

[1] The toxic effects of multiples of the lethal dose are abolished, and it is perhaps loose reasoning to assume by analogy that all effects will be abolished, for obviously they are not.

[2] Experiments in which such saturated erythrocytes were used for the primary injection yielded positive results in 6 out of 8 cases. The haemolytic power was, however, in three instances less than in the controls in which the same quantity of erythrocytes in their natural condition was injected. The reasons for this difference seem obvious. The anti-erythrocytic body produced is identical in both cases.

In view of the theoretical importance of these conclusions, it will be well to state that pains were taken to exclude fallacies, and that the same result was obtained (1) when the immune serum used to saturate the erythrocytes was obtained from one rabbit and the "neutralised" erythrocytes were injected into the peritoneum of another immune rabbit.

(2) When the immune serum was derived from the rabbit into which the injection was made.

(3) When the immune serum was derived from the animal into which the injection was made, the primary immunity obtained by subcutaneous injection, and the injection of "neutralised" erythrocytes was made into the peritoneum.

Under these circumstances there could be no question of the conference of passive immunity by means of a more highly haemolytic serum.

Summary of Haemolysis Experiments.

From the foregoing repetition of the experiments of Bordet and Ehrlich and Morgenroth, the conclusion is now drawn that by a process of immunisation, in the case of rabbits, a factor directed against the erythrocytes of the bullock is produced, and that this factor is in a wide sense of the term of an antitoxic nature, and in a narrower sense an "anti-erythrocytic body." The only real phenomena which are the concomitant consequences of acquired immunity are the production of the agent or agents directed against the erythrocytes and their reaction with the latter. The erythrocytes which have taken part in this reaction are laked in the presence of an agent present normally in serum, and we are thereby enabled to detect the existence of one of these concomitants. This seems to me to be the important fact, and without discussing the conflicting hypotheses bearing on the mechanism of the process, I take it as established that a means for exactly quantitatively estimating this antitoxic, or more correctly, anti-erythrocytic body, is afforded by the addition of a constant quantity of the serum of a normal rabbit[1].

[1] It is to be noted that throughout these experiments the normal serum used has been obtained from the same species as the immune serum, viz., the rabbit. It is customary to employ the normal serum of the guinea-pig. I used normal rabbit's serum to obviate introducing new factors in the conditions of experiment and consequently possible fallacies. The relations of the quantities of the immune and normal sera necessary to effect laking may be made to vary—a larger proportion of heated immune serum requires a smaller quantity of normal serum and *vice versâ*.

This holds good even if the presence of the serum of a normal rabbit do no more than provide conditions favourable to the solution of the membrane of the modified erythrocytes. Nor does it matter whether the anti-erythrocytic body has chemically united with constituents of the erythrocytes to provide new bodies soluble in the presence of normal serum, or combining to form products soluble in the constituents of normal serum. Even if, as some hold, the membrane of the erythrocytes is simply ruptured in consequence of its being no longer able to resist osmotic pressure previously withstood, the procedure is reliable for the estimation of the extent to which one concomitant feature has been developed in the immunity attained by an animal immunised against erythrocytes.

VII. *Conference of Passive Immunity to Erythrocytes.*

The basis of serum therapeutics is that in suitable cases the injection into another individual of the serum of one actively immune, confers on the former some of the characteristic accompaniments of the latter's immunity. The anti-erythrocytic serum of the rabbit readily confers its power on the serum of rabbits or guinea-pigs into which injections of this serum have been made. The passive acquisition of anti-erythrocytic power may be demonstrated without difficulty by the methods previously recorded, if the sera be examined soon after the injection. After a short lapse of time the power thus conferred is lost. In the rabbit it appears to gradually diminish without any attendant phenomena: but in the guinea-pig (*i.e.*, in a species different from that which produced the anti-erythrocytic body) the decline is accompanied by another phenomenon, viz. the appearance of agencies directed against the foreign anti-erythrocytic body injected. The demonstration of this phenomenon for erythrocytes is a somewhat complicated process and need not be detailed here, because in the case of ricin the experimental graphic demonstration is a much simpler process, and will subsequently be alluded to.

Immunity to Toxines.

As erythrocytes cannot be regarded as toxines in the strict sense of the term it is important to ascertain whether or not the phenomena described for acquired haemolysins are also characteristic of acquired immunity to the toxines proper. For this purpose, experiments have been performed with ricin, crotin, cobra venom, diphtheria, and tetanus toxines. The investigation has been most extensive for ricin. Ricin lends itself well to graphic demonstrations, and therefore the following topics are discussed with more especial reference to immunisation against ricin.

VIII. *A Mixture of Toxine and Antitoxine physiologically neutral in vitro, is also physiologically neutral in vivo*[1].

It is well known that ricin clumps the red corpuscles of the rabbit, and that the serum of a rabbit immunised against ricin possesses the power of abolishing this action by virtue of an antitoxine, which it is customary to call antiricin. The toxic effects of ricin on the living animal have nothing to do with its action on the red blood corpuscles, which in the following experiments are merely used as indicators.

The current belief that if a mixture of toxine and antitoxine be neutral *in vitro*, it will also be innocuous *in vivo*, is based on an experiment published by Ehrlich in 1898, at a time when the lethal action of ricin was believed to be due to clumping of the red blood corpuscles, and consequent thrombosis. I have been able to fully confirm Ehrlich's conclusions; but venture to qualify them by adding that the statement which heads this paragraph is only rigidly applicable to the one species of animal which has yielded the antitoxine and serves for the determination of the lethal dose. In Ehrlich's experiment erythrocytes were used to indicate where free ricin was present in a series of mixtures of ricin and antiricin; then a similar series of mixtures was tested on animals, and the conclusion was arrived at that the erythrocytes gave reliable indications of the physiological neutrality of such mixtures for living animals.

I have repeated Ehrlich's experiment in a different and more detailed manner. It was possible to devise a procedure whereby in a series of test-tube experiments the minimal agglutinating dose of ricin for erythrocytes was so adjusted that this quantity was also the minimal lethal dose for a rabbit of two kilogrammes, if subcutaneously injected. Therefore, in the experiments which follow on the relations between ricin and antiricin *in vitro*, these relations are also equally those which obtained when corresponding mixtures were injected into living rabbits. What is spoken of as complete clumping in the test-tubes is equivalent to death in a rabbit of two kilogrammes, and lesser degrees of clumping correspond to diminished degrees in the toxic effects of the ricin. In

[1] This is merely stating in other terms the experimental observations necessary for the study of the progressive augmentation of immunity to erythrocytes recorded on p. 49. A physiologically neutral mixture of toxine and antitoxine can only be determined with a large margin of error. I explained this matter fully in a paper on "The qualitative and quantitative relations of toxine and antitoxine" (*Lancet*, Oct. 17, 1903). For the purposes of immunisation by "neutralised" toxine or erythrocytes, I avoided an obvious fallacy by using more antitoxine than the erythrocytes indicated was necessary.

the same way complete abolition of clumping corresponds to complete abolition of the toxic effects, and imperfect abolition of clumping to diminution in toxicity.

Determination of a Physiologically Neutral Mixture of Toxine and Antitoxine.

(*a*) *The Unit Dose of Toxine.* (See Plate VII.) In a series of test-tubes, all containing the same total volume of fluid, with equal quantities of blood, but progressively diminishing quantities of ricin[1], it is possible to determine the smallest quantity of ricin which causes complete clumping of all the blood corpuscles. The experimental details are similar to those for haemolysis: 1 c.c. of a 5 % suspension of rabbit's erythrocytes which had been freed from their serum by washing, was used throughout. With adequate quantities of ricin the corpuscles may be caused to adhere to the wall of the test-tube as well as to one another, thus leaving the suspending fluid quite limpid. With lesser quantities of ricin the clumping diminishes and an increasing proportion of the corpuscles remains suspended in the fluid after the tubes have been shaken. It therefore follows that if equal portions of fluid be withdrawn from such a series of tubes and allowed to diffuse themselves on blotting-paper, the phenomena visible in the test-tubes will be transferred to a series of circles on the blotting-paper. It will be convenient for the purposes of demonstration to concede that within these circles corpuscles will be absent, if there be complete clumping. They will be present, and the extent and uniformity of their diffusion will increase as the degree of the clumping diminishes. As the quantitative relations are stated on Plate VII they need not be detailed here.

(*b*) *Determination of the Antitoxine equivalent of the Unit Dose of Toxine.* (See Plate VII.) Similarly, if a constant quantity of anti-ricin serum be added to a series of test-tubes otherwise identical with those above referred to, the extent to which the corpuscles diffuse after transference of samples of the fluid to blotting-paper will vary directly with the extent to which the action of the ricin has been abolished.

Thus on Plate VII, series 1, it is evident that down to tube 9, in the series, there was complete clumping, and that lower down the series the clumping diminished inversely as the diffusion of the corpuscles

[1] Exact figures are stated on Plate VII, and full details of similar experiments will be found in a paper in the *Journal of Pathology and Bacteriology*, Dec. 1903.

increased, till a degree of diffusion was reached as great as that manifested by corpuscles merely suspended in saline.

In series 2, uniformity in the diffusion of the corpuscles is present much higher up the series: for the action of the ricin was abolished by antiricin, complete abolition being effected up to tube 6, where the diffusion is as uniform as in the control at the end of series 1. Higher up the series the diffusion becomes less and less uniform as the degree of clumping increases and the action of the antiricin diminishes.

For toxines such as those of diphtheria and tetanus the above determinations can only be made on living animals. The principle is, however, the same.

IX. *Demonstration that the Antitoxine is specific for the species in which it is produced.*

The results of the two preceding experiments may now be reproduced at will, for one can be sure of (*a*) the quantity of ricin which will always completely clump a standard quantity of blood, and (*b*) of the quantity of antiricin serum which will as surely prevent this action In a series of tubes in each of which this equilibrium between ricin and antiricin has been established, it is possible to study the result of the introduction of any new factor into the experimental conditions. If a new factor added act upon the antiricin so as to deprive it of its power to abolish the action of the ricin, the latter will show that this has occurred by reproducing under the new conditions clumping of the corpuscles similar to that in series 1, Plate VII.

In all the tests represented in series 4 (Plate VII) constant quantities of ricin, antiricin and blood were present in the proportions needful for complete absence of clumping as determined above. The clumping in this series has been caused to appear under new conditions by the addition of the serum of a guinea-pig which was in a late stage of passive immunity. The serum of the passively immune guinea-pig was added in quantities increasing from left to right, and it will be noted the clumping is less perfect on the right-hand side. The passive immunity has been conferred on the guinea-pig by injecting the serum of an actively immune rabbit, and as the passive immunity declined, the serum of the animal acquired the peculiarity here demonstrated. This is really a reaction directed against the antitoxine, which although innocuous to the guinea-pig, is still dealt with as an agent foreign to its constitution.

When a new factor is introduced, it is also necessary to perform control experiments[1] designed to exclude fallacies, and especially such as may be introduced by the production of similar results by different mechanisms, *e.g.*, in this case the production of clumping by the two sera in the absence of ricin. Further, when one studies the influence of increasing quantities of the serum of a passively immune guinea-pig, on the above determined constant quantities of ricin and antiricin, it is necessary to control the observations by a similar series of observations in which normal guinea-pig serum replaces the serum of the passively immune guinea-pig in order to be able to be sure that the modifications produced are peculiar to passive immunity. Normal guinea-pig serum is shown to be indifferent to antiricin, which in its presence is quite able to abolish the action of ricin.

When rabbits have been rendered passively immune the phenomenon obtained for guinea-pigs does not present itself, or more correctly, I have not been able to detect its occurrence. It therefore seems possible that the antitoxine was specific for the species of animal in which it was produced.

The same phenomena can be demonstrated when a haemolytic immune serum of a rabbit is used to confer passive immunity on rabbits and guinea-pigs.

X. *Interference of Normal Rabbit's Serum with the Agglutinating Action of Ricin.*

The addition of normal rabbit's serum prevents the proper manifestation of the agglutinating action of ricin. In the test-tube reaction there is nothing to show that this interference is not identical with that exercised by the antiricin serum of an immunised animal. It appears to differ only in degree (cf. Plate VII, series 2 and 3). My experiments were so devised that the minimal agglutinating dose in the test-tube was also the minimal lethal dose for a rabbit. It was therefore an easy matter to determine that the interference of normal serum with the agglutinating action *in vitro* did not correspond to abolition of toxic action *in vivo*. For this purpose the quantity of normal serum which completely abolished the agglutinating action *in vitro* was taken and its action tested *in vivo*. The normal serum did have an influence upon the action of ricin when the latter was subcutaneously injected,

[1] The control experiments are discussed fully in a paper appearing in the *Journal of Pathology and Bacteriology*.

but at most this only amounted to diminution, and in some instances to abolition of the local necrotic action of ricin. The serum was not able to prevent the development of constitutional symptoms. This result showed that the interfering action of normal serum in the test-tube was not due to a quantity of "natural" antiricin too small to be effective, for in the latter case in experiments on the living animal it is the constitutional and not the local effects of ricin which are diminished. The interfering action of normal serum is therefore not due to naturally present antiricin, and in the acquirement of immunity to ricin, the antitoxine (antiricin) is something superadded to the serum of the immunised animal.

XI. *Augmentation of the Acquired Immunity to Ricin.*

Experiments similar to those recorded in connection with the course and augmentation of immunity to erythrocytes were performed with ricin. Erythrocytes can be used to determine that one has a physiologically neutral mixture of ricin and antiricin. With such mixtures experiments parallel to those performed with erythrocytes saturated with anti-erythrocytic body were made, and yielded confirmatory results. The power of the immunised animal's serum to neutralise ricin was increased when such mixtures were injected into an already immune animal, and therefore the reaction between ricin and antiricin had not modified the properties of the former in so far as its capacity to lead to the production of antiricin was concerned. It was also found that rabbits which had repeatedly received physiologically neutral mixtures of ricin and antiricin (derived from rabbits) slowly acquired immunity to ricin.

SUMMARY.

By means of the graphic records given on Plates II—VI and VIII the following facts have been illustrated.

Immunity to Erythrocytes.

Normal rabbit's serum is relatively innocuous for bullock's erythrocytes.

The serum of an immunised rabbit acquires the power to dissolve bullock's erythrocytes.

Besides acquiring the power to dissolve bullock's erythrocytes, an

immune serum may also acquire power to clump them, and it has been shown that the phenomena of haemolysis and of agglutination are independent.

The powers acquired by the immune serum can be artificially modified. The serum may be deprived of its powers by heat. Serum cautiously so deprived of its haemolytic power can have it restored by the addition of normal serum. The haemolytic power of the unheated serum is augmented if normal serum be superadded.

It has been shown that an immune serum only differs from a normal serum by its containing antitoxic bodies which are endowed with powers of specific reaction with the bullock's erythrocytes.

The mechanism by which erythrocytes are laked by an immune serum has been analysed, and it has been shown that the solution of the erythrocytes is effected through the intervention of an anti-erythrocytic body called forth by immunisation. The erythrocytes which have been subjected to the action of this product of immunity give indication of their reaction with it if they are subsequently or concomitantly placed under the influence of normal serum. The erythrocytes and normal serum together, therefore, form a combined indicator of the presence of the anti-erythrocytic body. The part played by normal serum has nothing to do with the acquisition of immunity.

The only conclusion drawn from the above observations is that in the production of immunity to erythrocytes the serum of the immunised animal acquires certain powers which are concomitant with, but are not necessarily the cause of the immunity. This special case of immunity to erythrocytes is therefore probably parallel to induced immunity to those bacterial toxines for which antitoxines are known to exist.

The course and progressive augmentation of artificial immunity to erythrocytes has also been illustrated, and it has been shown that erythrocytes saturated with anti-erythrocytic body retain the power to augment the immunity of an already immune animal.

The serum of an animal actively immunised has power to confer passive immunity upon other animals, and the course of this passive immunity differs in the two cases when it is induced in the same species and in a species alien to that providing the immune serum.

Immunity to Ricin.

The experiments with bullock's erythrocytes have been repeated in parallel observations with ricin in order to permit of the observations on haemolysis being utilised in drawing conclusions on the behaviour of bacterial toxines.

By adjusting the conditions of experiment in such a way that the minimal lethal dose for an animal was also the minimal agglutinating dose in test-tube experiments, it has been possible to give graphic records showing the parallelism between the processes when erythrocytes or living animals are used as indicators of the presence of free ricin. In this way it has been possible to illustrate the determination of the minimal lethal and minimal agglutinating doses of ricin and that quantity of antitoxine (antiricin) which is necessary to abolish the corresponding actions in the animal and in the test-tube, and to show that the mixture of toxine and antitoxine which is physiologically neutral *in vitro* is also physiologically neutral *in vivo* within the limitations imposed by the preliminary determinations.

The consequences of conferring passive immunity upon the guinea-pig by means of active immune serum of the rabbit have also been illustrated, and it has been shown that the alien antiricin serum leads to the production of agencies directed against itself.

Ricin neutralised by antiricin retains its power to produce immunity when injected into the species of animal which has yielded the antiricin.

In connection with the conference of immunity to erythrocytes and to ricin, the nature of the difference between normal and immune sera has been studied. Attention has been directed to the possession by normal sera of properties which simulate those possessed in more marked degree by the immune sera. In the case of haemolysis, it has not been possible to clearly demonstrate that the actions manifested by the normal and immune sera are distinct, although the weight of evidence is in favour of this view. In the case of ricin, however, it has been possible to demonstrate that the immune serum possesses properties which are quite distinct from those possessed by normal serum, and that the latter does not interfere with the action of ricin because of the natural presence of a trace of antiricin. In the case of immunity to ricin, the antitoxine is certainly something which has been superadded to the serum in consequence of the process of immunisation.

The facts ascertained in regard to artificial immunity to erythrocytes and to ricin completely agree. Only in one point is it impossible to be quite sure that the phenomena are identical, viz., in the simulation by normal serum of the powers characteristic of the immune serum; for the demonstration that the two are distinct has been possible for ricin, but open to doubt in the case of erythrocytes. My investigations have been extended to diphtheria and tetanus toxines and to cobra venom, kindly placed at my disposal by Sir Thomas R. Fraser. They have however been interrupted, but so far as they go they support fully the observations made on ricin and erythrocytes.

Some General Conclusions.

The following summary emphasises some points of importance, in a manner which is apt to give to the statements contained therein a definiteness which the results of further investigations may not justify. A subjective element necessarily enters into our judgment of the significance of these complex phenomena.

I. The acquirement of antitoxic power by the serum of an immune animal has been variously explained. The antitoxine may be regarded as a something normally present in the organism, but by a process of immunisation quantitatively increased (Ehrlich's side-chain theory); the antitoxine may be regarded as a new product, *i.e.* as a something superadded to the organism subsequent to the introduction of toxine into it, the mechanism responsible for its production has been differently conceived by Buchner, Fraser, and Metchnikoff. Professor von Behring holds a somewhat intermediate position by assuming that an antitoxic serum differs only from a normal serum in that the albuminous constituents of the latter have acquired a "force inseparable from them, and comparable to the conference of magnetism on previously non-magnetised iron." In my opinion the balance of evidence is in favour of the antitoxine being something superadded to the immune animal.

It is known that normal serum interferes with certain reactions manifested by various bodies *in vitro*. The bodies referred to are antitoxine-producing and not antitoxine-producing. For some bodies, which do not produce antitoxines, I have demonstrated that no increase in this power of serum to diminish a reaction can be obtained[1]. We have

[1] Further details are given in the *Journal of Pathology and Bacteriology* (March, 1902) and in the *Archives de Pharmacodynamie* (T. VIII. 1900).

no right to assume that antitoxine is naturally present in certain animals. The interfering power of serum *in vitro* is not explained by postulating the presence of antitoxine naturally. To prove that the power possessed by normal serum *in vitro* is truly antitoxic, it must be shown that the supposed antitoxic power *in vitro* corresponds to the abolition of the lethal action or an equivalent diminution of the lethal power *in vivo*. This I have found not to be the case. This power of serum *in vitro* has only the appearance of being an antitoxic reaction, it is really a pseudo-antitoxic reaction, and cannot be due to normally present antitoxine.

II. I cannot pretend to assert that the antitoxine is derived from the toxine. I can only advance the following results of experiment as having a bearing on the discussion of what is the source of antitoxine.

As shown in this paper, in the same species as that producing the antitoxine, union with the antitoxine does not abolish the efficacy of the toxine as an antitoxine-producing agent; therefore, under these circumstances, the antitoxine probably does not destroy the toxine, and in the combination (toxine and antitoxine) the toxine exists as such, and a simple explanation is afforded of how it comes about that in a highly immune animal the injection of small doses of toxine leads to further rises in the degree of immunity, although one cubic centimetre of the serum of such an immune animal would neutralise the toxicity of many multiples of the quantity of toxine injected. The view that the antitoxine does not destroy the toxine is no new one; Ehrlich has long held that the two unite with one another in a manner analogous to the formation of a double salt, but I believe these experiments give this view an amount of support which other investigations have failed to do. All sorts of hypothetical heightened affinities of cells, or cell "side-chains" for toxine; of liberation of toxine, allowing the latter to reach the cells; of slow combination of toxine with antitoxine, etc., have been advanced to explain the phenomenon that a small quantity of toxine injected into an immune animal leads to a further increase in the antitoxic power of this animal's serum. All this appears to me to be explained easily as above, assuming that both toxine and antitoxine retain their individuality in the species of animal producing the antitoxine, and that the latter is probably not lost to the organism in exercising its antitoxic function. It is a well-recognised fact that the early stages of those forms of artificial immunity which are accompanied by the appearance of antitoxine, are the most difficult to obtain, and

various devices, preliminary stomach administration, modification of the toxine by chemical means, are resorted to. If the early stages of immunity be once attained the production of further immunity proceeds without difficulty, and it may well be that the earliest and most difficult phase of immunity to attain is the production of antitoxine due to the presence of toxine alone, and that, in the later phases, toxine combined with antitoxine is the important factor in the production of the progressively increasing immunity.

In a species other than that which has yielded the antitoxine the toxine and antitoxine appear to have lost their individuality. The combination (toxine and antitoxine) may here react as one body, and its injection into a different species from that which yielded the antitoxine may lead to no production of antitoxine.

The fact that toxine neutralised by antitoxine can call forth both the earlier and the subsequent stages of immunity with antitoxine production has, however, another consequence of far-reaching importance. The strongest argument in support of the view that an antitoxine is a special secretion of cells, loses its significance if toxine neutralised by antitoxine can produce immunity. It has hitherto not been possible to meet the argument that the amount of antitoxine produced ought to be in proportion to the amount of toxine injected if the former is derived from the latter. It has been argued, that, because the amount of antitoxine produced has exceeded the amount of toxic bouillon injected (as estimated in terms of the number of lethal doses contained, in the ratio of, for example, 1 : 20,000 as found by Professor Woodhead), it was inconceivable that the antitoxine could be formed from the toxine. The number of lethal doses contained in a given quantity of toxic bouillon yields, however, no criterion of the antitoxine-producing capacity of the bouillon. The degree of antitoxine production is proportional to the sum total of the quantities of toxic bouillon injected, or more accurately, the greater the sum total of toxine injected, the greater is the amount of antitoxine produced. The results I have obtained with fully neutralised toxines demonstrate the utter unjustifiability of the above argument. In my cases, in terms of the toxicity introduced and antitoxine produced, the ratio has been as 1 to infinity. I hold it, therefore, to be established that it is erroneous and inadmissible to require that if the antitoxine be formed from the constituents of the toxic bouillon the ratio of the production of antitoxine should correspond to the estimated number of lethal doses of toxine injected. My remarks in this connection are,

of course, only based on the consequences of injecting toxine neutralised by antitoxine into the species of animal from which the antitoxine was derived and when the minimal lethal dose of toxine has also been determined on the same species.

There is, however, another matter which requires consideration in this connection. On the assumption that a toxine may suffer diminution in its poisonous properties without its capacity to unite with antitoxine being in any way diminished, there has been based the conception that in the toxine molecule there are present two independent atomic groups. One of these groups is regarded as being responsible for the production of the toxic symptoms, the other group is regarded as the one which combines directly with side-chains of the cells suffering under the poisonous action. It is alleged that it is only through the intermediation of this second atomic group that the toxic group comes to be able to exercise any influence whatsoever on the cells. In accordance with these conceptions it is held that a toxine, while losing its toxicity, can retain its power to neutralise antitoxine, and the inference is drawn that the two hypothetical atomic groups are absolutely independent of one another. Further, because a toxine which has become diminished in toxicity may still retain its power to lead to the production of antitoxine, it is concluded that the atomic group held to be responsible for the production of the poisonous action has nothing whatsoever to do with the production of antitoxine, and that therefore the latter is solely the consequence of the action of the other atomic group. The atomic group which is regarded as being responsible for the manifestation of toxic action, has been designated Toxophore. The atomic group which has been held solely responsible for the union with the "side-chains" of cells, has been designated Haptophore.

When a quantity of toxine is injected into an animal, we are only able to estimate the quantity by stating that a certain number of lethal doses has been injected. That is, adopting the phraseology of the above hypothesis, we estimate the quantity of toxine injected in terms of the toxophore group. When we estimate the power of a serum to neutralise toxine, we estimate its antitoxic power in terms of the haptophore group. According to the hypothesis explained in the preceding paragraph, the antitoxine is really an antihaptophore, and not an antitoxophore atomic group, and its underlying principle at the outset is that the toxophore group has nothing whatsoever to do with the haptophore group of the toxine, yet in estimating the amount of antitoxine produced we find ourselves actually estimating the one group in terms of the other. For, supporters of this view have demanded that if the antitoxine, or rather, antihaptophore group be a derivative of the toxine the quantity produced should be in direct proportion to the amount of toxine or, rather, toxophore groups injected, and in estimating the amount of antitoxine or, rather, antihaptophore, which is produced in consequence of an injection of toxine, it is their custom to state that the amount is equivalent to so many lethal doses or, in the special phraseology adopted, equivalent to so many toxophore groups. Obviously, there is a grievous confusion of thought in such a method of conceiving the relations obtaining here. At the outset, on injecting a certain number of lethal doses, the number of lethal doses which we have injected will give us, according to

this conception (that the toxophore and haptophore groups stand in no relation to one another), absolutely no idea of the number of haptophore groups which have been injected.

These considerations make it essential to more fully enquire into the extent to which the poisonous properties of a toxine may diminish, and its antitoxine neutralising power nevertheless remain constant; it will also be well to reinvestigate the power of modified toxines to lead to the production of antitoxine, and to exclude the possibility that this modification of a toxine is not a stage in the formation of the antitoxine. Knorr's experiments with tetanus toxine seem to indicate the proper line of enquiry in this connection.

III. I have adduced evidence pointing to antitoxines being specific in two distinct senses:

(1) As regards the toxine leading to its production.

(2) As regards the species in which it is produced.

These conceptions of a double specificity are intelligible if the antitoxine be a new product not present in an immunised animal previous to the injection of toxine. For if the antitoxine be a product derived from the toxine through the intermediation of cell activity, to some extent after the well-known views of Metchnikoff, or in the sense of Buchner or of Fraser, then the specificity in regard to the species of animal is explicable, as is also the specificity in regard to the toxine, because the power to react with the mother toxine will be the basis of the antitoxic function; and the divergence in the character of the antitoxines of differing species is certainly no more remarkable than the very marked divergence in the character of the different albuminous constituents of the sera derived from identical nutriment by differing species.

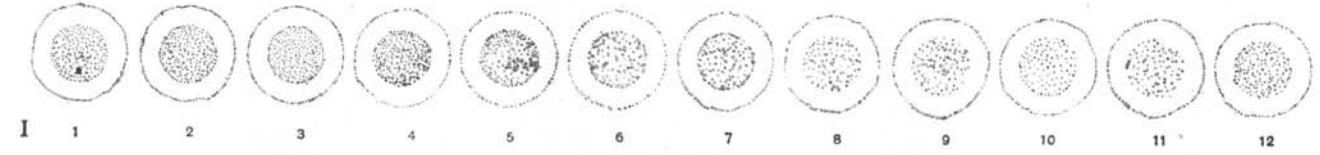
In conclusion, if the antitoxines have a common cause of origin, viz. the toxine, and have also the common property to neutralise the toxine, yet differ among themselves, *a priori* there would seem little objection to the hypothesis that the common cause of their origin should itself be the source, and that the antitoxine is in some way derived from the toxine. While making this statement I do not advance it as my belief, the evidence as yet by no means justifies its acceptance; the following considerations, however, give to it still further support.

IV. Our ideas on immunity have become to a large extent mere generalisations based on the evidence derived from special cases, *e.g.* diphtheria, tetanus, snake-poisoning. The study of other toxic diseases shows that different conditions may exist in these. The assumption which has for so long dominated our conceptions in this

field, viz., that *all bacterial* toxines must of necessity produce antitoxines, and that we only require to devise suitable means to obtain the latter, has tended to confine investigation to one narrow groove, and seems to me to have been a barrier to progress. The organism long ill of tuberculosis, acne, carbuncle, has, for example, lasting opportunity to produce antitoxine to the poisons of the tubercle bacillus and staphylococcus. Have we any evidence that the organism does so? In all sorts of new positions new foci of tubercular material may arise, during a long period of years many new acne pustules may develope. When healing occurs the evidence all points to this being a local process, as in the case of erysipelas. It is needless to multiply instances, in which a toxine appears to play a part in producing symptoms of illness, which do not, however, abate on account of anything of the nature of a true antitoxic reaction. Of all diseases tuberculosis seems to demonstrate that a patient in an advanced stage is able to support and deal with the products of unnumbered millions of tubercle bacilli, such a number as in an earlier stage of his illness would, if suddenly presenting themselves, have surely produced an acutely fatal disease. Is this relative degree of immunity due to antituberculin or other antitoxine? I am not aware that this idea has ever been entertained, and there is certainly nothing to justify it. The evidence does not justify the assumption that *all* bacterial toxines must of necessity produce antitoxines; and if the latter be products derived from the toxines, it may well be that, if the metabolic products consequent on the presence of the toxines in the organism be unable to react with the mother toxine, there is no basis for the antitoxic reaction. It seems not impossible that the generalisation from a few special cases of immunity will prove to have been unjustified, and that each case will have to be regarded from its own standpoint till our knowledge is much more extensive.

Finally, I desire to express my thanks to Dr G. H. F. Nuttall and Mr J. A. Murray for the advice and active assistance which has made the publication of this article and the preparation of the plates possible at this time. The work was performed chiefly in the laboratory of Sir Thomas R. Fraser, but also to some extent in that of Professor Oscar Liebreich, to them and also to Professor W. S. Greenfield I desire to acknowledge my indebtedness. The expenses incurred in connection with the illustrations accompanying this paper have been mainly defrayed by a grant from the Moray Fund for Research of the University of Edinburgh.

PLATE II. *illustrates in detail the experiments figured on Plate III*

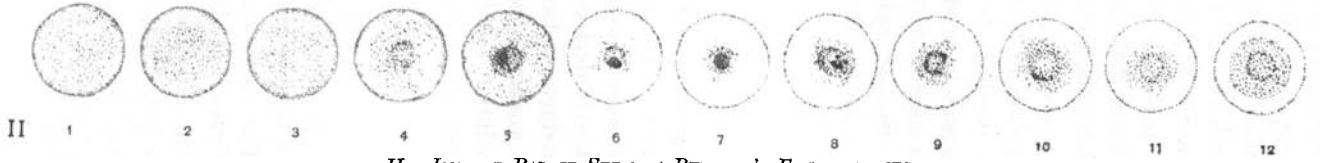


I. Normal Rabbit Serum + Bullock's Erythrocytes.

Control Series

Showing that normal rabbit serum exerts no haemolytic effect upon bullock's erythrocytes.

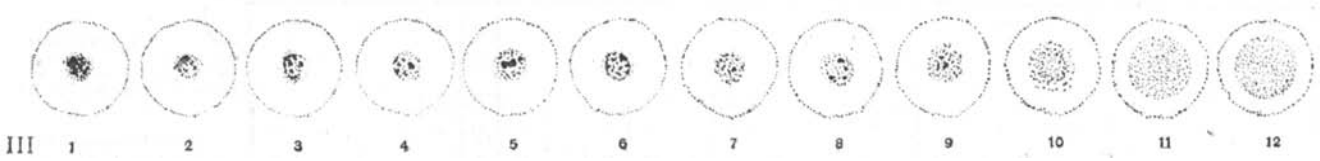
The circular spots are dark in the centre and show a broad clear margin throughout the series. The dark central area is due to intact erythrocytes which have not spread as far as the fluid in which they are suspended. The clear colourless zone shows that the normal serum dilution has not caused haemolysis, for if it had (see first numbers of next series), the whole area of diffusion of the fluid containing haemoglobin in solution would appear dark.



II. Immune Rabbit Serum + Bullock's Erythrocytes.

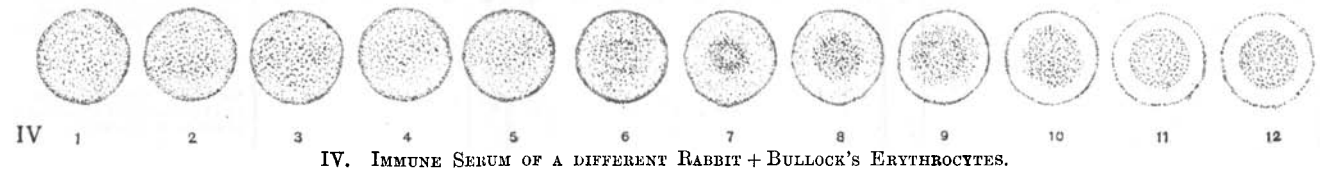
Showing that the serum of a rabbit immunised against bullock's erythrocytes exerts a haemolytic and agglutinative action on these erythrocytes.

The circular areas are uniformly dark where complete haemolysis has occurred. Where agglutination of the erythrocytes has taken place, they diffuse irregularly. Towards the end of the series the diffusion approximates to that in the series above.



III. HEATED IMMUNE SERUM + BULLOCK'S ERYTHROCYTES.

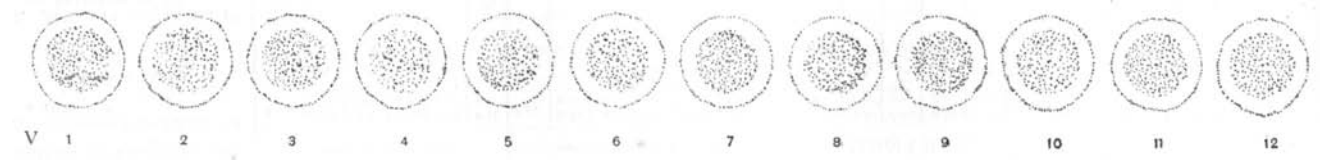
Shows that the haemolytic power has been abolished, but agglutinative power retained, and therefore proving that the two phenomena are independent, and that rapid haemolysis may mask agglutination as in series II above.



IV. IMMUNE SERUM OF A DIFFERENT RABBIT + BULLOCK'S ERYTHROCYTES.

Showing that haemolytic power may be acquired in the absence of agglutination.

The gradual diminution in the darkness of the area of diffusion is accompanied by an equally gradual appearance of erythrocytes near the centre, and ultimately the two zones characteristic of the control series above appear with the disappearance of haemolysis.



V. THE SAME IMMUNE SERUM AFTER HEATING + BULLOCK'S ERYTHROCYTES.

Showing that haemolysis had not masked agglutination in series IV and that haemolytic but not agglutinative power had been acquired. The serum appears to be as indifferent as that in the control series I above.

PLATE III. *Graphic record of the six fundamental experiments on the action of the serum of a rabbit which has been immunised against bullock's erythrocytes.*

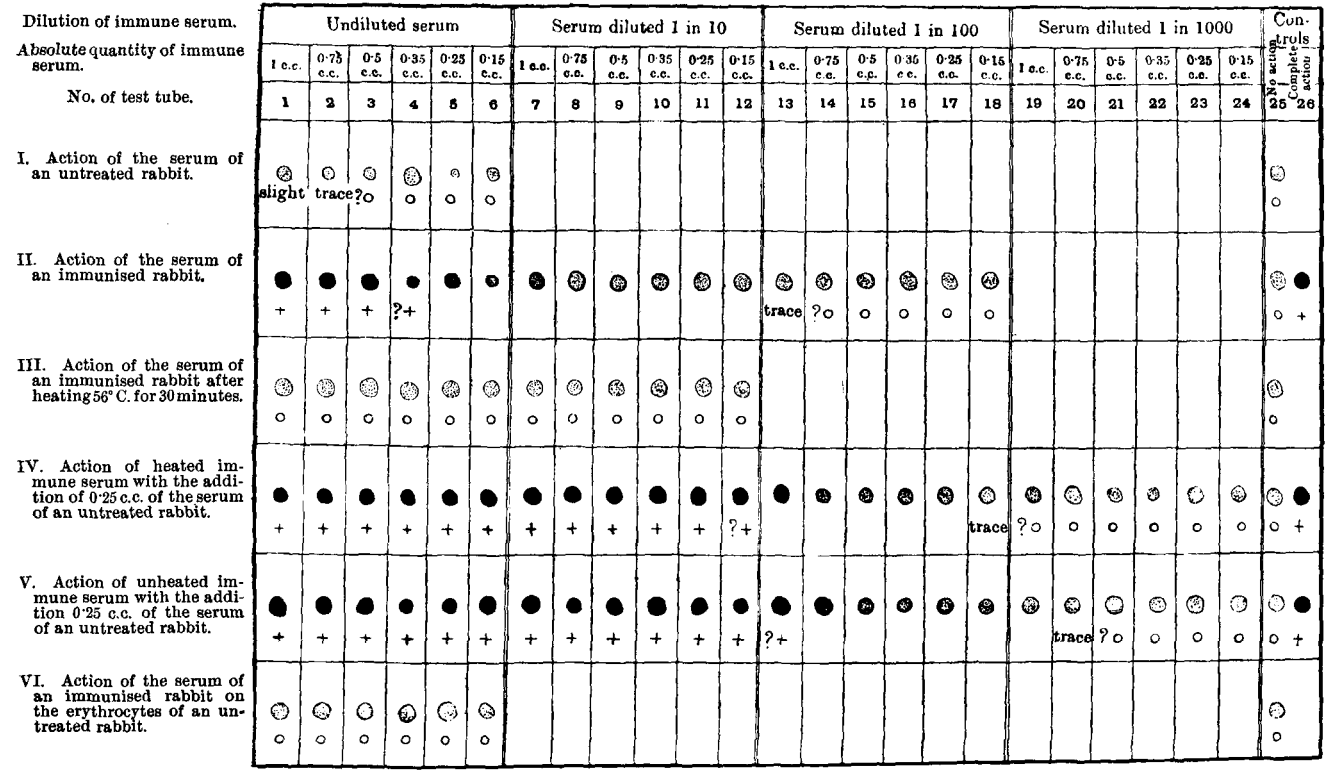


PLATE IV. *Graphic record of the experimental analysis of the fundamental phenomena of Haemolysis represented on Plates II. and III.*

Determination of Constants *.

Dilution of immune serum.	Undiluted serum						Serum diluted 1 in 10						Serum diluted 1 in 100						Serum diluted 1 in 1000					
Absolute quantity of immune serum.	1 c.c.	0·75 c.c.	0·5 c.c.	0·35 c.c.	0·25 c.c.	0·15 c.c.	1 c.c.	0·75 c.c.	0·5 c.c.	0·35 c.c.	0·25 c.c.	0·15 c.c.	1 c.c.	0·75 c.c.	0·5 c.c.	0·35 c.c.	0·25 c.c.	0·15 c.c.	1 c.c.	0·75 c.c.	0·5 c.c.	0·35 c.c.	0·25 c.c.	0·15 c.c.
No. of test tube.	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
I. Determination of the amount of normal serum which has no action of itself.	?o	o	o	o	o	o																		
II. Demonstration that the heated immune serum is inactive.	o	o	o	o	o	o																		
III. Determination of the limits of action of heated immune serum when combined with 0·25 c.c. of normal serum.	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+				trace	?o	o	o
IV. Determination of the limits of action of normal serum when combined with 0·0035 c.c. of heated immune serum.	+	+	+	+	+				trace	o	o	o												

Analysis of the part played by the two haemolytic factors.

V. Centrifugalised fluid. Inactivity of the fluid which had contained a haemolytic dose of immune serum before contact with bullock's erythrocytes.

VI. Centrifugalised erythrocytes. Haemolytic action of normal serum on bullock's erythrocytes after the latter have been in contact with a haemolytic dose of heated immune serum.

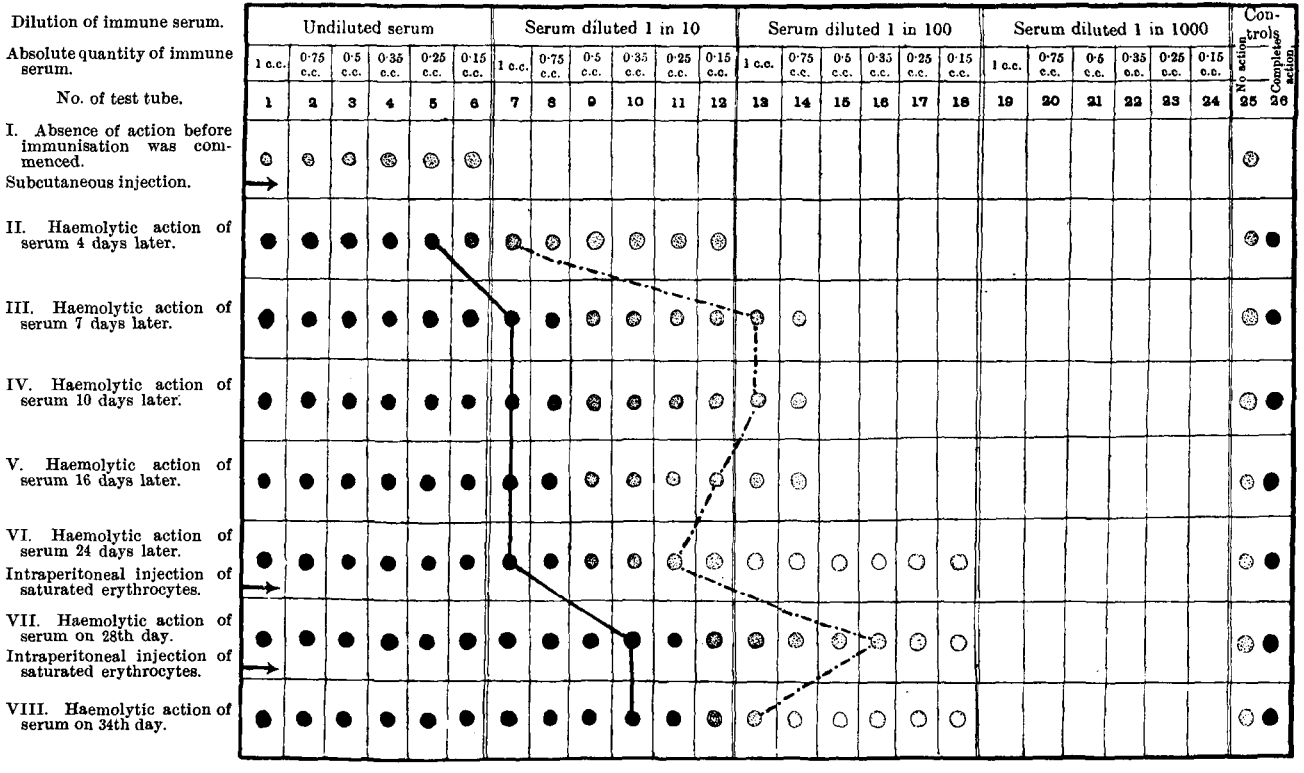
o	o	o	o	o	o	
+	+	+	?+	>		

VII. Centrifugalised fluid. Haemolytic action of the fluid which had contained 0·25 c.c. normal serum, when fresh erythrocytes and 0·0035 c.c. heated immune serum are added.

VIII. Centrifugalised erythrocytes. Inactivity of heated immune serum on bullock's erythrocytes after the latter have been in contact with 0·25 c.c. of normal serum.

+	+	+	+	?+	?+
o	o	o	o	o	o

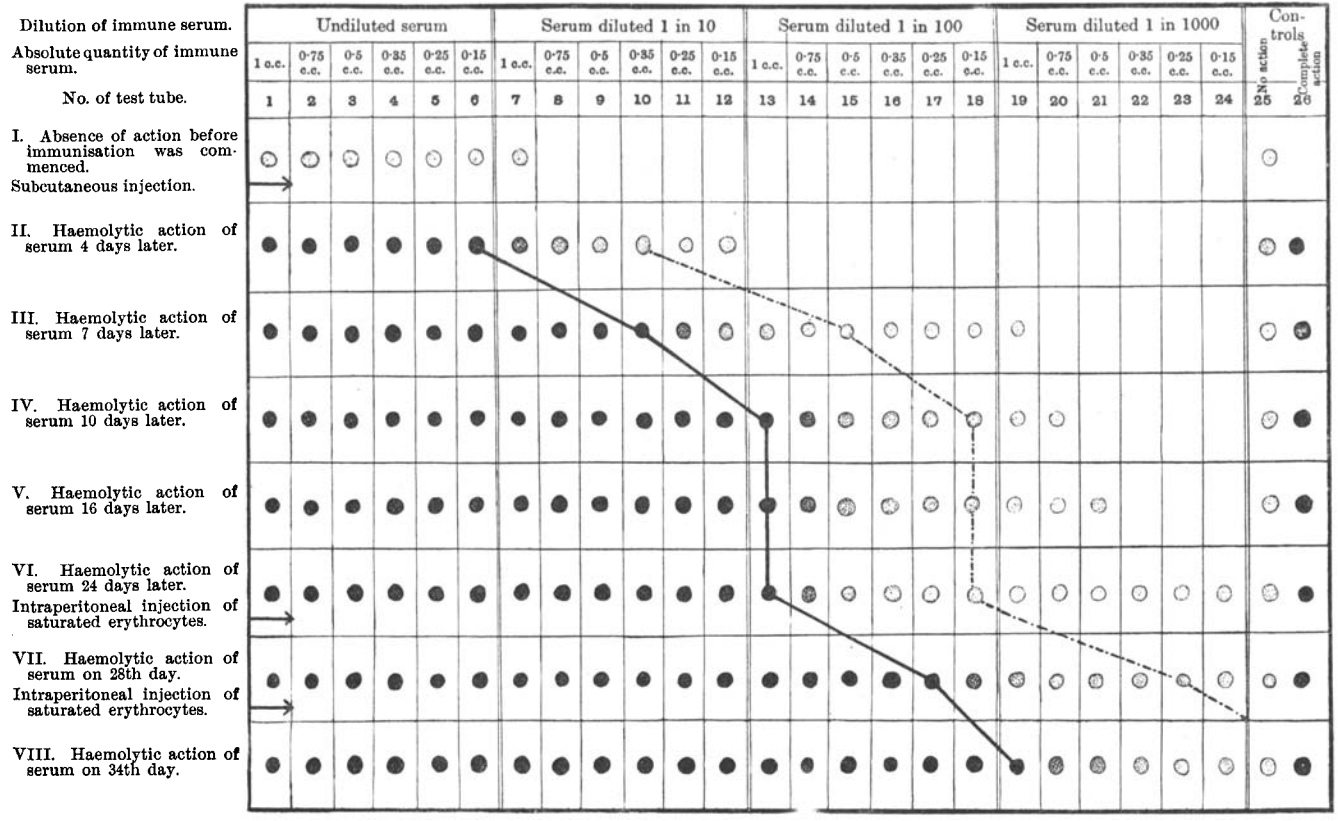
PLATE V. *Graphic record of the unaugmented haemolytic action of the serum of a rabbit immunised first by subcutaneous injection of bullock's erythrocytes and 24 days later rendered more highly immune by intraperitoneal injection of erythrocytes saturated with heated immune serum.*



——— The continuous line marks the limit of complete haemolysis.

•—•—• The dotted line passes through the tubes in which a trace of haemolysis was still present. The apparent retrocession of this limit is due to agglutination.

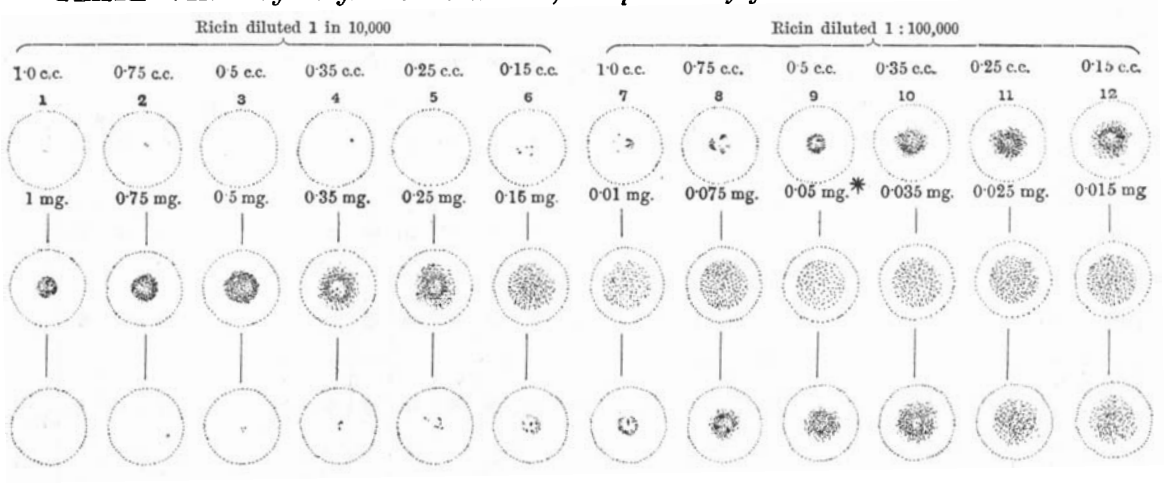
PLATE VI. *Graphic record of the augmented haemolytic action of the same rabbit's serum as on Pl. V. In this series of experiments the haemolytic action of the heated immune serum has been augmented by the addition of a normal rabbit's serum* (0·25 c.c.).
The agglutinative action no longer interferes with the limits of haemolysis.



——— The continuous line marks the limit of complete haemolysis.

•—•—• The dotted line passes through the tubes in which a trace of haemolysis was still present. The apparent retrocession of this limit is due to agglutination.

PLATE VII. *Erythrocytes as indicators of the presence of free ricin.*



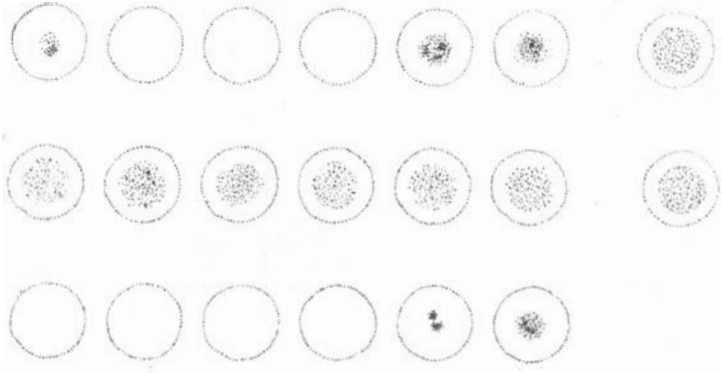
1. Determination of the minimal complete agglutinating dose of ricin*.

* 0·05 mg. was also the minimal lethal dose for a rabbit weighing 2 kilograms.

2. Limits of antitoxic action of 0·25 c.c. antiricin serum of rabbit.

3. Interference of 0·25 c.c. of normal rabbit's serum with agglutinating action of ricin. (Pseudo-antitoxic action).

Exp. 4, 5 *and* 6. *Ricin and antiricin present in constant quantities throughout.* (*Ricin* 0·05 *mg. Antiricin* 0·05 *c.c.*)



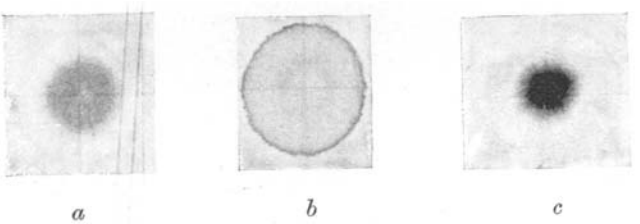
4. Antagonistic action of the serum of a passively immunised guinea-pig to the antiricin function of the serum of a rabbit actively immune.

Control. Saline replaces ricin. The combined sera do not produce clumping.

5. Absence of this antagonistic action in the case of a normal guinea-pig.

Control. Normal saline only. No action.

6. Consequences of mere dilution. Normal saline replaces guinea-pig serum in experiments 4 and 5.



DIFFUSION ON BLOTTING PAPER OF ERYTHROCYTES AND THEIR HAEMOGLOBIN CONTENTS.

(*a*) Erythrocytes suspended in an indifferent (non-haemolytic) serum.
(*b*) In a serum causing complete haemolysis.
(*c*) In a serum causing agglutination.

(Photograph, natural size).

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