RESEARCH PAPERS

THE EFFECT OF IMMUNE SERUM ON HÆMAGGLUTINATION BY RICIN

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The agglutination of red blood cells by ricin has been studied intensively since it was first noted by Stillmark[1]. Ehrlich[2], who discovered that an animal can be immunised against the toxic effects of ricin, showed that the agglutination of rabbit blood is inhibited by the addition of serum from an immunised goat, the inhibition of agglutination running parallel to the neutralisation of toxicity. Müller[3] found that immune rabbit serum prevented the agglutination of rabbit blood, but that the blood of an immune animal is agglutinated as easily as ordinary blood. He suggested that this apparent discrepancy was due to the difference in concentration of the serum. Kraus[4] showed that normal rabbit serum inhibited the agglutination of rabbit red cells by ricin, but that normal goat serum had no such action. Fraenkel[5] also noted the inhibitory action of normal serum, but found that it varied in intensity from species to species. Miessner and Rewald[6] made a somewhat similar observation, but found that it was impossible to generalise. Thus, while normal goat serum had little or no effect on the action of ricin on rabbit or bovine blood cells, in the case of dog cells it promoted agglutination, and with guinea-pig cells it had an inhibitory action. They also noted that immune serum has no anti-agglutinating effect unless it is mixed with the ricin solution and allowed to stand for 15 minutes before the red cells are added. di Macco[7] found that the addition of guinea-pig serum in optimum quantity promotes the agglutination of sheep red cells by ricin, too much having an inhibitory effect. Similarly Guest[8] showed that a trace of rabbit serum increases the agglutinating power of ricin towards goat red cells.

Moriyama[9,10] noted that normal rabbit serum inhibits the agglutination of horse red cells by ricin, but promotes the agglutination of bovine cells. On the other hand, anti-ricin serum in optimum concentration promotes the agglutination of equine red cells.

Karel[11] found that both rabbit and guinea-pig serum decreased the agglutinating power of ricin in respect to guinea-pig red cells, while Kabat, Heidelberger and Bezer[12] noted that a trace of normal rabbit serum increased the agglutinating power of crystalline ricin towards human red cells (group O). They also state that anti-ricin sera may be standardised by assay of their inhibition of the hæmagglutinating power of ricin.

The above summary contains a mass of inconsistencies and contradictions. This is partly because different workers have used widely

different experimental conditions, and partly because no adequately controlled experiments, in which the effect of immune serum is compared with the effect of normal serum of the same species of animal, have been carried out. The work described was undertaken to find whether there is a specific anti-agglutinin in anti-ricin serum, or whether the observed effects are due to some substance normally present.

Experimental

Materials. (1) Ricin. This was prepared by the method of Osborne, Mendel and Harris[13]. In view of the observation of Kabat *et al.*[12] that ricin loses nine-tenths of its agglutinating power on crystallisation, no attempt was made to crystallise it. Its lethal dose for a 25 g. mouse was about 0·5 μg. All solutions were in physiological saline.

(2) Serum. This was obtained by immunising 3 goats and some dozen rabbits. No significant difference was noticed in the effects of serum from different animals of the same species. Goat serum X, which was used in the following experiments, neutralised 200 mouse lethal doses (i.e. 100 μg.) per ml. Rabbit serum P neutralised 25 mouse MLD (12·5 μg.) per ml. All sera were preserved with 0·5 per cent. of phenol.

(3) Red blood cells. Fresh oxalated blood from several animal species was centrifuged, and the red cells washed 3 times with 20 times their volume of physiological saline solution. 2 ml. of the packed cells were suspended in 100 ml. of saline solution.

Technique of agglutination tests.

12 rows, each of 8 test-tubes 3 in. $\times$ $\frac{1}{4}$ in., were used. To each tube in the first row was added 1 ml. of physiological saline solution; to each tube in the second row 1 ml. of ricin solution of 2·5 mg./l. concentration; to each tube in the third row 1 ml. of a ricin solution of twice this concentration namely, 5·0 mg./l.; to each tube in the fourth row, 1 ml. of ricin solution of twice the last concentration, and so on, the concentration for the twelfth row being 2·56 g./l. In the case of animals whose red cells are difficult to agglutinate, concentrations 16 times as great as these were used. To the first tube in each row 1 ml. of saline solution was now added; to the second tube in each row 1 ml. of serum diluted with saline solution to a concentration of 0·01 per cent.; to the third tube in each row, 1 ml. of a serum solution of 4 times this concentration, and so on, the concentration for the last tube in each row being 41 per cent. The tubes were shaken and allowed to stand at room temperature for 15 minutes. To each tube was then added 0·5 ml. of a 2 per cent. suspension of red blood cells. The tubes were stirred and incubated at 37° C. for two hours, when the result was read.

Results

Complete agglutination, with clear supernatant liquid, is shown by $++++$; almost complete agglutination, but with supernatant liquid opalescent, is shown by $++$; and partial agglutination, as compared with

the controls, by +. The results obtained with dog red blood cells and normal goat serum are shown in Table I. There is no increase in the agglutinating power of ricin with increases of serum. Indeed, with quantities more than 1 per cent. inhibition takes place, more ricin being needed to bring about complete agglutination. The results with immune goat serum, shown in Table II, are very different. Small additions of serum cause an increase in the agglutinating power of ricin, the maximum, 32-fold, occurring with an addition of 0·064 per cent. of serum. This effect decreases with increasing volumes of serum, until finally an inhibitory action is noticed. Other experiments, in which quantities of

TABLE I

RICIN: NORMAL GOAT SERUM AND DOG RED BLOOD CELLS

Ricin	Serum per cent.							
p.p.m.	0	0·004	0·016	0·064	0·256	1·02	4·1	16·4
512	+++	+++	+++	+++	+++	+++	+++	+++
256	+++	+++	+++	+++	+++	+++	+++	+
128	+++	+++	+++	+++	+++	+++	++	—
64	++	++	++	++	++	+	—	—
32	+	+	+	+	—	—	—	—
16	—	—	—	—	—	—	—	—

There is no increase in the agglutinating power of ricin with increase of serum. When 1 per cent. is present inhibition takes place. (Concentrations of ricin above 512 and below 16 p.p.m. have been omitted.)

TABLE II

RICIN: IMMUNE GOAT SERUM AND DOG RED BLOOD CELLS

Ricin	Serum per cent.							
p.p.m.	0	0·004	0·016	0·064	0·256	1·02	4·1	16·4
265	+++	+++	+++	+++	+++	+++	+++	+++
128	+++	+++	+++	+++	+++	+++	+++	++
64	+	+++	+++	+++	+++	+++	+++	+
32	—	+	++	+++	+++	+++	+++	—
16	—	—	++	+++	+++	+++	—	—
8	—	—	+	+++	+++	++	—	—
4	—	—	—	+++	++	—	—	—
2	—	—	—	++	+	—	—	—
1	—	—	—	+	—	—	—	—
0	—	—	—	—	—	—	—	—

There is an increase in the agglutinating power of ricin with increase of serum with a peak at 0·064 per cent. followed by inhibition.

serum up to 64 per cent. of the total volume were added, showed that at higher concentrations the effect of normal and immune serum is exactly the same, the inhibitory action increasing with increasing percentage of serum.

The results shown in Tables I and II are depicted graphically in Figure 1. A, where the minimum concentrations of ricin necessary to bring about complete agglutination are plotted logarithmically as ordinates against the logarithm of the volumes of serum present as abscissæ. On the same figure are shown the results for dog red cells with normal and immune rabbit sera, which exhibit exactly the same effects as goat sera.

Figures 1. B and 1. C show the results obtained with cat and guinea-pig red cells, respectively. They show the same phenomena noticed with

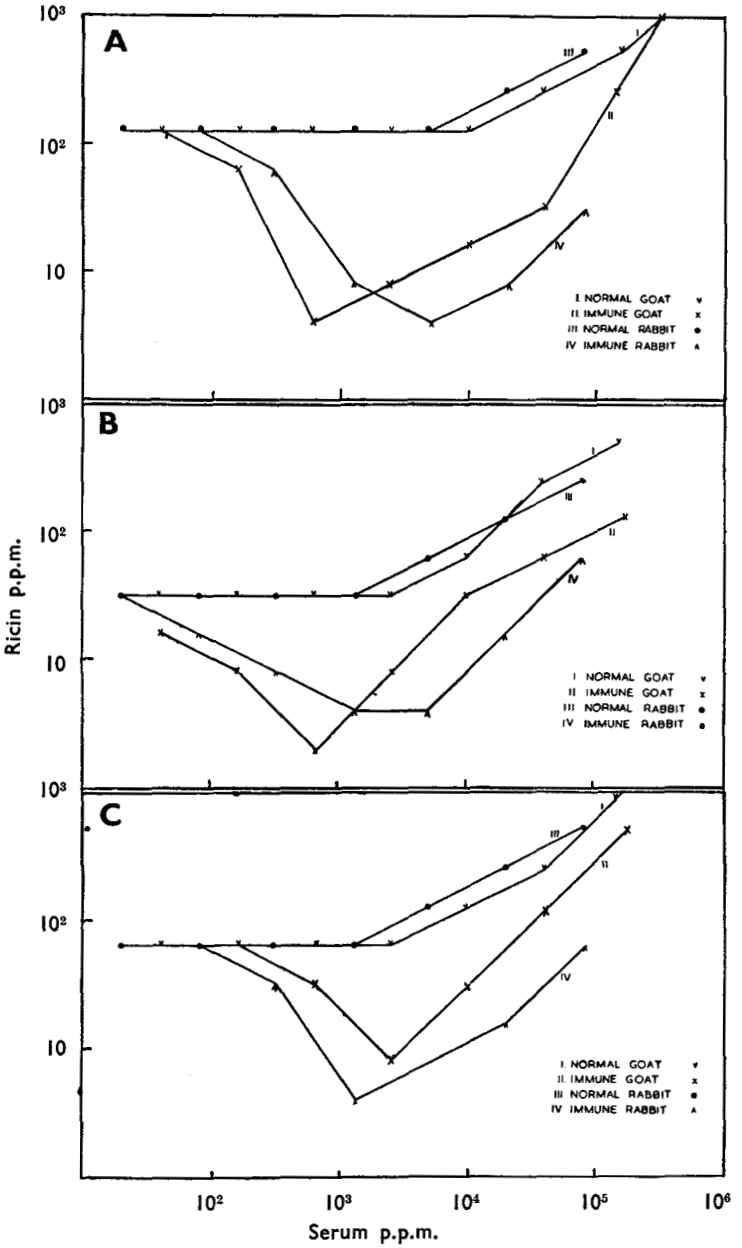

FIG. 1. A. The effect of concentration of goat and rabbit serum on the agglutination of dog red cells by ricin. There is promotion of agglutination only with immune sera at optimum concentration; both normal and immune sera show inhibition at high concentrations. B. The effect is similar with cat red cells and C with guinea-pig red cells.

The ordinates are the logarithms of the minimum concentration of ricin needed for complete agglutination. The abscissæ are the logarithms of the volumes of serum present.

dog cells, that is inhibitions at higher concentrations with both normal and immune sera, and marked promotion of agglutination with immune sera at optimum concentration. With rabbit cells (Fig. 2. A), rabbit serum behaves in the same way. With goat sera, however, although there is a similar picture with concentrations of sera up to 1 per cent. above this figure complete agglutination takes place even in the control tubes without ricin, owing to the presence in goat serum of some natural agglutinin for rabbit red cells.

With red cells which are less easy to agglutinate than the foregoing, somewhat different results are obtained. In the case of horse red cells (Fig. 2. B) both normal and immune rabbit sera have exactly the same effect, increasing the agglutinating power of ricin slightly when added to an extent of about 0·25 per cent. and inhibiting it when added in larger quantities. Normal and immune goat sera show a similar but slightly greater promotive action over a somewhat greater range, the effect being more pronounced for normal serum. Neither shows any inhibitory action when added in quantities of up to 8 per cent.

With bovine red cells results are very similar. Normal and immune goat sera, and normal rabbit serum promote agglutination when added in quantities between 0·1 and 4 per cent., above which the effect diminishes. With immune rabbit serum, however, this promotive action increases for increasing additions of serum up to 30 per cent.

In the case of goat red cells, Figure 2. C, an increase in the agglutinating power of ricin was observed for both normal and immune sera, the optimum quantities being 1 to 2 per cent. No inhibition of agglutination was noticed for additions of sera up to 16 per cent., though the graph suggests that further additions might cause it.

It was found impossible to agglutinate sheep red cells with ricin solutions of the concentrations employed (1·6 per cent.). Addition of serum, however, caused agglutination to take place over a narrow range for both rabbit and goat sera, the effect being the same for both normal and immune.

It will be seen from these experiments that in all cases immune serum causes a definite increase in the agglutinating power of ricin, this effect being greatest for additions of serum of the order of 1 per cent. Normal serum has a similar effect with blood of some species, while with that of other species it is without effect when present in small quantities. Inhibition only occurred in certain cases and then only when larger quantities of serum were added. In no case was the inhibition caused by immune serum greater than that caused by normal serum under the same conditions.

In order to determine whether this somewhat unexpected result was due to experimental conditions, a further series of experiments was carried out with dog red cells and rabbit sera under varying conditions. The following results were obtained:

1. *Red cell suspension.* Experiments similar to those described were carried out, but with 8 per cent., 4 per cent. and 1 per cent. suspensions of red cells instead of the 2 per cent. suspension employed previously.

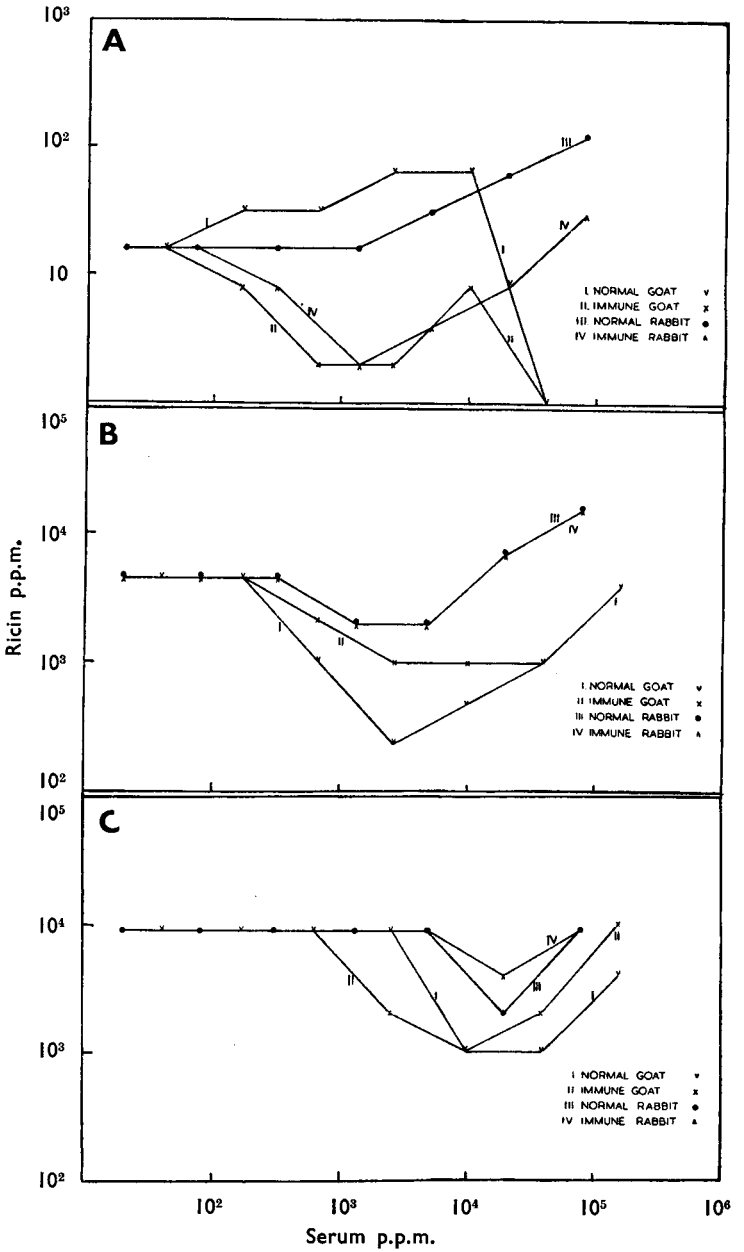

FIG. 2. A. The effect of concentration of goat and rabbit serum on the agglutination of rabbit red cells by ricin. There is promotion of agglutination only with immune sera at optimum concentration. Both normal and immune rabbit sera show inhibition at high concentrations. Above 1 per cent. goat serum agglutinates rabbit cells. B. The effect on horse red cells. Both normal and immune rabbit and goat sera first increase and then inhibit ricin agglutinating power. C. The effect on goat red cells. Both normal and immune rabbit and goat sera first increase and then inhibit ricin agglutinating power. The ordinates are the logarithms of the minimum concentrations of ricin needed for complete agglutination. The abscissæ are the logarithms of the volumes of serum present.

It was found that the observed results were independent of red cell concentration.

2. *Order of mixing.* In view of the statement of Miessner and Rewald[6], referred to above, that no anti-agglutination was noticed unless the immune serum was mixed with the ricin solution and allowed to stand before the red cells were added, a series of experiments was carried out as follows.

A. Ricin and serum were mixed and stirred, and incubated at 37° C. for half an hour, then red cells added.

B. Ricin and red cells were mixed and stirred, and incubated at 37° C. for half an hour, then serum added.

C. Serum and red cells were mixed and stirred, and incubated at 37° C. for half an hour, then ricin added.

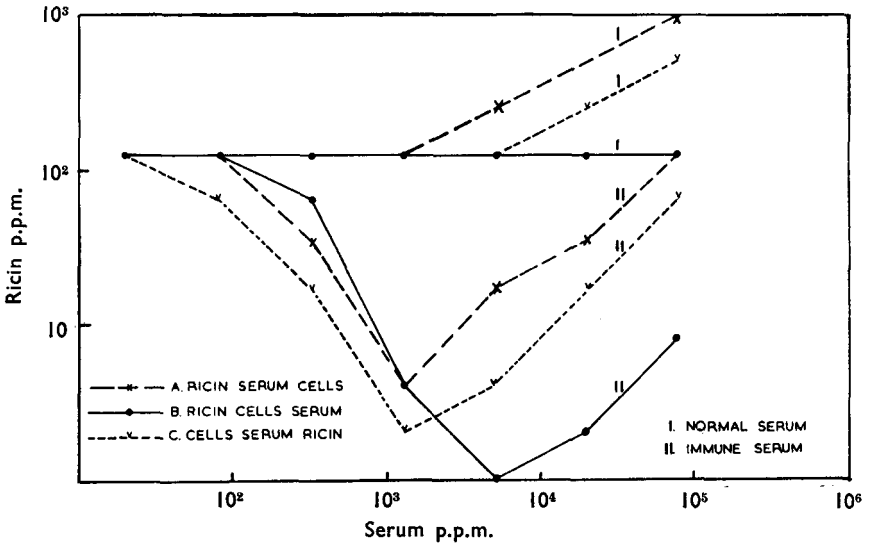

FIG. 3. The effect of the order of mixing the components upon the promotion of the agglutination of dog red cells by ricin in the presence of normal and immune rabbit serum. The ordinate is the logarithm of the minimum concentration of ricin needed for complete agglutination. The abscissa is the logarithm of the volumes of serum present.

The results are shown graphically in Figure 3. The inhibitory effect is greatest when ricin and serum are mixed and the cells added later, but the promotive effect of the immune serum appears to be least under these conditions. However, when the ricin and red cells are mixed first the promotion is at a maximum and the inhibition apparently least. Both effects have an intermediate value if the ricin is added last.

Use of goat serum instead of rabbit serum produced similar results. These results may be explained by postulating the presence of two factors in serum—an "inhibitory" factor, present in both normal and immune serum and a "promotive" factor, present in immune serum only. In experiment A the inhibitory factor has neutralised some of the ricin

before the red cells are added. Hence less agglutination takes place in the tubes containing the higher proportions of serum. In B the ricin has been adsorbed by the red cells, and subsequent addition of serum does not enable the inhibitory factor to neutralise it. In C the ricin, added last, is divided between the red cells and the serum, so that only a limited quantity is neutralised. It would appear that the promotive factor is independent of the order of mixing, as the apparent differences in intensity of its action are, in fact, due to the inhibition which takes place.

3. *Time and temperature.* As previous workers have employed temperatures ranging from 4° to 37° C. and have read the results after intervals varying from 10 minutes to 24 hours, it seemed essential to find the effect of varying these two quantities. The usual experiment was made at (*a*) 4° C., (*b*) 20° C., (*c*) 37° C. and (*d*) 45° C. Readings were taken after 0·5, 1, 1·5, 2, 4, 6 and 24 hours. Some typical results are shown in Figure 4. A and B. Agglutination took place more quickly at the higher temperatures, but in the absence of serum, eventually proceeded to a greater extent at lower temperatures. Thus at 37° C. in the control tubes without serum, 128 p.p.m. of ricin caused complete agglutination after 2 hours, no further increase being noticeable after 24 hours. At 4° C. however, it required 512 p.p.m. to cause complete agglutination after 4 hours, but after 24 hours, the value had fallen to 16 p.p.m.

In all cases agglutination was first noticed in tubes containing the larger quantities of serum, but these were also the first to reach equilibrium, i.e., the state at which no further agglutination took place as time went on. With both normal and immune sera, the concentration of ricin to cause complete agglutination in the tube containing the largest volume of serum employed (8 per cent.) was independent of temperature. In the case of immune serum, the same was true of the promotive effect at optimum concentration.

4. *Hydrogen ion concentration.* As agglutination by ricin is entirely dependent on the ions present, not taking place at all in the absence of electrolytes (Rona and Gyorgy[14]), it is impossible to investigate the effects of change in *p*H by adding buffer solutions, as one is unable to say how much of the observed effect is due to any small change in H-ion concentration and how much is due to any large changes in say, phosphate-ion concentration. By adding to each tube 1 drop of 0·01N hydrochloric acid or 0·01N sodium hydroxide, it was possible to bring the *p*H to 5·5 or 7 respectively, further additions of acid or alkali causing hæmolysis of the red cells. It was found that agglutination increased slightly with increasing acidity and diminished with alkalinity, but that these small changes in *p*H had no noticeable effect on either the inhibitory or promotive factors.

5. *Specificity.* Experiments were carried out with normal and immune anti-ricin sera and dog red cells, but with solutions of the phytotoxins abrin (from *Abrus precatorius*) and robin (from *Robinia pseudacacia*). No promotive effect was noticed, but there was some inhibition of

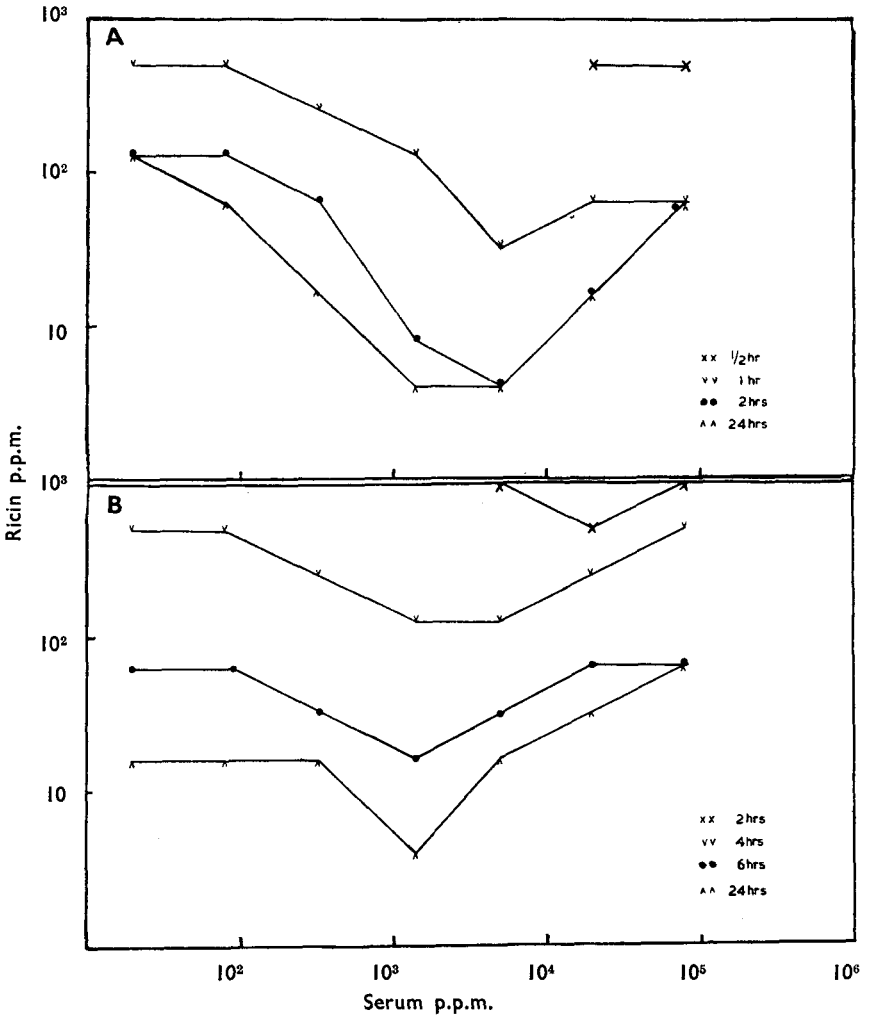

FIG. 4. A. The effect of contact time on the observed effect of agglutinating dog red cells by ricin in the presence of immune rabbit serum at 37° C. B. The effect of contact time on the observed effect of agglutinating dog red cells by ricin in the presence of immune rabbit serum at 4° C. The ordinates are the logarithms of the minimum concentration of ricin needed for complete agglutination. The abscissæ are the logarithms of the volumes of serum present.

agglutination with both normal and immune sera. This suggests that the promotive effect is specific, the inhibitory effect not.

DISCUSSION

It will thus be seen that, so far from there being a specific anti-agglutinin in anti-ricin serum, there appears to be a factor which, with the red cells of certain species at any rate, exerts exactly the opposite effect, bringing about a definite promotion of agglutination. Experiment showed that

this factor appeared in the blood at about the same time as the antitoxin and increased at a roughly parallel rate. It was found impossible to separate these two factors by procedures such as fractional precipitation with ammonium sulphate and similar reagents; any procedure which concentrated one factor concentrated the other and any method which destroyed the one destroyed the other. Both the promotive factor and the antitoxin could be clearly demonstrated in phenolised serum that had been stored at room temperature for 10 years. This would suggest that the promotive factor is some property of the antitoxin itself, the optimum concentration at which it exerts its maximum effect being akin to the zone phenomena encountered in precipitin reactions. So far no satisfactory explanation of ricin agglutination has been given, although several workers (Gunn[15], Northrop and Freund[16]), have suggested that it is a colloidal phenomenon. If this is so, one may possibly liken the "inhibitory" and "promotive" effect to the "protection" and "sensitisation" of one colloid by another. It would seem, however, that a complete explanation of these effects must await the elucidation of the mechanism of agglutination by ricin.

Summary

1. The literature dealing with the subject is briefly reviewed.
2. It was found impossible to demonstrate the presence of any specific anti-agglutinin in the serum of either goats or rabbits immunised against ricin.
3. Large additions of both normal and immune sera inhibit agglutination of the red cells of most species of animals tested.
4. When added in an optimum quantity of approximately 1 per cent. immune serum greatly increases the agglutinating power of ricin towards the red cells of dog, cat, rabbit and guinea-pig.
5. With the blood of horse, ox, goat and sheep, both normal and immune sera exert a slight promotive effect when added in optimum quantity.
6. The action of immune serum on dog red cells was investigated under varying conditions and the promotive action shown to be specific to ricin and independent of red cell concentration and of *p*H. It is also independent of temperature, provided sufficient time is allowed for equilibrium to be reached.
7. It is concluded that one cannot explain these phenomena until more is known of the mechanism of agglutination by ricin.

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