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A NOTE ON HÆMOLYSIN AND HÆMAGGLUTININ WITH REFERENCE TO THE WASSERMANN REACTION.

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A NOTE ON HÆMOLYSIN AND HÆMAGGLUTININ WITH REFERENCE TO THE WASSERMANN REACTION.

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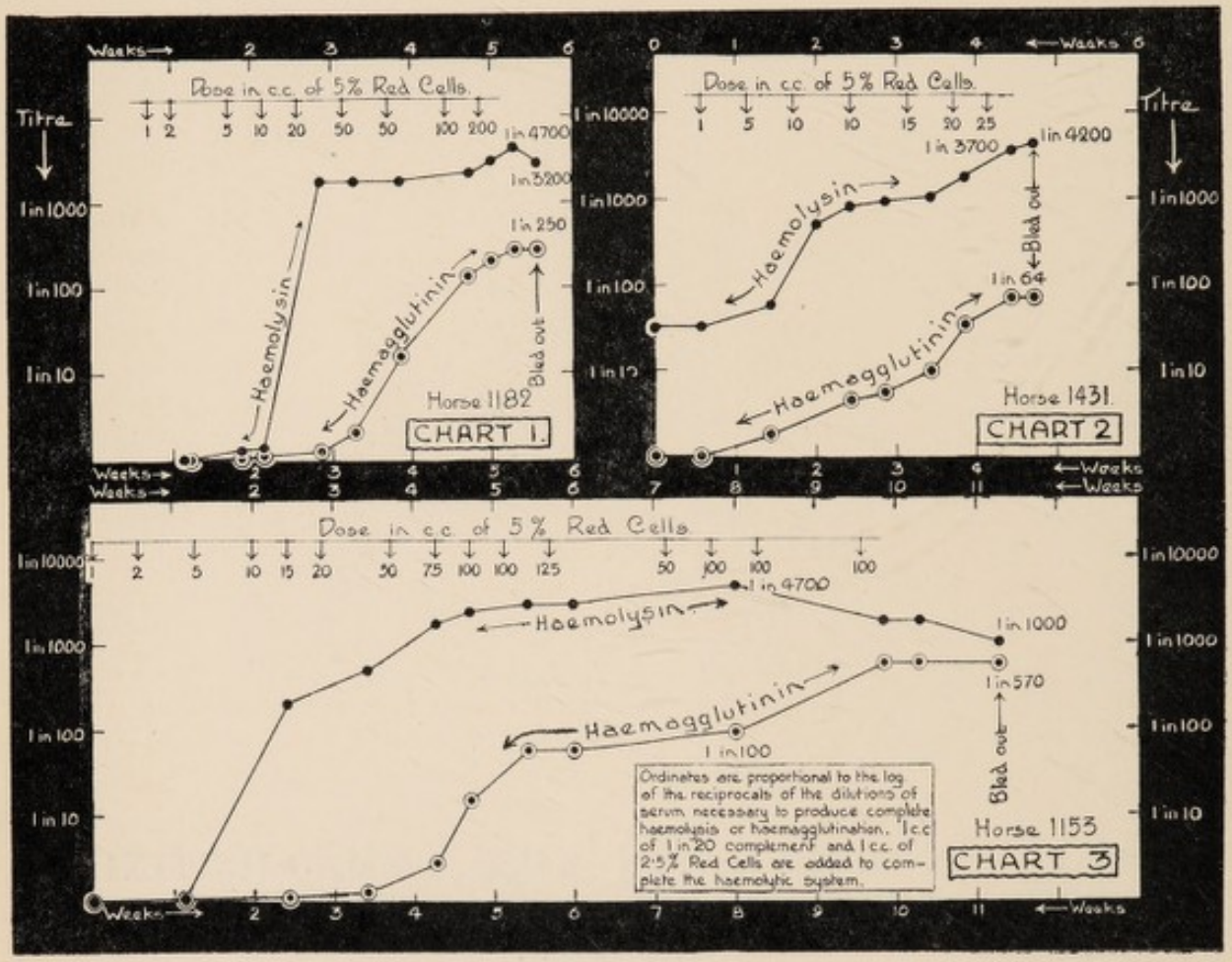
THE sequence of events observed in a horse or other animal immunized with sheep's red cells for the production of hæmolysin (amboceptor) has a practical bearing on the technique of the Wassermann and other complement—fixation tests.

Chart 1 illustrates the typical response of a horse injected with sheep's cells. Three separate phenomena connected with immunization are recorded on the chart, viz.—

(1) the production of hæmolysin (Bordet, 1898; Ehrlich and Morgenroth, 1899),
(2) the production of hæmagglutinin, and
(3) the gradually increasing tolerance of the animal to the injections, *i.e.* the development of immunity in the true sense. This is indicated, though only roughly, by the dosage of red cells recorded on the chart, since the reaction produced by each dose suggested that it was practically the maximum which could be tolerated by the animal at the time of injection without serious symptoms.



In our experience each progressive course of injections of red cells is followed by all three of these phenomena. It will be seen from the chart that, following the first small injection, there is a latent period of four to eight days, then a rapid rise of hæmolysin which may reach a titre of 1 in 1000 or more with hardly detectable agglutinin formation. A little later the agglutinins also begin to rise in titre, at first slowly, then much more rapidly.

Once the agglutinins begin to show rapid development the rate of increase of hæmolysin production tends to become relatively slow. Soon the hæmolytic titre shows no further rise in response to injections, and indeed it may begin to fall.

Chart 3 illustrates practically the same phenomena, but as a result of delay in making a full bleeding of the animal, the falling curve of the hæmolytic

titre and the rising curve of the hæmagglutinating titre tend to meet. As a consequence the serum from the final bleeding in this case proved unsuitable for hæmolytic tests, since hæmagglutination of the cells interfered with and masked hæmolysis.

As is well known, both horses and other animals occasionally contain naturally occurring anti-sheep hæmolysin in their serum. For example, Boerner (1915) reported the presence of natural anti-sheep hæmolysin in the serum of four of a series of two hundred horses which he examined; we have found it in two of a series of one hundred. Horses may also contain diphtheria antitoxin in their serum prior to immunization. Glenny and Sudmersen (1921) have shown that this normal antitoxin indicates the presence of active immunity, and that the animals react with the " secondary stimulus " response. In their experience also these horses are the most suitable for immunization. On analogy one would expect a more ready response to injections with sheep's red cells when natural anti-sheep hæmolysin is present.

Ruth Gilbert (1922) obtained large quantities of high-titre serum from a mule which before immunization contained in its blood natural hæmolysin for sheep's cells. We also have recently immunized a horse whose serum was naturally hæmolytic for sheep's cells in a titre of 1 in 32, and found that this animal responded exceptionally well to its immunization (Chart 2). No naturally occurring agglutinin for sheep's cells was detectable in this horse.

The test hæmolytic system consisted of 1 c.c. of serum dilution (in this case 1 in 32), 1 c.c. of 5·0 per cent. washed and re-suspended sheep's red cells, and 1 c.c. of 5 per cent. guinea-pig serum (complement) of normal activity. Readings were made after one hour in the water-bath at 37°C.

Cells washed with Ringer's fluid were used throughout for injection, as these appeared to cause less reaction than cells washed with normal saline.

The following series of red-cell doses was given: 1, 5, 10, 10, 15, 20 and 25 c.c., the interval between each dose being from three to five days. The horse showed remarkably little reaction to the gradually increasing doses of the injections, and only once had the same dose to be repeated owing to a rise in temperature. Within five weeks, after 86 c.c. in all of red cells had been injected, the hæmolytic titre had reached 1 in 4200 and the hæmagglutinative 1 in 64.

The details of the immunization of this horse are shown in Chart 2.

In connection with the occurrence of natural hæmolysin in animals, we thought it of interest to determine whether the natural hæmolysin present in this horse was of an isophile or heterophile type (Forssman, 1911; Friedemann, 1917). Following Taniguchi's technique (1921), we were unable to show any absorption of the hæmolysin by alcoholic and watery extracts of guinea-pig kidney and horse heart (heterophile antigens). Moreover, the hæmolysin present before immunization showed similar sensitiveness to heat as a hæmolytic horse-serum of the same titre of known isophile origin. This indicates that the natural hæmolysin present in this case was isophile in type. We are thus faced with a similar problem as in the case of the naturally occurring diphtheria antitoxin of the horse, where up to now it has been impossible to demonstrate the presence of any specific stimulus for its production.

We would like to emphasize the utility of a careful control of the injections by frequent experimental bleedings and the charting of the results. Inspection of the charts shows at once the all-important relation between the hæmolytic and the hæmagglutinating titres. The value of a hæmolytic serum obviously varies not only directly as its hæmolytic titre, but also inversely as its hæmagglutinating titre. When the agglutinating activity is small, it is easy to prepare "sensitized cells" containing a large number of doses of the hæmolysin, and although a system containing, say, two and a half M.H.D. of hæmolysin is probably quite as serviceable in the Wassermann reaction as one containing five or ten, many workers prefer a system with a large excess of hæmolysin.

The aim in immunization with red cells is therefore to produce hæmolysin with as little agglutinin as possible, and for this the following three methods suggest themselves:

1. To separate the hæmolysin from the hæmagglutinin in the serum.
2. To separate the hæmolytic antigen from the hæmagglutinative antigen.
3. So to arrange the course of immunization of the animal and the time of bleeding that the serum is strong in hæmolytic and weak in agglutinative properties.

With regard to the first method—separation of the serum—we have found, in agreement with most other observers, that lysin and agglutinin cannot be separated by fractionation of the serum proteins by the usual methods. Both lysin and agglutinin reside in the same fractions, viz. the globulins.

Attempts at partial absorption by red cells and filtration through various grades of filters have never in our hands been successful in separating the two elements.

With regard to 2, there seems to be no means of producing with certainty hæmolysin without hæmagglutinin by any antigenic modification so far employed (*cf.* Stewart, 1904).

The work of Coulter (1921) has shown that the same pH, viz. 5·3, corresponds with—

1. the iso-electric point of the serum globulin;
2. the reaction at which maximum agglutination of sensitized red cells takes place; and
3. the reaction at which the largest amount of hæmolysin is absorbed by cells.

This suggests a fundamental colloidal similarity between the lysin and the agglutinin, and it does not encourage us to hope for any chemical separation of the two elements.

We are left, then, with the third method, that of careful interpretation of the course of immunization and bleeding at the critical time, and it is this method that the charts illustrate. It seems probable that agglutinins correspond with the end immunity of the animal to the poison of the heterologous cells, and once they are available no further effort is made to produce hæmolysin. The classical methods of antitoxic immunization, which aim at the production of maximum tolerance to a foreign poison, do not therefore necessarily hold with regard to hæmolysin.

It may, then, be concluded that every high-grade hæmolytic serum is also to some extent hæmagglutinative. This, of course, is a matter of every-day importance in the Wassermann and other hæmolytic tests.

If a large excess of hæmolysin is used to sensitize cells, it may well happen that agglutination, more or less marked, may take place in the system of sensitized cells. If these cells are made up and used immediately, the agglutination may not be noticed, but even then it may upset the subsequent tests, causing blurred readings. The cells may, of course, appear agglutinated to the naked eye, and this is particularly likely to happen if the sensitized cells are kept overnight or for some time in a warm place. If they are shaken up they may appear to the naked eye to be completely re-suspended. The following experiment illustrates the danger of using cells shaken up after agglutination:

1 c.c. of the following dilutions of a hæmolysin (hæmolytic titre, 1 in 2000) and 1 c.c. of 5 per cent. red cells were put together. The usual method of 20 per cent. dilutions of hæmolysin was used, the hæmolysin in each tube being 20 per cent. more dilute than in the preceding tube. The agglutination titre is recorded below:

(Sensitising)—Agglutination Systems.

	$\frac{1}{50}$	$\frac{1}{62}$	$\frac{1}{78}$	$\frac{1}{97}$	$\frac{1}{120}$	$\frac{1}{150}$	$\frac{1}{187}$	$\frac{1}{234}$	$\frac{1}{292}$	$\frac{1}{365}$	$\frac{1}{456}$
I. 3 hours at 4° C.	(A)	a	a	a	o	o	o	o	o	o	o
II. 3 hours at 37° C.	A	A	A	(A)	(A)	(A)	a	a	a	o	o
III. 24 hours at 4° C.	A	A	A	A	(A)	a	a	a	o	o	o
IV. 24 hours at 15° C.	A	A	A	A	A	A	(A)	(A)	a	o	o

A = appearance of complete agglutination.
(A) = appearance of partial agglutination.
a = appearance of traces of agglutination.
o = no agglutination.

Each tube was shaken up until the cells appeared entirely re-suspended to the naked eye. 1 c.c. quantities of the mixtures were withdrawn from each tube, and to these 1 c.c. of 1/20 complement and 1 c.c. of saline were added. The following are the readings of the hæmolysis at the end of one hour in the water-bath at 37° C.:

Hæmolytic Systems.

	$\frac{1}{50}$	$\frac{1}{62}$	$\frac{1}{78}$	$\frac{1}{97}$	$\frac{1}{120}$	$\frac{1}{150}$	$\frac{1}{187}$	$\frac{1}{234}$	$\frac{1}{292}$	$\frac{1}{365}$	$\frac{1}{456}$
I	3 A	3 (A)	3 (A)	3 (A)	3 (A)	3	3	3	3	3	3
II	3 A	3 A	3 A	3 A	3 A	3 A	3 A	3 A	*3·2*	3	3
III	3 A	3 A	3 A	3 (A)	3 (A)	3 (A)	3 (A)	3 (A)	*3·2*	*3·2*	3
IV	2 A	2 A	2 A	2 A	2 A	2 A	2 A	2 A	2 A	2	2

3 = appearance of complete hæmolysis.
3·2 = appearance of almost complete hæmolysis.
2 = appearance of partial hæmolysis.
A = appearance of complete agglutination.
(A) = appearance of partial agglutination.

It will be seen that at dilutions 1/234 to 1/365 little or no agglutination is visible in the first part of the experiment. In the second part of the experiment after the addition of complement, though at these dilutions there is a large excess of hæmolysin present, for the titre was 1 in 2000, yet an appearance indistinguishable from incomplete hæmolysis was present in certain tubes

(shown in lines *II*, *III* and *IV* of the tables) between the stage of visible agglutination and complete hæmolysis. This is no doubt the explanation of the blurred readings which sometimes occur in Wassermann work when too large a dose of hæmolysin is used without regard to its agglutination titre. Similar results were obtained with rabbit hæmolysin.

With the ice-box method of fixation this point becomes of special importance, as the sensitized cells may have to be kept for twenty-four hours or so. As one can see by the tables, both the time and temperature factors are important.

In the initial testing of a hæmolysin it is always well to estimate its agglutinative as well as its hæmolytic titre under the conditions of concentration, time and temperature that will be required for the particular Wassermann method used. One such estimation only need be made on each batch of serum, as the hæmagglutinin is much more stable than the hæmolysin.

Too great an excess of hæmolysin should not, of course, be used in the Wassermann test, for the concentration of the hæmolytic serum might then be such that its hæmagglutinating properties are not inhibited. No hæmolytic serum of high titre in our experience is ever completely free from hæmagglutinins.

SUMMARY.

1. In order to obtain economically a large yield of useful hæmolysin in horses, it is important to control the immunization by frequent experimental bleedings, the results of which are charted in such a way as to show the relative production of hæmolysin and hæmagglutinin.

2. When the hæmagglutinin begins to show a marked rise relative to the hæmolysin it is advisable to bleed the horse for a maximum yield, as by delay the hæmolytic and hæmagglutinating titres may so approximate as to render the hæmolysin practically useless for hæmolytic work.

3. A horse showing natural hæmolysin for sheep's cells before immunization ran a particularly favourable course of immunization. The natural hæmolysin in this horse had the properties of isophile antibody (Forssman, Friedemann and Taniguchi).

4. Blurred hæmolysis where complete hæmolysis should be present in the titrations of the Wassermann reaction may be due to undue hæmagglutinating activity of the hæmolytic serum, *e. g.* by using too great a concentration of a high titre hæmolytic serum in the system of sensitized cells.

REFERENCES.

Boerner, F.—(1915) *J. Infect. Dis.*, **17**, 497.
Bordet, J.—(1898) *Ann. de l'Inst. Pasteur*, **12**, 688.
Coulter, C. B.—(1921) *J. Gen. Physiol.*, **3**, 309, 513.
Ehrlich, P., and Morgenroth, J.—(1899) *Berl. klin. Woch.*, 1.
Forssman, J.—(1911) *Biochem. Ztschr.*, **37**, 78.
Friedemann, U.—(1917) *Ibid.*, **80**, 333.
Gilbert, Ruth.—(1922) *New York State J. Med.*, **22**, 286.
Glenny, A. T., and Sudmersen, H. J.—(1921) *J. Hyg.*, **20**, 2.
Stewart, G. N.—(1904) *Amer. J. Physiol.*, **11**, 250.
Taniguchi, T.—(1921) *J. Path. & Bact.*, **24**, 217.

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