CXXXIX. THE SENSITISATION OF RED BLOOD CORPUSCLES IN HAEMOLYSIS.

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THE sensitisation of red blood cells by means of a specific amboceptor and their lysis by serum complement have been known to exist for a long time, but in recent years some cases have been found where this sensitisation can be produced by chemicals, though it cannot be definitely stated whether the analogy is only superficial or not. Sachs [1908] noticed a long time ago that when washed red blood cells were treated with low concentrations of soaps and then with normal serum, a marked acceleration of haemolysis took place. No attention was apparently paid to this type of work till Ponder [1922, 1923, 1924] described certain experiments on the accelerating effect on haemolysis of normal serum and other proteins when added to a suspension of washed cells previously treated with a bile salt. This observation enabled him to give a logical explanation of the percentage haemolysis curve for taurocholate haemolysis, where a great acceleration of haemolysis takes place after the haemolytic reaction has proceeded for some time—a fact due to the effect of liberated haemoglobin. Ponder also found that histamine could replace normal serum to a certain extent in this acceleration process. Recently he [1928] has repeated the work of Browning and Mackie [1914] and of Mackie [1919] on haemolysis by brilliant green and its acceleration by serum. In several previous papers [Sen *et al.* 1929, 1931] the present author studied this acceleration of haemolysis by different substances, and the view was expressed that the acceleration of haemolysis might be due to a sudden variation of the p_H at the cell surface which then induced further physico-chemical changes. The object of this paper is to give more evidence on this point. The present position of the problem, of great importance for the theory of haemolytic reactions in general, may be stated as follows. Since red blood cells can be sensitised by treatment with bile salts, soaps or dyestuffs like brilliant green, and then show a markedly lower resistance to normal serum, the question arises as to whether the action of normal serum is a continuation of the process started by the sensitising agent or not. It is known that all these sensitising agents are more or less adsorbed by the cells and are in themselves haemolytic at higher concentrations. It is to be assumed therefore that the adsorption of these substances, though not sufficient to cause lysis within the time given on account of the low concentration used, does actually change the cell surface

in such a way that some of the components of normal serum can act haemolytically. In studying the effect of normal serum on sensitised corpuscles, we can consider separately the effect of different major constituents of the serum—namely the proteins and the salts. From the work of Sachs, Ponder and others, it appears that the protein fraction is effective. Evidence is given in this paper that under many conditions the saline fraction of the serum solutions at the same p_H level is equally effective as the normal serum itself, and often the acceleration is determined by the amount of OH^- ions added.

EXPERIMENTAL.

In all the experiments recorded below, sheep's erythrocytes have been used after being defibrinated and washed four times with normal saline. The concentration is expressed in terms of cc. of corpuscles per 100 cc. of saline suspension. The sensitising substance (bile salt) was also freshly prepared in saline. The time of complete haemolysis was obtained by a colorimetric comparison with a standard.

As is well known, a haemolytic experiment in presence of normal serum can be made in one of the following three ways: (1) the serum may be added to the mixture of corpuscles and bile salt (sensitiser); (2) bile salt may be added to the mixture of corpuscles and serum; and (3) a mixture of bile salt and serum may be added to the corpuscles. The effect of serum in (2) always gives an inhibition of haemolysis, in (3) there may be inhibition or acceleration, but usually inhibition, and in (1) there also may be either inhibition or acceleration depending upon several variables. All these effects due to normal serum have been imitated by using a bicarbonate-Ringer solution instead of serum. As however we are more concerned with the procedure in (1), which is analogous to the amboceptor-complement reaction, we shall only deal with this. An example of the effect of serum obtained according to (1) and (3) may be given here in order to serve as a model for the presentation of results with substances other than serum. In Table I the concentration of cells is 1 % and of taurocholate 0.3 %. In each experiment 0.5 cc. cells and 0.5 cc. taurocholate in a total volume of 5 cc. have been used.

Table I.

(1)	Time of haemolysis of the control	3 mins. 21 secs.
(2)	0.5 cc. serum + taurocholate added to cells	No haemolysis in 2 hours
(3)	0.5 cc. serum added to cells + taurocholate ½ min. after adding the taurocholate to the cells	1 min. 4 secs.

This accelerating effect was analysed further by varying the amount of serum added, determining the effect of the time interval during which sensitisation is allowed to take place, the concentration of the corpuscles and of the sensitising agent, *etc.*, and it was found that all these factors had some effect on the observed acceleration. Under certain conditions, using the same technique, an inhibition instead of acceleration could be observed. Thus Fig. 1,

in which the experiments given in Table I (3) have been repeated with varying amounts of serum added to a sensitised corpuscle mixture after $\frac{1}{2}$ minute,

Fig. 1. Effect of serum on sensitised cells.

shows a well-marked inhibition and acceleration of haemolysis depending upon the amount of serum added. It was also found that the amount of serum necessary to induce the acceleration of haemolysis was much less when the time-interval given for sensitisation was greater. Both these effects of serum have been imitated by various substances with one common property, namely that of generating OH^- ions in solution. The experimental work falls mainly into three groups: (a) determination of inhibition-acceleration curves in presence of substances which give OH^- ions in solution, such as sodium hydroxide, ammonia, different amines and alkaline buffer systems; (b) measurements of the p_{H} of solutions after the addition of different amounts of these substances to sensitised cells in order to find out if there is any correlation between the change in the p_{H} and the observed inhibition and acceleration of haemolysis; and (c) the effect of time of sensitisation on the action produced by these different alkaline substances.

The general results are summarised as follows.

(a) If the concentration of the corpuscles and the amount of the sensitising agent (taurocholate in this case) are rightly chosen, and a suitable interval is given after which OH^- ions are added, it is easy to obtain both inhibition and acceleration with all the substances mentioned above. The concentrations can be easily adjusted by several trials. Some typical curves are shown in Figs. 2 and 3. In Fig. 2, the final cell concentration is 0.2 %, taurocholate approximately 0.01 %, and the time-interval after which ammonia is added is $\frac{1}{2}$ minute. In Fig. 3 and curve B of Fig. 6, the cell concentration is 0.1 %, taurocholate 0.03 % and the time-interval after which dipotassium hydrogen phosphate or Ringer-bicarbonate has been added is $\frac{1}{2}$ minute. It will be seen that the nature of the results is exactly the same as observed in the case of serum.

(b) Having determined the type of curves obtained by varying the amounts of the alkaline substances, it was decided to find out the relation, if any,

Fig. 2. Effect of ammonia on sensitised cells.

Fig. 3. Effect of dipotassium hydrogen phosphate on sensitised cells.

between the p_H change and the inhibition and acceleration of haemolysis. For this reason, the p_H of the final solutions after addition of normal serum or any of the above-mentioned substances was measured with a quinhydrone electrode. In these measurements, the final concentration of the cells was 0.1 %, and it was found that, with the concentration of haemoglobin derived from the cells, the error due to a drift in the potential could be neglected. The following solutions were used. Normal serum p_H 8.0, dipotassium hydrogen phosphate

$M/15$, Ringer-bicarbonate p_H 8.5, and 1 % gelatin in saline made alkaline with NaOH, p_H 8.5. Small amounts of these solutions were used in the actual experiments in order to vary the p_H , which was finally determined electrometrically. The corpuscle suspension had an initial p_H of 6.9 and the sensitised suspension had also almost the same p_H . In Figs. 4 and 5, two typical results

Fig. 4. Effect of p_H change on sensitised cells. Serum as accelerator.

Fig. 5. Effect of p_H change on sensitised cells. Potassium phosphate as accelerator.

with serum and phosphate are shown. The curves show clearly the range of p_H over which an inhibition of haemolysis is observed. This fact, that acceleration of haemolysis only results when the p_H change is sufficiently high, seems to indicate that the lytic activity of normal serum on sensitised corpuscles is due to its alkali content. Further experiments however show that the limit of p_H beyond which the acceleration is possible is not a constant one, and the phenomenon is more complex than was thought at first.

(c) It has already been stated that the time-interval after which the serum or the alkaline substances are added has a definite effect on the inhibition or

acceleration of haemolysis. A typical result obtained with Ringer-bicarbonate is shown in Fig. 6. The time of complete haemolysis has been determined after

Fig. 6. Ringer-bicarbonate as accelerator. *A*. Sensitisation time, 15 secs. *B*. Sensitisation time, 30 secs. *C*. Sensitisation time, 1 minute.

allowing variable periods to elapse before the bicarbonate solution was added to cells in the course of sensitisation by taurocholate. The p_H of the solutions was measured after the completion of the experiment. The following points in this figure are to be noted. When the time of sensitisation is 15 seconds (curve *A*), the addition of bicarbonate sufficient to raise the p_H to 7.6 does not accelerate the haemolysis, but retards it. When the time of sensitisation is 30 seconds (curve *B*), an acceleration is obtained as soon as the p_H exceeds 7.4. With a time-interval of 1 minute (curve *C*), the p_H limit comes down to 7.25, and with a time interval of 2 minutes (not shown in the figure) there is practically no inhibition, and the p_H range comes down to 7.05–7.1. It is to be noted here that this last time-interval is very close to the time necessary (3 minutes) for the haemolysis of the control. I have not been able to get any acceleration below p_H 7.0 under these experimental conditions, but it cannot be stated from these results that the acceleration is determined purely by the p_H change of the medium, as the magnitude of the change is sometimes very small. It is very likely that a large change in p_H is one of the factors by which we can obtain an acceleration of haemolysis, and it seems that during the sensitisation process the surface of the cells undergoes some irreversible changes making its stability conditions highly susceptible to p_H changes.

Attention may now be drawn to the effect of H^+ ions on sensitised cells. Thus it has been found that under certain conditions, with bile salt as sensitizer, a change of p_H from 6.9 to 6.4 made by the addition of acetate buffer would give an acceleration of haemolysis. No inhibition of haemolysis by acids could however be observed. It appears therefore that in bile salt sensitisation, the surface or some particular part of the surface of the corpuscle becomes so much changed, that a variation of p_H in either direction would cause a lysis

of the cells. This effect of acid is very marked when cells are sensitised by saponin, but with normal serum no sensitisation by saponin can be observed.

In conclusion two points of interest in connection with the acceleration of haemolysis in presence of normal serum may be mentioned. It has already been stated that though a mixture of normal serum and bile salt usually shows an inhibition of haemolysis, yet under some conditions this mixture might have a greatly increased haemolytic power. This fact of course is not solely dependent on the proteins, as a similar effect has been observed with a mixture of bile salt and either ammonia or ethylamine. The conditions under which this greatly increased haemolytic efficiency is observed have not been studied thoroughly, but it appears that there are at least three variables in this, namely, concentration of the cells, concentration of the bile salt and the concentration of serum or the alkaline substance used as the inhibitors. The second point of interest is the fact that though serum and certain other substances will haemolyse a sensitised suspension and some variables in this reaction have been mentioned, there is another variable, namely the condition of the cells themselves, which profoundly affects the results. This point has been previously emphasised by Ponder.

SUMMARY.

1. In a study of the effect of normal serum on corpuscles sensitised by bile salts, it is found that both inhibition and acceleration of haemolysis can be observed under suitable conditions.

2. It is found that this effect of normal serum is not solely due to the proteins, but the alkali-content of the serum must have some effect.

3. This effect of serum has been imitated by many substances which yield hydroxyl ions in solution.

4. Erythrocytes can be highly sensitised towards acids by previous treatment with bile salt or saponin.

5. The acceleration of haemolysis of corpuscles sensitised by bile salts due to the addition of serum or other acid or alkaline substances cannot be considered to be entirely dependent on the p_H change of the solution. The time allowed for the sensitisation to take place has a great influence on the effect of p_H change and acceleration of haemolysis.

6. Normal serum acts both as a haemolytic agent and an anti-haemolyte depending on the conditions of the experiment, and it appears that the haemolysis of sensitised cells by serum will have to be considered as a continuation of the action started by the sensitising substance.

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