THE EFFECT OF CERTAIN DRUGS, TOXIC SUBSTANCES AND MICROORGANISMS ON THE FRAGILITY OF THE RED BLOOD CORPUSCLES OF MAN AND ANIMALS.

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(With 10 Charts.)

Introduction.

Among the properties of the red blood corpuscles, that have from time to time been investigated, the resistance of this type of cell to the destructive action of various lytic agents occupies a place of some importance. Very many substances have been studied from this point of view, and no useful purpose would be served by enumerating them here; but it should be noted in passing that, as regards the great majority of them, we have no evidence that they play any part in blood destruction as it occurs 'in vivo' under either physiological or pathological conditions.

Ever since the idea first occurred to Donders of applying the work of De Vries on the action of certain salt solutions on plant cells to animal cells and especially to red corpuscles (an idea which was followed up in the long series of investigations by his pupil, Hamburger, and by von Limbeck) the phenomenon of the laking of erythrocytes by hypotonic salt solutions has occupied the attention of the greater number of the workers in this field of research. It is to the study of certain aspects of this type of cell destruction that this investigation has been devoted.

[1] The earlier part of these investigations was carried out at St Thomas's Hospital while holding the Salters' Research Fellowship.

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It will be more convenient to reserve all references to the work of the numerous investigators who have added to our knowledge of this subject for those portions of the paper in which the results and conclusions of each can be directly correlated with the experimental data obtained during the course of this research.

It is however desirable to enquire, at the outset, what significance may be attached to any alterations noted in the resistance of the erythrocytes to lysis of this type. The original conception put forward by Hamburger was that the red cell consists of an envelope having many of the properties of an impermeable membrane, containing within it certain salts and complex organic substances, among them haemoglobin, the whole suspension or solution exerting a definite osmotic pressure. Without the corpuscle is the serum, again consisting of a complex of salts and organic compounds, and which also exerts its particular osmotic tension, this tension being in excess of the tonicity of the corpuscular contents as measured against saline solutions. These comparatively simple physical conditions are implicitly assumed by Hamburger as the basis on which he explains his experimental results, and acting upon it, he was able to deduce that the isotonic value of a salt varied with its molecular weight, in which respect his work was soon after confirmed by von Limbeck. Von Limbeck, however, was far from believing that Hamburger's hypothesis sufficed to explain the phenomena observed. He notes that corpuscles are gradually lysed when allowed to remain in their native serum outside the body, and that therefore some chemical change must take place: secondly, that within the corpuscle there exists a fluid with much albumen and little salt, while in the serum there is usually less and quite different albumen, and more salt; so that either the albumen must affect the osmotic pressure or else part of the chlorides in the serum cannot enter into the osmotic action. He finds that, in the case of the blood of the horse, the isotonic value of the corpuscles measured against sodium chloride solution is 0·56 %, whereas the serum contains only 0·46 % of this salt, and therefore some other factor must be present to produce the isotonic relations. In human blood corpuscles, he found that the amount of sodium chloride was 0·2 %, their isotonic equivalent 0·4 % while the salt content of the serum was 0·62 %, again clearly proving the interposition of other factors. More recently, we have the extremely interesting work of Kiss, who endeavoured to demonstrate the intimate relation between the haemolytic action of the solutions of various salts and the position of their constituent atoms in the periodic system of the elements. He proves,

moreover, in the course of this investigation, that a lowering of the temperature results in an increased haemolysis in any given concentration of saline. This fact was independently observed by Lewis, and is, of course, exactly the reverse of that which would obtain were Hamburger's hypothesis correct. It may be taken, then, that the conception that hæmolysis in hypotonic saline solutions results from the rupture of a hypothetical cell membrane, produced by changes in the salt content and hence in the osmotic pressure within and without it, is definitely disproved.

We must now turn to the deeply interesting studies of Nolf. This investigator shows that all chemical substances, whose solutions produce haemolysis, may be divided into two classes. The members of one of these, such, for example, as weak solutions of urea, act as does distilled water. It suffices to add to them the amount of sodium chloride necessary to raise the tonicity of the solution to the usual limit to prevent haemolysis. The other class, and chief among its members is ammonium chloride, acts on the red cells and produces haemolysis even in the presence of sufficient sodium chloride to raise the tonicity of the solution to the normal value. Now, Nolf's conception is briefly this. He considers that there are three factors, the plasma within the cell with its salts and haemoglobin, the cell stroma and the serum, all of which possess a certain avidity for water, due both to their salt content and to other causes. Now, progressive dilution will increase the amount of water taken up by each of these three systems, and he believes that there is a definite critical point, beyond which absorption of water by the stroma produces a change in the physical relations between this constituent of the cell and the plasma and haemoglobin, which allows the diffusion of the latter. He particularly combats any idea of a mechanical rupture of any envelope or membrane. He considers that those chemical agents which belong to the same class as weak urea solutions act in the same way as distilled water; when their addition has brought about a certain dilution of the ambient fluid, and hence a certain definite "hydration" of the stroma, the critical point is reached and haemoglobin diffuses. Substances such as ammonium chloride, however, act in an entirely different manner, they specifically increase the power of the stroma to absorb water, so that a less dilution of the ambient fluid becomes necessary to produce the critical "hydration" of this element. Now, Nolf considers that all specific haemolysins, including specific haemolytic sera, act in this same manner. It is not possible to detail the experimental evidence that he adduces, but, briefly, he proves

that a haemolytic serum has no peptonising action on the red cells, and that increasing the tonicity of the saline solution in which the erythrocytes are suspended inhibits the action of both ammonium chloride and of a specific haemolysin; according to his hypothesis, by balancing the increased avidity of the stroma for water by the increased osmotic tension of the ambient fluid. However that may be, his observations on the inhibitory effect of increased saline concentrations on the action of specific haemolysins have been amply confirmed by Sutherland and McCay and also in the course of the present research. It is also of interest to note that ammonium chloride, alone of the chemical substances studied, ceases to exert its specific action at 0° C.

We must note that the validity of Nolf's hypothesis, or of some similar conception, is in no way affected by the observation of Peyton Rous, that alterations in the fragility of the red cells to hypotonic saline lysis on the one hand, and to haemolysis by a specific serum on the other, bear no definite relation to one another in the pathological conditions studied by him. Nolf does not state that haemolysis by a specific haemolysin and by hypotonic saline, or distilled water, is one and the same phenomenon; but that the action of the haemolysin produces a condition of the cell which enables a certain change to occur in a solution of high tonicity, which would in any event occur in a solution of low tonicity. It is, therefore, well within the bounds of possibility that in some cases, a change in the stroma which would render it more fragile, as regards the action of weak saline solution, would render it less liable to the action of a specific haemolysin. At all events, any change in the composition of the stroma might very well affect both factors, and there is not the least reason why it should affect both in the same way.

That hypotonic saline lysis is a change that has any place in the physiological or pathological processes of the body is in the highest degree improbable: that the effect of the tonicity of the body fluids on the action of haemolytic agents of other types may be a factor of considerable importance, is, on the other hand, an undoubted possibility.

In studying alterations in the fragility of the erythrocytes to weak saline solutions, we are dealing with a phenomenon which occurs, well-marked, in certain definite states of disease, and hence a condition concerning which any further information is of definite value.

In the present investigation, an effort has been made to obtain information on the following points,

1. The effect of the administration of certain arsenical compounds on the red cell fragility.

2. The effect of bile and of certain of its constituents.

3. The effect of certain pathogenic micro-organisms, known to be associated with haemolytic phenomena.

4. The effect of specific haemolytic sera.

Whenever, in the following pages the term 'fragility' occurs unqualified it is intended to denote fragility to hypotonic saline solutions. The term 'resistance' is used in an exactly inverse sense.

Technique.

The methods employed by different workers have varied in every possible direction; in the method of preparation of the saline solutions, in the use of washed or unwashed blood corpuscles, in the ratio between the volume of blood or red cells and the volume of saline solution to which they are added, and in the method of estimating the degree of haemolysis produced.

It was realised that the present research would involve a large number of readings, taken often at very short intervals, as, for instance, following the intravenous inoculation of a highly toxic substance; and hence it was essential to employ some method which would allow reasonably rapid readings and would not involve long and laborious examinations during each estimation. The method adopted, therefore, was the determination of the point of commencing haemolysis, *i.e.* the exact strength of the saline solution in which a faint trace of haemolysis occurred, the solution next higher in the series remaining entirely untinged. This was combined with such information regarding the degree of haemolysis in the lower concentration of saline as could be obtained from observation of the series of tubes put up in each case. The investigation, therefore, resolved itself in the main into the determination of the fragility of the cells of minimal resistance. The maximum resistance of the red cells would be determined by noting the strength of saline in which complete haemolysis occurred, but this point is very much harder to determine than the point of commencing haemolysis. There is a marked retardation in the relative number of cells destroyed in successive tubes as the saline concentration increases, so that several tubes intervene between that in which almost all the cells are destroyed and that in which every cell is lysed, and as the tints produced are indistinguishable, the determination has to be made on the presence of

a microscopic deposit after centrifugalisation or the detection of a very faint opalescence on shaking. The appearance of the first faint tinge of haemolysis in the series of tubes is however much more easily and certainly determined. Wherever possible the determinations were made in bright daylight, since this illumination is much the most satisfactory. In those cases in which artifical light had to be employed it was found that the use of a background possessing a slight greyish tint gave better results than one of a dead white colour.

It is not suggested that this method is so satisfactory as that followed for instance by Smith and Brown, in which the degree of haemolysis in each of a long series of tubes was accurately noted, using standard solutions of haemoglobin and suspensions of cells prepared from the actual corpuscles under examination, and the results plotted in curves giving the amount of haemolysis at each dilution. But it is changes at the lower end of the scale of resistance that have been the subject of most careful study; and it is almost certain that it is such changes which are the significant factors in actual pathological processes, if the fragility to hypotonic saline lysis really indicates any increased tendency to blood destruction in the body; since those cells of minimal resistance will obviously offer the first point of attack. The actual technique employed varied little from that adopted by Butler in his recently published observations on the fragility of the red blood cells in various pathological conditions, and this in its turn was a modification of the method employed by Dudgeon. The exact details of these methods will be found in the original papers. The details of the technique employed in the course of the present investigation are as follows:—

Preparation of saline solutions.

The basis of all the standard solutions was an accurate 1 % solution of pure sodium chloride (Kahlbaum) in freshly distilled water, which was renewed at frequent intervals. The purity of the salt is obviously a matter of the first importance, and the routine use of Kahlbaum's preparation was found to be the only satisfactory method of ensuring this. Several samples supplied from other sources as "Pure Sodium Chloride," and employed in a few of the earlier experiments gave discordant results. The 1 % saline solution was then placed in a 50 c.c. burette and a similar burette was filled with freshly distilled water. Two covered glass vessels were then taken and into one was run, say 5 c.c. of the 1 % saline and 5 c.c. of distilled water, into the other 7 c.c.

of saline and 3 c.c. of distilled water, thus giving a 0·5 % and 0·7 % saline solution respectively. A series of small glass tubes of 2 c.c. capacity were then placed in a rack and into the first was placed 10 volumes of the 0·5 % saline, into the second 9 volumes of 0·5 % saline and 1 of 0·7 % saline, into the third 8 volumes of 0·5 % saline and 2 of 0·7 % saline, and so on, the last tube containing 10 volumes of 0·7 % saline. Thus, a series of solutions was prepared differing by 0·02 % and ranging from 0·50 %, 0·52 %, 0·54 % etc. to 0·70 % saline. In Butler's method, solutions varying by 0·025 % were prepared directly from the burettes and used in making up the dilutions of blood. The experience obtained during the course of these experiments has convinced me that a difference of about 0·02 % approximates to the degree of sensitiveness of which this method is capable, and hence the method adopted has no advantage in point of accuracy over that employed by Butler, and it involves an additional stage of preparation. It possesses however one very real advantage. It was often necessary to be able to record a rapid change in fragility, the extent of which could not be foretold in advance, and hence a series of tubes, which would give results of the required accuracy immediately before an injection, would have to be extended to a cumbersone length if the possible variation was to fall within the limits of a second series, the individual solutions of which varied by a like amount. By varying this difference between successive tubes, this difficulty was obviated. Thus, using a 0·50 % and a 0·60 % solution a first series giving readings to 0·01 % could be employed immediately before an injection. If previous experience suggested that a rapid alteration would probably follow, the second series could be prepared from a 0·50 % and a 0·80 % solution, giving differences of 0·03 % between successive solutions, while, when the range of variation was determined, a series of tubes varying by 0·01 % or 0·02 % could be rapidly prepared, in which a range of eleven tubes would be certain to include the critical strength of solution and allow an ample margin on either side. This elasticity of arrangement gives a great advantage where several series of tubes of differing values are required during a short space of time. It is cumbersome and clumsy to prepare less than 10 c.c. of a saline solution accurate to two places of decimals per cent. from a burette, employing 1 % saline and distilled water as the parent solutions, and it is useless to prepare 10 c.c. when very possibly only 1 c.c. of that particular strength will be needed. As regards the use of capillary pipettes with a single mark, using an air-bubble to measure off volumes, as against the employment

of graduated pipettes; the former method was at first adopted, but it was afterwards found that the use of 1 c.c. pipettes, graduated in tenths, added much to the rapidity and comfort of the manipulation where many tubes had to be put up.

Addition of blood or red cells.

The great majority of the estimations were made on blood, as such, and here $\frac{1}{20}$th of the volume of saline to be used as diluent was employed. Dudgeon employed $\frac{1}{11}$th of the total volume, Butler $\frac{1}{10}$th and the method here adopted gives $\frac{1}{21}$th. This measure was employed, since for any given total volume it necessitates a smaller quantity of blood, and this in the case of a moribund rabbit is a very great advantage. Thus, in the very great majority of these experiments, where 1 c.c. of saline was present in each tube, 0·05 c.c. of blood withdrawn in a fine graduated pipette, was added. Where washed corpuscles were employed, they were received into saline and citrate, deposited after an interval by centrifugalisation, washed again in saline and citrate, then once in saline, and centrifugalised at high speed for several minutes, the thick deposit of cells from the last washing being employed for putting up the dilutions. In all cases sedimentation of the cells, so that the degree of tingeing of the supernatant fluid could be examined, was produced by centrifugalisation. As regards the time allowed to elapse between collecting and mixing the blood or red cells and centrifugalising the mixtures; a series of preliminary experiments showed that, while slight changes occurred in the degree of haemolysis during the first 30 minutes, there was very little alteration after that time until two to three hours had elapsed, whereas, after long periods the degree of haemolysis in all tubes steadily increased. An uniform interval of 30 minutes was therefore allowed to elapse between preparing the mixtures and centrifugalising them. The major part of this investigation has taken the form of observations on the fragility of the red blood cells of animals following inoculation with various toxic and other substances. The animals employed throughout have been rabbits. The marginal auricular vein of these animals affords an excellent site both for venipuncture for obtaining blood for examination, and for intravenous inoculation. In almost every case the marginal vein of one ear was employed for administering the injection, that of the other ear for withdrawing the blood. The blood was obtained by simple veni-

puncture. 0·05 c.c. was immediately drawn up into a calibrated pipette and expelled into one of the tubes of saline solution, and then mixed by drawing up and expelling the suspended red cells two or three times. In cases in which the cells were to be washed previous to examination the blood was allowed to flow straight into saline and citrate solution and was thereafter treated as stated above.

Red cell fragility in normal rabbits.

It may be noted that the normal fragility of rabbits' red cells was found to correspond to commencing haemolysis in 0·54 to 0·58 % saline. Of 41 rabbits whose blood was examined during the course of this investigation, five only showed any marked variation from these figures. All these alterations were in the direction of abnormally low values for the saline corresponding to the first trace of lysis, which in two cases was 0·46 %. In some of the experiments, in which low readings were obtained, a specimen of "Pure Sodium Chloride" was employed that was by no means above suspicion, but this would hardly account for such wide departures as the above. The figures obtained in the great majority of cases for commencing haemolysis, agree closely with those mentioned by most of the workers who have employed rabbits' red cells, von Limbeck—0·55 % saline, Cornwall—0·58 % saline[1],—etc.

On the other hand, French workers seem to have obtained consistently lower values, Nolf—0·46 % saline and Foix and Salin—0·41 % to 0·46 % saline. In this connection it is of considerable interest to recall earlier experiments of Theobald Smith, in which the abnormal corpuscular fragility met with, in certain of the anti-toxin horses he examined, led him to believe that the repeated abstractions had produced this variation, while widely extended researches, carried out in conjunction with H. R. Brown, convinced him that he was in reality dealing with an individual peculiarity. A considerable series of preliminary experiments were carried out in order to determine whether the corpuscular fragility of the rabbit is a non-variable factor. Repeated examinations carried out over many weeks on any individual rabbit yielded remarkably constant results, variations greater than those corresponding to 0·02 % saline being very unusual.

[1] This observer found that the average range of lysis for all the animals examined corresponded to 0·144 % NaCl. He gives the mean lytic point for the rabbit as 0·51 % NaCl.

Effect of injecting normal saline, etc.

Similar determinations were made on animals which were subjected to intravenous and intraperitoneal inoculations of normal saline, and on animals which had suffered a few blood abstractions of moderate amounts, but no variation was noted. In view of the importance which has recently been attributed to the absolute purity of the distilled water used for preparing saline solutions which are employed in animal inoculation, a certain number of experiments were made with saline prepared from distilled water which had stood in an iron tank for some eight months and was obviously grossly contaminated. It produced no variation whatever in the corpuscular fragility. The distilled water actually employed in the whole of the following series of experiments was freshly distilled from a glass vessel, using an ordinary glass condenser which was kept perfectly clean.

The effect of arsenic and of certain arsenical compounds on the fragility of the red cells of man and animals.

Gunn, as the result of certain 'in vitro' experiments, suggests that the administration of arsenic produces an increased resistance of the erythrocytes to hypotonic saline haemolysis, and believes that the beneficial effect produced by compounds of this element in Pernicious Anaemia may be explained on these lines. The results obtained by the various workers who have estimated the red cell fragility in this disease have been somewhat discordant, and Butler, who examined four cases without finding any appreciable variation from the normal in this respect, has suggested that the administration of arsenic which is so generally resorted to in these patients, may introduce a disturbing factor and mask the true state of affairs.

For this reason, and also because of the common employment of arsenical preparations in blood diseases, it appeared to be of some importance to gain further knowledge of the effect of the administration of drugs of this class on the corpuscular fragility.

1. *Administration of arsenic (arsenious acid) to human beings.*

The red cells of several patients, who were taking this drug in ordinary therapeutic doses as treatment for various conditions, were examined without finding the slightest departure from the normal in any case. In no instance had the drug been pushed far enough to produce any toxic effect.

2. *Administration of salvarsan (dioxydiamidoarsenobenzol) to human beings.*

The blood of several patients who received injections of this drug was examined before its administration and at intervals varying from ten minutes to six days afterwards. In the majority of cases the drug was inoculated intravenously, and in all of these only one injection of 0·6 gramme was given. In one case the drug was administered intramuscularly, and here repeated inoculations were given. The blood cells of this patient were examined on 14 different occasions during a period of one month. They showed throughout a somewhat high fragility, commencing haemolysis occurring with 0·42 % to 0·47 % saline, but this abnormality was present before the administration of the drug was commenced and showed no constant increase during the period of examination. There is, therefore, no evidence that the increased fragility was in any way connected with the mode of treatment.

The patient was suffering from a Syphilitic Transverse Myelitis. In every other case of this series the results obtained fell within normal limits.

3. *The inoculation of arsenic (arsenious acid) into rabbits.*

Experiment.

A small rabbit was selected and the fragility of its red cells determined on two separate occasions. A 1/400 solution of arsenious acid in sterile saline was prepared, just sufficient hydrochloric acid being added to produce complete solution. Varying amounts of this solution were injected intraperitoneally and the red cell fragility was determined at intervals. The results obtained are tabulated on p. 203.

The animal showed only slight wasting until 30. 1. 13 when it became obviously ill, and eventually died a few hours after the last injection. On 31. 1. 13 and at all subsequent examinations a slight degree of haemoglobinaemia was present, so that the colouration decreased not to a completely colourless fluid but to one faintly tinged with haemoglobin. Since, however, a long series of tubes was employed at each examination, it was not difficult to observe the first tube in which the added tinge, due to the hypotonic saline haemolysis, was present.

The result of this experiment is shown graphically in Chart 1.

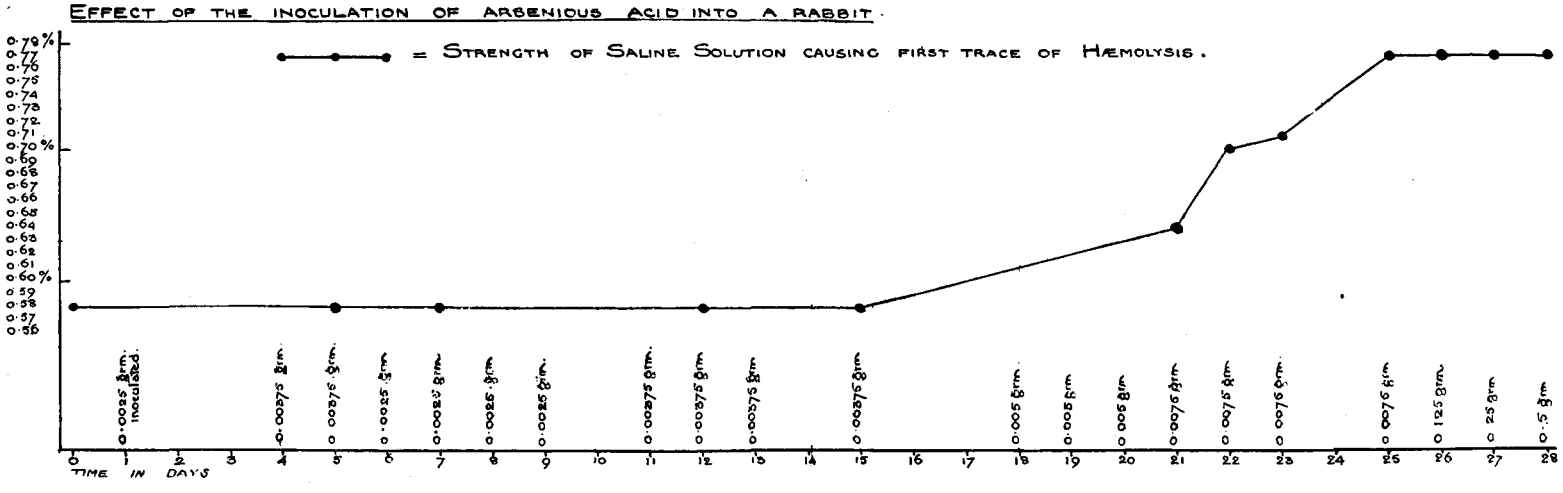

Chart 1.

Date of examination	Amount injected	Strength of saline solution causing first trace of haemolysis
Before injection		0·58 %
10. 1. 13	0·0025 grm.	—
13. 1. 13	0·00375	—
14. 1. 13	0·00375	0·58
15. 1. 13	0·0025	—
16. 1. 13	0·0025	0·58
17. 1. 13	0·0025	—
18. 1. 13	0·0025	—
20. 1. 13	0·00375	—
21. 1. 13	0·00375	0·58
22. 1. 13	0·00375	—
24. 1. 13	0·00375	0·58
27. 1. 13	0·005	—
28. 1. 13	0·005	—
29. 1. 13	0·005	—
30. 1. 13	0·0075	0·64
31. 1. 13	0·0075	0·70
1. 2. 13	0·0075	0·71
3. 2. 13	0·0075	0·77
4. 2. 13	0·0125	0·77
5. 2. 13	0·025	0·77
6. 2. 13	0·5	0·77

Experiment.

A second rabbit received a single inoculation of 0·25 gramme of arsenious acid intraperitoneally. Its blood cells were examined before the inoculation and at intervals of 45 minutes and three hours afterwards, with the constant result that haemolysis commenced in 0·58 % saline. Two hours later the animal developed acute toxic symptoms and died within 15 minutes, and, as is so often the case in moribund animals, only a drop of blood could be obtained from the veins of the ear. Immediately after death blood was obtained from the axillary vessels and this showed the first trace of haemolysis in 0·68 % saline.

As a result of these experiments it will be seen that it is only when the arsenic is pushed to highly toxic doses that any effect is produced, and that this is in the direction of increased fragility.

4. *The effect of atoxyl (meta-arsenic-anilide) on the fragility of rabbits' red cells.*

A certain number of 'in vitro' experiments were first performed to test the action, if any, of atoxyl on the washed red cells; the action was tested in solutions varying from 1 % to 0·001 % of the drug in

normal saline. In some series of experiments the cells were washed after the drug had been allowed to act for 30 minutes at 37° C., in others the mixtures were centrifugalised at high speed after incubation, the supernatant fluid pipetted off and the red cells immediately added to the saline solution. The results are quite without interest and need not be elaborated here.

Experiment.

A rabbit received an injection of 0·2 gramme of atoxyl into the lateral auricular vein. The red cells were examined before the injection, 5 minutes, 1 hour, 3 hours, 24 hours and 48 hours afterwards without showing any alteration worthy of note. On the day following the inoculation the rabbit showed slight toxic symptoms, but these passed off completely in three days. On the seventh day after the first injection a second, consisting of 0·3 gramme of atoxyl, was given. The blood was examined immediately before the inoculation, 5 minutes, 15 minutes, 1 hour and 3 hours afterwards without showing any alteration. The effect on the rabbit however was almost instantaneous and of a most marked character. It became obviously ill within 15 minutes of the inoculation, respiration becoming extremely rapid and the animal being unable to stand, death in fact appeared imminent. Severe gastro-intestinal symptoms appeared within 12 hours and on the second day the hind limbs were completely paralysed. All symptoms except the paralysis passed off in seven days, during which time however the animal wasted considerably. In connection with these toxic symptoms a marked change occurred in the fragility of the red cells.

The percentage of sodium chloride in the tube which showed commencing haemolysis at the beginning of the experiment was 0·57. 24 hours after the second injection haemolysis occurred in 0·63 % saline. This value fell daily during the following week and on the seventh day was again 0·57 %. 10 days after this injection 0·5 gramme of atoxyl was administered. As previously the blood cells were examined before the inoculation and at varying intervals afterwards. No alteration in fragility occurred and the rabbit displayed no toxic symptoms of any kind. The blood was examined on several subsequent occasions but showed no change in the red cell fragility, and the rabbit recovered completely. The results of this section would appear to show that arsenical compounds administered in therapeutic doses have no action on the fragility of normal red corpuscles.

The effect of bile and of certain of its constituents on the fragility of the red blood cells of the rabbit.

Among all the pathological conditions affecting the human subject which have been studied from the point of view of alterations in red cell fragility, none have given more interesting and constant results than those associated with Jaundice. Von Limbeck first drew attention to the decreased corpuscular fragility in Obstructive Jaundice, and his results have been confirmed by Chauffard, Chalier, Vaquez and Ribierre, Peyton Rous, McNeil, Butler and others. This phenomenon seems to be closely associated with the Jaundice itself, since, in Butler's cases for example, the actual causative conditions included Gall-stones, Carcinoma of the pancreas, Pancreatitis, Secondary Carcinoma of the liver and Cirrhosis. It is also noteworthy that a decreased fragility has been recorded in cases of Septic Jaundice.

If now we consider another, much less common, condition associated with a certain degree of Icterus, namely Congenital Family Cholaemia, we are met with a wide variation in the opposite direction, *i.e.* with a markedly increased susceptibility to hypotonic saline haemolysis.

This interesting phenomenon, first noted by Chauffard, has been frequently confirmed by Peyton Rous, Tileston and Griffin, Chalier, Le Gendre and Brulé, Cade, Hawkins and Dudgeon, Hutchison and Panton and Butler.

It becomes, therefore, a matter of interest and importance to try and determine what are the factors involved.

Von Limbeck originally offered the tentative suggestion that in Obstructive Jaundice the bile-acids might cause the haemolysis of the more fragile cells, leaving those of greater resistance. He abandoned this idea, however, on two grounds: (1) that it assumes that the tonicity of the serum sinks below the normal isotonic limits when bile-acids are present, while he himself found the opposite to be the case, (2) that it would involve an Oligocythaemia, which does not occur.

McNeil, in his communication on Saponin Haemolysis, suggests that the cause of the decreased fragility is a result of the hypertonicity of the serum. His reasoning is difficult to follow, and the results of his experiments on this point are in direct contradiction to those of Butler, with whose observations my own are in entire agreement. It is also, I think, the common experience of all, who work with red blood cells, that their tendency to haemolysis steadily increases when they are kept in saline solution after removal of the plasma by washing.

As regards the heightened tendency to saline haemolysis in Congenital Cholaemia, there is general agreement in attributing it to an inherent abnormality of the erythrocytes, evidenced in other ways by their altered shape, size and staining reaction.

The methods adopted during the course of this part of the investigation were as follows:

(1) The inoculation of rabbits' bile into rabbits.

(2) The inoculation of sheeps' bile into rabbits.

(3) The inoculation into rabbits of bile-salt (sodium taurocholate), together with certain 'in vitro' experiments with this substance.

(4) The inoculation of cholesterin into rabbits.

(1) *The inoculation of rabbits' bile into rabbits.*

The bile employed was collected from the gall-bladder immediately after death. It was heated at 58° C. for thirty minutes to ensure sterility, and this sterility was proved by culture before injection. Two experiments were made.

A rabbit received 2·5 c.c. of a $\frac{1}{50}$ dilution of rabbits' bile in sterile saline intravenously, and a second 2·5 c.c. of the same dilution 24 hours later. Repeated examinations of the red cells revealed no alteration in their fragility.

A second rabbit received 3 c.c. of a $\frac{1}{4}$ dilution of rabbits' bile intravenously. The red cells were examined at various intervals ranging from 5 minutes to 7 days after the inoculation, but no change in fragility occurred. Marked haemoglobinaemia was present 5 minutes after the injection but this gradually decreased and entirely disappeared in 24 hours.

(2) *The inoculation of sheeps' bile into rabbits.*

A rabbit received, on four successive days, injections of 2 c.c., 2 c.c., 3 c.c. and 2 c.c. of undiluted, sterile sheeps' bile intraperitoneally. Severe toxic symptoms supervened a few minutes after each injection but rapidly passed off. Repeated determinations of the red cell fragility showed no alteration. The bile in this case was not heated prior to inoculation.

(3) *The inoculation into rabbits of bile salt (sodium taurocholate) together with certain 'in vitro' experiments with this substance.*

Twelve rabbits were subjected to single or repeated injections of this salt, in doses which varied from amounts which caused no symptom of any kind to those which caused almost instantaneous death. The injections were given both intravenously and intraperitoneally.

No alteration of importance was noted in any case. In those cases where the doses given were so large as to cause rapid death, blood obtained post-mortem showed a moderately increased fragility, but the intrusion of this factor deprives these results of any value.

No useful purpose would be served by giving details of this series of experiments which involved a very large number of determinations. One experiment, however, may be briefly noted. A rabbit received repeated inoculations of sodium taurocholate over a period of 38 days. During this time 23 inoculations of this salt were given, commencing with 1 c.c. of a 0·5% solution administered intravenously, and terminating with 3 c.c. of a 10% solution administered intraperitoneally. The animal showed practically no toxic symptoms throughout the whole experiment and repeated estimations of its red cell fragility gave constant results.

A considerable number of 'in vitro' experiments were performed, consisting in the subjection of washed red cells, both human and rabbits', to the action of varying amounts of sodium taurocholate for different periods of time, both at 37° C. and at room temperature. The results were uniformly negative, so far as any change in fragility was concerned.

(4) *The inoculation of cholesterin into rabbits.*

McNeil has given reasons for believing that the lessened resistance of the red cells in Jaundice to Saponin Haemolysis is dependent on the presence in them of an abnormally large amount of cholesterin; and Thiele and Embleton have noted, in a recent study on the factors involved in Wassermann's Reaction, that cholesterin in some way alters red cells which have been in contact with it, so that more amboceptor-complement compound is required to haemolyse them, even after they have been centrifugalised and washed. It was thought, therefore, that it might be this constituent of bile which was especially concerned in the altered resistance to hypotonic saline haemolysis noted in jaundiced conditions.

The cholesterin was injected intravenously as a fine suspension in sterile saline. Three out of four injections made in this way produced no ill effects, the fourth caused death within an hour. The toxic effect in this case was probably due to the physical condition of the suspension since no more cholesterin was administered in this case than in the other three.

Intraperitoneal injections were given as fine suspensions in saline and as solutions in olive oil, and a solution prepared in this way was also inoculated intramuscularly. The amount of cholesterin administered at one injection varied from 0·05 to 0·2 grammes. One rabbit received two intravenous injections, another three intraperitoneal and one intramuscular injection. In no case was any change in red cell fragility observed.

Thus, in the whole series of experiments made with the bile and its constituents the results were almost entirely negative.

Alterations in the fragility of the red blood cells of rabbits following the inoculation of certain micro-organisms.

A considerable amount of data has been collected regarding the influence of acute bacterial infections on the fragility of the red blood corpuscles of man.

Von Limbeck records an increased fragility in Pneumonia, Erysipelas and Typhoid Fever, and quotes Cavazzani as obtaining a similar result in Typhoid after the fifteenth day of illness.

Dudgeon, in one case of Erysipelas, found an increased fragility comparable to that met with in cases of Congenital Cholaemia.

Butler examined six cases of acute bacterial infection, including three of Septicaemia, two of which proved to be due to a *Streptococcus*, one Streptococcal Arthritis, one streptococcal infection of a Hernia wound, and one case of Pyaemia. Four out of the six cases showed a slightly decreased fragility. One case of Erysipelas and one of Cellulitis showed a normal degree of fragility, while nine cases of Pneumonia showed a uniform slight decrease. In acute Rheumatism, this observer found the fragility to fall within normal limits.

G. B. Bianchi-Mariotti injected filtered cultures of various micro-organisms into rabbits and subsequently tested the fragility of their red cells with the following results:—filtered cultures of *B. anthracis*, *B. pyocyaneus*, *Streptococcus pyogenes* and *V. cholerae*, when injected in small or moderate doses, increase the fragility of the red cells, though often only to a slight degree. Moderate doses of a filtered culture of

B. typhosus produced a decreased fragility in 42 hours. Injections of large amounts of filtrate constantly caused a fall in the fragility. The author defines "moderate" doses of the filtrate as being, according to the nature of the organism and the age of the culture, from 3 to 6 c.cm. per kilo of the animal's body-weight. No full details of the experiments are given nor any numerical results stated.

Thus the evidence as regards the effect of micro-organisms and their products on the fragility of the red blood cells of man and of animals is of a peculiarly conflicting nature.

In the experiments to be described, attention was particularly directed towards those bacteria whose toxic products usually possess a certain haemolytic power; and in all cases living organisms were employed for inoculation.

1. *Inoculation of cultures of Streptococci.*

Experiment.

The fragility of the red cells of a small black rabbit was examined and found to be such that haemolysis commenced in 0·56 % saline. 3 c.c. of a saline emulsion of a *Streptococcus* of the *pyogenes* type were injected into the muscles of the left thigh. The red cells were examined at intervals of $2\frac{1}{2}$, $5\frac{3}{4}$, $7\frac{3}{4}$, 26, 29, 50 and 74 hours after the injection; but no deparature from the normal fragility was observed. The rabbit became temporarily ill, and some swelling of the thigh occurred, but this rapidly passed off, and complete recovery took place.

Experiment.

A small brown and white rabbit was selected and the fragility of its red cells determined. 1·2 c.c. of a saline emulsion of a 24 hours' agar culture of a *Streptococcus*, known to produce haemolysis, was injected intravenously. The red cells were examined at the following intervals and with the results shown.

Time of examination	Strength of saline solution causing first trace of haemolysis
Before inoculation	0·55 %
$2\frac{1}{2}$ hours after inoculation	0·55
6 ,, ,, ,,	0·56
$7\frac{3}{4}$,, ,, ,,	0·54
26 ,, ,, ,,	0·61
29 ,, ,, ,,	0·59
50 ,, ,, ,,	0·55
74 ,, ,, ,,	0·56

The animal became obviously ill about 24 hours after the inoculation but gradually recovered.

Experiment.

A large rabbit was selected and the usual preliminary investigation was made. 3 c.c. of a 48 hours' broth culture of an actively haemolytic *Streptococcus* were injected intravenously. The red cells were then examined at various intervals. At each of these examinations a tube of blood was collected, allowed to stand for 30 minutes at 37° C., and then centrifugalised and the supernatant serum examined for any tingeing with haemoglobin. This precaution was taken because this Streptococcus had produced haemoglobinaemia in experiments performed by Dr Walter Macleod, to whose kindness I am indebted for the cultures used in this and in the preceding experiment. It will be seen that after eight hours, the serum became faintly tinged with haemoglobin; so that it is clear that in this case one was not really working to a completely untinged solution; but it will be remembered that the serum is diluted at least $\frac{1}{40}$th, and the faintly-tinged serum diluted to this extent gave no colour observable by the eye or by the spectroscope, and the demarcation, between the tube in which the additional tinge caused by saline haemolysis was present and that in which it was absent, yielded no difficulty. The following were the results obtained:

Time of examination	Strength of saline solution causing first trace of haemolysis	Serum
Before injection	0·55 %	Nil.
1 hour after injection	0·67	Nil.
2 hours ,, ,,	0·67	Nil.
8 ,, ,, ,,	0·73	Faint tinge.
22 ,, ,, ,,	0·73	Faint tinge.
51 ,, ,, ,,	0·73	Very faint tinge.
95 ,, ,, ,,	0·70	Nil.
13 days ,, ,,	0·58	Nil.

The animal became acutely ill within two hours after injection, and at 24 hours it appeared moribund, but afterwards it gradually recovered, and at the end of a week appeared perfectly normal. The results of the earlier part of the experiment are shown in Chart 2.

Experiment.

A rabbit was selected and a preliminary determination made of the fragility of its red cells. The first trace of haemolysis was found to occur in 0·54 % saline. One c.c. of a saline emulsion of a 24 hours' agar culture of the *Streptococcus pyogenes* was injected intravenously. The fragility of the red cells was examined after 3, 6, 26, 29, 54

and 74 hours, the result in each case being identical with that obtained before the injection. The animal showed a marked indisposition, lasting from 12 to 24 hours, and then completely recovered. The Streptococcus employed in this case was isolated from an infected hernia wound.

EFFECT OF THE INTRAVENOUS INJECTION OF 3 C.C. OF A 48 HRS. BROTH CULTURE OF A VIRULENT *STREPTOCOCCUS.*

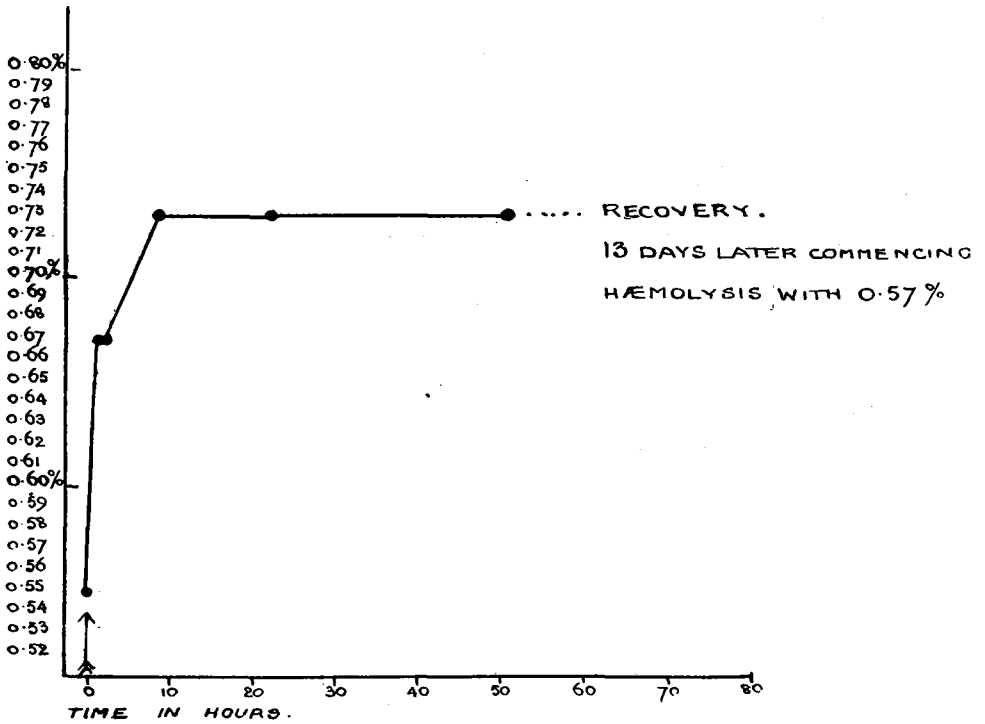

Chart 2.

Thus, out of four rabbits inoculated with different strains of Streptococci, two showed no alteration in their red cell fragility, one showed a slight rise, and one a very considerable increase persisting for several days. It is noteworthy that this occurred in the rabbit inoculated with a 48 hours' broth culture, which thus contained in addition to the organism itself the haemolysin which Macleod has shown to be present in filtered cultures. Another noteworthy circumstance is the ultimate recovery of all four animals, and also the fact that the rabbit exhibiting the most marked rise in red cell fragility became the most acutely ill.

2. *Inoculation of cultures of the Staphylococcus aureus.*

Experiment.

A small white rabbit was injected intravenously with 1 c.c. of a saline suspension of a 24 hours' culture of the *Staphylococcus aureus.* Before inoculation the first trace of haemolysis was observed in 0·46 % saline. Half-an-hour later the tube containing 0·47 % saline showed distinct tingeing. The rabbit, unfortunately, became acutely ill and died within a few hours, before another estimation was made. The very slight rise here observed renders the result of little value.

Experiment.

A small brown rabbit was selected and a preliminary determination of its red cell fragility made. Various inoculations of saline suspensions of the *Staphylococcus aureus* were performed, an agar culture 24 to 48 hours' old being used in each case in the preparation of the suspension. The results obtained were as follows:—

Time of examination	Strength of saline solution causing first trace of haemolysis
Before inoculation	0·49 %
Inoculation of 2 c.c. of saline suspension of the *Staphylococcus aureus*, intravenously.	
6 hours after inoculation	0·51
26 ,, ,, ,,	0·51
30 ,, ,, ,,	0·51
48 ,, ,, ,,	0·51
54 ,, ,, ,,	0·55
76 ,, ,, ,,	0·58
Inoculation of 2 c.c. of saline suspension of the *Staphylococcus aureus*, intravenously.	
90 hours after first inoculation	0·57
100 ,, ,, ,,	0·57
120 ,, ,, ,,	0·62
Inoculation of 2·5 c.c. of saline suspension of the *Staphylococcus aureus*, subcutaneously.	
124 hours after first inoculation	0·63
144 ,, ,, ,,	0·61
216 ,, ,, ,,	0·60
240 ,, ,, ,,	0·61
Inoculation of 0·5 c.c. of saline suspension of the *Staphylococcus aureus*, intravenously, and 2 c.c. intraperitoneally.	
264 hours after first inoculation	0·63
268 ,, ,, ,,	0·63
282 ,, ,, ,,	0·60
286 ,, ,, ,,	0·60
310 ,, ,, ,,	0·58
334 ,, ,, ,,	0·58
358 ,, ,, ,,	0·57

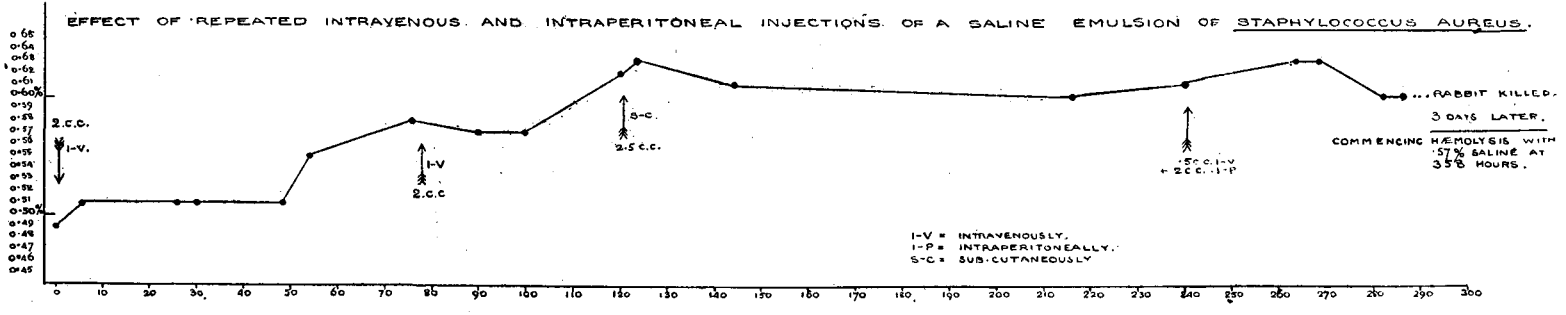

Chart 3.

Two subcutaneous abscesses developed towards the end of the experiment and the rabbit wasted rapidly; it was therefore killed on the 16th day.

The results of the earlier part of this experiment are shown in Chart 3.

3. *Inoculation of cultures of the Bacillus pyocyaneus.*

Experiment.

A small brown rabbit was selected and after the fragility of its red cells had been determined, 1 c.c. of a saline emulsion of a stock laboratory culture of the *B. pyocyaneus* was injected intravenously. The results were as follows:—

Time of examination	Strength of saline solution causing first trace of haemolysis
Before inoculation	0·46 %
½ hour after inoculation	0·46
1 hour ,, ,,	0·47
19 hours ,, ,,	0·50

When the last estimation was made the animal was 'in extremis,' and, as is so often the case under these conditions, it was impossible to obtain more than a drop of blood from the veins of the ear. The rabbit was killed and the subclavian artery immediately cut across, the blood taken from this source being employed in the tests. This detracts somewhat from the value of the results.

Experiment.

A small brown and white rabbit was selected and after the usual determination had been made, 1·5 c.c. of saline emulsion of a stock laboratory culture of *B. pyocyaneus* were injected intravenously. The results obtained were as follows:—

Time of examination	Strength of saline solution causing first trace of haemolysis
Before inoculation	0·50 %
2½ hours after inoculation	0·53
6 ,, ,, ,,	0·53
26 ,, ,, ,,	0·57

Death occurred within a few hours of the last estimation. The results of this experiment are shown graphically in Chart 4.

EFFECT OF THE INTRAVENOUS INJECTION OF 1·5 C.C. OF A SALINE SUSPENSION OF *B. PYOCYANEUS*.

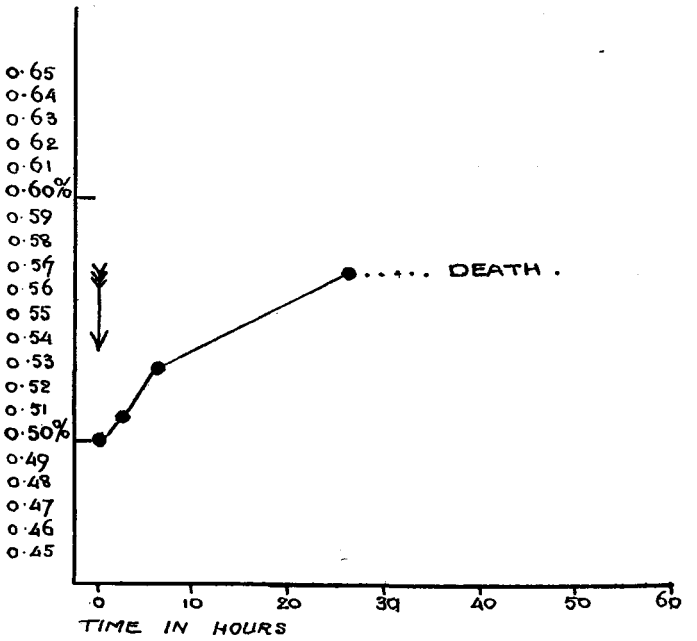

Chart 4.

4. *Inoculation of cultures of the Bacillus of Danysz.*

Only one experiment was performed with this organism.

Experiment.

Small brown rabbit.

Intravenous injection of 2 c.c. of a saline suspension of a young agar culture of the bacillus of Danysz.

Time of examination	Strength of saline solution causing first trace of haemolysis
Before inoculation	0·58 %
3 hours after inoculation	0·62
6 ,, ,, ,,	0·64
26½ ,, ,, ,,	0·62

Death occurred within a few hours of the last examination.

The results of this experiment are shown graphically in Chart 5. Thus, we may summarise the results of this part of the investigation by saying, that of certain of the bacteria, known to cause haemolysis under various conditions, almost all the strains examined produced, on inoculation, an increasing fragility, usually slight, but sometimes well marked.

The effect of haemolytic sera on corpuscular fragility.

The relation between hypotonic saline haemolysis and haemolysis due to specific haemolysins has been already considered, and the important work of Nolf, Peyton Rous, Kiss, Sutherland and McCay and others briefly referred to. We must now consider, in greater detail, the actual effect produced on the red blood cells by haemolytic sera 'in vivo' and 'in vitro,' and it will be more convenient to commence with the former.

EFFECT OF THE INTRAVENOUS INJECTION OF 2 C.C. OF A SALINE SUSPENSION OF *B. DANYSZ.*

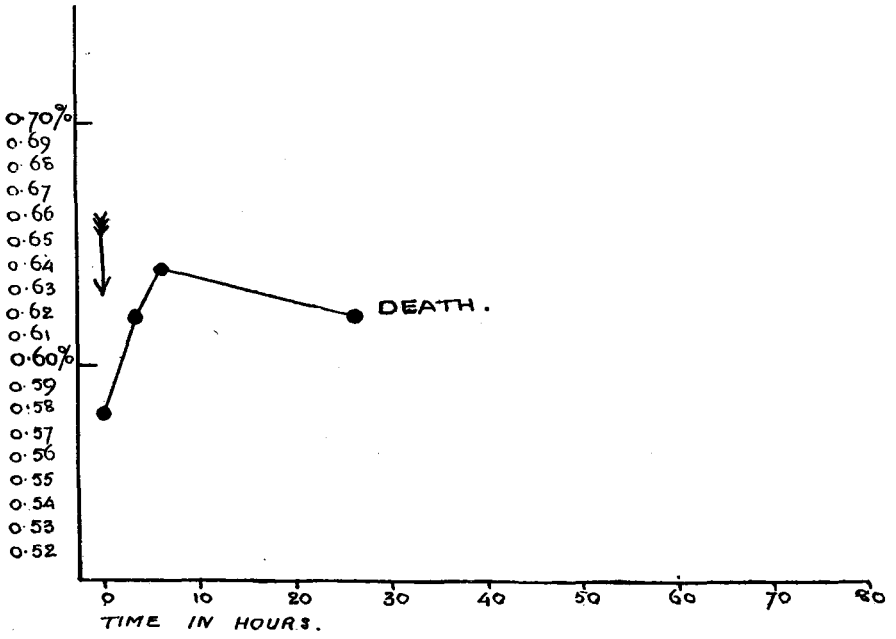

Chart 5.

1. *The inoculation of specific haemolytic sera into rabbits.*

We already possess definite evidence that the injection into animals of specific haemolysins results in an increased fragility of the red blood cells. Christophers and Bentley, in the course of their valuable research on Blackwater Fever, injected into dogs considerable quantities (15 c.c.) of the serum of goats, which had been immunised against dogs' red cells, and obtained variations in fragility corresponding sometimes to 0·3 % or

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even 0·4 % NaCl. They also inoculated Daboia Venom into rabbits and obtained similar results, while the specimen of Cobra Venom employed by them was much less active. These workers, however, in this part of their research, employed solutions showing wide differences of concentration from tube to tube (0·05 % to 0·10 %), and they consider such alterations as that indicated by the occurrence of commencing haemolysis in 0·45 %, 0·52 %, and 0·40 % saline in successive determinations made during the course of a few hours as unimportant. Certain points brought out by them are of great interest. They note that increased fragility occurs in the complete absence of either haemoglobinaemia or haemoglobinuria, and they also point out that the resistance of the red cells regains its normal limit after several days in those animals which survive, and that at this time large cells with polychromatophilic staining are present. More recently Foix and Salin, in a paper dealing with the experimental study of Paroxysmal Haemoglobinuria, have found that a constant increase in fragility followed the injection into rabbits of fresh and heated human serum, though this rise is seldom considerable in degree. The actual values they obtained differ very considerably from my own, since they give a normal maximum resistance corresponding to 0·41 % to 0·46 % saline for rabbits' red cells, figures which differ widely from those obtained by the majority of workers; on the other hand, they correspond closely with those mentioned by Nolf. In view of the importance of this question, it seemed desirable to study these phenomena in more detail by the methods described above.

The specific haemolytic sera employed were, in all cases, prepared by repeated intraperitoneal injections of the washed red cells of rabbits into guinea-pigs. In some cases the serum was heated before use, in others it was employed in the fresh state.

Experiment.

A serum haemolytic for rabbits' red cells was prepared in the above manner, and labelled serum '*α*.'

No determination of its precise strength, when acting 'in vitro,' was made, and the serum was not heated to destroy complement. A normal rabbit was selected and the fragility of its red blood corpuscles determined. One c.c. of haemolytic serum '*α*' was then injected intravenously. The following were the results obtained:—

Time of examination	Strength of saline solution causing first trace of haemolysis
Before injection	0·54 %
3 hours after injection	0·60
6 ,, ,, ,,	0·63
26 ,, ,, ,,	0·69
30 ,, ,, ,,	0·70
54 ,, ,, ,,	0·69
74 ,, ,, ,,	0·66
96 ,, ,, ,,	0·67
108 ,, ,, ,,	0·62

The animal showed no clinical manifestation of ill-health during the whole course of the experiment. Thus, the injection of 1 c.c. of this serum intravenously was followed by a rise in fragility corresponding to 0·14 % saline in 6 hours, and to 0·16 % saline in 30 hours.

Experiment.

A second rabbit received 0·25 c.c. of the same serum intravenously. The results were as follows:—

Time of examination	Strength of saline solution causing first trace of haemolysis
Before injection	0·54 %
4 hours after injection	0·57
24 ,, ,, ,,	0·63
48 ,, ,, ,,	0·60

As in the last experiment, the animal remained to all appearances perfectly well.

Thus, comparatively small doses of a haemolytic serum, judged from the clinical effects, produced in rabbits a marked rise in corpuscular fragility.

It was most natural to suppose that these changes were produced by the actual action of the haemolysins on the red cells circulating in the blood, but certain other possibilities were present which had to be eliminated.

Muir and McNee, in an extremely interesting paper on the Anaemia produced by a haemolytic serum, describe the effect caused by inoculating rabbits intravenously with a goat *v.* rabbit immune serum. They found that an enormous reduction occurred in the number of red cells in the peripheral circulation, commencing immediately but only reaching its maximum about the third day in the majority of cases. This phenomenon was usually accompanied by such evidences of blood destruction as haemoglobinaemia and haemoglobinuria, although in one case it is

specifically mentioned that although haemoglobinuria was found to be present on two examinations, within a few hours of the injection of the serum, the rabbit's own serum showed no trace of tingeing. The point, however, which is of especial moment in connection with the present research, is that the blood destruction, in Muir's and McNee's experiments, was in all cases associated with the appearance in the peripheral circulation of various types of abnormal red cells, basophilic cells and nucleated red cells of all types, often in enormous numbers. They usually appeared late, about the third day, but sometimes much earlier, and then rapidly increased in numbers. It obviously becomes a question of moment whether the increased fragility, observed in the corpuscles of the peripheral blood, in such cases, is in reality due to the entrance into the circulation of abnormal cells possessing an abnormal fragility. To determine this point, a series of experiments were instituted, in which simultaneous determinations were made of the fragility of the red cells, the number of corpuscles per cmm. of peripheral blood, the morphological characteristics of the cells present and the presence of any tingeing of the serum. The haemolytic serum employed in this series of experiments was prepared in the same way as serum 'α.' It was employed unheated and a previous experiment showed that 0·5 c.c. of a 1/20 dilution of the serum in the presence of excess of complement completely haemolysed 0·5 c.c. of a 1/20 suspension of rabbits' red cells in 1 hour at 37°C. This serum will be referred to as serum 'β.'

Experiment.

A rabbit was selected and the fragility of its red cells determined. A red cell count was made and two blood films were prepared and stained with Leishman's stain. These last showed the scanty polychromatophilic cells normally present in the rabbit's blood. Another sample of blood was taken, allowed to stand for 30 minutes at 37° C., and the clot separated by centrifugalisation. It showed no trace of tingeing with haemoglobin. All these observations were repeated at intervals after the injection of 1 c.c. of Serum 'β' intravenously. After 46 hours, a second intravenous injection of 1 c.c. of this serum was given and the observations were continued. The results obtained were as follows:—

Time of examination	Strength of saline solution causing first trace of haemolysis	Red cells per cmm.	Serum	Stained films
Before injection	0·56 %	5,340,000	Nil	Normal.
After injection				
1 hour	0·59	4,650,000	Nil	Normal.
4 hours	0·59	3,930,000	Nil	Normal.
24 ,,	0·63	—	Nil	Slight increase in polychromatophils and a few nucleated red cells.
46 ,,	0·58	4,360,000	Nil	Normal.
		Further injection of 1 c.c.		
47 ,,	0·64	5,510,000	Nil	Normal.
52 ,,	0·63	—	Nil	Nucleated red cells more numerous.
55 ,,	0·68	4,600,000	Nil	Nucleated red cells numerous.
72 ,,	0·73	3,700,000	Very slight tingeing	Nucleated red cells very numerous.
100 ,,	0·72	3,680,000	Very slight tingeing	Nucleated red cells very numerous.
220 ,,	0·67	4,050,000	Nil	Nucleated red cells scanty.

The animal showed only slight signs of distress during the earlier part of the experiment and made a complete recovery. The results are shown graphically in Chart 6. The examination of the table, or better still the chart, shows several points of interest. It is clear that increased fragility has gone hand-in-hand with blood destruction; the lowest counts correspond with the highest saline values. There is also, however, a certain correspondence between the increase in fragility and the number of abnormal red cells present, so that we cannot, as a result of this experiment, exclude this factor. It became necessary, therefore, to perform further experiments in which the action of the serum should be less marked and slower and also in other cases more rapid and severe.

Experiment.

The rabbit was selected and the same preliminary examinations made as in the last experiment. 2 c.c. of Serum 'β' were injected intraperitoneally, and the resulting changes recorded at intervals.

Time of examination	Strength of saline solution causing first trace of haemolysis	Red cells per cmm.	Serum	Stained films
Before injection	0·56 %	4,560,000	Nil	Nil
After injection				
1 hour	0·59	—	Nil	Nil
4 hours	0·59	4,610,000	Nil	Nil
24 ,,	0·62	3,540,000	Nil	Nil
53 ,,	0·64	4,240,000	Nil	Nil
101 ,,	0·62	—	Nil	Nil

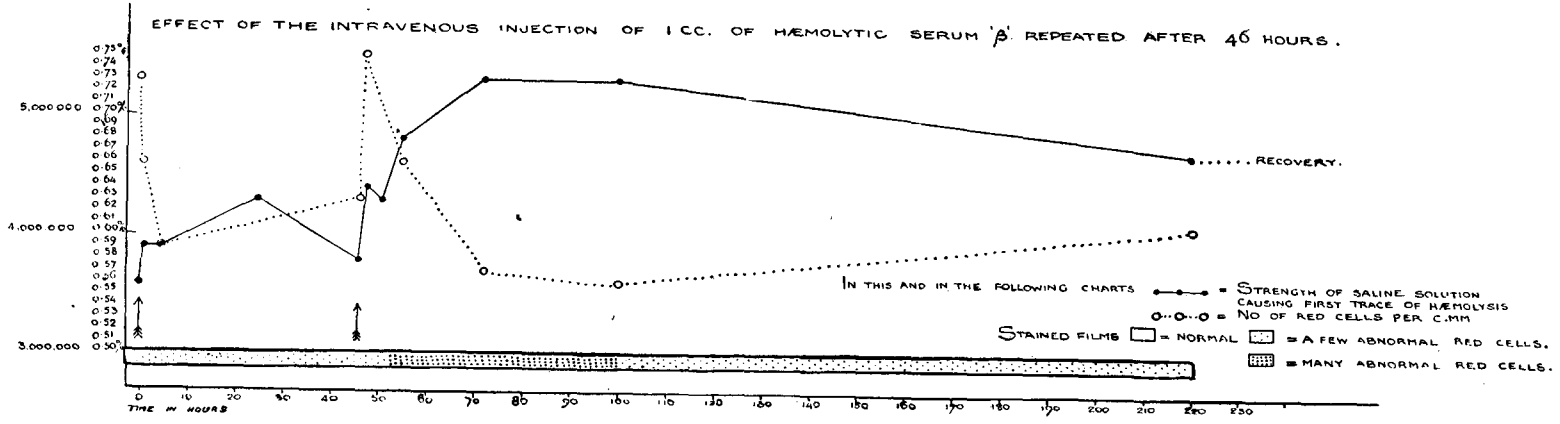

Chart 6.

Here, too, the same general correspondence between blood destruction and increased fragility appears, while it is proved that an increase corresponding to 0·08 % saline may occur without any abnormal red cells being present in the peripheral circulation.

In the following experiments a third haemolytic Serum 'γ' was employed. It consisted in the mixed sera of two guinea-pigs, immunised as described above, but with somewhat larger doses of rabbits' red cells. It was heated before use, and was found to be slightly more powerful than Serum 'β' as the result of titration.

Experiment.

Employing exactly the same procedure as in the two preceding experiments, a rabbit was inoculated intravenously with 5 c.c. of Serum 'γ.' The various examinations carried out gave the following results:—

Time of examination	Strength of saline solution causing first trace of haemolysis	Red cells per cmm.	Serum	Stained films
Before injection	0·57 %	6,050,000	Nil	Nil.
After injection				
5 mins.	0·68	5,000,000	Slight tinge	Nil.
20 ,,	0·72	3,750,000	Deep tinge	Nil.
40 ,,	0·72	3,500,000	Deep tinge	A few nucleated red cells.

Death occurred within the hour.

The occurrence of haemoglobinaemia of a marked degree caused the saline in every tube to be tinged with haemoglobin, so that it was necessary to work to a uniform pink-tinged fluid instead of to a completely colourless one. As it was possible, however, to arrange the series of solutions so that a long range beyond the saline corresponding to the maximum fragility was employed, a considerable number of tubes showing the exact tint due to the serum were obtained, and no difficulty was experienced in picking out the tubes in which the additional tinge due to hypotonic saline lysis was present. The results are shown graphically in Chart 7. This experiment entirely confirmed the results obtained in the two preceding ones, and points to the conclusion that the fragility increases directly with the blood destruction and is entirely unrelated to the appearance of abnormal red cells in the general circulation.

A second experiment was made, using this same serum, and inoculating intravenously, but with a smaller quantity, 2 c.c. The results are shown in Chart 8, and the full details need not be

EFFECT OF INTRAVENOUS INJECTION OF HAEMOLYTIC SERUM 'γ.'

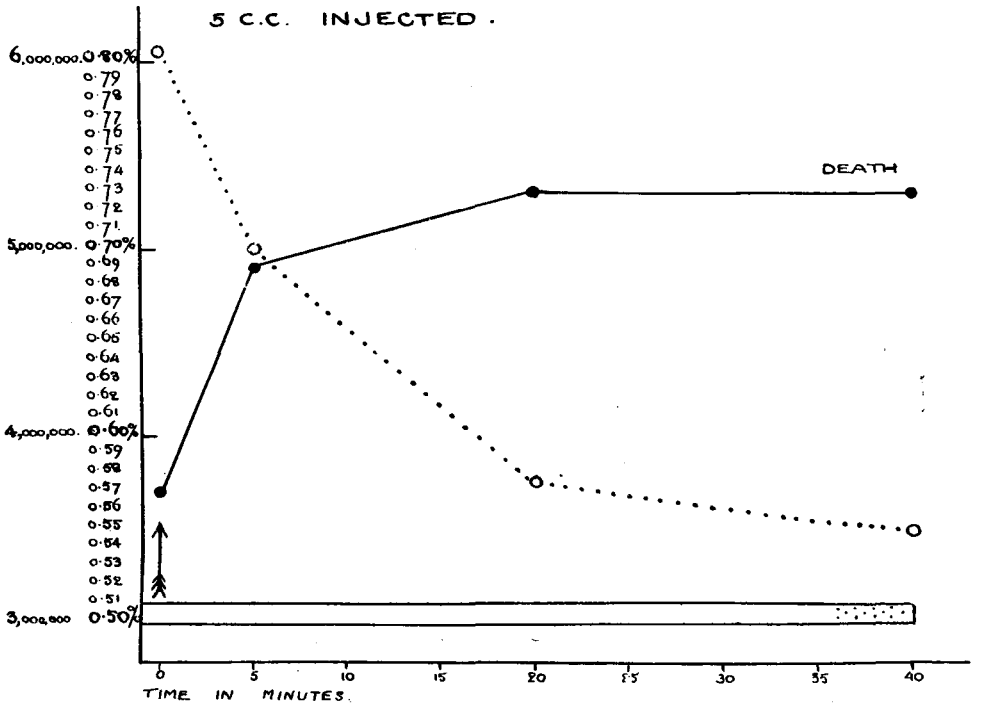

Chart 7.

EFFECT OF INTRAVENOUS INJECTION OF HAEMOLYTIC SERUM 'γ.'

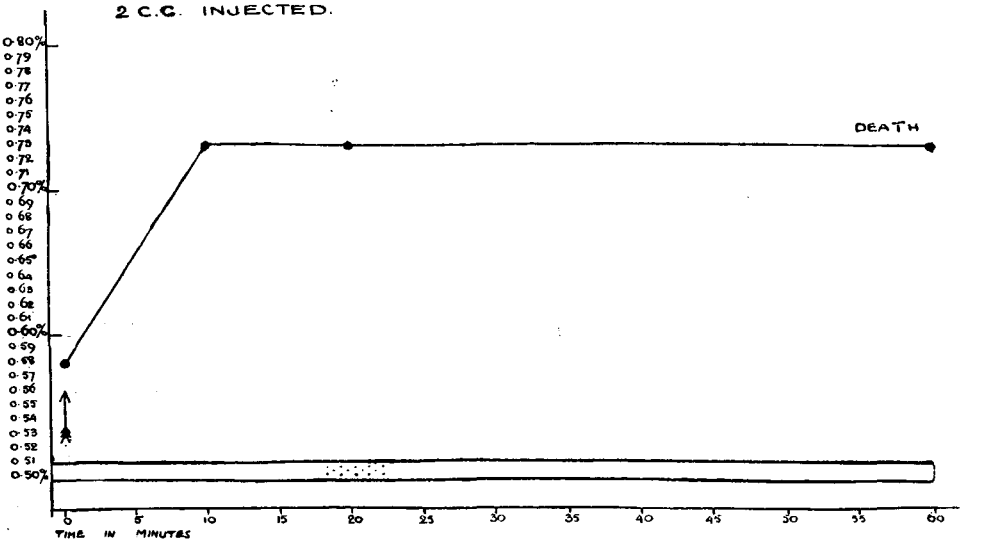

Chart 8.

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tabulated here. The fragility rose rapidly, so that within 10 minutes its maximum value, which was originally equivalent to 0·58 % saline became equivalent to 0·73 % saline. Haemoglobinaemia was marked at this first examination and the animal died in little over one hour. At the 20 minutes' examination, stained films showed a slight increase in basophilia and the presence of a few nucleated red cells, but at each of the other examinations they showed nothing abnormal.

Finally, the same serum was employed using the intraperitoneal route.

Experiment.

The usual estimations were made and 2·5 c.c. of Serum 'γ' was then injected. The results were as follows:—

Time of examination	Strength of saline solution causing first trace of haemolysis	Red cells per cmm.	Serum	Stained films.
Before inoculation	0·56 %	5,000,000	Nil	Nil.
After inoculation				
½ hour	0·59	4,790,000	Nil	Nil.
¾ ,,	0·65	4,987,000	Nil	Nil.
1¾ hours	0·65	4,050,000	Nil	Nil.
2¼ ,,	0·62	3,900,000	Nil	Nil.
8 ,,	0·71	3,600,000	Nil	Nil.
22 ,,	0·58	—	Nil	Nil.
51 ,,	0·62	4,420,000	Nil	A few nucleated red cells.
95 ,,	0·56	4,987,000	Nil	A few nucleated red cells.

The animal never showed any marked sign of distress, and eventually made a complete recovery. The results are shown graphically in Chart 9. Here again there is the obvious correspondence between increased fragility and blood destruction, while abnormal cells only appeared on the scene when the fragility was returning to normal limits.

It would be an additional demonstration of the independence of the two phenomena, if we could produce marked abnormalities in the morphology of the peripheral blood without a corresponding alteration in fragility. This was attempted by making use of the well-known alteration which may be produced in the blood by repeated severe haemorrhages. The effect of a single severe, or repeated slight haemorrhages, had already, in a series of preliminary experiments, been found to be nil. There was a strong probability that a similar result would follow from repeated large abstractions of blood, since Theobald

Smith, first alone and later in conjunction with H. R. Brown, had already studied exhaustively the red cell fragility in a large series of horses, repeatedly bled for the purpose of obtaining anti-toxic sera. These papers have been referred to above and it will be remembered that these workers finally concluded that there was no evidence that any of the variations which occurred depended on the blood abstraction. The blood losses in this series of experiments, however, though severe, were not extremely frequent and there is no evidence as to their effect on the morphological characters of the peripheral blood, so that it was necessary to carry out a special series of observations bearing on this point.

EFFECT OF THE INTRAPERITONEAL INJECTION OF 2·5 C.C. OF HAEMOLYTIC SERUM 'γ.'

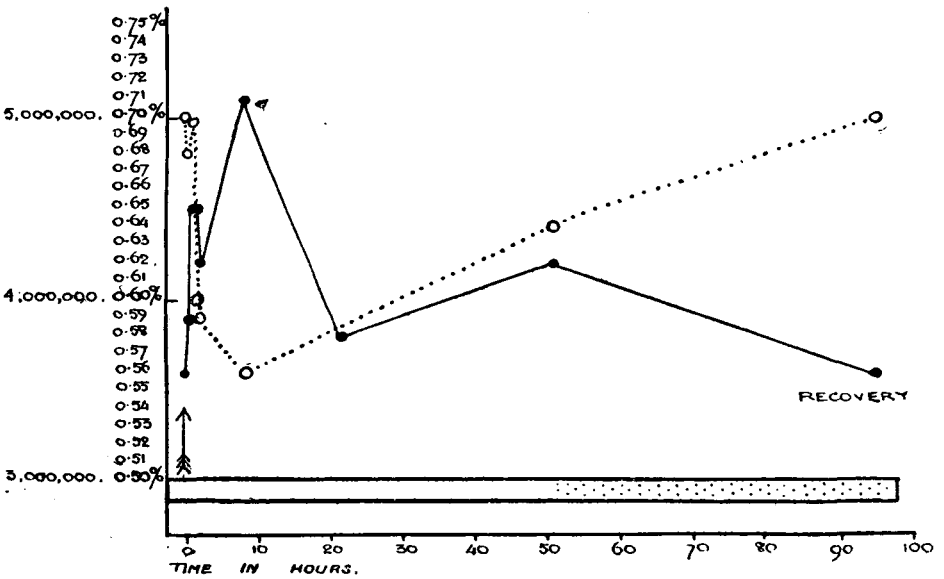

Chart 9.

Experiment.

A rabbit was selected and a preliminary determination made of its red cell fragility and of the morphology of its peripheral blood. It was then bled to large amounts, first on alternate, and then on successive days. Each day before the blood was abstracted the corpuscular fragility was determined, and two blood films were prepared. These

were afterwards stained by Leishman's method and any abnormalities carefully searched for. The results obtained were as follows:—

Day	Amount of blood extracted	Strength of saline solution causing first trace of haemolysis	Stained films
1st	10 c.c.	0·58 %	Nil.
3rd	20	0·55	Nil.
5th	55	0·56	Nil.
7th	40	0·55	Increased basophilia.
10th	40	0·58	,, ,,
11th	40	—	,, ,,
12th	10	0·56	A few nucleated red cells.
13th	5	—	,, ,,
14th	50	0·56	,, ,,
15th	—	0·55	,, ,,
16th	50	0·56	,, ,,
18th	50	0·55	Nucleated red cells numerous.
20th	—	0·56	Very few nucleated red cells.

The rabbit was somewhat distressed immediately after each of the larger bleedings, but the symptoms always passed off within a few minutes, and at the end of the experiment the animal appeared to be in perfect health. The results of this experiment are shown graphically in Chart 10. It is clear that the alterations produced are slight and of no permanent character, mostly falling within the limits of experimental error, and probably partly accounted for by the alteration in the tonicity of the serum which must result from the repeated and frequent abstraction of so large a proportion of the animal's total blood. Thus it is possible to cause the appearance in the peripheral circulation of the rabbit of a considerable number of morphologically abnormal red cells, without altering the maximum fragility to hypotonic saline, thus affording additional evidence of the complete independence of these two phenomena.

Having thus established the fact that a specific haemolytic serum is able to act on red blood corpuscles 'in vivo,' in such a manner as to increase their fragility towards hypotonic saline solutions, it becomes necessary to examine, as far as possible, the means by which this change is brought about.

A vast number of 'in vitro' experiments have yielded a considerable mass of precise information regarding the manner in which a specific haemolysin produces its effect, and a certain number of observations were undertaken in order to determine whether certain factors, which have been proved to be involved in the actual lysis of red cells, are concerned

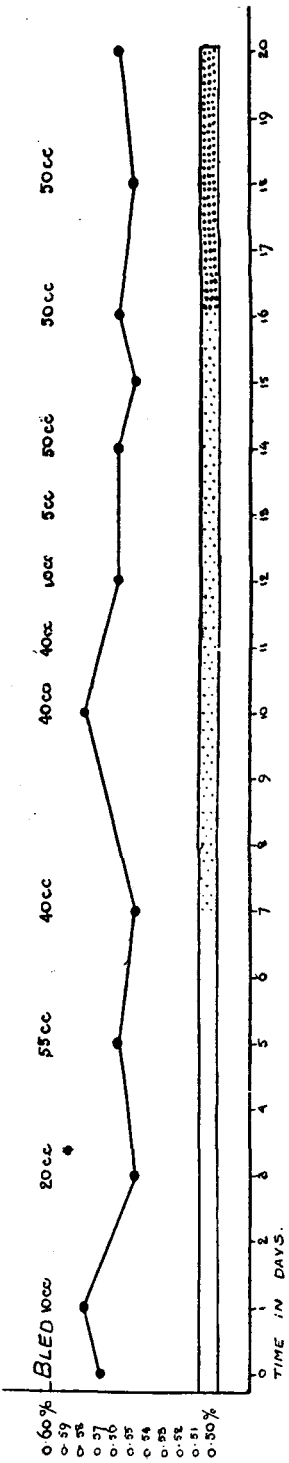

Chart 10.

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also in producing alterations in their fragility. The first point investigated was as to whether the haemolytic amboceptor, apart from complement, had any effect on the corpuscular fragility. In the initial series of experiments human red cells were employed, together with the serum of a rabbit which had received a large number of intraperitoneal injections of washed human corpuscles. The human red cells were collected and treated in the routine manner, and the thick deposit from the last washing, during which centrifugalisation was carried out for a prolonged period and at high speed, was employed for the tests. The rabbit's serum was obtained in the usual manner and heated at 58°C. for 30 minutes to destroy complement. This serum, in addition to the specific haemolysin, contained an extremely powerful haemagglutinin, as is indeed usually the case in rabbit *v.* human haemolytic sera, and this fact rendered the preliminary treatment of red cells by the serum and their subsequent washing and testing impracticable, since the violence necessary to break up the firmly clumped red cells, in order to subject them first to washing and then to the action of the standard saline solutions, produced a certain degree of mechanical haemolysis which vitiated the result of the test.

The following method was therefore adopted. The requisite series of saline solutions were set up and then to each tube was added, first the usual amount of red cells, and then an equal quantity of the heated immune serum, or saline, or normal serum used as controls. The whole series were then placed in an incubator at 37°C. for 15 minutes and then centrifugalised as usual. The shorter time was adopted on account of the rapid settling of the cells in the tube containing the immune serum.

Experiment.

Contents of tubes		Strength of saline solution causing first trace of haemolysis
Standard Saline + Red Cells + Normal Saline	=	0·42 %
,, ,, + ,, + Heated Immune Serum	=	0·40 %

Thus the red cells appeared to be less fragile in the presence of the heated immune serum containing the haemolytic amboceptor. This effect might have been due to the binding of the amboceptor to the red cells, but it was equally possible that it resulted from the mechanical action of the haemagglutinins in rapidly removing the red cells from the action of the saline, since clumps of massed corpuscles formed almost instantaneously in the tubes containing the immune serum. A third possibility was that the variation was caused by the difference between the tonicity of the serum and that of the normal saline.

Experiment.

The serum was obtained from a normal rabbit and heated as above to destroy complement. It was tested for the presence of a haemolytic amboceptor towards human red cells with a negative result. It was then tested for the presence of haemagglutinins by mixing one volume of the heated serum, one volume of washed human red cells and ten volumes of saline in a capillary pipette, and incubating at 37°C. for 15 minutes. Marked haemagglutination occurred. The following tests were put up with the results shown.

Contents of tubes							Strength of saline solution causing first trace of haemolysis
Standard Saline	+	Washed Red Cells	+	Normal Saline	=		0·41 %
,,	,,	+	,,	,,	+ Heated Immune Serum	=	0·40 %
,,	,,	+	,,	,,	+ Heated Normal Rabbit Serum	=	0·38 %

Thus, the heated serum of the normal rabbit which possessed no haemolytic amboceptor had a greater effect than the specific haemolytic serum.

To exclude the normal protective action of the serum the following series of tests were made.

Experiment.

The washed red cells taken from a normal human subject 'A' were used throughout. The sera employed were as follows:—

(1) 'A's Heated Serum.
(2) The Heated Serum of another normal human subject, 'B.'
(3) The Heated Serum of a normal rabbit.
(4) The Heated Immune rabbit Serum.

A preliminary test for the presence of haemagglutinins was made in the manner described above with the following results.

A's Serum and B's Serum produced no agglutination. The two rabbits' Sera as before agglutinated the red cells to a very marked degree. The following tests were then put up and the results recorded.

Contents of tubes							Strength of saline solution causing first trace of haemolysis
Standard Saline	+	A's Washed Red Cells	+	Normal Saline	=		0·40 %
,,	,,	+	,,	,,	+ A's Heated Serum	=	0·39 %
,,	,,	+	,,	,,	+ Heated Normal Serum (Rabbit)	=	0·37 %
,,	,,	+	,,	,,	+ Heated Immune Serum (Rabbit)	=	0·37 %

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Another experiment employing a non-agglutinating human serum and an agglutinating rabbit's serum yielded corresponding results. It appears, from these experiments, that the amboceptor present in a rabbit *v.* human haemolytic serum has, by itself, no power of increasing the fragility of the red blood cells. The haemagglutinins present cause an apparent decrease in fragility, due probably to purely mechanical causes.

Nolf, however, in the communication referred to above, states that, whereas the normal erythrocytes of the fowl show no haemolysis in 0·45 % saline, they may, when sensitized by a specific serum, show a faint trace of haemolysis in 0·55 % saline or in even more concentrated solutions.

Turning now to the effect of a complete haemolytic system, amboceptor plus complement working 'in vitro,' the difficulties involved in the technique become very great. The most satisfactory procedure would obviously be to submit washed red cells to the action of both amboceptor and complement in amounts which fail to produce haemolysis, separate and wash the cells and then test them in the various saline solutions. The large number of washings and the considerable manipulation of the cells, involved in this process, have, however, a very appreciable effect. The amboceptor and complement, moreover, once bound to the cells, continue to act upon them and, thus, minute traces of these substances, which produced no trace of haemolysis during the usual incubation period, caused a slight degree of lysis during the somewhat lengthy operations which followed. Experiments in which the incubation was carried on for three hours, to allow the haemolysin to exert its full influence before the cells were washed, did not overcome this difficulty. The effect of carrying out all the operations, subsequent to incubation, as nearly as possible at 0° C. in order to prevent the further action of the haemolysin, yielded even less satisfactory results, since the effect of cold on the red cells was even more marked than it is on normal erythrocytes, which are bathed in hypotonic saline solution, and the fluid in all the tubes showed a strong tinge of haemolysis. For this reason, it is of little advantage to give full details of the experiments performed, but the general technique and results may be noted. Two combinations were studied (*a*) rabbits' red cells and a guinea-pig *v.* rabbit haemolytic system, (*b*) human red cells and a rabbit *v.* human serum. In all cases a $\frac{1}{10}$ dilution of normal guinea-pig serum was employed as complement and the haemolytic immune body was diluted to a point considerably above that which gave the last trace of haemolysis in

a preliminary experiment. The red cells were washed in the usual manner and a $\frac{1}{20}$ suspension prepared. Considerable quantities of such a suspension were subjected to the action of immune body + complement, immune body + saline, complement + saline, and a double volume of saline alone. Incubation was carried on for periods of one to three hours and the mixtures were then centrifugalised and the deposited red cells washed twice in normal saline. The deposit from the last washing was added to the test solutions in quantities of 0·05 c.c. to each tube. In almost all experiments there was, at any given dilution, most marked haemolysis in the tube containing red cells which had been subjected to the action of amboceptor + complement, the red cells which had been suspended in normal saline showed less destruction, while the tubes containing those cells which had been in contact with immune serum alone or complement alone, showed the least tingeing of the supernatant fluid. For this reason, a similar method to that employed when testing the action of amboceptor alone was resorted to.

Experiment.

Sheep's red cells were washed in the usual manner. Six saline solutions of different strengths were prepared, and with each of these a $\frac{1}{20}$ suspension of the red cells, a $\frac{1}{10}$ dilution of fresh guinea-pig serum and varying dilutions of a heated rabbit *v.* sheep haemolytic serum were made up. In every tube was then placed 0·5 c.c. red cell suspension, 0·5 c.c. complement and 0·5 c.c. diluted immune body. The whole series were then incubated for 30 minutes at 37°C. after which the tubes were centrifugalised and the degree of haemolysis noted. The results were as follows:—

Haemolysis produced by increasing dilutions of immune body.

Strength of saline solution	$\frac{1}{20}$	$\frac{1}{40}$	$\frac{1}{80}$	$\frac{1}{160}$	$\frac{1}{320}$	$\frac{1}{640}$	$\frac{1}{1280}$	$\frac{1}{2560}$	$\frac{1}{5120}$
0·85 %	Complete	Complete	Complete	Almost complete	Marked	Slight	Trace	–	–
0·83 %	Complete	Complete	Complete	Complete	Complete	Almost complete	Marked	Trace	–
0·81 %	Complete	Complete	Complete	Complete	Complete	Marked	Slight	Trace	–
0·79 %	Complete	Complete	Complete	Complete	Complete	Complete	Slight	Trace	Trace
0·77 %	Complete	Complete	Complete	Complete	Complete	Almost complete	Slight	Trace	Trace
0·75 %	Complete	Complete	Complete	Complete	Complete	Complete	Almost complete	Slight	Trace

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This observation is, however, by no means a new one. As long ago as 1900, Nolf, in the article already referred to, showed that the haemolytic action of specific sera was retarded by the presence of certain metallic salts, and that this retardation increased with increasing concentration of the solution. In this connection he studied the action of the sera of the ox, dog, and rabbit on the corpuscles of the rabbit, horse, dog and fowl. As the result of a considerable number of experiments he found that the salts of the alkaline metals opposed haemolysis, in proportion to the concentration of the solution used, while the salts of the metals of the alkaline earth series inhibited the action of a haemolytic serum in all dilutions.

More recently, Sutherland and McCay have made similar observations, using anti-sera derived from rabbits, fowls and a goose, which had been immunised against sheep's red cells. They employed the chlorides of sodium and calcium, and found that the action of a haemolytic serum varied inversely with the concentration of the saline solution, while the presence of calcium salts had a most marked inhibitory action, thus confirming exactly the results obtained by Nolf. They could not obtain evidence that the haemolysin itself was affected, and assumed the difference observed to be due to variations in the resisting power of the erythrocytes. Nolf's hypothesis has been dealt with above.

Now it is obvious that experiments of the above type may be regarded as indicating either that the hypotonicity of the saline increases the fragility of the red cells towards the action of the serum, or that the action of the serum increased the liability of the corpuscles to hypotonic saline lysis. These considerations also involve a question as to the validity of the results obtained by injecting haemolytic sera into animals, since it might be argued that, in adding the blood of the animal as such to the various saline solutions, one in reality added both red cells and a minute quantity of the specific haemolysin, which might be considered to be present in the blood stream. Hence, the results might be obscured by a lytic action due to this trace of haemolysin in the hypotonic saline solutions. Various considerations, however, completely negative this idea. In the first place, the ultimate dilution of the haemolysin arrived at by injecting a small quantity, 1 c.c. for instance, of a haemolytic serum into the general circulation of an adult rabbit, and then withdrawing 0·05 c.c. of the animal's blood and adding it to 1 c.c. of saline, is a considerable one. The comparatively weak haemolytic sera employed would produce no lytic action in this dilution,

when acting for 30 minutes at room temperature. Again, if the action of this minute trace of haemolytic serum were a disturbing factor, it would obviously operate most strongly immediately after the injection, when it would be present in the highest concentration. As a fact, the effect with small or moderate doses rises to a maximum at some point between the second and fourth days. Finally, such an action would be a very gradual one, especially at room temperature; whereas, with alterations in fragility of the degree observed in the series of experiments in question, the change is obvious immediately one adds the blood to the saline solutions, by the partial laking of the corpuscles in tubes which before showed no such rapid lysis, although the particular tube which corresponds to a complete cessation of haemolysis can only be determined after centrifugalisation. It is thus clear, that the changes noted after the injection of haemolytic sera into rabbits correspond to an increased fragility towards hypotonic saline lysis, and this lends additional support to the assumption of Sutherland and McCay that it is the red cell itself and not the haemolytic system which is affected by the saline concentration in the 'in vitro' experiments.

CONCLUSIONS.

1. Of the various arsenical compounds studied (Arsenious Acid, Atoxyl and Salvarsan) none produced 'in vivo' any decrease in red cell fragility; while the two former in highly toxic doses produced a pronounced increase.

2. The injection into rabbits of foreign bile, of bile obtained from animals of the same species, of bile salts (Sodium Taurocholate) and of Cholesterin resulted in no change in fragility worthy of note.

3. Various pathogenic organisms known to be associated with haemolytic phenomena (*Streptococcus pyogenes*, *Staphylococcus aureus*, Bacillus of Danysz and *Bacillus pyocyaneus*) produced in the majority of cases, when inoculated into rabbits, a rise in fragility of varying degree, most marked in the case of certain strains of Streptococci.

4. The injection into rabbits of specific haemolytic sera, heated or unheated, constantly produced an increase in fragility, in many cases of a most marked type.

5. This increased fragility was not due to the appearance in the peripheral blood-stream of cells of abnormal type, but resulted from the direct action of the haemolytic serum on the erythrocytes.

It was directly proportional to the degree of blood destruction which occurred.

6. The action of a haemolytic amboceptor alone did not, in the case of a rabbit *v.* human serum, produce any increased fragility. There was indeed an apparent decrease, but this was due to the mechanical action of the coexisting haemagglutinins.

7. 'In vitro' experiments on the effects of specific haemolysins on red cell fragility were unsatisfactory, on account of the extreme technical difficulties involved; but such evidence as could be obtained confirmed the 'in vivo' findings.

8. The relation between the action of the more specialised haemolysins and that of weak saline solutions, or distilled water, has never yet been definitely established; but the effect produced by the action of certain agents of one class results in an altered fragility to agents of the other.

It only remains for me to acknowledge my indebtedness to Dr L. S. Dudgeon for many kind and helpful suggestions.

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