**OBSERVATIONS ON THE INHIBITORY INFLUENCE
EXERTED BY HYPERTONIC SALINE SOLUTIONS AND
CALCIUM CHLORIDE SOLUTIONS ON THE ACTION OF
SPECIFIC HAEMOLYSINS, WITH SUGGESTIONS AS
TO THE THERAPY OF BLACKWATER FEVER**

BY MAJOR W. D. SUTHERLAND, M.D., I.M.S., *on special duty at
the Medical College, Calcutta*, AND CAPTAIN D. McCAY, M.B.,
I.M.S., *Professor of Physiology, Medical College, Calcutta*.

From the Physiological Laboratories, Medical College, Calcutta

(Received October 23rd, 1909)

We have decided to place on record the following contribution to the action of specific haemolysins and their inhibition, not because the research is by any means complete but because we believe that the results that have been obtained are of considerable interest and of great practical importance, we publish them in the hope that other workers in the same field may be able to follow the side-issues in detail, and that they may be put to the test by practical application.

One of us has already published certain papers (1) giving an account of some experimental work on the haemosozic value of the blood serum; the haemosozic value being understood to indicate those constituents present in the blood serum which preserve the red blood corpuscles from solution in the living body. In the final paper on this subject the significance of a modification of the haemosozic value of the serum by drugs, and the bearing which any lowering of it would have on the etiology of blackwater fever, was discussed.

A certain amount of evidence was brought forward to show that drugs such as mineral acids or acid radicles, which on absorption had a tendency to deplete the plasma or the blood of bases, caused a lowering of the haemosozic value, and therefore diminished the resisting power of the erythrocytes; while drugs whose action on absorption was to increase the number of inorganic ions of the plasma, such as the sodium and calcium chlorides, had the opposite effect, in that they raised the resisting power of the erythrocyte. It was pointed out that if this view were correct, the raising of the haemosozic value of the serum by the administration of chlorides, such as quinine hydrochloride, sodium or calcium chloride, would appear to be a rational indication in the prophylaxis and treatment of the blackwater fever.

The method introduced by Wright (2) for the determination of the haemosozic value was made use of in all these observations, except for the slight but important modification, that the haemolysis obtained with each particular dilution was compared with a standard dilution of one volume of blood in two volumes of distilled water, one in which, therefore, complete haemolysis had taken place. Absolutely comparable results were thus obtained, and the method seemed perfectly satisfactory. However, owing to the criticisms of Christophers and Bentley (3) that Wright's method was not accurate enough to show slight variations in haemolysis produced with different dilutions, a method was devised in which the personal factor was absolutely eliminated.

This brings us to a consideration of the light shed on the problem by the later work we have been able to carry out. These researches we shall take up under two distinct and separate headings:—

A. The evidence afforded by a change in the size of the erythrocytes induced by the administration of sodium and calcium chlorides, acids and drugs. For this section one of us (McCay) alone is responsible.

B. The evidence afforded by the effect of different strengths of saline solution and of calcium chloride on the action of a specific haemolysin. This part of the work has been carried out by both of us in collaboration, but mainly by one of us (Sutherland) who prepared the specific haemolysins.

PART A. THE CHANGE IN THE SIZE OF THE ERYTHROCYTES INDUCED BY THE ADMINISTRATION OF SODIUM AND CALCIUM CHLORIDES, ACIDS AND DRUGS.

If our contention is correct, that an increase of the inorganic ions present in the plasma accompanies the absorption of salts not foreign to the blood, then, other things being equal, there should be a rise in the osmotic tension of the plasma and a decrease in the size of the erythrocytes by exosmosis. Further, if a decrease in the inorganic ions of the plasma accompanies the absorption of acid salts foreign to the blood, then there should be a fall in the osmotic tension of the plasma and an increase in the size of the erythrocytes from endosmosis. We are not concerned at present with the question as to whether the outer layer of the erythrocyte acts as a semipermeable membrane or not; whatever the explanation may be, we know that a decrease in the number of inorganic ions present in the plasma or suspension fluid is accompanied by an increase in the size of the erythrocyte and vice versa. For example, if, after giving a dose

of sodium chloride and calcium chloride to an animal, we find that the size of the erythrocytes is distinctly decreased, and if, after giving a mineral acid or acid salt, we find the erythrocyte distinctly increased in size, we are justified in assuming that the inorganic ions sodium, calcium, chlorine, are present in excess in the first case while in the second they are diminished, or that some change as suggested by Benjamin Moore, such as adsorption, followed by physical and chemical changes in the haemoglobin, has occurred and is accompanied by an increase in the volume of the red blood corpuscles. Further, in the first case the plasma in which the erythrocytes float will require a greater degree of dilution with distilled water before haemolysis takes place, and in the second case the plasma will require a lesser degree of dilution to produce haemolysis: in other words, the resisting power of the erythrocytes is raised by an increase in the number of inorganic ions present in the plasma and lessened by their decrease. Resistance here means their resistance to haemolysis caused by dilution of the plasma with distilled water.

In the papers already referred to we had put forward evidence to show that the ingestion of chlorides did cause an increase in the resisting power of the erythrocytes, while the ingestion of sulphates had the opposite effect; it therefore remained to prove that with increased resistance of the erythrocyte there was a decrease in its size, and with decrease in its resisting power there was an increase in the size of the erythrocyte.

In order to obtain definite information on this point, after rejecting different methods such as actual measurements of the erythrocytes, percentage volume of erythrocytes and plasma, the following method was devised and the results obtained appear to be fairly free from all inherent sources of error.

From an animal, usually a rabbit, two c.c. of blood was drawn off from a vein. The blood was quickly defibrinated, and a known volume sucked into a large-bore haematocrite tube and the corpuscles centrifugalised in a high-speed electric machine until the volume of corpuscles became constant. This volume was read off and noted.

The clear plasma was now pipetted off and a solution of known composition introduced (N/10 sodium chloride was always used) the corpuscles were shaken up in, and thoroughly mixed with, the saline and allowed to stand for exactly one hour. The emulsion of erythrocytes was again centrifugalised to a constant volume and this volume noted.

The percentage increase in volume due to the substitution of N/10 sodium chloride for the plasma was then calculated. These observations were carried out for two, three or four days for each rabbit, in order to obtain the average increase in the volume of the erythrocytes under normal conditions.

Having obtained this the animal was given the drugs, sodium chloride, calcium chloride, quinine bihydrochloride, quinine sulphate, sulphuric acid, &c., whose effects were under investigation, and exactly the same procedure was followed. In this way any increase or decrease in the volume of the individual corpuscles at once became evident. The necessity of substitution of a known saline solution for the normal plasma was early forced on us by the marked variations met with in the percentage volumes of the plasma and corpuscles when these drugs were given. Thus when an animal was given sodium and calcium chlorides the percentage of corpuscles fell very considerably, the plasma increasing proportionately; at first this was thought to be due to a decrease in the size of the red blood corpuscles, but a little consideration showed it to be really caused by an increase in the percentage of the plasma from a retention of fluid to keep the plasma as nearly isotonic as possible. However, there is a relative as well as an absolute increase in the inorganic ions under the above conditions, the retention of fluid not being able to keep pace with the absorption of ions, so that the osmotic tension of the plasma rises and the size of the erythrocyte falls. The drugs were given to the anaesthetised animals used by means of the stomach tube, so that we knew exactly how much of each particular drug was administered.

Observations were also made on the haemosozic value of the serum during the experiments.

Table I shows the effects of the administration of sodium chloride and calcium chloride on the erythrocytes and plasma.

This investigation shows that, in a rabbit, the administration of chlorides is followed by:—

1. A decrease in the salt content equivalent of the serum, i.e., the corpuscles are able to stand a greater and greater dilution of a normal salt solution before haemolysis takes place, showing an increased resisting power. In the table we find that haemolysis normally occurs when one volume of blood is mixed with two volumes of N/45 sodium chloride, but after the administration of the third dose of chlorides haemolysis only took place when N/70 sodium chloride was substituted for the N/45 sodium chloride. As a sequela of this we also find the haemosozic value of the serum much increased after the administration of chlorides.

ACTION OF SALINES ON HAEMOLYSIS

5

TABLE I

Date	Percentage salt content equivalent	Percentage haemosozic value of serum	Percentage volume of corpuscles	Percentage volume of corpuscles in 0.1 N sodium chloride	Normal percentage increase due to 0.1 N sodium chloride	} This shows an average increase in volume of the red corpuscles when N/10 sodium chloride is substituted for the plasma of 12.28 per cent.		
27.1.08	0.130	0.94	41.45	47.06	13.29			
28.1.08	0.137	0.98	46.40	51.42	10.81			
11.2.08	—	—	45.06	50.56	12.26			
12.2.08	0.130	1.04	47.80	53.90	12.76			
AFTER DRUGS								
Date	Drugs	Percentage salt content equivalent	Percentage haemosozic value of serum	Percentage volume of corpuscles	Percentage volume of corpuscles in 0.1 N sodium chloride	Percentage increase due to 0.1 N sodium chloride	Percentage decrease due to the increased salinity of serum from drugs	
28.1.08	Cal. Chlor. grains v	0.123	1.23	37.63	?	?	?	2 hours after first dose was given.
29.1.08	Sodium Chlor. grains xxx	0.123	0.984	39.57	46.28	16.95	4.67	24 hours after first dose of drugs.
29.1.08	Dose (above)	0.097	1.16	35.42	41.98	18.52	6.24	2 hours after second dose of drugs.
30.1.08	Nil	0.116	1.16	33.40	39.75	19.00	6.72	24 hours after second dose of drugs.
30.1.08	Dose (above)	0.097	1.63	26.80	33.33	24.37	12.09	2 hours after third dose of drugs.
31.1.08	Nil	0.083	1.83	22.80	28.42	24.65	12.37	24 hours after third dose of drugs.
1.2.08	Nil	0.093	1.12	31.61	37.38	17.93	5.65	48 hours after last dose of drugs.
3.2.08	Nil	0.1016	1.16	30.26	35.52	17.38	5.10	72 hours after last dose.
4.2.08	Nil	0.116	0.95	32.90	37.03	12.55	0.27	96 hours after last dose.
6.2.08	Nil	0.123	0.97	38.56	43.71	13.35	1.07	144 hours after last dose.
11.2.08	Nil	—	—	45.06	50.56	12.26	Nil	11 days after last dose.
—	Nil	0.130	1.04	47.80	53.90	12.76	0.48	12 days after last dose.

The effects of the drugs are well marked up to from 48-72 hours after stopping their use.

2. The next point of importance brought out by the investigation is the very great decrease in the percentage volume of the corpuscles with a corresponding increase in that of the plasma. Thus in the rabbit normally the relations of corpuscular elements to plasma are 45 and 55 per cent. As soon as the chloride treatment was begun a fall in the percentage volume of the corpuscles took place, and after the third dose (in all calcium chloride 15 grains and sodium chloride 90 grains had been ingested) the volume of the corpuscles had fallen to 23 per cent., and that of the plasma had risen to 77 per cent. This can only be explained by retention of water in the attempt to maintain the isotonicity of the plasma, and, perhaps to some extent, by more corpuscles being removed during defibrination—the formation of fibrin having been increased by the increased calcium content of the plasma.

3. Under the conditions of the experiment we found that the average increase in volume of the corpuscular elements when N/10 sodium chloride was substituted for the plasma was normally 12·28 per cent. Reference to the table will show that we obtained a very much greater increase than this after chlorides had been administered. Thus twenty-four hours after the third dose the increase in the volume of the corpuscles, on substituting N/10 sodium chloride for the plasma, reached the high figure of 24·65 per cent. The explanation of this is fairly obvious. Suppose in the accompanying diagram that the circle A represents the size of the normal erythrocyte in its own plasma, substitution of N/10 sodium chloride for this plasma causes a 12 per cent. increase in the volume of the corpuscle, which we shall say is represented by the circle C. The rabbit is then given large doses of sodium chloride and calcium chloride, and it is found that substitution of N/10 sodium chloride for the plasma now produces a 24 per cent. increase in the

volume of the corpuscle: therefore, since C represents the volume in N/10 sodium chloride and A represents a 12 per cent. reduction in the volume, the circle B will represent a 24 per cent. reduction, that is, we have practical proof that the administration of chlorides does cause a decrease in the size of the red blood corpuscle, and that this smaller decrease in the size of the red blood corpuscle, and, as we have already shown, this smaller corpuscle is much more difficult to break up by the dilution method than the normal sized corpuscle.

We may therefore conclude that the results obtained by this method afford confirmatory evidence of the increase in the resisting power of the erythrocyte caused by the ingestion and absorption of chlorides, and that the administration of sodium and calcium chloride is accompanied by an increase in the relative number of the inorganic ions present in the plasma.

Another series of observations was made in which 20 grains of sodium chloride and 4 grains of calcium chloride were given daily to a rabbit; the results, while not so marked, undoubtedly pointed to the same conclusion, for the same increased resistance of the corpuscles to a dilution of the plasma, and the same reduction in the volume of the erythrocytes followed the ingestion of these drugs.

Further proof that the administration of calcium chloride is accompanied by an increase in the calcium content of the blood is afforded by the fine piece of research on this subject by Boggs (4). This observer experimenting on dogs has proved that calcium given by the mouth does cause a great increase—up to 36 per cent.—of the calcium content of the blood, and that this increase is maintained for several days after cessation of the administration of the salt.

The experiments of Grube on the alteration of the molecular concentration brought about by the ingestion of hypotonic and hypertonic mineral waters would appear to put it beyond all doubt that the osmotic tension of the blood can be very considerably modified: a decrease following the ingestion of hypotonic and an increase the ingestion of hypertonic mineral waters (5).

As we go to press further confirmation of the above views comes in an article by Mendel and Benedict (6), who conclude that a considerable quantity of the excess of calcium introduced (intravenously) may be retained for some time in the body.

Another point of importance in connection with the therapeutic use of calcium in blackwater fever is its diuretic effect when given intravenously with sodium chloride solution, suppression of urine being often a marked sign in severe cases of blackwater fever.

TABLE II

NORMAL							
Date	Percentage salt content equivalent	Percentage haemosozic value of serum	Percentage volume of corpuscles	Percentage volume of corpuscles in 0.1 N sodium chloride	Normal percentage increase due to 0.1 N sodium chloride	{ From these and other observations the normal percentage increase in the volume of the corpuscles on substitution of N/10 sodium chloride for the plasma = 12.28 per cent.	
11.2.08	0.135	0.98	45.06	50.56	12.26		
12.2.08	0.130	1.04	47.80	53.90	12.76		
AFTER DRUGS							
Date	Drugs	Percentage salt content equivalent	Percentage haemosozic value of serum	Percentage volume of corpuscles	Percentage volume of corpuscles in 0.1 N sodium chloride	Percentage increase due to 0.1 N sodium chloride	Percentage decrease due to the increased salinity of serum from drugs
13.2.08	Cal. Chlor. grains iv Sod. Chlor. grains xxii	0.1016	1.17	46.34	52.44	13.16	0.88 24 hours after first dose.
14.2.08	"	0.0974	1.168	41.96	49.14	17.11	4.83 24 hours after second dose.
15.2.08	"	0.0974	1.18	41.92	49.94	19.84	7.56 24 hours after third dose.
16.2.08	"	0.087	1.25	41.18	50.00	21.40	9.12 24 hours after fourth dose.
17.2.08	"	0.0873	1.09	39.63	48.50	22.13	9.85 24 hours after 5th dose.
18.2.08	Nil	0.085	1.24	35.82	42.87	19.68	7.40 48 hours after 5th dose.
19.2.08	Nil	—	—	—	—	—	—
20.2.08	Nil	0.1016	1.176	41.44	47.36	14.28	2.00 96 hours after fifth dose.

Working by the same method, after the administration of acid sulphates and sulphuric acid we got distinct evidence that these drugs have an effect directly opposed to that of chlorides on the volume and resisting power of the erythrocytes. Owing, however, to the pressure of other duties, these observations had to be stopped before they had reached completion. Table III gives some of the typical results obtained.

Comparison of the results of Table III with those of Tables I and II shows very distinct differences under the three headings:—

1. There is no decrease, but an increase, in the salt content equivalent of the blood, so that the erythrocytes are unable to withstand identical dilutions of the plasma with distilled water before and after the administration of sulphates.

2. There is no fall in the percentage volume of the corpuscles, but on the contrary a tendency to increase—a very marked difference from what was found to be the case with chlorides.

3. When N/10 sodium chloride is substituted for the plasma before and after the administration of sulphates, the increase in the volume of the corpuscles, instead of showing the normal average for healthy erythrocytes, is considerably less than the normal, and this again contrasts very markedly with the effects of chlorides. The following figure will make this clear:—

Suppose the circle X represents the volume of the normal corpuscle before any drugs are given, and Z represents it when the plasma is replaced by N/10 sodium chloride. Now we find that after sulphates have been given the increase in volume of the corpuscles is not nearly so great on substituting N/10 sodium chloride for the plasma as when no sulphates have been given; therefore the size of the corpuscle will lie between normal and its size in N/10 sodium solution, i.e. at Y.

TABLE III
THE ACTION OF SULPHATE ON THE ERYTHROCYTES

NORMAL

Date	Percentage salt content equivalent	Percentage haemosozic value of serum	Percentage volume of corpuscles	Percentage volume of corpuscles in 0.1 N sodium chloride	Normal percentage increase due to 0.1 N sodium chloride
20.1.08	0.130	0.92	42.50	46.25	8.82
21.1.08	0.135	0.912	36.04	40.10	11.26
21.1.08	0.135	0.85	40.77	44.07	11.00
25.1.08	0.116	1.16	34.57	38.30	10.79

This gives an average increase in
the volume of the corpuscles
when N/10 sodium chloride is
substituted for the plasma of
10.47 per cent.

AFTER DRUGS

Date	Drugs	Percentage salt content equivalent	Percentage haemosozic value of serum	Percentage volume of corpuscles	Percentage volume of corpuscles in 0.1 N sodium chloride	Percentage increase due to the decreased salinity of serum from drugs
21.1.08	Quin. sulph. grains ii Mag. sulph. grains. ii s.s. Ac. sulph. dil. min. ii s.s.	0.135	0.85	40.77	44.84	0.51
22.1.08		0.146	1.02	38.35	39.93	6.25
22.1.08		0.123	0.921	40.83	42.75	5.77
23.1.08		0.146	1.02	40.59	—	—
24.1.08		0.130	0.97	39.52	39.71	9.98
25.1.08		0.116	0.87	42.76	43.38	9.02

We are justified from these observations in concluding that the administration of sodium, calcium, chlorine, etc., has a marked influence in increasing the resisting power of the erythrocyte to haemolysis by dilution, while at the same time causing a very distinct decrease in its size; and that the administration of sulphates and mineral acids has the opposite effect, though not to such a marked extent.

As it appeared to be likely that in blackwater fever there is something present in the blood that tends to lower the resisting power of the erythrocytes, it was reasoned that in this disease sulphates and mineral acids should not be given, but that the administration of chlorides and inorganic basic salts is indicated, in the hope of causing thereby an increase of this resisting power. It was considered that even if this 'something' be a specific haemolysin,¹ the chlorides would have a good effect on cases which are, so to speak, on the brink, and likely to be precipitated into the abyss if their equilibrium be upset: if the cells are just able to withstand the amount of haemolysin present, on the ingestion of chlorides their power of resistance would be increased, whereas the ingestion of sulphates or mineral acids would cause them to succumb. Again, supposing that the patient were suffering from an attack of blackwater fever, the ingestion of sulphates or mineral acids would tend to turn the scale in favour of the haemolysin and against the resistant powers of the organism, and so would hasten the fatal issue. It was not suggested at the time that the specific action of the haemolysin would be interfered with by an increase in the amount of inorganic ions present in the blood.

After a year and a half the opportunity came of testing the effect of different strengths of solutions of saline and of calcium chloride on the action of specific haemolysins.

In the meantime the valuable research on blackwater fever by Christophers and Bentley appeared, in which these observers also favour the view of the presence of an haemolysin as the causative agent.

They made two most important observations, which are decidedly confirmatory of the views we have expressed, and which in turn our work on specific haemolysins recorded below has corroborated. These are:—

1. 'In comparing the drawings of blood films it became evident in several instances that in a series of films taken at short intervals within

1. That a specific haemolysin is the cause of blackwater fever is rendered very probable by the fact that in paroxysmal haemoglobinuria a specific haemolytic amboceptor has been demonstrated by various observers, including Meyer and Emmerich (*Deut. Archiv. f. klin. Med.*, June, 1909.)

the first twenty-four hours of the onset of blackwater fever, all the elements of the blood, both red cells and leucocytes, were on a larger scale in those taken later in the attack than in the earlier ones.' (7) This is confirmatory of our view that the resisting power of the corpuscle being decreased either by the action of a specific haemolysin, or by depleting the plasma of inorganic ions, an increase in the size of the corpuscle will take place.

2. They found that there was a marked raising of the isotonic point of the erythrocytes after the injection of haemolytic sera or snake-venom into animals (8). However, they say they have not found any marked raising of the isotonic point in blackwater fever, although the figures in the three cases seen early in the disease hardly bear out this contention (9).

They consider a specific haemolysin to be the most probable cause of blackwater fever, and we shall be able to produce strong evidence of the inhibitory influence of saline hypertonic solutions and of calcium salts on the action of haemolysins. This in turn will again confirm the view which we have expressed as to the superiority of the chloride salts of quinine over the sulphate, and of the value of therapy by which inorganic ions are introduced into the plasma in the prophylaxis and treatment of blackwater fever.

B. THE INHIBITORY EFFECT OF HYPERTONIC SALINE SOLUTIONS AND CALCIUM SALTS ON THE ACTION OF A SPECIFIC HAEMOLYSIN

That a specific haemolysin *sui generis* is present in blackwater fever may justifiably be assumed from the results of Myers and Emmerich's work on paroxysmal haemoglobinuria quoted above. As we had no case of blackwater fever before us, we have not been able to demonstrate the presence of this haemolysin. But we have been able to carry out a considerable amount of work with a haemolysin for the erythrocytes of the sheep, and we believe that the results obtained by us *in vitro* are valuable indications of what may be expected to occur *in vivo*.

At first we examined the question whether any modification of the action of a haemolytic system occurs when the saline solution used varies from that usually employed. For reasons which will at once occur to our readers we preferred a definite haemolytic system to a normal serum that had a haemolytic action on sheep's erythrocytes. We believe that we have established beyond doubt that the results of

the action of the haemolytic system on the erythrocytes vary inversely with the degree of salinity of the medium, and that the presence of excess of calcium salts has a most marked inhibitory effect on this action.

The inhibitory influence of calcium and hypertonic saline solutions on haemolysis we assume to be due to an increase of the resisting power of the erythrocytes, on the ground of the observations detailed above. We have not been able to obtain evidence that the haemolysin itself is affected; but even assuming that it is because the haemolysin is affected that its specific action is inhibited, the *therapeutic indication* to be drawn from our observations remains the same.

In the following experiments we employed haemolytic antisera obtained from rabbits, fowls, and a goose that had been immunized by means of injections of washed sheep's erythrocytes. The erythrocytes were washed three times for the injections, and for making the indicator, as we found that this amount of washing was enough to deprive them of all serum. Standardization of the power of the haemolytic antiserum was carried out in the usual way, the antiserum being heated to 56° C. for half an hour to render it inactive, and then reactivated at the time of experiment by the addition of fresh guinea-pig serum. We have found that 0·08-0·1 c.c. of fresh guinea-pig serum is enough, and that this will not of itself cause any haemolysis of the indicator, which was in every case a 5 per cent. suspension of thrice-washed sheep's erythrocytes, the medium in which these were suspended being indicated in the case of each experiment. For standardization, of course, a 0·85 per cent. sodium chloride solution was used.

Experiment I.—Twice the minimum haemolytic dose of amboceptor A (anti-sheep erythrocyte, rabbit's serum), whose minimal haemolytic dose was 0·0035 c.c., was used in each tube, as complement 0·08 c.c. of fresh guinea-pig's serum, and the total volume made up to 1 c.c. with the various concentrations of sodium chloride salines shown in the table as follows:—

Indicator—1 c.c. 5 per cent. suspension in 0·85 per cent. sodium chloride.

1. N/2	No haemolysis
2. N/3	Haemolysis slightly inhibited
3. N/4	Haemolysis complete
4. N/5	„
5. N/6	„
6. 0·85 per cent.	„
7. N/8	„
8. N/9	„

Experiment II.—Amboceptor 0.0035 c.c. = 0.7 c.c. of 1 : 200 dilution. Other conditions as in Experiment I.

1.	N/3	No haemolysis
2.	N/4	Haemolysis slightly inhibited
3.	N/5	Haemolysis complete
4.	N/6	"
5.	0.85 per cent.	"
6.	N/8	"
7.	N/9	"
8.	N/10	"

Experiment III.—Haemolytic amboceptor B minimal haemolytic dose = 0.0075 c.c. = 0.75 c.c. of 1 : 100 dilution. Complement : 0.1 c.c. guinea-pig's serum in each tube.

Indicator 5 per cent. suspension washed sheep's erythrocytes in the various strengths of salines used. The dilution of amboceptor and the making up of the quantities of this to 1 c.c. were carried out with 0.85 per cent. sodium chloride.

Amboceptor c.c.	N/4	N/5	N/6	0.85 per cent.	N/10
0.01	Inhibition fair	Trace of inhibition	Haemolysis complete	Haemolysis complete	Haemolysis complete
0.0075	Inhibition greater	Inhibition greater	Some inhibition	Haemolysis complete	Haemolysis complete
0.005	Still greater inhibition	Inhibition greater	Greater inhibition	Some inhibition	Nearly complete haemolysis
0.0025	Scarcely any haemolysis	Inhibition still greater	Greater inhibition	More inhibition	Some inhibition
0.0015	Trace of haemolysis	Inhibition still greater	Slight haemolysis	More inhibition	More inhibition
0	No haemolysis	No haemolysis	No haemolysis	No haemolysis	Slight haemolysis

Thus we have slight haemolysis caused by the complement alone in the last tube of the N/10 series, in which series all the tubes show more haemolysis than the corresponding tubes of other series. We note too, that N/6 (0.98 per cent. sodium chloride) inhibits the action of 0.0075 c.c. amboceptor, which quantity caused complete haemolysis in the 0.85 per cent. and N/10 series.

Experiment IV.—Amboceptor C minimal haemolytic dose = 0.01 c.c. = 1 c.c. of 1 : 100 dilution. Complement : guinea-pig's serum 0.1 c.c.

Indicator 5 per cent. suspension of washed sheep's erythrocytes in various strengths of sodium chloride solution shown.

The dilutions of amboceptor and the making up of the quantities of this to 1 c.c. in each tube were carried out with the various strengths of sodium chloride solution noted at the head of each column.

Amboceptor c.c.	N/4	N/5	N/6	0.85 per cent.	N/10
0.01	No haemolysis	About three-quarters haemolysed	Nearly complete haemolysis	Complete haemolysis	Complete haemolysis
0.0075	No haemolysis	About half haemolysed	About three-quarters haemolysed	Nearly complete haemolysis	Complete haemolysis
0.005	No haemolysis	Trace of haemolysis	About half haemolysed	About three-quarters haemolysed	Complete haemolysis
0.0025	No haemolysis	No haemolysis	Some haemolysis	Some haemolysis	About three-quarters haemolysed
0.0015	No haemolysis	No haemolysis	Some haemolysis	Trace of haemolysis	About half haemolysed
0	No haemolysis	No haemolysis	No haemolysis	No haemolysis	Some haemolysis

From this it is evident that any solution of sodium chloride that contains more than 0·85 per cent. of this salt is able to inhibit haemolysis in proportion to the percentage of salt that it contains, while any solution that contains less than 0·85 per cent. permits haemolysis to take place with ease proportionate to the lessening of its salt content.

The haemolytic action of a specific haemolysin varies then inversely with the strength of the saline solution in which the corpuscles of the indicator float. Thus the corpuscles' resisting power is so increased by strong saline that a specific antiserum which can cause complete haemolysis of corpuscles in 0·85 per cent. has little or no effect when the corpuscles are suspended in a 1·46 per cent. solution, and only half its effect when they are suspended in a 1·17 per cent. solution.

Experiment V.—In this experiment we endeavoured to find out what minimum amount of calcium chloride would inhibit the action of the specific haemolytic amboceptor.

Amboceptor C minimal haemolytic dose 0·01 c.c. = 0·1 c.c. of 1 : 10 dilution in each tube. Complement 0·1 c.c. of fresh guinea-pig's serum.

Indicator 1 c.c. of 5 per cent. suspension of washed sheep's erythrocytes in 0·85 per cent. sodium chloride solution.

Dilutions of calcium chloride, of which the required quantities were taken, and where necessary made up to 1 c.c. with 0·85 per cent. sodium chloride.

Tube	Strength of calcium solution used	Amount of calcium solution added c.c.	Result	Contents of tube 2·2 c.c. Amount of calcium chloride present
1	1 : 100 in 0·85 per cent. sodium chloride	1·0	Very little haemolysis	0·01 grm.
2	[Quantities made up to 1 c.c. with 0·85 per cent.]	0·75	A little more haemolysis	0·0075
3	—	0·5	Still more haemolysis	0·005
4	—	0·25	Still more haemolysis	0·0025
5	—	0·15	About half haemolysed	0·0015
6	1 : 1000	1·0	More haemolysed	0·001
7	—	0·75	Still more haemolysis	0·00075
8	—	0·5	Still more haemolysis	0·0005
9	—	0·25	Still more haemolysis	0·00025
10	—	0·15	About three-quarters haemolysed	0·00015
11	—	0	Complete haemolysis	0
12	No amboceptor in this tube	0	Practically no haemolysis	0

From this the inhibitory power of calcium chloride *per se* is evident, for even so small a quantity as 0·0015 gm. of this salt in 2·2 c.c. of tube contents lessened the haemolytic power of the amboceptor by half, and the tenth of this quantity had a distinct inhibitory effect.

Experiment VI.—Haemolytic amboceptor of which minimal haemolytic dose as yet undetermined. A 1 : 100 dilution of this with various solutions was used in varying quantities, and these were made up to 1 c.c. with the various solutions, where necessary.

In each tube 0.0015 grm. of calcium chloride, save where indicated.

Complement = 0.1 c.c. fresh guinea pig's serum.

Indicator 1 c.c. of 5 per cent. suspension of washed erythrocytes in the various solutions.

Amboceptor dilution 1 : 100	N/5	N/6	0.85 per cent.	N/10
1.0 c.c.	Complete haemolysis	Complete haemolysis	Complete haemolysis	Complete haemolysis
0.75 "	Two-thirds com- plete haemolysis	Complete haemolysis	Complete haemolysis	Complete haemolysis
0.5 "	Half complete haemolysis	Three-quarters complete haemolysis	Complete haemolysis	Complete haemolysis
0.25 "	Quarter complete haemolysis	Half complete haemolysis	Complete haemolysis	Complete haemolysis
No calcium chloride and no amboceptor	No haemolysis	No haemolysis	No haemolysis	Trace of haemolysis

On standardising the amboceptor used in this case, it was found that its minimal haemolytic dose was 0.001 c.c. = 0.1 c.c. of 1 : 100 dilution. Notwithstanding the fact that much more than this had been used in the experiment, the inhibitory power of hypertonic salt solution containing calcium chloride is very evident, and is confirmed by the following experiment :—

Experiment VII.—Haemolytic amboceptor D minimal haemolytic dose = 0.0004 c.c. = 0.4 c.c. of 1 : 1000 dilution with various solutions.

Complement : 0.1 c.c. guinea-pig's serum.

In each tube, save where noted, was 0.0015 grm. of calcium chloride.

Indicator 5 per cent. suspension of erythrocytes in the various strengths of saline solution.

Quantities of amboceptor made up where necessary to 1 c.c. by addition of the required solution.

Amboceptor 1 : 1000 dilution	Calcium chloride 0.0015 grm. in each tube				No calcium chloride 0.85 per cent.
	N/5	N/6	N/10	0.85 per cent.	0.85 per cent.
0.8 c.c.	Half complete haemolysis	Complete haemolysis	Complete haemolysis	Complete haemolysis	Complete haemolysis
0.6 "	Half complete haemolysis	Half complete haemolysis	Complete haemolysis	Three-quarters complete haemolysis	Complete haemolysis
0.4 "	Quarter complete haemolysis	Half complete haemolysis	Complete haemolysis	Half complete haemolysis	Complete haemolysis
0.2 "	Practically no haemolysis	Trace of haemolysis	Nearly complete haemolysis	Trace of haemolysis	Three-quarters complete haemolysis
0.1 "	No haemolysis	No haemolysis	Less complete haemolysis	Slight trace of haemolysis	Half complete haemolysis
0	No haemolysis	No haemolysis	Trace of haemolysis	No haemolysis	No haemolysis

The following experiment gives a further control for the above results.

Experiment VIII.—Amboceptor not standardised diluted 1:1000 with various solutions.

Complement: 0.1 c.c. guinea-pig's serum.

Control 1 c.c. of 5 per cent. suspension of erythrocytes in the various solutions.

Quantities made up to 1 c.c. where necessary with various dilutions.

Series A. Calcium chloride 0.0015 grm. in each tube. Series B. No calcium chloride.

Amboceptor 1:1000 dilution		N/5	N/6	N/10	0.85 per cent.
A	0.8 c.c.	Complete haemolysis	Complete haemolysis	Complete haemolysis	Complete haemolysis
	0.6 „	No haemolysis	Complete haemolysis	Complete haemolysis	Complete haemolysis
	0.4 „	No haemolysis	Partial haemolysis	Complete haemolysis	Partial haemolysis
	0.2 „	No haemolysis	No haemolysis	Partial haemolysis	No haemolysis
	0.1 „	No haemolysis	No haemolysis	No haemolysis	No haemolysis
	0 „	No haemolysis	No haemolysis	No haemolysis	No haemolysis
B	0.8 „	Complete haemolysis	Complete haemolysis	Complete haemolysis	Complete haemolysis
	0.6 „	Complete haemolysis	Complete haemolysis	Complete haemolysis	Complete haemolysis
	0.4 „	Partial haemolysis	Complete haemolysis	Complete haemolysis	Complete haemolysis
	0.2 „	No haemolysis	Partial haemolysis	Complete haemolysis	Complete haemolysis
	0.1 „	No haemolysis	No haemolysis	Complete haemolysis	Complete haemolysis
	0 „	No haemolysis	No haemolysis	No haemolysis	No haemolysis

Amboceptor standardised and minimal haemolytic dose found to be 0.00008 c.c. = 0.08 c.c. of 1:1000.

Having thus established the inhibitory effect of hypertonic saline solutions and calcium on the action of the haemolytic system, we attempted to discover whether this inhibition was due to any modification of the haemolytic system. To this end we carried out the following experiment.

In series A the tubes contained amboceptor which had been acted upon by the N/5 solution for an hour; in series B the corpuscle-suspension used as indicator had been prepared with N/5 solution an hour before the experiment was carried out; and in series C the guinea-pig serum had been for an hour subjected to the action of N/5 solution, being otherwise under the same conditions as that used for the other series.

The control is furnished by the tubes of series D, in which the haemolytic amboceptor's standardization was, so to speak, confirmed. This amboceptor had been obtained from a goose, and was, as is evident, of but slight potency.

We may note that so far we have had only small success in the immunization of geese, whereas fowls have yielded excellent antisera of very high potency, when immunised with cell-free serum or washed erythrocytes.

Experiment IX.—

	A	B	C	D
Amboceptor	N/5	N/5	N/5	0.85 per cent.
0.8 c.c.	No haemolysis	No haemolysis	No haemolysis	Complete haemolysis
0.6 „	No haemolysis	No haemolysis	No haemolysis	Half complete haemolysis
0.4 „	No haemolysis	No haemolysis	No haemolysis	Quarter complete haemolysis
0.2 „	No haemolysis	No haemolysis	No haemolysis	Trace of haemolysis
0.1 „	No haemolysis	No haemolysis	No haemolysis	No haemolysis
0.1 c.c. guinea-pig's serum as complement.				

1 c.c. 5 per cent. suspension of erythrocytes in N/5 for A, B, and C, and in 0.85 per cent. sodium chloride for D, as indicator. Control tube for complement. No amboceptor. No haemolysis.

Obviously no conclusion can be drawn from this experiment, save that it confirms the results of the others as to the inhibitory power of N/5. Nor did repetition of the experiment, carried out by us with the very greatest care, shed any further light on the subject, although the progress of haemolysis in the tubes was noted every fifteen minutes.

SUGGESTIONS AS TO THE THERAPY OF BLACKWATER FEVER

On the basis of the results obtained from the administration of sodium and calcium chlorides in increasing the resisting power of the erythrocyte and of diminishing its size, also from the proved influence of hypertonic saline solutions and of calcium in inhibiting the action of specific haemolysins, the natural deductions are obvious.

Christophers and Bentley have shown that in blackwater fever the corpuscles, both red and white, are larger than normal and, therefore, according to our view, more vulnerable.

They have also proved that the action of specific haemolysins is

accompanied by a raising of the isotonic points,¹ i.e., the red blood corpuscles instead of being able to resist dilutions of the plasma down to what corresponds to 0·47 per cent. saline, as they can normally, are dissolved in solutions of 0·6, 0·7, 0·8, and even in 0·9 per cent. in some cases. Their negative finding of any raising of the isotonic point in the cases of blackwater fever investigated may be explained by the presence of an anti-haemolysin, or by the difficulty of knowing when haemolysis due to the saline begins to take place in a condition where haemoglobinaemia is already present. But we cannot accept their results, shown on page 130, Scientific Memoirs, No. 35, as proving their contention that there is no raising of the isotonic point. In every case they examined on the day of onset there was haemolysis with N/12 sodium chloride, and even with lower dilutions—the average for normal individuals being N/14 or N/15 sodium chloride. This fact is well brought out in the records of the several cases [XX, XXVI and XXVII] where comparisons with controls were carried out. In every case of haemoglobinuria the blood was more easily broken down than the normal blood by dilutions of saline. We think that further work will bring the condition of the isotonic point in blackwater fever into line with the acknowledged rise that takes places in other conditions which are brought about by the action of a specific haemolysin. It is not stated whether death took place in any of these recorded cases; of course it is to be expected that in recovery there would be no rise of the isotonic point but that, on the contrary, this would fall. Further, in most of the cases in which they determined the isotonic point the case was 'mild,' or the observations were made some days after the onset of the disease—it may well be that the condition of affairs in these circumstances is by no means that which obtains just before, or at, the onset of the attack. Besides it is very possible that the presence of anti-haemolysins in all the cases they had under observation, might go far to obscure the results.

However, leaving all this aside, the facts that the corpuscles are enlarged, and that the isotonic point is disturbed in blackwater fever, afford strong evidence that the condition of the erythrocytes is one of increased vulnerability. To whatever this may be due, we have shown that it is possible by increasing the inorganic ions in the plasma to prevent

1. We do not agree with the meaning that these observers seem to attach to the term 'isotonic point.' The haemolytic point would appear to be the correct term, or, as might be said in German, the 'Haemolyse-Anfangspunkt.'

haemolysis in conditions where otherwise haemolysis would have taken place, and this even in the case of a specific haemolysin.

The suggestions we make with regard to the treatment of blackwater fever are:—

That any drug having a tendency to decrease the number of inorganic ions present in the plasma be eschewed. Such drugs as sulphates, acid salts, mineral acids, and even magnesium sulphate—unless given in doses sufficient to cause purgation and as a consequence to prevent its absorption—would come into this category.

That the administration of drugs that tend to increase the number of inorganic ions present in the plasma is indicated.

Such drugs are sodium chloride or carbonate, neutral salts of vegetable acids, calcium chloride, lactate or carbonate, or, if necessary, quinine bihydrochloride, hydrobromide or tannate.

In all cases where blood destruction is at all marked and where the patient is seriously ill, medication by the mouth will probably be of little avail. In such cases we are firmly of the opinion that *intravenous injection* of hypertonic saline to which a little calcium has been added, or of Ringer's fluid to which extra sodium chloride has been added, will be of the greatest service. In combination with the intravenous injection, medication by the mouth may be tried if vomiting is absent, but it is very doubtful indeed if any adequate absorption from the intestinal tract can take place in patients suffering severely from a condition like blackwater fever; and we know, from Dixon's (10) work, that it is only when it is subcutaneously administered that calcium chloride can be expected to cause a rise of the calcium content of blood, for when given by the mouth the salt is eliminated *pari passu* with its absorption.

The solution we recommend is this:—

Sodium chloride	1·2 grammes.
Calcium chloride	0·025 grammes.
Aquae destill	100 c.c.

For ordinary work, where it would be impossible to have these quantities accurately determined in time, two tea-spoonsful of common salt in a pint of boiled water can be made use of; if available three grains of calcium chloride should be added, or injected hypodermically, in saline solution.

The amount to be given should not be less than one pint, and may

be determined by the condition of the patient. Repeated injections of small amounts would probably be found to give the best results.

We have shown that the smallest trace of calcium has a marked effect, and that the raising of the salinity of the blood plasma to 1 per cent. will absolutely inhibit haemolysis due to specific haemolysins, even when these are present in sufficient amount to cause complete haemolysis of a known suspension of corpuscles.

Now in blackwater fever there is never, even in the most virulent case, a sufficient quantity of the haemolysin present to cause complete haemolysis of all the erythrocytes in the body; so that our task of preventing haemolysis should be much easier than in the experiments above detailed. It would not therefore be necessary to raise the salinity of the plasma to anything like 1 per cent. in order to inhibit the action of the active agent in blackwater fever. For this reason we recommend that treatment should be carried out by beginning with one pint of 1·2 per cent. saline intravenously, the physician being guided by the condition of the patient as to the necessity for repeating the injection.

Corroborative evidence of the accuracy of these views is afforded by the success of the Hearsey method of treatment, which is the only method that can really claim comparative success.

This consists in giving sodium bicarbonate and perchloride of mercury, and is, after all, only another method of increasing the inorganic salts of the plasma—the end aimed at in our treatment. It is remarkable and well worthy of note that Dr. Hearsey forbids all *acid* drinks and lays stress on the *antacid* action of his combination. This method would fail, of course, in the very conditions in which treatment is most necessary, viz., in those cases which are so severe that absorption from the alimentary canal does not take place, and in those in which vomiting is present and persistent.

Acting on the views herein detailed Dr. R. L. Patterson, of Assam, reports several successful results with calcium chloride, and one very suggestive case treated with intravenous injection of hypertonic saline solution.¹ He writes that the patient was dying, 'I at once opened a vein and transferred several pints of hypertonic saline. There was a temporary rally, and the urine drawn off with a catheter a few hours later was clear, but the patient died the same night. I hope to catch my next case early, and transfuse as once.'

1. Dr. Patterson is the first, to our knowledge, who has treated cases of blackwater fever in this way.

LITERATURE

1. *The Lancet*, June, 1907.
2. *The Indian Medical Gazette*, 1907, 1908.
3. *The Bio-Chemical Journal*, Vol. III, No. 3, 1908. See also Wright and Kilner, *Lancet*, 1904. Wright and Ross, *Lancet*, 1905. *Scientific Memoirs, Government of India*, No. 35.
4. T. R. Boggs, M.D., 'Variations in the calcium content of the blood following therapeutic measures,' *Johns Hopkins Hospital Bull.*, Vol. XIX, No. 208, July, 1908.
5. *Ueber den Einfluss der Mineralwässer auf das Blut : Zschrft. f. diät. u. phys. Therapie*, 7 Bd., 255, 1904.
6. *American Journal of Physiology*, September, 1909.
7. *Scientific Memoirs*, No. 35, p. 76.
8. *Loc. cit.*, p. 117.
9. *Loc. cit.*, p. 130.
10. *Brit. Medical Journal*, August 28th, 1909.