OBSERVATIONS ON THE HAEMOLYTIC ACTION OF CERTAIN BILE DERIVATIVES

By HUGH MACLEAN, M.D., *Carnegie Research Fellow, University of Aberdeen*, AND LANCELOT HUTCHINSON, M.D. (*Liverpool*).

From the Bio-chemical Laboratory, University of Liverpool

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Our knowledge of the processes involved in the laking of red blood corpuscles is at present in an obscure and unsatisfactory condition, and despite the amount of research carried out on haemolysis and the number of theories advanced to account for different results, many cases still remain unexplained.

In connection with this subject, one very interesting point has lately been advanced by St. Faust and Tallqvist, and afterwards by Moore; these observers found that haemolytic action, when brought about by such bodies as the fatty acids, occurred only in cases where the acid employed was one of an unsaturated variety containing in its molecule one or more double bonds, while the corresponding saturated bodies produced no effect. In view of this observation, it is likely that the state of combination of the carbon atom constitutes an important factor in many cases of haemolysis produced by chemical agents.

The strong probability, on chemical grounds, of certain bile products being constituted by bodies of an unsaturated nature suggested the present investigation; the curious results obtained do not seem capable of explanation by any of the theories put forward at present.

SUBSTANCES USED

The present paper deals with the results of haemolysis by three bile derivatives—the sodium salt of glycocholic acid, cholalic acid and choleic acid. These were prepared as follows:—

SODIUM GLYCOCHOLATE

Fresh ox bile was freed from pseudo-mucin by treatment with alcohol; after the evaporation of the alcohol, neutral acetate of lead was added, the resulting precipitate separated off and decomposed by heat in

the presence of a sodium carbonate solution. The mixture was evaporated to dryness and the residue extracted with alcohol; the alcoholic extract was filtered, the filtrate evaporated to dryness and the residue dissolved in water. This watery solution of sodium glycocholate was now decolorised by animal charcoal, and the free glycocholic acid thrown out by means of a dilute solution of hydrochloric acid. The acid was thoroughly washed with water, then dried and again dissolved in alcohol. To this alcoholic solution sodium carbonate was added, and the whole again evaporated to dryness. Residue was dissolved in a little cold alcohol, and the mixture filtered. This process of purification by dissolving in alcohol was repeated; the alcoholic solution was then evaporated to dryness and the residue dissolved in a little water, filtered, evaporated to dryness over the water bath, and ultimately dissolved in normal saline solution, and in this solution utilised for the experiments.

CHOLALIC ACID

Ox bile was boiled for thirty hours with one-fifth of its volume of 30 per cent. caustic soda, the total volume of the mixture being kept constant by the addition of water from time to time. The solution was then saturated with carbon dioxide and evaporated almost to dryness. Residue was extracted with 96 per cent. alcohol, and the extract diluted with water so as to contain not more than about 20 per cent. of alcohol; it was then treated with a solution of barium chloride, the precipitate filtered off, the filtrate treated with weak hydrochloric acid, and the precipitated cholalic acid separated off and thoroughly washed with water. This acid was now changed into the sodium salt, and the above process of treatment with barium chloride, etc., repeated twice. The free acid ultimately obtained was dissolved in alcohol, and by the addition of sodium carbonate again changed into the sodium salt; the alcoholic solution was filtered, evaporated to dryness, the residue dissolved in a little cold alcohol and filtered; filtrate was evaporated to dryness, residue dissolved in water, filtered, again evaporated to dryness and final residue utilised for experiments.

CHOLEIC ACID

Choleic acid was prepared from the precipitate obtained by barium chloride in the preparation of cholalic acid: this precipitate, which consisted of an impure salt of barium choleate, was purified on the lines described above; it was also utilised in the form of the sodium salt.

EXPERIMENTS

The first set of experiments was carried out with the sodium salt of cholalic acid. In all cases red blood corpuscles from the sheep were used; these were carefully washed four times in the ordinary way with normal salt solution, and then made into a 5 per cent. emulsion with isotonic saline.

For convenience in preparing the somewhat varied strengths of cholalic acid utilised in the experiments, two solutions were made—one a deci-molecular and the other a centi-molecular; in each case the molecular weight of the salt was taken as corresponding to the formula $C_{24}H_{39}O_5Na$, all solutions being made in normal saline.

For each experiment a series of tubes, each containing 1 c.c. of the above emulsion of sheep's red blood corpuscles, was taken, and to this was added a measured amount of sodium cholalate. By the addition of the necessary amount of normal saline, the final volume of each tube was maintained constant, and in all our experiments amounted to 5 c.c. The tubes were then placed in the incubator at $37^{\circ}C.$, and observations constantly taken at short intervals.

As the result of repeated experiments, it was found that this salt displayed well-marked haemolytic properties, but at the same time exhibited some peculiarities so striking as to amount to what may be suggestively termed a haemolytic paradox.

In tubes containing a moderately strong dose of sodium cholalate, the corpuscles were completely laked in intervals of from one to two hours; when the substance was present in somewhat smaller quantities, haemolysis was delayed for three or four hours; on still further reduction of the amount of substance, however, the peculiar fact was observed that haemolysis took place in a shorter and shorter space of time, until a point was reached at which a minimum dose gave a maximum effect. With such an amount this *minimal-optimum* dose often caused complete laking of the corpuscles in six minutes, or even less; while on the other hand, a dose several times as great might take hours to produce a similar effect.

When exhibited in amounts smaller than this minimal-optimum dose, the effect became gradually less, until ultimately a strength was used beyond which no further haemolysis could be obtained.

The accompanying chart gives a good idea of the results obtained; in a long series of experiments the laking always took place as indicated.

CHART No. I

SODIUM CHOLALATE

Time during which sodium cholalate had been acting	Minutes											
	5	13	20	30	40	53	65	80	115	155	320	
1 c.c. sheep's r. b. c. + + 4 c.c. $\frac{M}{5}$ sodium cholalate	-	+	+	+	+	+	+	+	+	+	+	+
„ „ + 1 c.c. saline..... + 3 „ „	-	-	-	+	+	+	+	+	+	+	+	+
„ „ + 1 „ „ + 3 c.c. $\frac{M}{10}$ sodium cholalate	-	-	-	-	-	-	-	+	+	+	+	+
„ „ + 2 „ „ + 2 „ „	-	-	-	-	-	-	-	-	+	+	+	+
„ „ + 3 „ „ + 1 „ „	-	-	-	-	-	-	-	-	-	+	+	+
„ „ + 3·1 „ „ + 0·9 „ „	-	-	-	-	-	-	-	-	-	+	+	+
„ „ + 3·2 „ „ + 0·8 „ „	-	-	-	-	-	-	-	-	-	-	+	+
„ „ + 3·3 „ „ + 0·7 „ „	-	-	-	-	-	-	-	-	-	+	+	+
„ „ + 3·4 „ „ + 0·6 „ „	-	-	-	-	-	-	-	-	+	+	+	+
„ „ + 3·5 „ „ + 0·5 „ „	-	-	-	-	-	-	-	-	+	+	+	+
„ „ + 3·6 „ „ + 0·4 „ „	-	-	-	-	-	-	+	+	+	+	+	+
„ „ + 3·7 „ „ + 0·3 „ „	-	-	-	-	-	+	+	+	+	+	+	+
„ „ + 2 „ „ + 0·2 „ „	-	-	+	+	+	+	+	+	+	+	+	+
„ „ + 3 „ „ + 0·1 „ „	+	+	+	+	+	+	+	+	+	+	+	+
„ „ + 3·5 „ „ + 0·05 „ „	-	-	-	+	+	+	+	+	+	+	+	+
„ „ + 3·6 „ „ + 0·04 „ „	-	-	-	-	-	-	+	+	+	+	+	+
„ „ + 3·7 „ „ + 0·03 „ „	-	-	-	-	-	-	-	+	+	+	+	+
„ „ + 3·8 „ „ + 0·02 „ „	-	-	-	-	-	-	-	-	-	-	-	-
„ „ + 3·9 „ „ + 0·01 „ „	-	-	-	-	-	-	-	-	-	-	-	-
„ „ + 4 „ „ (control)	-	-	-	-	-	-	-	-	-	-	-	-

NOTE.—The emulsion of red corpuscles was rapidly added last of all, the difference in time between the first and last tube being barely 4 minutes. Immediately after the corpuscles were added, the tubes were placed in a thermostat [37° C.] where they were observed.

The times were calculated from the moment when the tubes were placed in the thermostat.

In measuring amounts of sodium cholalate less than 0·2 c.c., an $\frac{M}{100}$ solution was used.

◆ Denotes point at which laking became complete.

The results are also shown graphically plotted in fig. 1, in which the ordinates represent time of complete haemolysis in minutes, and the abscissae, the concentrations of the sodium cholalate, in c.c., as indicated, of 0·1 M solution added to a total volume of 5 c.c. when completely made up.

FIG. 1. SODIUM CHOLALATE

Each test tube contained 1 c.c. sheep's r. b. c. (5% emulsion) + amounts, as indicated above, in cubic centimetres of $\frac{M}{10}$ sodium cholalate + sufficient normal saline to maintain a constant volume of 5 c.c.

The following reproduction also shows these results; the tubes were left to stand for one hour and then centrifuged; by this means a more suitable condition for photographic purposes was obtained. Haemolysis is distinctly seen in tubes 1, 6, 7 and 8, the remainder being quite free from laking, with the exception of Nos. 2 and 9, where a slight action has taken place.

From these results, it is obvious that the haemolytic power of the so-called minimal-optimum dose is much more marked than that obtained when much larger amounts are used, and that between these two points there lies a sort of neutral ground where the haemolytic power is very much reduced. In the experiment recorded in the chart it is seen that

1 2 3 4 5 6 7 8 9 10

PHOTOGRAPH SHOWING HAEMOLYTIC PARADOX

Tubes with black appearance are those in which laking has occurred.

The following table indicates the amount of sodium cholalate in each tube in the photograph:—

No. 1.	3 c.c.	$\frac{M}{5}$	Sodium	cholalate	No. 6.	0.3 c.c.	$\frac{M}{10}$	Sodium	cholalate
„ 2.	„	$\frac{M}{10}$	„	„	„ 7.	0.2 c.c.	„	„	„
„ 3.	1 c.c.	„	„	„	„ 8.	0.5 c.c.	$\frac{M}{100}$	„	„
„ 4.	0.8 c.c.	„	„	„	„ 9.	0.3 c.c.	„	„	„
„ 5.	0.5 c.c.	„	„	„	„ 10.	0.2 c.c.	„	„	„

0.1 c.c. of 0.1 M. sodium cholalate lakes completely in five minutes, whereas it takes thirteen minutes for 4 c.c. of 0.2 M solution to produce the same effect; in other words, a certain minimum dose gives in *five* minutes an effect equivalent to that produced by a dose about *eighty* times as great in *thirteen* minutes.

In another series of experiments performed under the same conditions, but using the sodium salt of choleic acid, similar results were obtained.

When a strength of 4 c.c. 0.1 M sodium choleate was used, haemolysis was complete in about *six* minutes; in this strength laking was quickly followed by an action on the liberated haemoglobin, as indicated by a dark brown coloration of the liquid. With 0.8 c.c. 0.1 M solution the full effect was in evidence in about *eleven* minutes; when, however, only 1 c.c. 0.01 M was used, haemolysis was almost instantaneous, the corpuscles being completely laked within *one* minute.

This salt is a much more powerful agent than the corresponding compound with cholalic acid. Whereas with sodium cholalate a solution of 0.3 c.c. 0.01 M required about eighty minutes for complete laking, and a strength corresponding to 0.2 c.c. 0.01 M gave only the merest indication of a positive reaction after twenty hours' observation, with sodium choleate in strength of 0.7 c.c. 0.001 M complete haemolysis was obtained in one hundred and sixty minutes, and with 0.6 c.c. 0.001 M in twenty hours: even with 0.5 c.c. 0.001 M, traces of haemolysis were evident after this time.

The full results of one experiment are indicated in Chart 2.

Similar experiments, giving in the main the same kind of result, were performed with sodium glycocholate. Here, however, there was not the same marked difference between the haemolytic action of large and small amounts as was seen in the case of the above described salts; this is due to the fact that the action of the minimal-optimum dose is in this case not nearly so rapid. In other respects, as indicated by the chart, the same general result is in evidence. The results are shown also in fig. 3.

On the other hand, haemolysis was ultimately obtained with smaller amounts than in the case of sodium cholalate, though, even in this respect, it did not appear to be so powerful as the choleic salt.

A few observations were also made with free cholalic acid; considerable difficulty was experienced in procuring a suitable method of application owing to its lack of solubility in inert substances. We succeeded, however, in emulsifying a small amount with normal saline, and with this emulsion similar results to those obtained with the sodium compound were noticed; that is to say, with a certain small amount of emulsion a much more marked haemolysis was produced in a given period than was in evidence after the exhibition of perhaps nine or ten times as much in a much longer space of time.

CHART No. II

SODIUM CHOLEATE

Minutes

Time during which sodium choleate had been acting	1	2	3	6	7	8	9	11	20	31	50	105	140	160	240	1200
1 c.c. sheep's r. b. c. + + 4 c.c. $\frac{M}{10}$ sodium choleate	-	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+
„ „ + 1 c.c. saline + 3 „ „	-	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+
„ „ + 2 „ „ + 2 „ „	-	-	-	-	+	+	+	+	+	+	+	+	+	+	+	+
„ „ + 3 „ „ + 1 „ „	-	-	-	-	-	-	+	+	+	+	+	+	+	+	+	+
„ „ + 3.1 „ „ + 0.9 „ „	-	-	-	-	-	-	+	+	+	+	+	+	+	+	+	+
„ „ + 3.2 „ „ + 0.8 „ „	-	-	-	-	-	-	+	+	+	+	+	+	+	+	+	+
„ „ + 3.3 „ „ + 0.7 „ „	-	-	-	-	-	-	+	+	+	+	+	+	+	+	+	+
„ „ + 3.4 „ „ + 0.6 „ „	-	-	-	-	-	-	+	+	+	+	+	+	+	+	+	+
„ „ + 3.5 „ „ + 0.5 „ „	-	-	-	-	-	+	+	+	+	+	+	+	+	+	+	+
„ „ + 3.6 „ „ + 0.4 „ „	-	-	-	-	-	+	+	+	+	+	+	+	+	+	+	+
„ „ + 3.7 „ „ + 0.3 „ „	-	-	-	-	-	+	+	+	+	+	+	+	+	+	+	+
„ „ + 3.8 „ „ + 0.2 „ „	-	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+
„ „ + 3.9 „ „ + 0.1 „ „	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
„ „ + 3.5 „ „ + 0.75 c.c. $\frac{M}{100}$ „	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
„ „ + 3.6 „ „ + 0.4 „ „	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
„ „ + 3.7 „ „ + 0.3 „ „	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+
„ „ + 3.8 „ „ + 0.2 „ „	-	-	-	-	-	-	+	+	+	+	+	+	+	+	+	+
„ „ + 3.0 „ „ + 1.0 c.c. $\frac{M}{1000}$ „	-	-	-	-	-	-	-	-	-	+	+	+	+	+	+	+
„ „ + 3.2 „ „ + 0.8 „ „	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+	+
„ „ + 3.3 „ „ + 0.7 „ „	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+
„ „ + 3.4 „ „ + 0.6 „ „	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+
„ „ + 3.5 „ „ + 0.5 „ „	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	○
„ „ + 3.6 „ „ + 0.4 „ „	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
„ „ + 3.7 „ „ + 0.3 „ „	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
„ „ + 4 „ „ (control)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

✦ Indicates complete haemolysis.

NOTE.—The same conditions existed during this experiment as in the preceding Chart except that the tubes were not placed in the thermostat for 8 minutes after adding the corpuscles. The corpuscles were added during 2½ minutes, and the times are calculated from the time when the last tube received its corpuscle.

○ Trace of haemolysis.

These results are shown graphically in Fig. 2, where ordinates show as in Fig. 1.

FIG. 2. SODIUM CHOLEATE

Each test tube contained 1 c.c. sheep's r. b. c. (5 % emulsion) + amounts in cubic centimetres, as indicated above, of $\frac{M}{10}$ sodium choleate + sufficient normal saline to maintain a constant volume of 5 c.c.

The curve shows well the paradoxical result that a small amount of the haemolyser is more efficient than a larger amount.

Fig. 3. SODIUM GLYCOCHOLATE

Each test-tube contained 1 c.c. sheep's r. b. c. (5% emulsion) + amounts in cubic centimetres, as indicated above, of $\frac{M}{10}$ -sodium glycocholate + sufficient normal saline to maintain a constant volume of 5 c.c.

CHART No. III

SODIUM GLYCOCHOLATE

Times in minutes		25	30	35	40	44	50	60	80	20 hours
1 c.c. sheep's r. b. c. + 1 c.c. saline + 3 c.c. $\frac{M}{10}$ sodium glycocholate		+	+	+	+	+	+	+	+	+
"	" + 3 " " + 1 " "	-	-	-	-	+	+	+	+	+
"	" + 3.25 " " + 0.75 " "	-	-	-	-	-	+	+	+	+
"	" + 3.5 " " + 0.5 " "	-	-	-	-	-	+	+	+	1st
"	" + 3.6 " " + 0.4 " "	-	-	-	-	-	+	+	+	2nd
"	" + 1 " " + 3.0 " $\frac{M}{100}$ "	-	-	-	-	+	+	+	+	+
"	" + 2 " " + 2.0 " "	-	-	-	+	+	+	+	+	+
"	" + 3 " " + 1.0 " "	-	-	+	+	+	+	+	+	+
"	" + 3.1 " " + 0.9 " "	-	-	+	+	+	+	+	+	+
"	" + 3.2 " " + 0.8 " "	-	-	+	+	+	+	+	+	+
"	" + 3.3 " " + 0.7 " "	-	+	+	+	+	+	+	+	+
"	" + 3.4 " " + 0.6 " "	-	-	+	+	+	+	+	+	+
"	" + 3.5 " " + 0.5 " "	-	-	-	+	+	+	+	+	+
"	" + 3.6 " " + 0.4 " "	-	-	-	-	+	+	+	+	+
"	" + 3.7 " " + 0.3 " "	-	-	-	-	-	+	+	+	+
"	" + 2 " " + 2 " $\frac{M}{1000}$ "	-	-	-	-	-	-	+	+	+
"	" + 3 " " + 1 " "	-	-	-	-	-	-	-	-	+
"	" + 3.1 " " + 0.9 " "	-	-	-	-	-	-	-	-	Partially laked
"	" + 3.2 " " + 0.8 " "	-	-	-	-	-	-	-	-	Trace of laking
"	" + 3.3 " " + 0.7 " "	-	-	-	-	-	-	-	-	Trace of laking
"	" + 3.4 " " + 0.6 " "	-	-	-	-	-	-	-	-	-
"	" + 4 " " (control)	-	-	-	-	-	-	-	-	-

+ = Complete laking.

Times calculated from time at which tubes were placed in thermostat.

Of late years, it has been repeatedly demonstrated that the serum of an animal is able to exert a well-marked protective action against haemolytic agents on behalf of its own red blood corpuscles. We therefore tried the effect of substituting 1 c.c. of fresh sheep's serum in place of 1 c.c. normal saline in a series of tubes in which sodium cholalate in various strengths was used as a haemolytic agent; here it was found that the serum exercised a very marked inhibitory action on the haemolytic power of this substance. The results of such an experiment are seen in Chart 4.

It was found, for instance, that 1 c.c. of 0.1 M sodium cholalate, which when used alone was able to completely haemolyse the 1 c.c. of sheep's red corpuscles within five minutes, was, when used in conjunction with 1 c.c. of sheep's serum, absolutely devoid of haemolytic action. Such a result indicates very marked power indeed in the inhibition and prevention of haemolysis by the protective action of normal serum.

In examining the chart, one apparent contradiction will be noticed; in the case of the tube containing 3 c.c. 0.1 M solution of sodium cholalate, haemolysis was complete in twenty-two minutes. Such an amount, however, when used alone, is capable of completing haemolysis only after eighty minutes, so that in this particular case the serum has increased instead of diminished the haemolytic action.

This result is easily explained in the light of our former observations; if we assume that the amount of sheep's serum used was insufficient to neutralise the effect of the total amount of choleic acid salt present, but had neutralised its equivalent amount, it is obvious that in this case a reduced amount of sodium cholalate would now be present in an active form, and since a weak dose (within certain limits) is more powerful than a stronger one, an increased action might naturally be expected.

Some sheep's serum was then inactivated by heating it at 56° C. for one hour; it was then tested to make sure that the process was complete; this was done by means of several controls against inactivated pig's serum which normally lyses sheep's red corpuscles. In a series of tubes similar to those used in the preceding experiment, 1 c.c. of the inactivated serum was substituted for the fresh material; it was found that the protective action was in no way diminished as the result of inactivation; indeed, if anything, it seemed more marked. It may, therefore, be assumed that in these cases the presence or absence of complement is immaterial in so far as the protective action of the serum is concerned.

A similar series of experiments were carried out using the sodium salt of choleic acid, and similar results obtained. Chart 4 shows these results epitomized.

CHART No. IV

Description of Tubes			Results
1 c.c. sheep's r. b. c. + 1 c.c. sheep's serum +	$\frac{M}{10}$ sodium cholate...	...	Complete haemolysis in 22 minutes
" " + " " + 2 c.c. saline + 1 c.c. "	" "	...	Complete haemolysis after 5 hours
" " + " " + 2.5 " " + 0.5 "	" "	...	Very slight haemolysis after 18 hours
" " + " " + 2.7 " " + 0.3 "	" "	...	No haemolysis
" " + " " + 1 " " + 2.0 $\frac{M}{100}$ "	" "	...	No haemolysis
" " + " " + 2 " " + 1.0 "	" "	...	No haemolysis
1 c.c. sheep's r. b. c. + 1 c.c. inact. sheep's serum +	$\frac{M}{10}$ sodium cholate...	...	Complete haemolysis in 25 minutes
" " + " " + 2 c.c. saline + 1 " "	" "	...	Almost complete haemolysis after 18 hrs.
" " + " " + 2.5 " " + 0.5 "	" "	...	Were trace of haemolysis after 18 hours
" " + " " + 2.7 " " + 0.3 "	" "	...	No haemolysis
" " + " " + 1.0 " " + 2.0 $\frac{M}{100}$ "	" "	...	No haemolysis
" " + " " + 2.0 " " + 1.0 "	" "	...	No haemolysis
1 c.c. sheep's r. b. c. + 1 c.c. sheep's serum + 1 c.c. saline + 2 c.c. $\frac{M}{100}$ sodium cholate	" "	...	Complete haemolysis in 113 minutes
" " + " " + 2 " " + 1 " "	" "	...	No haemolysis
" " + " " + 2.5 " " + 0.5 "	" "	...	No haemolysis
1 c.c. sheep's r. b. c. + 1 c.c. inact. sheep's serum + 1 c.c. saline + 2 c.c. $\frac{M}{100}$ sodium cholate	" "	...	Complete haemolysis in 140 minutes
" " + " " + 2 " " + 1 " "	" "	...	No haemolysis
" " + " " + 2.5 " " + 0.5 "	" "	...	No haemolysis
Controls	" "	...	No haemolysis

NOTE.—The observations on these tubes extended over 24 hours.
After the sheep's r. b. c. were added the tubes were placed in a thermostat at a temperature of 37° C.

According to Liebermann, this inhibitory power of the blood serum is dependent on the serum-albumin. Iscovesco,¹ on the other hand, attributes it to the cholesterol normally present in blood serum. Gerard and Lemoine² have studied the anti-toxic action of cholesterol as applied to tubercular poisons. Ranson³ has also demonstrated the anti-haemolytic power of cholesterol on certain chemical substances.

In view of these results, we have tried the effect of cholesterol on the haemolytic action of sodium cholalate. Using an 0.002 M emulsion of cholesterol, it was found that even in strengths of 0.0008 M solution it exercised a considerable anti-haemolytic action, and in the presence of weak doses of sodium cholalate was sufficient to entirely prevent a laking effect.

These results are demonstrated in Fig. 4, Chart 5. When using 0.4 c.c. 0.1 M sodium cholalate, the amount of cholesterol used (2 c.c. 0.002 M) was sufficient to delay the usual laking effect for about thirty minutes; with 0.5 c.c. 0.01 M there was a delay of seventy minutes, and in the case of 0.4 c.c. 0.01 M haemolytic action was totally abolished.

In all these experiments, the cholesterol emulsion and the sodium cholalate were incubated together at 37° C. for one hour previous to the addition of the red blood corpuscles.

The above are some of the results obtained with the substances mentioned; it is hoped to extend them at a later period. No theoretical considerations with regard to them have been advanced, since much more work must be done before any satisfactory explanation can be forthcoming; they are merely given as facts obtained as the result of repeated experiments.

CONCLUSIONS

Such bile derivatives as the sodium salts of cholalic, choleic, and glycocholic acids are capable of producing haemolysis in the ordinary way when strong doses are used, but exhibit marked peculiarities when present in considerably weaker amounts:—

1. It was found that under similar conditions the same haemolytic effect can be produced in a given time by widely divergent amounts of the salts. Between these two points lies what may be termed a more or less

1. 'Les Lipoides,' 1908.

2. Congrès de Médecine, 1907. *Soc. Médic. des Hospitaux*, Paris, 1907.

3. *Deutsche med. Wochenschr.*, 1901.

neutral zone, in which haemolysis is very considerably delayed depending on the relative amounts of haemolytic agent employed. The minimum dose giving the maximum effect in the shortest time we have designated the 'minimal-optimum haemolytic dose.' For instance, it was found that this minimal-optimum dose in the case of sodium cholalate was 0.1 c.c. 0.1 M solution; this quantity, mixed with 1 c.c. of 5 per cent. sheep's red corpuscles (the total volume being made up to 5 c.c.), gave complete haemolysis within five minutes. *Stronger* solutions gave a much *less* marked effect, a solution eight times as strong requiring one hundred and fifty-five minutes for a similar result, while a dose eighty times as large as the minimal-optimum dose required thirteen minutes to complete haemolysis.

2. In all these cases the addition of cholesterin produced a marked anti-haemolytic effect.

3. In a similar manner, the addition of fresh sheep's serum exercises a markedly anti-haemolytic action; in some cases, however, an apparent augmentation is in evidence. This is obviously due to the fact that the amount of serum used was unable to completely neutralise the laking action of the bile salts; this would result in a smaller relative amount of active haemolytic agent being present, and hence a more powerful effect would be seen (within certain limits), in accordance with the above observations.

4. This inhibitory action of serum does not depend on the presence of complement; an inactivated serum acts equally well.

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