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THE ISOLATION OF A HAEMOLYTIC SUBSTANCE FROM ANIMAL TISSUES AND ITS BIOLOGICAL PROPERTIES

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Attempts to explain the mechanism of erythrocyte destruction under normal and pathological conditions on immunological principles were not successful except in the case of paroxysmal haemoglobinuria (Donath & Landsteiner, 1904, 1905) and the anaemias involving the *Rh*-factor. Korschun & Morgenroth (1902) were the first to suggest that the destruction of erythrocytes might be due to the presence of a haemolytic substance in the blood affecting the erythrocytes directly without the interaction of an immunological factor, such as complement. They showed that a number of animal tissues contain an ether-soluble, heat-stable, non-antigenic fraction which is haemolytic *in vitro*. Their results have been confirmed and elaborated by Wölfel (1905), Levaditi (1903, 1905) and Friedemann (1909). Different results were reported by Belfanti (1924, 1925, 1928) who obtain an *ether-insoluble* haemolytic fraction from pancrease and, more recently, by Bergenhem & Fähraeus (1936), Bergenhem (1938), and Fähraeus (1939), who also obtained haemolytic activity in the ether-insoluble fraction of serum, the active principle of which they claimed to be either identical with, or closely related to, lysolecithin. The lecithinase responsible for its production in the body was supposed to manifest its activity in the spleen. Their theory has been accepted by a number of authors [Bogaert (1937), Gripwall (1938–9), Singer (1941), and Singer, Miller & Damaschek (1941)], especially in view of the clinical success of splenectomy in acholuric jaundice. On the other hand, Mann & Castle (1940), Foy & Kondi (1943–4), Gillespie (1944) and Maizels (1944–6) were either unable to confirm the lysolecithin nature of the haemolytic substance (H.S.) as described by Bergenhem & Fähraeus or rejected their evidence. It has, furthermore, to be realized that neither lysolecithin nor lecithinase has ever been isolated from plasma or tissue extracts. Lastly, the methods used for preparing the ether-insoluble H.S. are often open to criticism. Bogaert (1937), for instance, states that the lysolecithin-containing ether precipitate from serum was 'quickly dried at room temperature'

before using it for the haemolysis test. This procedure of drying either precipitates is unsatisfactory, as the resulting haemolysis from such preparations may easily be caused by the remaining traces of ether and not necessarily by the inherent haemolytic activity, a possibility which was not excluded by Bogaert.

In this paper the isolation of an ether-soluble H.S. (or a group of substances) from animal tissues is described, the biological properties of this substance are examined and its possible role in normal and pathological destruction of erythrocytes is discussed. Preliminary accounts of parts of this investigation have previously been given elsewhere (Laser & Friedmann, 1945; Laser, 1948).

METHODS

Preparation of erythrocyte suspension. Horse erythrocytes obtained from defibrinated blood of healthy animals, which were bled for serological work, have been used. A few ml. of erythrocytes were twice washed with saline or 0·15 M-phosphate buffer pH 7·3 and 5 of 10 % (v/v) suspensions prepared. A given suspension was divided into several parts and a fresh suspension used for each series to prevent protracted incubation at 37° before zero time. Erythrocytes in serum keep for 4–5 days in the cold, whereas washed erythrocytes are unreliable after the first day.

Suspension media and isotonic solutions of substances tested for their effect on haemolysis. The following molar concentrations were used according to the data given by Hitchcock & Dougan (1935) and Wilbur & Collier (1943): NaCl, 0·163 M; KCl, 0·15 M; $CaCl_2$, 0·11 M; $MgSO_4$, 0·155 M; NaF, 0·165 M; phosphate buffer (from Na_2HPO_4 and KH_2PO_4), 0·15 M; pyrophosphate, $Na_4P_2O_7$, 0·163 M in 0·163 N-HCl; metaphosphate, 0·15 M; sodium citrate, 0·109 M; sodium oxalate, 0·127 M; glucose, 0·296 M. The procedures adopted for preparing and testing certain organic substances were as follows. Crystalline albumin, globulin, haemoglobin and denatured globin were prepared according to the standard methods. Globin and stromatin were finely dispersed by vigorous shaking. Cholesterol (twice recrystallized) and lecithin (B.D.H.), dissolved in a small amount of ethanol, were added to the medium. The ethanol was then removed by boiling. Bilirubin, biliverdin, haematin, haematoporphyrin and protoporphyrin were dissolved in tribasic phosphate solution and neutralized with KH_2PO_4. They then remained in solution at neutral pH.

Preparation of stromata. These were obtained by high-speed centrifugation of laked (previously washed) erythrocytes or of washed erythrocytes laked with ice and ether (100 ml. erythrocytes + 30 ml. ice + 30 ml. ether + a few g. NaCl), whereby the stromata rise to the surface. They are then separated and, as far as possible, washed free from haemoglobin and dried *in vacuo*.

Measurement of haemolysis. All experiments, except those for the determination of the percentage haemolysis curve, were done in test-tubes at 37° in a water-bath having glass walls at the front and back. The test-tubes were placed in a metal stand which at its back held a transparent glass plate with horizontally etched and blackened lines at about 5 mm. distance apart. The test-tubes were viewed against a light source. End-points of haemolysis were reached when all cloudiness in the test-tube had disappeared and the lines on the glass plate became distinctly visible. Viewing the tubes through a red filter facilitated the determination of the end-point. The suspension medium, the H.S. in relatively high concentration, and the erythrocyte suspension were separately incubated in test-tubes until they attained the temperature of the bath. The haemolytic substance was then added to the test-tubes containing the medium, the erythrocytes being added last, at zero time. The contents of each test-tube were mixed by reversing the tube.

Qualitative test. A given amount of any fraction of the H.S. to be tested was taken up in a few ml. ether, or in 1 ml. ethanol, which was boiled down to about 0·2 ml. After addition of a known amount of water (if ether was being used the tubes were vigorously shaken) the tubes were immediately placed in boiling water for 5 min. The active material then formed a finely dispersed emulsion, a given amount of which was brought to isotonicity. Control tubes containing the same

amount of ether or ethanol were put up at the same time in order to check the complete removal of the solvent. This method was not applicable for quantitative determinations requiring known dilutions because after cooling some of the H.S. stuck to the walls of the test-tubes and to the pipettes, entailing an unknown loss.

Quantitative test. A known amount of the H.S. was dissolved with warming in 0·3 M-phosphate (K_3PO_4 or Na_3PO_4) or in 0·32 N-NaOH (or KOH). Stock solutions containing 0·5–1.0 mg./ml. were prepared. The stock solutions in alkali were stable in the cold, but on account of their marked thixotropy they had to be warmed before pipetting. To prevent a pH shift of the medium used for the measurement of haemolysis 0·163 ml. 0·15 M-KH_2PO_4 or 0·1 ml. 0·32 N-HCl were added per 0·1 ml. of the H.S. dissolved in K_3PO_4 or NaOH respectively.

Percentage haemolysis curves were determined in optical cuvettes at room temperature by means of a photoelectric cell measuring the transmitted light (King's colorimeter; red filter).

RESULTS

Attempts to demonstrate haemolytic activity in the ethanol-soluble and ether-insoluble fraction of serum, following the method of Bergenhem & Fähraeus (1936) failed, if care was taken to remove from the precipitate all traces of ether in a vacuum in the presence of shavings of paraffin wax. The ether precipitate from serum was, moreover, not found to be water soluble, as stated by Bergenhem & Fähraeus, and it was therefore tested in suspension. However, the ether-soluble fraction of serum possessed haemolytic activity and it was this fraction which was used for the isolation of the H.S.

Isolation of the haemolytic substance

Table 1 summarizes the general procedure of the isolation of H.S., which, in the first instance, was effected from serum. Later on, large-scale preparations were made from brain. The material obtained by high vacuum distillation, which is partly crystalline and partly oily at room temperature, is strongly haemolytic. This fraction has been used without further purification for the following activity tests and for examination of its biological properties.

TABLE 1. Isolation of the haemolytic fraction

(1) Dehydration of material with acetone or by freeze-drying
(2) Hot ethanol extraction of dried material
(3) Removal of ethanol. Dry residue obtained
(4) Ether extraction of residue suspended in water acidified with H_2SO_4 (blue to Congo red)
(5) Transference of alkali-soluble material from ether extract (4) into 1·5 % KOH solution
(6) Ether extraction of KOH fraction (5) acidified with H_2SO_4 (blue to Congo red)
(7) Transference of K_2CO_3-soluble material from ether extract (6) into 2·4 % K_2CO_3 solution
(8) Ether extraction of K_2CO_3-fraction (7) acidified with H_2SO_4 (blue to Congo red)
(9) Transference of alkali-soluble material from ether extract (8) into 1·5 % KOH solution
(10) Neutralization of KOH-fraction (9) with acetic acid and precipitation at neutral pH with 5 % lead acetate
(11) Drying of lead precipitate *in vacuo* and extraction with ether
(12) Decomposition of ether-soluble lead salts with dilute H_2SO_4
(13) Removal of ether. Dry residue obtained
(14) Molecular distillation of residue at high vacuum at as low a temperature as possible (about 60°)

A separate account of the further purification and chemical constitution of the H.S. will be given elsewhere in collaboration with Dr I. D. Morton, of the Chemical Laboratory, Cambridge. The purified substance, which was found

to contain only carbon, hydrogen and oxygen, is a monocarboxylic, mono-unsaturated fatty acid with the chain length C_{18}. (See note at the end of the paper.)

Distribution of haemolytic substance

H.S. has been found widely distributed in the body (Table 2). In every case purification has been carried through to the ether-soluble lead salt. High vacuum distillation has been carried out with the material from human plasma,

TABLE 2. Distribution of haemolytic substance in different tissues

Tissue	Relative content of H.S.
Plasma (human)	+
Serum (horse)	+
Spleen (horse)	+
Liver (horse)	+
Leucocytes (human pus)	+ +
Roe, male and female (herring)	±
Spermatozoa (hog)	−
Erythrocytes (horse)	+
Stromata	+
Remainder of cell	−
Erythrocytes (monkey) malaria parasitized*	+ + +
Brain—grey matter (human, horse)	+ + +
Brain—white matter (human, horse)	+ + +
Optic nerve (horse)	−
Anterior and posterior spinal roots (dog)	−

−, ±, +, + +, + + + = None, trace, small, medium and large yield.
* Laser (1948).

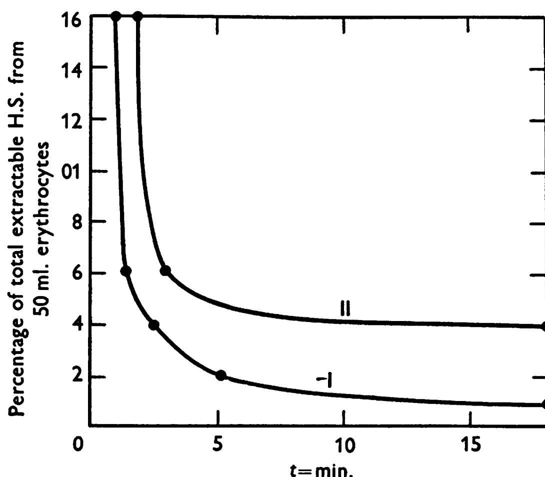

Fig. 1. Effect of incubation of erythrocytes (16 hr., 37°) on the yield of H.S. from stromata. In this and the following figures the abscissae give the time in minutes required for complete haemolysis. I, H.S. from normal erythrocytes; II, H.S. from incubated erythrocytes.

horse brain and malaria-infected monkey erythrocytes. While the content of H.S. in different tissues varied greatly, the haemolytic activity of the isolated material from different sources was of the same order. The data given in Table 2,

however, are only approximate, as they are based on comparatively rough overall calculations. Sperm (hog) was devoid of H.S. and herring roe contained only traces, which might be derived from the interstitial tissue. Leucocytes and brain (grey and white matter) gave the highest yield. The optic nerve (horse), however, and the anterior and posterior spinal roots (dog) had no recoverable H.S. In erythrocytes H.S. appeared to be bound to the stromata, the remainder of the cell having none. Incubation of erythrocytes at 37° prior to the separation of stromata decreased the amount of recoverable H.S. from the stromata (Fig. 1).

Aggregation of haemolytic substance at neutral pH

The activity of a given amount of H.S. was found to decrease with time after neutralization. This is probably the result of aggregation of H.S., as such preparations become slightly cloudy on standing. Deterioration of neutralized H.S. is more rapid the higher the concentration (Table 3). This fact has to be taken into account when evaluating the effect of inhibitors of H.S. In all subsequent experiments, therefore, the erythrocytes were added to each test-tube exactly 3 min. after the H.S.

TABLE 3. Deterioration of activity of haemolytic substance after neutralization

Time between addition of erythrocytes and neutralization (min.)	Vol. in which neutralized (ml.)	Haemolysis time (min.)
0	10	2·25
15	10	3·5
15	0·3	6·0
150	0·3	23·0

H.S., 1:200,000, final concentration in 10 ml. 0·1 ml. 7·5 % erythrocyte suspension.

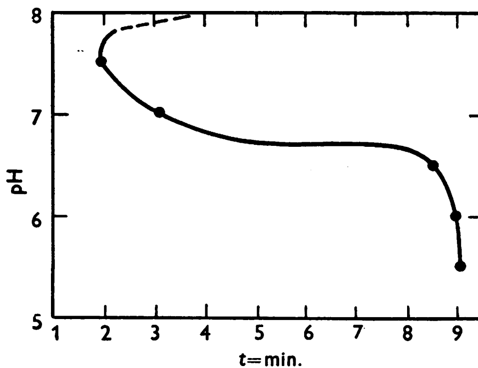

Fig. 2. Effect of pH on the rate of haemolysis by H.S. H.S., 1:120,000 in 10 ml. 0·15 M-phosphate buffer; 0·1 ml. 7·5 % erythrocyte suspension.

Effect of pH

Fig. 2 shows the dependence of the activity of H.S. on the pH of the medium. In view of the aggregation, with consequent deterioration of the activity of

H.S. at and below neutral pH, it cannot be decided whether the variations of haemolysis time with pH are real or apparent, because it is possible that aggregation of H.S. occurs faster at lower pH. However, no further delay of haemolysis time occurs between pH 6 and 5, furthermore, towards alkaline pH haemolysis again tends to slow down. At still higher pH values lysis takes place in the absence of H.S. Regardless of interpretation, however, the data prove the necessity of controlling the pH in *in vitro* experiments with haemolytic substances.

Effect of phosphate concentration

The rate of haemolysis increases with increasing phosphate concentration in saline in a manner shown in Fig. 3. The effect is equally obtained if orthophosphate is replaced by either pyrophosphate, metaphosphate or adenosine-

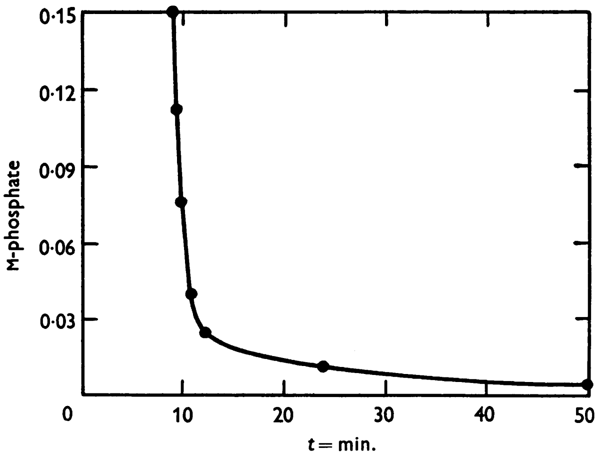

Fig. 3. Effect of phosphate concentration on the rate of haemolysis by H.S. in an isotonic medium containing phosphate buffer and sodium chloride. H.S., 1:200,000 in 10 ml. isotonic medium; 0·1 ml. 10 % erythrocyte suspension.

triphosphate. A certain concentration of phosphate does seem to be necessary for optimal activity of H.S., although no definite ratio of phosphate to the amount of H.S. or of erythrocytes has been established. The phosphate effect can be demonstrated with regularity only with low concentrations of H.S. acting on fresh erythrocytes. A similar phosphate effect has been obtained with saponin as the haemolytic agent (Fig. 4), a result which is also evident in the experiments of Maizels (1944–6).

Effect of other substances

The substances examined are listed in Table 4. Since H.S. used for this series had been dissolved in K_3PO_4, all tubes contained a small amount of phosphate buffer (final concentration 0·004 M, pH 7·3), which by itself, however, is too small to produce the phosphate effect. The tested substances can be classified

in three groups: (*a*) those which do not affect the rate of haemolysis compared with that in saline: these are KCl and glucose; (*b*) those which slow down the rate of haemolysis: these are $CaCl_2$ and $MgSO_4$; (*c*) those which accelerate

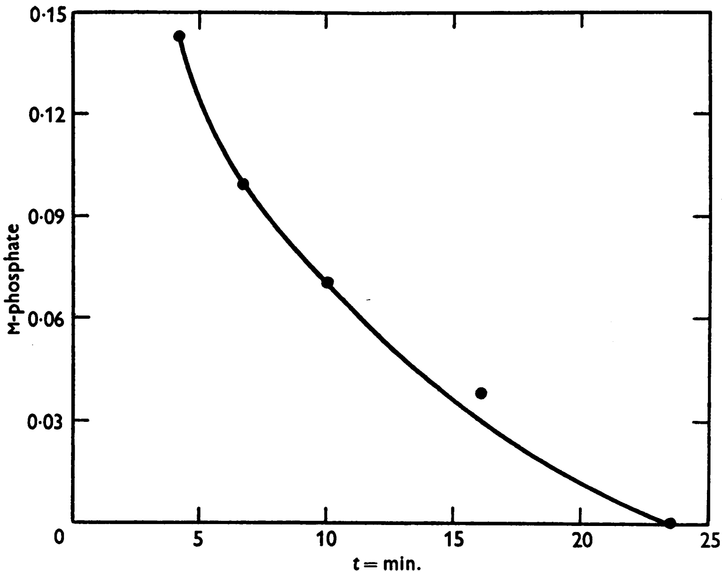

Fig. 4. Effect of phosphate concentration on the rate of haemolysis by saponin in an isotonic medium containing phosphate buffer and sodium chloride. Saponin, 1:125,000 in 10 ml. isotonic medium; 0·2 ml. 10 % erythrocyte suspension.

haemolysis to about the same extent as phosphate in optimal concentration: these are Na-citrate and oxalate. The acceleration caused by Na-fluoride was less pronounced.

TABLE 4. Effect of various substances on haemolysis time

Substance tested	Haemolysis time (min.)
—	29
Phosphate	8
KCl	33
Glucose	38
$CaCl_2$	∼
$MgSO_4$	90
Na-citrate	7
Na-oxalate	5
Na-fluoride	15

H.S. 1/200,000 final concentration. 0·2 ml. 5 % erythrocyte suspension. The samples contained 0·004 M-phosphate buffer pH 7·3 (for dissolving and neutralizing H.S.), and 1·2 ml. isotonic solution of the substance to be tested in 10 ml. isotonic saline.

The inhibitory effect of Ca and Mg arises probably from the formation of insoluble Ca or Mg salts of H.S., while the speeding up caused by citrate, oxalate and fluoride is probably the result of the combination of these substances with

Ca on the cell surface, and its removal as a natural brake on the rate of haemolysis. A similar acceleration of saponin haemolysis by Na-oxalate and citrate has also been observed by Wilbur & Collier (1943), who explain the effect as due to initial shrinkage of the erythrocytes in Na-oxalate and citrate solutions, a condition which accelerates haemolysis. Their measurements were done in isotonic Na-oxalate or citrate solutions; in the experiments just described isotonic Na-oxalate and citrate solutions had been diluted eight times. It seems therefore desirable, when studying haemolysis, to refrain from using anticoagulants such as sodium citrate or oxalate, but to use either defibrinated blood or heparin, which does not affect the haemolysis rate.

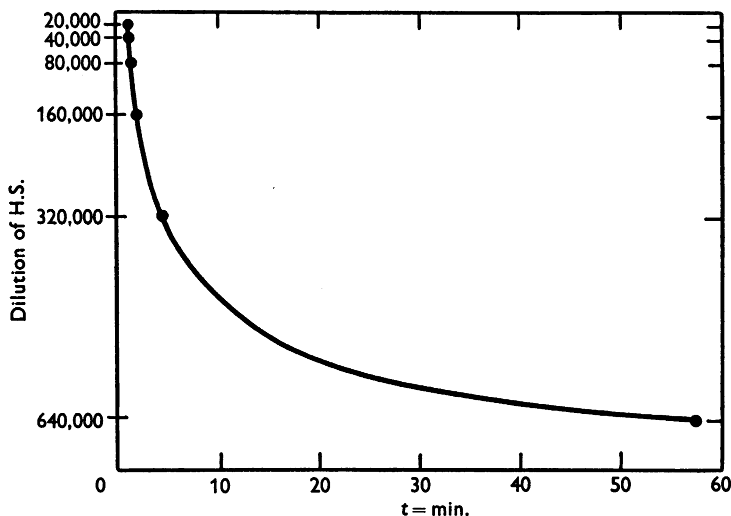

Fig. 5. Relationship between dilution of H.S. and rate of haemolysis. 10 ml. phosphate buffer pH 7·3; 0·1 ml. 5 % erythrocyte suspension.

Relationship between dilution and haemolysis time

Time-dilution curves have been obtained by varying either the amount of erythrocytes acted upon by the same amount of H.S. or by varying the amount of H.S. acting on the same amount of erythrocytes (Fig. 5). The curves follow the same pattern as described by Ponder (1934) in experiments with saponin.

It has been observed by several workers that a given amount of a haemolytic substance which haemolyses x ml. erythrocytes in y min. haemolyses consecutively added fractions of x ml. erythrocytes in the aggregate in a longer time (Ponder, 1932). The haemolysis by H.S. behaves in a similar way (Fig. 6). It is assumed that some products set free by haemolysis, such as stromata and haemoglobin act as inhibitors. This is borne out by a test, in which the haemolysis time was measured in presence of a laked 10 % erythrocyte suspension with and without the removal of the stromata. The haemolysis time of the

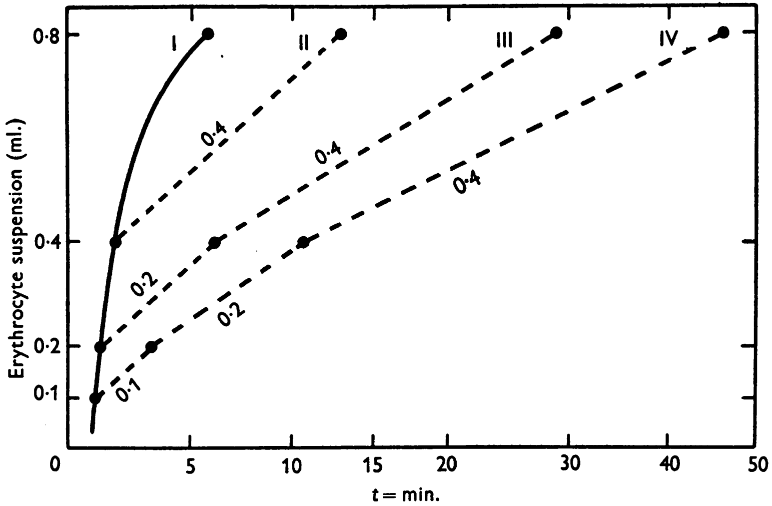

Fig. 6. Relationship between constant amount of H.S. and varying amounts of erythrocytes, when added in fractions. H.S., 1:100,000 in 10 ml.; phosphate buffer pH 7·3; 5 % erythrocyte suspension. Dotted lines indicate consecutive addition of erythrocyte suspension, each following addition being made immediately haemolysis of the preceding amount of erythrocytes has been completed. I, addition to four different tubes of 0·1, 0·2, 0·4 and 0·8 ml. erythrocyte suspension (solid line); II, consecutive addition to the same tube of 0·4 and 0·4 ml. erythrocyte suspension; III, as II with 0·2, 0·2 and 0·4 ml. erythrocyte suspension; IV, as II with 0·1, 0·1, 0·2 and 0·4 ml. erythrocyte suspension.

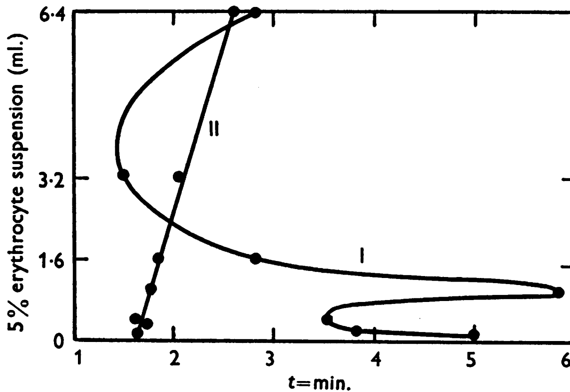

Fig. 7. Effect of the addition of laked erythrocytes on the time of haemolysis with increasing amounts of erythrocytes and constant amounts of lysolecithin. Lysolecithin, 1:4000 in 10 ml. phosphate buffer pH 7·3; 5 % erythrocyte suspension.

Tubes ...	1	2	3	4	5	6	7
Series I: erythrocyte suspension (ml.)	0·1	0·2	0·4	0·8	1·6	3·2	6·4
Series II: erythrocyte suspension (ml.) + laked	0·1	0·2	0·4	0·8	1·6	3·2	6·4
erythrocytes (ml.) (5 % suspension)	6·3	6·2	6·0	5·6	4·8	3·2	0

control (0·1 ml. 10 % erythrocyte suspension in 10 ml. phosphate pH 7·3, H.S. 1:100,000) was 1·7 min.; in presence of 0·4 ml. laked erythrocytes it was 16 min.; while in presence of 0·4 laked erythrocytes with stromata removed it was 4 min. Hence both the stromata and some constituents of the erythrocytes decreased the rate of haemolysis.

It is interesting to note that the time-dilution curve with lysolecithin differs fundamentally from that obtained with H.S. The rate of haemolysis shows an optimum with low concentrations of lysolecithin relative to the amount of erythrocytes, i.e. a given amount of lysolecithin lyses a large amount of erythrocytes more quickly than a small amount (Fig. 7, curve I). It thus seems that the products of haemolysis accelerate erythrocyte destruction by lysolecithin, contrary to their effect on H.S. haemolysis. This is borne out by the experiment shown in Fig. 7, curve II, where the addition of haemolysed erythrocytes increases the lysolecithin haemolysis rate. The mechanism of lysolecithin haemolysis is therefore different from that operating with H.S.

The percentage haemolysis curve

A calibration curve was first established by measuring the light transmitted when using erythrocyte suspensions and haemolysed erythrocytes in varying proportions. Details of the procedure for making up standards of a given

TABLE 5. Procedure for obtaining erythrocyte suspensions representing various percentage of haemolysis for determining the percentage haemolysis curve (calibration curve) with the King colorimeter

Sample	0·5 % erythrocyte in phosphate buffer (ml.) (1)	Water (ml.) (2)	Twice isotonic phosphate buffer buffer (ml.) (3)	Isotonic saline (ml.) (4)
I	3·0	—	—	12·0
II	2·5	7·5	7·5	2·5
III	5·0	10·0	10·0	5·0
IV	7·5	12·5	12·5	7·5
V	3·0	12·0	—	—

Sample	0·5 % erythrocyte in phosphate buffer (ml.) (5)	Isotonic saline (ml.) (6)	Cuvette mixture from (1)+(2)+(3)+(4) (ml.) (7a)	Cuvette mixture from (5)+(6) (ml.) (7b)	Representing percentage haemolysis of 0·1 % erythrocyte suspension (8)
I	—	—	5·0	—	0
II	5·0	15·0	2·0	3·0	25
III	4·0	12·0	3·0	2·0	50
IV	2·5	7·5	4·0	1·0	75
V	—	—	5·0	—	100

Five samples (I–V) representing the required percentage of haemolysis (column (8)) were obtained by the addition of varying amounts of erythrocyte suspensions to isotonic solutions of haemolysed erythrocytes of varying concentration. This was effected by separately mixing for each sample (I–V) the volumes stated in columns (1)+(2)+(3)+(4) and (5)+(6). The optical cuvettes, which held 5 ml. were then filled with varying amounts of these mixtures, as indicated in column (7a) and (7b).

erythrocyte suspension which contain known percentages, say 50 % of intact and 50 % of haemolysed cells in isotonic solution, are given in Table 5. The calibration curve thus obtained was found to be a straight line. H.S. was added to an erythrocyte suspension of the same concentration and galvanometers readings taken at convenient intervals, depending on the speed of the reaction. The readings were then converted into percentage haemolysis on the basis of the calibration curve. Fig. 8 shows the results obtained with different amounts of H.S.

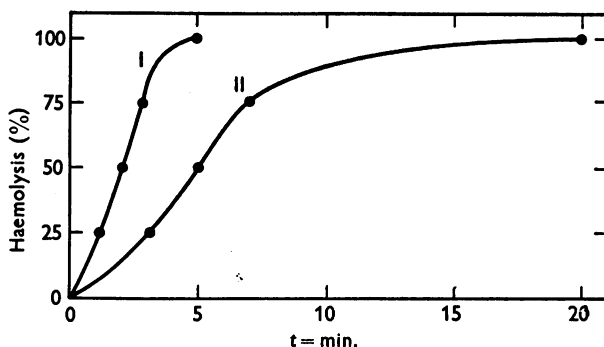

Fig. 8. Percentage haemolysis curves, determined photometrically with the King colorimeter. $T = 17°$. Empirical amounts of H.S.: concentration in test I about three times that in test II. 5 ml. 0·1 % erythrocyte suspension in phosphate buffer pH 7·3.

Inhibitors of haemolytic substance present in plasma

Normal plasma contains H.S., and relatively large amounts of H.S. can be injected intravenously without any apparent haemolysis. It is therefore likely that plasma contains inhibitors of H.S. The following physiological components of plasma have been examined: albumin, globulin, bilirubin and cholesterol. The proteins and cholesterol proved to be strong inhibitors, bilirubin had a slight inhibitory effect in fairly high concentration (> 1/10,000). Globulin inhibits less than albumin (Fig. 9). The strong inhibition by calcium has already been mentioned. In the case of cholesterol it is difficult to decide whether the inhibitory effect is specific or a result of the physical properties of the suspension of cholesterol which adsorbs H.S. in an unspecific way. However, even adsorbed H.S. should come into contact with erythrocytes. As at a concentration of cholesterol of 1/100,000 inhibition was infinite, there is no reason to doubt its specific inhibitory effect, which has also been observed in saponin haemolysis by Ranson (1901) and Stocks (1919–20). The inhibitory effect of serum on the H.S. haemolysis, determined quantitatively by serial dilutions *in vitro*, was found to correspond closely to the effect produced by the equivalent amount of albumin contained in the serum. The inhibition of H.S. by plasma *in vivo* seems, therefore, to be accounted for by its protein content and not by cholesterol.

Inhibitors of haemolytic substance present within the erythrocytes

Since H.S. occurs in normal erythrocytes and can be extracted from their stromata after washing and laking it must be assumed that H.S. is inactive while bound to the stromata. In order to test the possibility that other constituents of the erythrocytes might act as inhibitors of H.S., the following substances were examined: haemoglobin, lecithin and biliverdin, the last-named being the substance most closely related to verdohaematin, the first normal breakdown product of haemoglobin (Lemberg, Cortis-Jones & Norrie, 1938*a*, *b*). Biliverdin did not affect the rate of haemolysis by H.S., while

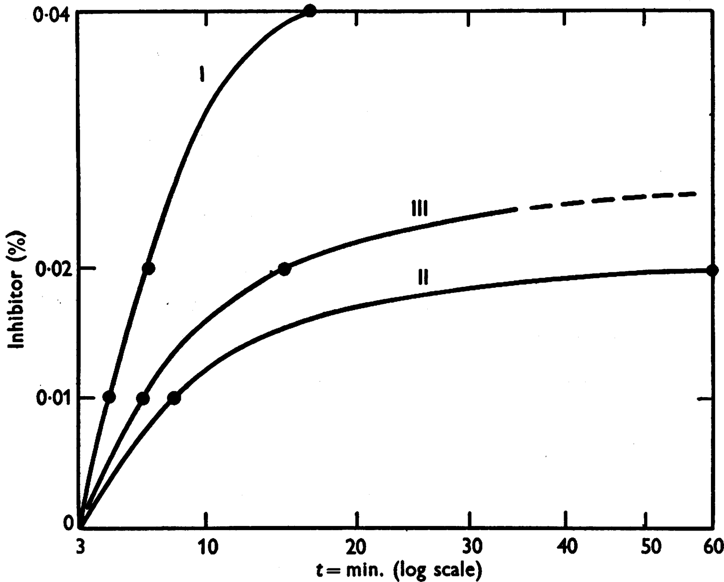

Fig. 9. Relationship between concentration of inhibitor and time of H.S. haemolysis. H.S., 1/50,000 in 10 ml. phosphate buffer pH 7·3; 0·5 ml. 5 % erythrocyte suspension. I, globulin; II, albumin; III, serum, calculated as protein equivalent.

haemoglobin and lecithin were found to act as inhibitors. Under the experimental conditions (H.S. 1/200,000) lecithin inhibited only at a concentration > 1/100,000, while haemoglobin was effective at a concentration > 1/100,000. Assuming a molecular weight of H.S. of about 300, the inhibitory effect of lecithin seems to occur at roughly equimolar concentration of lecithin and H.S., while the inhibition by haemoglobin was manifest at a much lower molar concentration. The inhibition by lecithin was not apparently caused by impurities in the lecithin preparation, as these would mainly be free fatty acids derived from lecithin which are known to have the reverse effect. In fact, several samples of old or less pure lecithin either increased the rate of haemolysis by H.S. or were haemolytic in absence of H.S.

Haematin and porphyrins

Haematin, which is not a physiological breakdown product of haemoglobin, was found by itself to be haemolytic *in vitro* down to relatively low concentra-

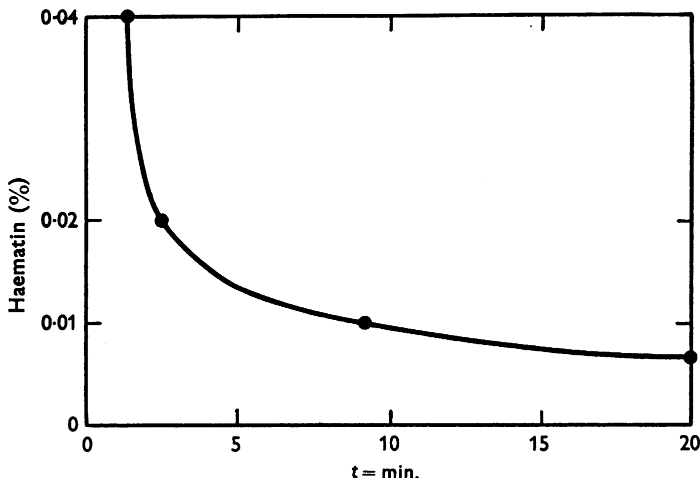

Fig. 10. Haemolytic effect of haematin. Relationship between concentration of haematin and haemolysis time. 0·1 ml. 5 % erythrocyte suspension in 10 ml. phosphate buffer pH 7·3.

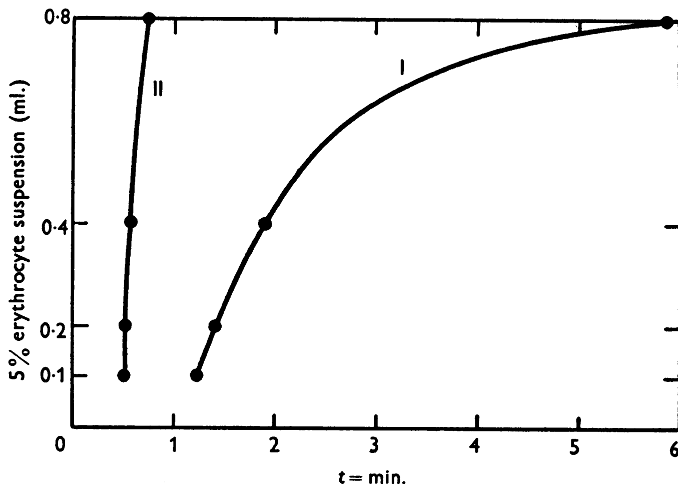

Fig. 11. Effect of subhaemolytic concentration of haematin on H.S. haemolysis. H.S. 1/100,000 in 10 ml. phosphate buffer pH 7·3; 5 % erythrocyte suspension. I, time of H.S. haemolysis with increasing amounts of erythrocytes; II, as I in the presence of haematin 1/100,000.

tions (Fig. 10). In subhaemolytic concentrations it considerably increased the rate of haemolysis by H.S. (Fig. 11). This effect was enhanced in the presence of lecithin (Table 6) although lecithin itself acted as an inhibitor. In high

concentration haematin abolished the inhibition of H.S. haemolysis by serum (Table 7). Cyan-haematin and glyoxaline-haematin act like haematin, while haemato-porphyrin and protoporphyrin had a similar but less pronounced effect requiring higher concentrations. The effect of haematin and its power to increase H.S. haemolysis were dependent upon the presence of phosphate.

TABLE 6. Effect of haematin in subhaemolytic concentration of haemolytic substance haemolysis in presence of added lecithin

	Haemolysis time (min.)
H.S.	13
H.S. + haematin	2
H.S. + lecithin	>90
H.S. + haematin + lecithin	0·66

H.S., 1/200,000 in 10 m. phosphate buffer pH 7·3. Lecithin, 1/40,000. Haematin, 1/100,000. 0·125 ml. 10 % erythrocyte suspension.

TABLE 7. Effect of haematin on inhibition of haemolytic substance haemolysis by serum

	Haemolysis time (min.)
H.S.	3
H.S. + serum (200)	~
H.S. + haematin (2,500)	0·5
H.S. + haematin (5,000)	0·75
H.S. + haematin (10,000)	1·0
H.S. + serum (200) + haematin (2,500)	0·5
H.S. + serum (200) + haematin (5,000)	1·3
H.S. + serum (200) + haematin (10,000)	7·5

H.S., 1:100,000 in 10 ml. phosphate buffer pH 7·3. 0·4 ml. 5 % erythrocyte suspension. Figures in brackets denote dilutions.

Phenylhydrazine

Phenylhydrazine is known to produce an increase in the number of siderocytes. These are aged erythrocytes which are easily disposed of in the body by phagocytosis. Phenylhydrazine, therefore, accelerates ageing and increases the osmotic fragility of erythrocytes. In high dilutions (1/100,000–1/200,000) it was found to accelerate the rate of H.S. haemolysis by about 50 %. No further acceleration could be produced by increasing the concentration or by previous incubation of erythrocytes with phenylhydrazine. The effect of phenylhydrazine was furthermore found to be irreversible.

DISCUSSION

At the time when Korschun & Morgenroth (1902) described the existence in extracts of animal tissues of an ether-soluble haemolytic substance, which was not antigenic and acted without complement, the main interest connected with problems of haemolysis lay in the field of immunology. Their work has therefore not been followed up. After the elucidation of the haemolytic effect of cobra venom, which causes lysolecithin to be formed by means of a lecithinase

(Delezenne & Fourneau, 1914), attempts were made to demonstrate the existence of lysolecithin in certain tissue extracts (Belfanti, 1924) and in incubated serum (Bergenhem & Fähraeus, 1936). These claims, however, have not been supported by more recent investigations. For instance, Gillespie (1944) found that the addition of lysolecithin to suspensions of erythrocytes in serum produced neither an increase in volume nor in osmotic fragility of erythrocytes, effects which are the characteristic of incubation with serum. There is, in fact, no convincing evidence that it is possible to extract from tissues or from normal or incubated serum a haemolytic substance of the nature of lysolecithin. In the present experiment it has also not been possible to obtain an ether-insoluble haemolytic fraction showing the chemical or biological properties of lysolecithin.

The haemolytic substance described in this paper was first isolated from the ethanol—and ether-soluble fraction of plasma (Laser & Friedmann, 1945). It has also been found to occur in other tissues, the largest yield being obtained from brain.

The chemical constitution of the substance has not yet been established beyond the fact that it is a mono-unsaturated, mono-carboxylic fatty acid with a chain length C_{18}. (See note at the end of the paper.) Fatty acids are known to cause haemolysis *in vivo*, as in *Bothriocephalus* anaemia (Faust & Tallquist, 1907), and also to be haemolytic *in vitro*. However, the minimal haemolytic concentration of oleic acid and of the acid isolated in these investigations are of a different order, the latter substance being considerably more powerful.

The optimal activity of H.S. *in vitro* was found to be dependent on the presence of phosphate. This effect might be regarded as similar to that of citrate or oxalate, i.e. removal of the inhibition by Ca through formation of an insoluble Ca salt. Analysis of the conflicting claims for the absence or presence of Ca in erythrocytes seems to justify the conclusion that they contain Ca in very much lower concentration than the plasma. A similar suggestion of the importance of intracellular phosphate for saponin haemolysis has already been put forward by Port (1910), Höber & Nast (1914), and Orahovats (1926). Ponder (1927), however, has rejected the possibility of a relation between P content and haemolysis rate as, according to his mathematical treatment of haemolysis as a simple reaction, the amount of any substance which is involved in a haemolytic process should not be directly but inversely proportional to its degree of lability. Yet the phosphate effect could clearly be demonstrated with H.S. as well as with saponin. There appears to be no reason to doubt that an increased intracellular content of P should similarly facilitate haemolysis. The objection of Ponder may therefore not hold, since a system, which involves accelerators of haemolysis, is a complex one, to which the mathematical treatment of a simple haemolytic reaction does not apply.

At present it is difficult to make any assumptions regarding the physiological significance of the presence of H.S. in relatively large amounts in different organs, especially brain, or regarding its distribution in the central nervous system. However, the existence of H.S. inside the erythrocyte lends support to the assumption that H.S. is concerned in the normal destruction of erythrocytes. Owing to the large excess of inhibitors of H.S. in the plasma, notably albumin, it cannot be assumed that any circulating H.S. is concerned in normal erythrocyte destruction, which takes place as an inherent function of the erythrocytes, as evidenced by the linear destruction of donor erythrocytes when transfused into normal patients or into patients suffering from anaemias, including pernicious anaemia and congenital familial acholuric jaundice (Brown, Hayward, Powell & Witts, 1944; Callender, Powell & Witts, 1945) or the linear decay of sulphaemoglobin erythrocytes in T.N.T. workers (Jope, 1946). However, in the reactions which determine the life span of the erythrocyte H.S. is probably an important factor. The yield of H.S. recoverable from stromata decreases on incubation of the erythrocytes in serum. This may be explained on the assumption that bound H.S. is liberated on autolysis from the stromata into the corpuscle. In a similar manner bound inactive H.S. might be liberated *in vivo* into the ageing corpuscle. If this were to happen at or near the surface of the erythrocyte, its increased fragility or haemolytic destruction might easily result.

None of the normal constituents of plasma or erythrocytes so far tested accelerated H.S. haemolysis, some were ineffective, while most of them were inhibitory, especially haemoglobin and albumin. A qualitatively different result was obtained with haematin, haematoporphyrin and protoporphyrin which do not occur under physiological conditions in the body. In certain concentrations they proved to be haemolytic by themselves and in subhaemolytic concentration to accentuate the effect of H.S. The acceleration of H.S. haemolysis by haematin in subhaemolytic concentration may be specific for H.S. and substances of similar constitution since it was shown that under similar conditions saponin haemolysis was delayed.

Haematin has been found in the plasma in a number of cases of pernicious and haemolytic anaemias (Heilmeyer, 1932, 1943) and porphyrin has been isolated in a case of haemolytic anaemia by Fischer & Zerweck (1924*a*, *b*) and by Müller (1931). According to Heilmeyer the occurrence of haematin is not due to excessive destruction of erythrocytes but to a particular disturbance of the normal degradation process of haemoglobin, leading to abnormal products. The formation of haematin in these cases is generally believed to occur in the plasma from liberated haemoglobin. The evidence brought forward in the present paper that haematin is haemolytic by itself, that in subhaemolytic concentrations it increases the haemolytic power of H.S., and that this effect is strongly potentiated by the presence of lecithin, may have a certain bearing

on those pathological conditions of increased erythrocyte destruction which are associated with the appearance of haematin and, possibly, some porphyrins. It is suggestive to assume that in these cases the occurrence of haematin might not only be the result of erythrocyte destruction but one of its causes.

SUMMARY

1. A haemolytic substance of high activity has been isolated from plasma and a number of animal organs.

2. Chemically the substance appears to be a mono-unsaturated, mono-carboxylic fatty acid with the chain length C_{18}.*

3. The haemolytic substance is widely distributed in the body. The relative content varies considerably in different organs and in different parts of the same organ, as, for example, in the central nervous system. In erythrocytes the substance is bound to, and inactivated by, the stromata.

4. Optimal haemolytic activity of the substance *in vitro* is dependent upon the presence of phosphate.

5. The body contains a large number of inhibitors of the haemolytic activity of the substance, notably proteins, cholesterol, lecithin and calcium, while none of a large number of normal constituents of plasma or erythrocytes accentuates its effect.

6. Haematin was found to be haemolytic by itself and in subhaemolytic concentration considerably to increase the effect of the haemolytic substance, especially in the presence of lecithin.

7. The presence of the haemolytic substance inside the erythrocytes is assumed to be related to their normal life span.

8. The existence of lysolecithin in the body, which has been claimed, has not been confirmed.

* Note added on 26 August 1949. The acid has been identified by Dr I. D. Morton as *cis*-11–12 octadecenoic acid [$CH_3.(CH_2)_5CH{=}CH.(CH_2)_9.COOH$] and this has been confirmed by synthesis.

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