

TISSUE HEMOLYSINS AS LYSIN-INHIBITOR COMPLEXES*

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(Received for publication, October 17, 1950)

The most unsatisfactory feature of the many attempts to establish that hemolysis *in vivo* may be due, in part at least, to the occurrence of circulating lysins derived from tissues is not the lack of descriptions of lytic substances, but that the accounts which have been given of their lytic activity are so widely different that they do not seem to refer to the same substance or even to the same group of substances. This is partly the result of some of the lysins having been obtained by extracting tissues, either as minces or as slices, with saline (Weil, 1907; Maegraith, Findlay, and Martin, 1943 *a, b*; Ponder, 1944; Brückmann and Wertheimer, 1945; Gross, 1947; Tyler, 1949), while others have been obtained by extracting with alcohol, acetone, and ether (Bergenheim and Fahraeus, 1936; Singer, 1940, who identified an alcohol-soluble and ether-insoluble lytic material as lysolecithin or a closely related substance, an identification which has been questioned by several investigators;¹ Laser, 1950, who has identified an ether-soluble substance, reported to be present in various tissues by a number of earlier workers, as *cis* 11-12 octadecenoic acid). While most observers are agreed that the lysins are not species specific, the descriptions are further complicated by different tissues having been used; *e.g.*, Weil used dog kidney and human tumors, most of Maegraith, Findlay, and Martin's work was done with human, monkey, and guinea pig tissues, Gross used mouse and human tumors, Singer obtained lysolecithin from plasma, and Laser extracted his ether-soluble lysin from human plasma and a variety of animal tissues.

Speaking generally, the lysins extracted with saline are weak; they take hours to produce complete hemolysis, and seem entirely different from the lysins extracted with alcohol and particularly from those extracted with ether, in that the latter produce hemolysis in very much shorter times. The purpose of this paper is to develop the simplifying idea that the lytic substances of tissues and plasma exist as enzymatically produced and feebly hemolytic lysin-inhibitor complexes from which highly lytic components can be extracted with

* This work was carried out under a grant from the American Cancer Society through the Committee on Growth of the National Research Council.

¹ Extensive references to the earlier literature will be found in the papers by Weil (1907) and by Laser (1950).

various solvents; these highly lytic substances are, however, laboratory creations in the sense that they result from the breaking up of naturally occurring complexes.

General Description of Method

Although the details of the methods used vary with the different aspects of the problem, there are certain general features which can be described here in order to avoid repetition.

Tissues.—Mouse liver (with care to avoid including the gall bladder), mouse kidney, and mouse lung have been used almost exclusively, although a few experiments have been repeated with rabbit liver and kidney, guinea pig liver, and human tissues obtained at operation so as to establish that the results are not peculiar to the mouse. The mice used are of the C3H strain, selected because this strain develops spontaneous mammary tumors which contain the hemolytic material described by Gross. The animals can be anesthetized with chloroform, ether, or nembital without affecting the results. The liver and kidneys are removed aseptically, and 0.5 to 1.0 ml. of heart blood is withdrawn into heparinized saline. After rinsing in saline, the liver or kidney tissue is weighed; it may then be homogenized or cut into pieces either in the fresh state, immediately after being quickly frozen (Tyler), or after having been kept at -10°C . for periods as long as several days. These methods of treatment affect the result, the lytic material obtainable by saline extraction of fresh liver being considerably more active, or present in greater quantity, than that obtainable from frozen liver.

Homogenates are made in the homogenizer described by Wilbur and Skeen (1950), the amount of the medium (usually NaCl-buffer) being 5 times the weight of the tissue; this gives a "20 per cent homogenate." Slices are easily prepared by cutting out the tissue with a sharpened No. 3 cork borer, the cylinders which result being again cut with parallel razor blades set 2 mm. apart; uniform disks are particularly easily made if the tissue is frozen. Each such disk weighs about 80 mg.

Red Cell Suspensions.—These are usually made by suspending the thrice washed red cells of 1 ml. of heparinized mouse blood in 10 ml. of NaCl buffer at pH 7.0; this gives a "4 per cent" suspension. Mouse red cells tend to hemolyze on standing, so the suspensions are kept at 4°C . until required; the addition of 100 mg./100 ml. of glucose also improves the stability of the suspensions. It is not necessary to use the red cells from the same mouse as that from which the tissues (even when these are spontaneous mammary tumors) are taken, but it is usually convenient to do so.

Precautions as to Sterility.—Since long times are involved in the preincubation of homogenates and of tissue disks and also in the hemolysis of subsequently added red cells, it is important to guard against the systems being contaminated with hemolytic bacteria. All glassware, solutions, and instruments are sterile, and all manipulations are carried out by a technic which uses vaccine-capped tubes equipped with air vents attached to air filters, syringes of various capacities for transferring fluids, sterile mincers, and homogenizers, etc. More than ordinary precautions are necessary if a satisfactory degree of sterility is to result.

1. The Appearance of Lytic Substances during Preincubation

Freshly prepared saline extracts of normal mouse tissues are not hemolytic, but if the tissue is incubated in saline at 37°C. ("preincubation"), lytic substances appear in the surrounding medium. These substances can be detected, and their concentration can be measured, by adding red cells to the medium after removal of the tissue. The tissue may be in the form of slices, of a mince, or of a homogenate. Systems containing tissue slices added to red cell suspensions (such as those studied by most of the investigators who have worked on the lysins associated with tissue slices) are not ideal for quantitative work, since the effects of preincubation, which results in the production of the lytic material from the tissue, overlap the hemolytic effects of the material on the red cells of the systems. The effects of preincubation can be better studied in systems in which they are allowed to occur before the cells are added, as in the following procedures.

1. Lytic Activity as a Function of Time of Preincubation.—A 20 per cent homogenate is prepared from several mouse livers in NaCl-buffer at a pH between 6.5 and 7.0. It is placed in a water bath at 37°C., and from time to time 3 ml. samples are withdrawn and centrifuged. The ease with which a clear supernatant fluid is obtained depends on two related changes which occur during preincubation; the first is a coagulation effect which usually appears after about 6 hours, and the second is a decrease in pH, which usually falls to between 4.5 and 5.5, the extent of the fall being quite variable from preparation to preparation. These two effects are either a part of, or are accompanied by, a fermentation process. Until the coagulation begins, even vigorous centrifuging does not give a clear supernatant fluid. Two kinds of hemolytic system are prepared by adding 0.1 ml. of a 4 per cent mouse red cell suspension to 0.5 ml. of the supernatant fluids; in the first, the cells are added to the supernatant fluids without any adjustment of pH, and in the second the pH of the supernatant fluid is brought to 6.5 by adding small amounts of M/10 NaOH in M/10 Na₂HPO₄ buffer. The adjustment is conveniently made in the glass electrode vessel. The time for complete hemolysis in each system is measured at 37°C.

Fig. 1 shows the typical course of events, although the fall in pH is often smaller. The pH of the preincubated homogenate begins to fall almost immediately, and after about 6 hours falls more sharply. At this time there is evidence that a fermentation process is occurring; centrifuging a sample of the homogenate now gives a clear supernatant fluid, whereas centrifuging before this stage is reached gives a milky supernatant fluid which is non-lytic, the occurrence or non-occurrence of hemolysis being very difficult to determine because of the milkiness. Once clear supernatant fluids are obtained, it is easy

to show that they are very hemolytic when the pH is unadjusted (curve marked A, representative time for complete hemolysis 10 to 15 minutes) and much less hemolytic when the pH is adjusted to 6.5 (curve marked B, representative time for complete hemolysis, 1 to 2 hours). After long periods of preincubation, the time for complete hemolysis increases slightly both in unadjusted

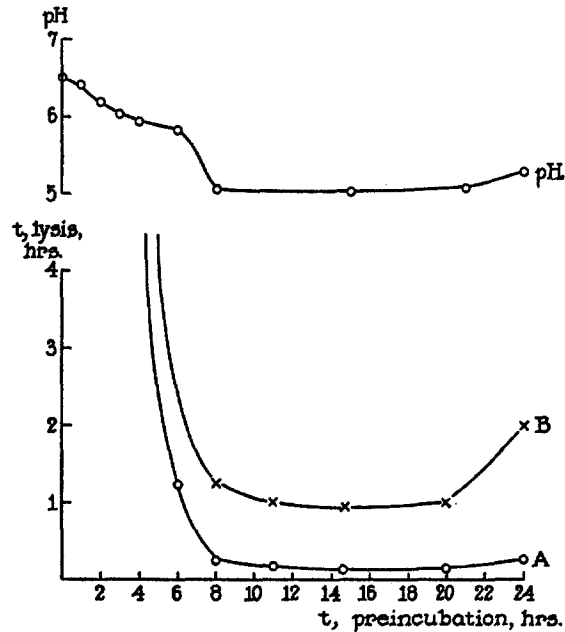


FIG. 1. To show the course of events occurring during preincubation of mouse liver homogenate.

Upper graph, change in pH of homogenate with duration of preincubation (abscissa).

Lower graph, ordinate, time required for complete hemolysis of mouse red cell suspension by supernatant fluid of homogenate incubated for various lengths of time (abscissa), A, with pH unadjusted, B, with pH adjusted to 6.5.

systems and at constant pH; in the case of a few homogenates, the pH has also been observed to increase by about 1 pH unit after long times.

It will be clear from the foregoing that no meaningful statement can be made about the concentration or activity of the lysins which are liberated into systems containing tissue slices, etc., unless the pH of the system is either specified or controlled. The lowest terms in which these systems could be described would therefore be those of lysin-accelerator systems (the variable acceleration being supplied by the variable decrease in pH). It will be seen below that in reality they are more complex than this because the lysin exists

in association with inhibitors. Quantitative treatment is therefore possible only in terms of the properties of lysin-accelerator-inhibitor systems.

2. *Effect of pH on Production of Lytic Material.*—Mouse liver homogenates (40 per cent) are made in NaCl and are added to equal volumes of phosphate buffers at pH 8.0, 7.5, 7.0, 6.5, and 6.0. The mixtures (now 20 per cent homogenates) are preincubated for 8 to 12 hours. The supernatant fluids are removed and their final pH's are determined; they are usually about 6.5, 6.0, 5.6, 5.2, and 4.8, the preincubation resulting in considerable acid production. Each supernatant fluid is brought to pH 7.0 by adding a few drops of $m/10$ NaOH in $m/10$ Na_2HPO_4 ; 0.5 ml. of each is taken, 0.1 ml. of a 4 per cent suspension of mouse red cells added to complete the hemolytic system, and the time for complete hemolysis is determined at 37°C.

The most active lytic material is found in the supernatant fluid of the homogenates with initial pH of 8.0 and 7.5. The supernatant fluids of homogenates with initial pH's on the acid side of neutrality have much less hemolytic activity. A pH optimum obviously cannot be satisfactorily determined in a system in which the pH falls by as much as 1.0 to 2.0 pH units; all that can be concluded is that the optimum for the production of the lytic material is situated on the alkaline side of pH 7.0, and probably on the alkaline side of pH 7.5. This must not be confused with the pH optimum for the lysis of mouse red cells by the material, which is situated at low pH's.

3. *Effect of Heat, Iodoacetate, etc.*—Lytic material is not found in the supernatant fluids of homogenates which have been heated to 100°C. for 5 minutes (*cf.* Tyler, 1949); heating also stops the fall in pH on preincubation. With a view to stopping the production of lytic material, homogenates may be heated to 100°C. for 5 minutes after they have been preincubated for various lengths of time. This results, however, in the disappearance of most of the lytic material which would have been found in the supernatant fluid of the homogenate, had it not been heated. The lytic material is probably carried down with the heat-coagulated protein. All observers agree that the effect of heat is due to the inactivation of an enzyme system; the step in the process which is affected will be discussed below.

Alternatively, sodium iodoacetate may be added, in sufficient quantity to give a 0.005 m concentration in the system, to volumes of homogenate after they have been allowed to preincubate for various lengths of time. An even smaller concentration of iodoacetate has been reported to stop the increase in lytic activity which occurs during preincubation (Tyler, 1949); it appears to do so, however, principally by reducing the pH shift. If a liver homogenate is prepared in 0.005 m sodium iodoacetate instead of in saline, a lytic supernatant fluid can still be obtained after 8 to 12 hours' preincubation, but the lytic activity is not as great as that in the supernatant fluid of a saline homogenate which has been preincubated for the same length of time. The pH, how-

ever, is about 1 unit higher in the iodoacetate homogenate, and the supernatant fluid obtained is usually not clear. The difference between the hemolytic activity of the fluid from the preincubated saline homogenate and that from the preincubated iodoacetate homogenate almost disappears if the fluids are adjusted to pH 6.5 before the red cells are added. This points to the effect of sodium iodoacetate being principally one on enzyme systems (the system involved in the fermentation reaction, for example, since gas formation is greatly reduced in systems containing iodoacetate) other than that which is directly involved in the production of the lytic material.

The effect of NaF is similar to that of iodoacetate. KCN in buffer at pH 7.0 and in a concentration of 0.02 M accelerates the production of lytic material in preincubated mouse liver homogenates.

2. Existence of Lysin-Inhibitor Complexes

It is helpful to think of the lysins which can be extracted from tissues, plasma, etc., as existing together with a mixture of proteins, lipids, and other substances which are inhibitors (or, in a few cases, accelerators) of the hemolysis which the isolated lysins produce. These inhibitors combine with the lysins to form partially reversible lysin-inhibitor complexes, some of the lysin being rendered inert and some of it being left free and capable of reacting with subsequently added red cells. The relations between the quantities bound and the quantities left free under a variety of circumstances have been extensively investigated (Ponder, 1948), but only one aspect of these relations bears on the present problem. It can be explained by considering the results which are obtained by the following experimental procedure.

Unit volume of a preincubated homogenate is extracted with fat solvents, and the extracted material is dissolved in unit volume of saline at a selected pH. A time-dilution curve, which may for the sake of clearness be thought of as a time-concentration curve ($c = 1/\delta$), is plotted to show the relation between various dilutions or quantities of the extracted material and the time which each takes to produce complete hemolysis (Fig. 2; this shows the results of an actual experiment²). Suppose that the undiluted extracted material takes time t_1 to produce complete hemolysis; let this time correspond to $c_1 = 1.0$ or to $\delta = 1.0$ on the curve. Unit volume of the supernatant fluid obtained by centrifuging the homogenate is extracted in an identical manner, the extracted material is dissolved in unit volume of saline at the same pH, and the time taken for it to produce complete hemolysis is again determined. Suppose that this time is t_2 , corresponding on the time-concentration curve to c_2 ; the fraction of the total extractable lytic material present in the supernatant fluid is then given

² Sometimes these time-dilution curves show a zone phenomenon, lysin being slower in high concentrations than in somewhat lower ones.

by the value c_2 . The time t_2 is nearly always found to be longer than t_1 , and c_2 is nearly always found to be smaller than c_1 . The hemolytic activity of the supernatant fluid itself is now determined at the same pH. Suppose that complete lysis takes a time t_3 , always longer than t_2 , and that t_3 corresponds to a quantity c_3 . The fraction of the total quantity of lysin which is free in the supernatant fluid is c_3 itself, and, on the assumption that the extraction does not give rise to an entirely new lysin which was not present, even in a state of combination, in the supernatant fluid, the quantity $\Delta = c_2 - c_3$ is a measure of the amount of lysin held in an inactive lysin-inhibitor complex in the supernatant fluid of the homogenate. Alternatively, the fraction $(1 - c_3/c_2)$ of the lysin

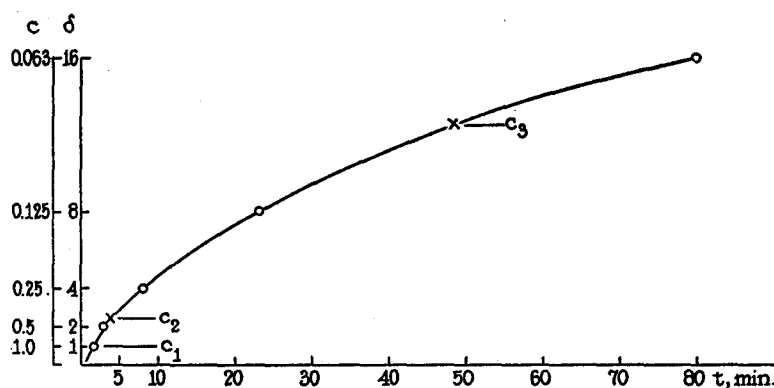


FIG. 2. Time-dilution curve for alcohol- and ether-soluble material from homogenate. Ordinate, concentration or dilution of lytic material in arbitrary units; abscissa, time for complete hemolysis by material extracted from homogenate (c_1), by material extracted from supernatant fluid of homogenate (c_2), and by the supernatant fluid itself (c_3). For further explanation see text.

in the supernatant fluid of the homogenate exists in the form of an inactive lysin-inhibitor complex.³

Procedure.—Mouse tissue (usually about 2 gm. of mouse liver) is homogenized in 5 times its weight of NaCl-buffer at pH 7.0 and the homogenate is preincubated at 37°C. for a time which can be varied but which is usually between 8 and 12 hours. A small volume (about 3 ml.) is set aside for extraction, and the remainder is centrifuged. About 3 ml. of the supernatant fluid is set aside for extraction; the remainder is used for the estimation of its pH and of its hemolytic activity. To determine the

³ The assumption is made that the combination of lysin and inhibitor is a Δ -reaction (Ponder, 1948), and that the lysin exists in either an active or an inactive state with no intermediate states of activity. This assumption is probable enough and is convenient, although not absolutely necessary, for the interpretation of the course of events.

latter, 0.1 ml. of a 4 per cent suspension of mouse red cells in NaCl-buffer at pH 7.0 is added to 0.5 ml. of the supernatant fluid, and the time for complete hemolysis is measured. This is t_3 , usually several hours.

The extraction of the homogenate is carried out by adding 3 ml. to 10 ml. of absolute alcohol, allowing to stand for about 1 hour at 25–30°C., filtering, and evaporating to dryness at the same temperature. Three ml. of ether are added to the dry residue with as thorough mixing as possible; after adding 7 ml. of acetone, the mixture is filtered and the filtrate is again evaporated to dryness at the same temperature. The extraction with 3 ml. of ether and 7 ml. of acetone is repeated, and the final dry residue is taken up in 3 ml. of alcohol. This is transferred to a 16 × 90 mm. Whatman diffusion shell, which is arranged to dip into a large vessel containing NaCl-buffer of the same pH as that found for the supernatant fluid of the homogenate (usually between pH 5.5 to 6.5). Care must be taken to lower the shell into the NaCl-buffer so that the level of the fluid inside it is the same as that outside. Diffusion is allowed to proceed for 24 hours at 4°C. The opalescent fluid inside the shell, which contains the lytic material, is removed, and its hemolytic activity is determined by adding 0.1 ml. of the 4 per cent suspension of mouse red cells in NaCl-buffer at pH 7.0 to 0.5 ml. of various dilutions, in powers of 2, of the fluid. This gives the time-dilution (or time-concentration) curve for the extracted hemolytic material of the homogenate, the units being such that the concentration of the undiluted material is represented by $c_1 = 1.0$; the time which this concentration takes to produce complete hemolysis is t_1 , usually only a few minutes.

The hemolytic material in the supernatant fluid derived from the preincubated homogenate is extracted similarly, starting with 3 ml. of supernatant fluid and ending with 3 ml. of opalescent fluid in the diffusion shell. In practice, the extraction of the homogenate and of its supernatant fluid is carried out side by side. The activity of the extracted hemolytic material of the supernatant fluid is determined in the same way as that of the extracted material of the homogenate; if it takes a time t_2 (nearly always $> t_1$, but usually a matter of minutes) to produce complete hemolysis, reference to the time-dilution curve shows that this corresponds to a concentration c_2 . Finally, reference to the time-dilution curve shows that the long time t_3 , which the supernatant fluid itself takes to produce complete hemolysis, corresponds to a still smaller concentration c_3 .⁴

Table I shows typical values of c_2 and c_3 found in the cases of homogenates of mouse liver and mouse kidney, mouse liver homogenate heated for 10 minutes at 100°C., and mouse liver prepared in 0.005 M sodium iodoacetate. The following conclusions can be drawn from the observations.

(1) The values of c_2 are always of the same order as those of c_1 (put equal to unity throughout), the variations being due to inequalities in the distribu-

⁴ If hemolysis does not occur at all in the supernatant fluid, the concentration c_3 will be the same as the asymptotic concentration of the time-dilution curve. Under these conditions all that can be said is that c_3 is less than a certain value; it may, however, be very much less, and may be zero.

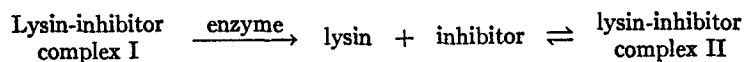
tion of the ether-soluble lysin between the clear supernatant fluid and the undispersed material which is thrown down by the centrifuge. (2) The values of c_3 are very much less than those of c_2 , a fraction amounting to between 0.76 and 0.93 of the lysin being apparently inactive. This fraction exists as a lysin-inhibitor complex which, to distinguish it from its precursor, will be called lysin-inhibitor complex II; it is characterized by existing side by side with free lysin in concentration c_3 , with which it is probably in partially reversible equilibrium. (3) In the case of the homogenate heated to 100°C., the value of c_3 is virtually zero. The supernatant fluid of this heated homogenate accordingly does not contain lysin-inhibitor complex II, although it contains a lysin-inhibitor complex from which ether-soluble lysin can be extracted (value of

TABLE I

Material	c_1	c_2	c_3	$(1 - c_1/c_2)$
Mouse liver homogenate	1.00	0.43	0.08	0.82
	1.00	0.25	0.06	0.76
	1.00	0.60	0.12	0.80
Mouse kidney homogenate	1.00	0.70	0.05	0.93
Mouse liver homogenate, heated 100°, 10 min.	1.00	0.55	0.015	0.097*
Mouse liver homogenate, in 0.005 IAA	1.00	0.75	0.03	0.96

* May lie between 0.97 and 1.00.

$c_2 = 0.55$). Call this latter lysin-inhibitor complex I; it is distinguished from lysin-inhibitor complex II by the lysin being completely bound. (4) The enzyme which is destroyed by heat is concerned with a conversion of the following type,



If the enzyme is supplied to the non-lytic supernatant fluid of a heated homogenate by the addition of small quantities (0.05 ml. or even less) of unheated homogenate, the mixture being maintained at 37°C. for some hours, the supernatant fluid of the heated homogenate becomes hemolytic; *i.e.*, lysin-inhibitor complex II is formed from lysin-inhibitor complex I. (5) The supernatant fluids of homogenates made with sodium iodoacetate give values of c_3 which are small, but not so small that they may be zero. The enzymatic process responsible for the conversion of complex I to lysin plus inhibitor is accordingly not com-

pletely inhibited by iodoacetate; indeed, most of the apparent iodoacetate inhibition is due to an effect on the pH of the system (see above).

Little can be said at the present time about the nature of the components of these lysin-inhibitor complexes. The lysin is evidently ether-soluble and may be identical with the lysin described by Laser (*cis* 11-12 octadecenoic acid, probably as its Na salt; *i.e.*, as a soap.⁵ As regards the inhibitors it is not necessary that the inhibitory component of complex I should be the same as the inhibitory component of complex II; indeed, it is likely that they are different. In complex I the lysin may be bound as a fatty acid is in a glyceride, or as fatty acids are in lipoproteins; the bond seems to be one which requires an enzyme to break it. Complex II, on the other hand, may be thought of as the relatively loose and partly reversible lysin-inhibitor complex which occurs familiarly in mixtures of lytic soaps with protein or lipoprotein. In the tissues, the lytic substance seems to be firmly bound as complex I; at any time, however, large quantities of free lysin can be extracted with alcohol and ether from either complex I or complex II. An important point which must be left undecided in the meantime is whether the unpreincubated tissue contains complex II in equilibrium with small amounts of free lysin; all that is certain at present is that complex II and free lysin are produced enzymatically from complex I during incubation, but it is reasonable to suppose that the same process may go on, on a much smaller scale, in tissues *in vivo*.

The general idea of the existence of these lysin-inhibitor complexes is supported by observations made with the ultracentrifuge (Tyler, personal communications), for the lytic material of adult guinea pig liver is found in the supernatant fluid after centrifuging at 100,000 *G* for 1 hour (microsome fraction), and is inhibited by material which is thrown down at a lower centrifugal force (25,000 *G*, mitochondrial fraction). Even the lytic material in the microsome fraction, however, must still be combined with the inhibitory components of a complex, for the lytic activity of this fraction, great as it is, is much less than that of the lytic components which can be extracted with alcohol and ether. Both the microsome fraction and the mitochondrial fraction are rich in lipoproteins, which could be the source of both lysins and inhibitors.

There is evidence that more than one identifiable species of lysin-inhibitor complex are present in tissues and in plasma (see section 4, below).

3. Diffusibility of the Lytic Material

Brückmann and Wertheimer (1945) have observed that shaking a hemolytic system containing tissue slices and red cells results in an inhibition of the hemol-

⁵ It may also be any one, or a mixture, of a large number of ether-soluble saturated and unsaturated fatty acids (palmitic, stearic, oleic, linolic, linolenic, arachidonic, myristic, or their soaps) which have been obtained from tissue (principally from fat) by Ellis and his coworkers (Ellis and Haskins, 1925; Ellis and Isbell, 1926.) Brinkman (1922) identified a lysin obtained from serum as linolenic acid.

ysis, and have suggested either that the red cells are more fragile in unshaken systems (the effect of stasis described by Ham and Castle, 1940 *a, b*) or that shaking lessens the probability that a red cell will come into contact with lysins liberated in the immediate neighborhood of the tissue slice. In systems containing tissue disks, it is easy to observe that lysis begins in close proximity to the disk; this in turn suggests that the lytic material liberated from the tissue has only a small diffusibility, as might be expected from its being a lysin-inhibitor complex which is probably of considerable size. This small diffusibility can be demonstrated in several ways.

One method uses glass 2-way stopcocks, which are ordinarily supplied with several centimeters of glass tubing, about 5 mm. in bore, on either side of the stopcock proper. A small cylinder of mouse liver or kidney, of the same size as the bore of the tubing, is introduced and arranged so as to lie about 10 mm. from the stopcock; by using a number of stopcocks each with its cylinder of tissue, the distance of the tissue from the stopcock can be varied from 2 mm. to 20 mm. A 1 per cent suspension of washed mouse red cells is then introduced into the tubing so as to occupy the space between the tissue and the stopcock and also so as to make a column about 20 mm. long above the cylinder of tissue; the stopcock is closed, and the tubing is placed at 37°C. either vertically or horizontally. When it is placed vertically, the cells in the suspension medium below the tissue fall away from it, leaving a column of clear fluid between them and the cylinder of tissue; the cells in the suspension medium above the tissue fall on to it, leaving a column of clear fluid above them. After some hours, lysis of the cells above the tissue, and in contact with it, nearly always occurs, but there is rarely lysis of the cells below the tissue even although the lytic material would have to diffuse downwards through only a few millimeters of saline. This shows that the lytic material is either slowly diffusible or of low density, or both. When the tubing is placed horizontally, the red cells fall to form a narrow strip running along its whole length on either side of the cylinder of tissue. Lytic material does not diffuse outward from the tissue, however, sufficiently far to produce lysis in even the few millimeters of this strip nearest to the tissue.

Alternatively, disks of tissue can be placed in small Petri dishes which are then filled to a depth of several millimeters with a 1 per cent mouse red cell suspension. The dishes are kept at 37°C.; the cells sediment to form a layer on the bottom of the dish and around the tissue. If lytic material were to diffuse from the tissue, one would expect it to hemolyze the cells in a ring around the disk; this is almost never observed even after times as long as 18 to 24 hours, and so it may be concluded that the diffusibility of the lytic material is small.

4. The Extraction of Lysolecithin-Like Substances

Lytic substances which are alcohol-soluble but insoluble in cold ether can be extracted from mouse liver and from a variety of other tissues, as well

as from serum, by the method described by Singer (1940). The ether precipitate should be washed several times with ether to remove traces of ether-soluble lysins, dried under a fan, redissolved in a small amount of water, dried again, and finally dissolved in saline. Although they have not been completely identified, these are the lysolecithin-like substances referred to by Bergenheim and Fahraeus, Bogaert, Singer, Gillespie, and others.⁶ Some investigators, particularly Brückmann and Wertheimer, Maizels, and Laser, have been unable to find them, possibly because a zone phenomenon is sometimes associated with the lysins, high concentrations being less lytic than lower concentrations.

The quantity of the lysolecithin-like substances extractable from the supernatant fluid of preincubated mouse liver is such that 0.5 ml. of a 1 in 16 dilution of the extract (prepared so as to contain all the alcohol-soluble but ether-insoluble substances derived from 1 gm. of liver in 5 ml. of the final extract) gives complete hemolysis of 0.1 ml. of a 4 per cent mouse red cell suspension in 60 to 90 minutes at 37°C.; *i.e.*, 0.1 ml. of this suspension is hemolyzed in this time by the lysolecithin-like substances of about 6 mg. of preincubated liver. This is a much greater activity than that present in the supernatant fluid of the preincubated liver homogenate, and so, as in the case of the ether-soluble lysins, the lysolecithin-like lysins must exist in association with inhibitors.

5. *The Color Changes in the Systems*

These are very conspicuous when the observations are extended over more than a few hours, but they have not been commented upon by other investigators who have worked with tissue lysins. In systems containing mouse liver disks and mouse red cells, lysis is either accompanied or followed by the conversion of oxyHb to metHb, and this is either accompanied or followed by the appearance of an opacity and then of a precipitate of denatured protein. This often makes it difficult to determine the end-point corresponding to complete hemolysis. Addition of 0.1 per cent ascorbic acid in NaCl-phosphate buffer (2 parts 0.75 per cent NaCl and 1 part M/15 phosphate buffer at pH 7.0) greatly delays the appearance of metHb but increases the amount of denatured protein;

⁶ The suggestion has been made that the lysis which Bogaert obtained was due to remaining traces of ether (Laser, 1950). Even 1 per cent ethyl ether in isotonic saline does not hemolyze human red cells at 37°C. It may be true, so far as isolation is concerned, that neither lysolecithin nor lecithinases have been isolated from plasma or tissue extracts, but the presence of these substances seems to have been satisfactorily demonstrated by the type of evidence usually accepted in enzyme chemistry (see Sumner and Somers, 1947, for references). The objections of Foy and Kondi and of Ham and Castle are directed against Bergenheim and Fahraeus' theories regarding *in vivo* hemolysis by tissue lysins, and not against the demonstration that ether-insoluble substances can be extracted; Gillespie confirmed Bergenheim and Fahraeus as to the results of extraction, and again his objections are concerned with the proposed hemolytic mechanism. Maizels could not confirm the results of extraction.

if added after the metHb has been formed, it reduces the latter to oxyHb within 15 to 30 minutes, but the denatured protein continues to precipitate. If the systems containing ascorbic acid are allowed to stand for 12 hours or more at 37°C. (preferably after removal of the liver disks), they become green, large quantities of denatured protein being precipitated. The reactions occurring in these systems are probably the same as those involved in the coupled oxidation of ascorbic acid and oxyHb, a peroxide intermediary entering into one reaction in which oxyHb is reversibly oxidized to metHb, and into another consecutive reaction which results in the formation first of choleglobin, and later of biliverdin, denatured globin, and a labile form of ferric iron (Lemberg and Legge, 1949).

The color changes are particularly interesting because Lemberg and Callaghan (personal communication from Dr. Lemberg) have shown that red cells containing choleglobin, prepared by incubating washed red cells with ascorbic acid in a slightly hypotonic medium, are unstable; some hemolysis occurs during the incubation, and the cells obtained at the end of it are easily hemolyzed by centrifuging. These cells contain about 12 per cent of choleglobin.

Experimental.—Systems containing 4 ml. of sheep, human, or mouse washed red cells in 50 ml. of 0.8 per cent NaCl-phosphate buffer at pH 7.2, to which 50 mg. of ascorbic acid is added, are warmed to 37°C. for 2 hours. At the end of this time, the cells show the dark color of choleglobin, the amount of which can be measured spectrophotometrically (Lemberg, Legge, and Lockwood, 1941); under these conditions the concentration is about 10 per cent. The cells are thrown down by gentle centrifuging, washed once with 0.8 per cent NaCl-buffer, and resuspended to a final volume of 4 ml. Systems treated similarly except that no ascorbic acid is added are used for comparison.

The resistance to hemolysis by hypotonicity, and in some cases the tonicity-volume relations, were investigated by methods already described (Ponder, 1950). The mechanical fragility was measured in a rotator similar to that described by Shen, Castle, and Fleming (1944) the rotator being run at a rate of 90 R.P.M. for 1 hour at 25°C. Resistances to saponin and to sodium taurocholate were measured by plotting time-dilution curves at 37°C. in the usual way.

In the case of each of the three species of red cell the choleglobin-containing cells were found to be less resistant to hypotonic hemolysis (a difference of 0.3 to 0.5 in the value of p at $T = 0.35$) than the cells containing oxyHb only. No differences were observed in the form of the tonicity-volume relation. Similarly, the choleglobin-containing cells were more mechanically fragile (a difference of 10 to 15 per cent hemolysis) than the cells containing oxyHb only. The presence of choleglobin in the cells did not make any difference, however, to the resistances to hemolysis by either saponin or sodium taurocholate.

These results confirm those of Lemberg and Callaghan. Lemberg and his collaborators suggest that the slow formation of choleglobin is the process by means of which the red cell ages, and that the accumulation of choleglobin to-

gether with its denatured product leads to such a state of instability that it ends the life of the cell. The accumulation of choleglobin is inhibited by catalase, and the duration of the life of the red cell may be related to the amount of catalase which it contains (Foulkes and Lemberg, 1949). It is possible that a similar process, occurring with much greater rapidity, contributes towards the hemolysis which occurs in the presence of tissue disks. As an extreme example, in a buffered system containing mouse red cells and 0.1 per cent ascorbic acid, the choleglobin-containing cells hemolyze spontaneously; in a system containing liver slices and mouse red cells, there is accordingly a hemolytic mechanism present by virtue of the ascorbic acid, reduced glutathione, and other hydrogen donors which the slices contain. While the hemolytic activity of such a mechanism is probably very small as compared to that of the lysin-inhibitor complexes described above, it is possible that *in vivo* hemolysis by the latter might be accelerated by the former.⁷

SUMMARY

1. Lytic substances are enzymatically produced at 37°C. from tissue slices or homogenates (mouse liver, kidney, etc.) and appear in the medium in which the tissue fragments are suspended. Their concentration increases with the time during which the tissue is kept at 37°C. (preincubation), and is accompanied by pH changes, so that the lytic activity as finally measured is a function of both the time of preincubation and of the pH. The optimum pH for lysin production is above 7.0, but the lysins, once produced, hemolyze red cells more rapidly at low pH's than at high ones. The enzyme system which produces the lysins is inactivated by heating to 100°C. for 5 minutes. Sodium iodoacetate and fluoride interfere with lysin production principally by reducing the concomitant pH shift; KCN accelerates the production of lytic material in mouse liver homogenates.

2. Comparison of the lytic activity of the supernatant fluid of a preincubated homogenate with the much greater lytic activity of the substances which can be extracted from the same supernatant fluid by alcohol and ether points to these extractable substances existing in the supernatant fluid as lysin-inhibitor complexes of relatively low lytic activity. These complexes are formed enzymatically during preincubation from non-lytic complexes in the tissue. The latter may be lipoproteins, and the highly lytic ether-extractable substances may be fatty acids or their soaps.

⁷ Granick has described swollen but intact red cells, found in freshly teased horse spleen, which take on a smooth grey appearance when treated with H₂S. This is due to a breakdown of heme and the release of iron within the red cell, no doubt as a stage in its destruction in the tissue concerned. The same phenomenon can be recognized, with less certainty, as occurring in horse marrow, and in the spleens of rabbit, guinea pig, and man.

3. The diffusibility of the lysin-inhibitor complexes is small.
4. Lytic substances which are ether-insoluble can be extracted with alcohol from tissues as well as from serum. These "lysolecithin-like" substances exist in the supernatant fluids of homogenates as lysin-inhibitor complexes.
5. Lysis of mouse red cells by substances contained in mouse tissue (liver and kidney) is often accompanied by the formation of methemoglobin and choleglobin. Mouse red cells containing choleglobin are abnormally fragile both osmotically and mechanically, and it is possible that a process involving the production of choleglobin, accompanied or followed by globin denaturation, is one which contributes towards the hemolysis which occurs in systems containing tissue slices or homogenates.

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