FACTORS AFFECTING THE HEMOLYTIC ACTION OF "LYSOLECITHIN" UPON RABBIT ERYTHROCYTES*

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The effect of various factors upon the hemolytic action of lysolecithin with washed rabbit erythrocytes is described in this paper. Wilbur and Collier (1943) used a photoelectric method to investigate the hemolytic action of lysolecithin; and the present investigation is a continuation of their work, with improved methods.

The term "lysolecithin" has been used to describe the hemolytic substance formed by the action of snake venoms on egg yolk; but as pointed out by Levene, Rolf, and Simms (1924) the product contains a small proportion of lysocephalin as well as lysolecithin. Throughout the present paper the abbreviation LL will be used to designate this mixture of lysolipids.

Methods

Lysolipid was prepared by the action of rattlesnake venom upon egg yolk, according to the method of King and Dolan (1933). The reprecipitated and dried product had a total phosphorus content of 5.16 per cent, as compared with the range of 5.19 to 5.72 per cent found by King and Dolan in their various fractions. (The theoretical P content of pure palmitic lysolecithin is 6.04 per cent and of stearic lysolecithin is 5.73 per cent.) The product was dissolved in absolute ethanol as a stock 1:1000 solution and this was diluted to 1:4000 with absolute ethanol for daily use.

The method of measuring hemolysis was that of Wilbur and Collier (1943), with certain modifications as described below. Rabbit blood was obtained by heart puncture and heparinized. The cells from 1 ml. of blood were washed three times in the centrifuge with isosmotic phosphate-buffered saline of pH 7.3, and were made up to 50 ml. volume with the saline. Unless otherwise stated, 1 ml. volumes of this 1:50 dilution were added to 4 ml. volumes of the buffered saline, in a series of small test tubes in a water bath at 25°C. The resultant 1:250 dilution of washed erythrocytes is referred to as the "standard suspension." It contained approximately 2.4×10^7 cells per ml. and gave a transmittancy of about 50 per cent in the photometer. Increasing volumes of the 1:4000 LL were added to the tubes, and after mixing they were allowed to stand for 15 minutes at 25°. (The volumes added were usually in the range of 20 to 100 μl., and these small amounts of ethanol were found to have no influence upon the action of the lysin or the fragility of the cells.)

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At the end of the 15 minute period the opacities of the cell suspensions were determined in the lumetron photometer, model 401, fitted with the red filter B660. The estimations of percentage hemolysis are based upon the assumption that the observed opacity of a cell suspension (expressed in extinction units, $2 - \log T$) is proportional to the concentration of unlysed cells, after deduction of a small correction for light absorption by hemolysate. Actual cell counts upon partial hemolysates gave excellent correlation with photometric readings, showing that this assumption was justified. On the other hand, centrifugation, followed by determination of free hemoglobin in

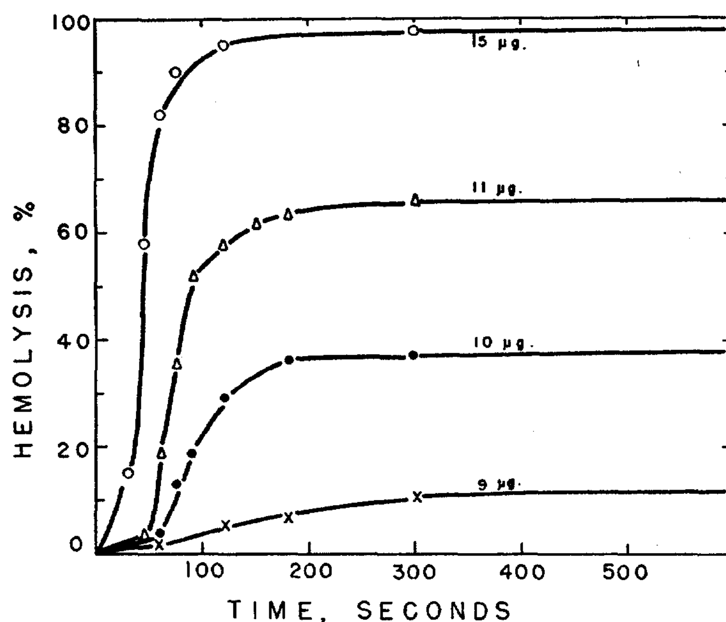

FIG. 1. The time course of hemolysis by lysolecithin. The amount of LL added to 5 ml. of standard cell suspension is indicated on the curves.

the supernatant, gave results that were consistently too high. Evidently centrifugation causes a slight additional hemolysis.

In the study of the effect of various factors on the hemolytic action of LL, 50 per cent hemolysis was taken as the end-point. Degree of hemolysis was plotted against the amount of lysin added, and the amount required for 50 per cent lysis was obtained by interpolation. Thus the mean resistance of the erythrocytes could be expressed in micrograms of LL per 5 ml. of standard suspension.

RESULTS

1. Time Course of Hemolysis.—Varying amounts of LL were added to standard cell suspensions, and the time course of the lytic action was followed photometrically. Typical curves are given in Fig. 1.

The curves are sigmoid, with a short latent period, then a period of very

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rapid action followed by an abrupt cessation of hemolysis. The action was virtually completed in 10 minutes or less, at room temperature. Gorter and Hermans (1943) made the same observation and suggested that the LL is not released following hemolysis, as otherwise the lytic action would continue.

The fact that hemolysis virtually ceases in about 10 minutes led us to modify the method of Wilbur and Collier (1943). They had noted the time required for 50 per cent hemolysis with a fixed lysin concentration; and Collier and Wil-

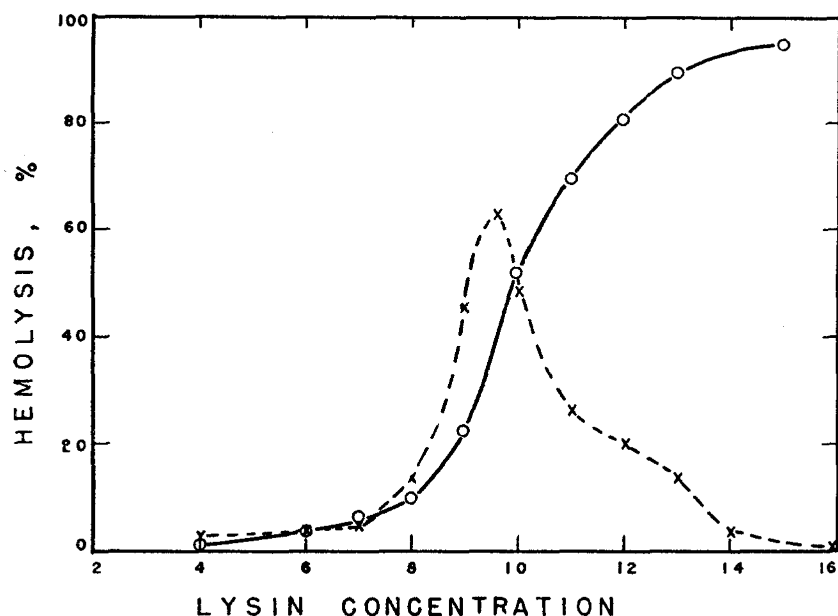

FIG. 2. The relationship between percentage hemolysis and lysin concentration (solid line). Lysin concentration is in μg. per 5 ml. of standard cell suspension. The broken line, indicating the distribution of cell resistance to lysis, is obtained by graphical differentiation of the solid line.

bur (1944) had determined the concentration of LL required to cause 50 per cent lysis in 60 seconds. It is obvious that these procedures did not measure equilibrium values because of the short intervals employed, and the present method was therefore adopted. This consisted in allowing varying concentrations of LL to act for 15 minutes at room temperature—when it could be assumed that the action had virtually ceased—and then measuring the percentage hemolysis. The former procedure will be referred to as the "rate" method, the present as the "equilibrium" method.

2. *Concentration of Lysin.*—To a series of tubes containing standard cell suspension were added increasing amounts of LL, and after 15 minutes the degree of hemolysis in each tube was measured. Fig. 2 illustrates the type of

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curve obtained. Graphical differentiation of the percentage hemolysis curve gives the resistance distribution of the cell population, indicated in the figure as a broken line.

Ponder (1948, p. 209) has given several examples of similar resistance distribution curves. Gorter and Hermans (1943), on the other hand, obtained straight lines for their percentage hemolysis curves with LL, which would be possible only if all the cells had the same resistance to lysis.

3. *Cell Concentration*.—The concentration of the cell suspension was varied, and the amount of LL required for 50 per cent hemolysis was determined, with the results shown in Table I. It is evident that, over the limited range employed, the amount of LL required per cell varied markedly with the cell concentration. Since the more dilute suspensions required relatively more LL it appears that the lysin adsorbed on to the cell surface was in equilibrium with that dissolved in the medium. Ponder (1948, p. 192) has given a mathematical analysis of this phenomenon.

TABLE I

The Effect of Erythrocyte Concentration on the Action of Lysolecithin

Cell concentration	LL for 50 per cent hemolysis	
millions per 5 ml.	*μg. per 5 ml.*	*μμg. per cell*
62	12.7	0.205
83	12.8	0.154
124	17.7	0.143
155	19.0	0.123

Gorter and Hermans (1943), using relatively concentrated cell suspensions, found that the number of erythrocytes hemolyzed by a given amount of LL was independent of the concentration of the cell suspension.

4. *Temperature*.—Varying amounts of LL were added to three series of tubes in water baths at 3, 20, and 37°C., and for each temperature and each lysin concentration the time course of hemolysis was followed. At 20 and at 37° the action was complete in less than 15 minutes, but at 3° an interval of about 3 hours was required. The results are summarized in Table II in which only the final equilibrium values are recorded. The amounts of LL required for 50 per cent hemolysis at each temperature were obtained by interpolation from the equilibrium values.

It is clear from these experiments that the *rate* of hemolysis is decreased as the temperature is lowered, but that the *extent* of hemolysis is increased. Apparently more lysin is adsorbed on to the cells at lower temperatures, and less at higher temperatures.

Maizels (1946) found that saponin, oleate, and bile salts varied markedly in

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their response to temperature changes. Ponder (1948, p. 190) has discussed the very complex effects of temperature upon saponin hemolysis.

5. Tonicity Changes.—The rate method of Wilbur and Collier showed that decreasing tonicity of the medium, accompanied by swelling, markedly accel-

TABLE II

The Effect of Temperature upon the Degree of Hemolysis by Varying Concentrations of Lysolecithin

Temperature	LL added	Time	Hemolysis	LL for 50 per cent hemolysis
°C.	*μg. per 5 ml.*	*min.*	*per cent*	*μg. per 5 ml.*
3	7.5	170	34	
3	8.8	170	73	8.1
3	10.0	170	84	
20	10.0	15	44	
20	11.2	15	69	10.3
20	12.5	15	85	
37	11.2	15	18	
37	12.5	15	41	13.3
37	15.0	15	64	

TABLE III

The Effect of Tonicity of the Medium upon Erythrocyte Resistance to Lysolecithin

Tonicity, fraction of isotonic	LL for 50 per cent hemolysis	Opacity of cell suspension
	μg. per 5 ml.	
0.7	22.0	0.123
0.8	21.2	0.173
0.9	22.0	0.183
1.0	22.0	0.198
1.2	21.8	0.190
1.7	22.4	0.225
2.2	22.4	0.252

erated the action of LL. Maizels (1946) obtained similar results with his method of measuring per cent lysis at fixed lysin concentration.

With the equilibrium method the concentration of LL required to cause 50 per cent hemolysis at various tonicities was determined. Table III shows that the cell resistance, under these conditions, was virtually independent of tonicity and initial cell volume.

6. Effect of H Ion Concentration.—Cell suspensions in buffered saline were adjusted to various pH values by the addition of HCl or NaOH and the con-

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centration of LL required for 50 per cent hemolysis was then determined. Table IV gives the results of a typical experiment. The opacity readings of the cell suspensions indicate changes in volume with pH, such as were reported by Wilbur and Collier (1943). Increased opacity represents decreased mean cell volume. The hemolysis readings are not corrected for volume changes, as it has been shown above that cell resistance to LL is independent of cell volume under these conditions. It is concluded, therefore, that the H ion concentration, within the range tested, has little effect upon the resistance of the cells to LL.

Wilbur and Collier (1943) using the rate method observed a maximum rate of hemolysis at about pH 6, within the range of pH 5.4–7.9. Maizels (1946) obtained a minimum degree of hemolysis at pH 7–8, when he incubated human erythrocytes for 1 hour with LL. He then varied the concentrations of his buffers to give constant cell volume, and within the range of pH 6–8 found that hemolysis was decreased as the pH was raised. This agrees with our finding that,

TABLE IV

The Effect of pH upon Hemolysis by Lysolecithin

pH	Opacity of cell suspension	LL for 50 per cent hemolysis
		μg. per 5 ml.
5.52	0.197	8.7
6.32	0.206	9.5
6.80	0.217	10.7
7.27	0.234	11.2
7.80	0.250	11.2

as the pH is raised, more lysin is required for the same degree of hemolysis. (It should be noted that the rather marked changes in degree of lysis as recorded by Maizels would correspond to very small changes in effective lysin concentration, if the values fell upon the steep portion of the concentration-hemolysis curve.)

7. *Effect of Cupric Ions.*—Jacobs and Corson (1934) showed that exceedingly low concentrations of copper salts inhibited the penetration of glycerol into the human erythrocyte. In the present investigation a standard cell suspension was made in unbuffered isosmotic NaCl and 10^{-5} M $CuSO_4$ was added. The LL required for 50 per cent hemolysis of 5 ml. of suspension was 16.9 μg., both in the presence and in the absence of the copper salt. Hence the cupric ions had no apparent effect upon the action of LL.

8. *Effect of Calcium and Magnesium Ions.*—Mayer, Osler, Bier, and Heidelberger (1946) found that certain divalent cations, such as Mg^{++}, Ca^{++}, Ni^{++}, Co^{++}, were necessary for the hemolytic action of the C′ component of complement, and that Mg^{++} showed the greatest activity.

The veronal-buffered saline of these workers was used by us to investigate

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the effect of Ca and Mg ions on the action of LL. Mg ions had little effect. Ca ions caused a marked increase in the opacity of the cell suspensions in the presence of LL, but not in its absence. This opacity increase was not due to an inhibition of hemolysis but was the result of a shrinkage of the erythrocytes, accompanied by a slight acceleration of hemolysis.

Calcium chloride added to a standard cell suspension to a concentration of 10^{-3} M caused a cell shrinkage in the presence of LL. In order to measure this volume change accurately, however, a stronger suspension was employed (3×10^9 cells per 5 ml.). To this was added $CaCl_2$ solution to 0.025 M and a proportionately higher concentration of LL. The percentage hemolysis was determined by cell counts, and the mean corpuscular volume by centrifuging in Bauer-Schenck tubes (Kimble No. 46815). Opacity was measured after a 1:25 dilution. The results of such an experiment are found in Table V.

TABLE V

The Effect of Calcium Ions upon the Cell Volume and Degree of Hemolysis by Lysolecithin

0.20 mg. of LL acting for 15 minutes upon 3×10^9 cells in a total volume of 5 ml.

Calcium ion concentration	Cell opacity after dilution	Mean cell volume	Hemolysis
mM		μ^3	*per cent*
0	0.321	84	13.4
25	0.403	60	19.6

It was also observed that 0.010 M $CaCl_2$ in the presence of LL caused a great increase in the opacity of a standard cell suspension in unbuffered NaCl, pH 6.4. The actual cell volumes, however, were not measured.

The role of the Ca ions in shrinking the erythrocytes in the presence of LL is a matter for speculation. It has been suggested by Alexander, Teorell, and Åborg (1939) that the bivalent Ca ions might bind together adjacent phosphate groups in cephalin layers, and such an action might cause shrinkage of the erythrocytes. Evidently the adsorption of LL is necessary for the action of the Ca ions.

9. Acetylphenylhydrazine.—Acetylphenylhydrazine is a well known *in vivo* hemolytic agent. Collier (1947) observed an acceleration of LL hemolysis by 10^{-3} M acetylphenylhydrazine, and obtained an R value of 0.78 by Ponder's method of calculation (1948, p. 260). No changes in shape or color of the cells were observed. As reported by Chen and Collier (1951) the drug reacts with protein and lipoprotein monolayers, which may explain its action in accelerating hemolysis.

10. Effect of LL on Osmotic Properties of the Erythrocyte.—To test the effect of LL on osmotic fragility, cell suspensions were made up in buffered saline of varying tonicity, and a non-lytic concentration of LL was added to each tube,

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5 μg. to 5 ml. After 30 minutes the degree of hemolysis was estimated photoelectrically. The results are recorded in Table VI and it is evident that the osmotic resistance was actually increased in the presence of LL; *i.e.*, the fragility was decreased.

Ponder (1948, p. 104) observed a similar effect with lecithin and discussed possible explanations. Since many lysins cause prolytic loss of K^+, such a change may decrease the cell fragility, through loss of osmotically active salt.

In order to measure the prolytic loss of K^+, 0.10 mg. of LL was added to 10 ml. of a 1:20 cell suspension (about 3×10^9 cells). The amount of potassium was then determined in the treated cells, and in an aliquot of untreated cells. The control was found to contain 177 μg. of K, and the treated cells 178 μg., after a correction for about 5 per cent hemolysis. The method of potassium determination, which gave a precision of about ± 2 per cent, was as follows:—

TABLE VI

The Effect of a Sublytic Concentration of Lysolecithin upon Hypotonic Hemolysis

Tonicity, fraction of isotonic	Hemolysis	
	No LL	LL, 5 μg. per 5 ml.
	per cent	*per cent*
0.4	100	100
0.5	98	88
0.6	38	19
0.7	19	10
1.0	0	0

The cell suspension was treated with copper tungstate as a protein precipitant, according to the method of Weichselbaum, Somogyi, and Rusk (1940), and the potassium in the filtrate was precipitated with cobaltinitrite. Complete precipitation of the very small amounts of K was achieved only by standing overnight in the presence of ethanol, as recommended by Stephenson (1945). The precipitate was washed with the water-alcohol-ether mixture of Harris (1940); it was then dissolved in NaOH, diazotized with sulfanilamide, and coupled with naphthylethylenediamine, as described by Looney and Dyer (1942). The color thus developed was compared photometrically with the colors of K standards which were run simultaneously through the whole procedure.

Penetration of thiourea into LL-treated cells was observed by the method of Jacobs, Stewart, Brown, and Kimmelman (1949). The lumetron photometer was used as a direct reading instrument and galvanometer deflections, to indicate opacity, were observed every 10 seconds. It was found that addition of 3 ml. of 0.3 M thiourea to 2 ml. of a 1:100 cell suspension (about 1.2×10^8 cells) resulted in complete hemolysis in about 3 minutes. The presence of sublytic

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quantities of LL greatly increased the rate of thiourea penetration, as illustrated in Table VII.

TABLE VII

The Effect of a Sublytic Concentration of Lysolecithin upon Rate of Thiourea Penetration

2 ml. of cell suspension + 3 ml. of 0.3 M thiourea.

Time	Opacity of cell suspension	
	No LL	LL, 5 μg. per ml.
sec.		
10	0.236	0.229
20	0.225	0.114
30	0.194	0.041

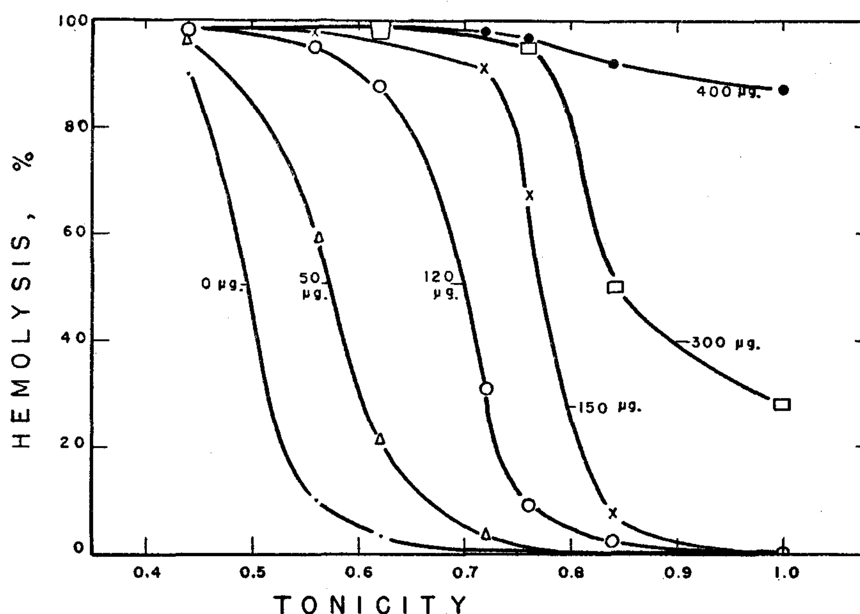

FIG. 3. Plots, by the Wilbrandt technique, of hemolysis of cells subjected to varying concentrations of lysin and varying tonicities. The amount, in μg., of LL added to 5 ml. of standard suspension is indicated for each curve.

Finally, the technique of Wilbrandt (1941) was used to elucidate further the nature of the action of LL upon erythrocytes. Cells treated with varying concentrations of LL were placed in buffered saline solutions of varying tonicity, and after 30 minutes the degree of hemolysis was measured photoelectrically. The curves thus obtained are given in Fig. 3. (We have plotted percentage

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hemolysis rather than photocurrent.) The curves resemble those given by Wilbrandt to represent "colloid-osmotic" hemolysis, as distinct from non-osmotic hemolysis.

DISCUSSION

Our observations confirm those of Gorter and Hermans (1943) who found that the hemolytic action of LL is rapid for the first few minutes and then ceases abruptly. This is in contrast with a lysin such as saponin, whose action may continue for hours. Gorter and Hermans suggested that the LL which is adsorbed by the cell is not released following hemolysis, and therefore is not able to attack other cells. Thus, under given conditions, a certain amount of LL hemolyzes a fixed number of erythrocytes.

We have found that the amount of LL required to lyse a given number of erythrocytes depends upon the concentration of the cell suspension: this points to an equilibrium between the LL in solution and that adsorbed on the cell. The fact that hemolysis by LL is more extensive as the temperature is lowered is a further indication of the importance of adsorption of the lysin in the hemolytic process.

Gorter and Hermans (1943) estimated that the amount of LL required for complete hemolysis was just sufficient to form a monolayer on the surface of the cells. We have confirmed this estimate from our own measurements. However, this correspondence between the area of the LL, as a monolayer, and the surface area of the erythrocyte may be of no significance, since it is believed that LL acts primarily by combining with the free cholesterol of the cell membrane. As pointed out by Collier and Chen (1950) the amount of LL required for complete lysis of washed rabbit erythrocytes is roughly equivalent, on a molar basis, to the cholesterol content.

There is insufficient cholesterol in the red cell to form a monolayer on the surface, although it should be pointed out that Ponder's estimate (1948, p. 131) of 10^3 A^2 of cell surface per cholesterol molecule is too great by a factor of 10. Since rabbit erythrocytes contain about 10^{-10} mg. of cholesterol per cell, and the limiting area of the cholesterol molecule is about 39 A^2, the cholesterol should occupy an area of 62 μ^2. The average surface area of the rabbit erythrocyte is 110 μ^2, hence the cholesterol could not occupy much more than one-half of the cell surface. In any case there is evidence that the erythrocyte surface is a mosaic of lipid and protein structures, rather than an area completely covered by a layer of lipid (Ponder, 1948).

Wilbur and Collier (1943) found that tonicity and cell volume markedly influenced the *rate* of hemolysis by saponin and by LL. The use of the equilibrium method, on the other hand, has demonstrated that the resistance to LL is independent of the initial volume. Possibly the erythrocyte surface, irrespective of its shape, requires a fixed amount of LL for hemolysis, provided that the critical volume has not been reached. In the macrocytosis following hemoly-

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tic anemia in rabbits, Collier and Chen (1950) found that the resistance of the erythrocytes to LL varied with the mean volume, and therefore with the cell surface.

The marked shrinkage of the erythrocytes that is induced by calcium ions in the presence of LL is of considerable interest,[1] and recalls the well known observation of Leathes (1925), that calcium salts inhibit myelin formation by lecithin. Alexander, Teorell, and Åborg (1939) have suggested that calcium ions may bind adjacent acid groups; and according to Furchgott and Ponder (1941) the phosphate groups of cephalin are probably the chief acid groups of the cell surface. Whatever the action of the calcium ions may be, the manner in which LL might make the acid groups available to these ions is not clear. Studies on a possible binding of calcium ions by the cell surface are now under way.

The resistance curves obtained by the technique of Wilbrandt (1941) indicate that the hemolytic action of LL is of the "colloid-osmotic" type. The actual hemolysis is believed to be preceded by cation permeability, and Jacobs and Stewart (1947) have provided a general discussion of this phenomenon. We failed to detect loss of potassium in the presence of a sublytic amount of LL; but further experiments on possible cation exchange should be carried out. The slight decrease in osmotic fragility in the presence of LL does suggest loss of electrolyte.

Whether or not LL increases cation permeability, there is no doubt that sublytic concentrations cause definite alterations in the erythrocyte membrane. The discs become spheres; hypotonic fragility is decreased; the rate of penetration of thiourea is greatly increased; and calcium ions cause considerable shrinkage.

SUMMARY

1. Lysolipid was prepared by the action of snake venom on egg yolk, and a study was made of the factors affecting its hemolytic action upon rabbit erythrocytes.

2. Lysis proceeded very rapidly at first, then ceased within a few minutes at room temperature. A given amount of lysin appeared to hemolyze a fixed number of cells, under specified conditions.

3. The more dilute erythrocyte suspensions required relatively more lysin per cell, for 50 per cent hemolysis of the suspension. There may be an equilibrium between the lysin dissolved in the medium and that adsorbed on the cells.

[1] Collier and Barschel (unpublished observations) have recently noted that ghosts prepared by hypotonic hemolysis and precipitated by dilute acid in the presence of calcium ions pack to a smaller volume than the ghosts precipitated in the absence of calcium.

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4. The degree of hemolysis for varying lysin concentrations was measured, and the cells showed a typical distribution of resistance to hemolysis.

5. As the temperature was lowered lysis was more extensive. Adsorption of the lysin on the cell surface was apparently increased.

6. The resistance of the erythrocytes to lysis increased slightly as the pH was raised from 5.5 to 7.8.

7. Resistance to lysis was independent of the tonicity of the medium and of initial cell volume. The magnitude of the cell surface was probably the determining factor.

8. A marked shrinkage of the erythrocytes was observed in the presence of calcium ions and lysin, but not in the absence of the lysin.

9. Hemolytic resistance curves obtained by the Wilbrandt technique were of the "colloid-osmotic" type. However, there was no evidence of prolytic loss of potassium ions.

10. Hypotonic fragility of the cells was slightly increased in the presence of the lysin. The rate of penetration of thiourea was greatly increased.

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REFERENCES

Alexander, A. E., Teorell, T., and Åborg, C. G., 1939, *Tr. Faraday Soc.*, **35,** 1200.

Chen, H. L., and Collier, H. B., 1951, *J. Gen. Physiol.*, **35,** 17.

Collier, H. B., 1947, *Fed. Proc.*, **6,** 245.

Collier, H. B., and Chen, H. L., 1950, *Canad. J. Research*, sect. E, **28,** 289.

Collier, H. B., and Wilbur, K. M., 1944, *J. Lab. and Clin. Med.*, **29,** 1123.

Furchgott, R. F., and Ponder, E., 1941, *J. Gen. Physiol.*, **24,** 447.

Gorter, E., and Hermans, J. J., 1943, *Rec. trav. chim. Pays-bas*, **62,** 681.

Harris, J. E., 1940, *J. Biol. Chem.*, **136,** 619.

Jacobs, M. H., and Corson, S. A., 1934, *Biol. Bull.*, **57,** 325.

Jacobs, M. H., and Stewart, D. R., 1947, *J. Cell. and Comp. Physiol.*, **30,** 79.

Jacobs, M. H., Stewart, D. R., Brown, W. J., and Kimmelman, L. J., 1949, *Am. J. Med. Sc.*, **217,** 47.

King, E. J., and Dolan, M., 1933, *Biochem. J.*, **27,** 403.

Leathes, J. B., 1925, *Lancet*, **1,** 957.

Levene, P. A., Rolf, I. P., and Simms, H. S., 1924, *J. Biol. Chem.*, **58,** 859.

Looney, J. M., and Dyer, C. G., 1942, *J. Lab. and Clin. Med.*, **28,** 355.

Maizels, M., 1946, *Quart. J. Exp. Physiol.*, **33,** 183.

Mayer, M. M., Osler, A. G., Bier, O. G., and Heidelberger, M., 1946, *J. Exp. Med.*, **84,** 535.

Ponder, E., 1948, Hemolysis and Related Phenomena, New York, Grune and Stratton.

Stephenson, N. R., 1945, *J. Lab. and Clin. Med.*, **30,** 80.

Weichselbaum, T. E., Somogyi, M., and Rusk, H. A., 1940, *J. Biol. Chem.*, **132,** 343.

Wilbrandt, W., 1941, *Arch. ges. Physiol.*, **245,** 22.

Wilbur, K. M., and Collier, H. B., 1943, *J. Cell. and Comp. Physiol.*, **22,** 233.

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