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HEMOLYSIS INDUCED BY *PRYMNESIUM PARVUM* TOXIN KINETICS AND BINDING

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SUMMARY

Rates of hemolysis of rabbit erythrocytes induced by *Prymnesium parvum* toxin (prymnesin) have been measured colorimetrically at 25.5°. The data have been treated as consecutive first-order rate processes associated with the prolytic and lytic period for which two specific rate constants were obtained (k' and k_ψ, respectively). Both are a function of concentration and temperature, though only the first constant has a linear Arrhenius relationship. The variation of the second constant with concentration of prymnesin follows Michaelis–Menten kinetics. Both constants are decreased by inhibitors (cholesterol and cephalin (approx. 10 μM)) at a given concentration of prymnesin, and the effect is ascribed to reduction in the effective concentration of prymnesin owing to the formation of a toxin–inhibitor complex. The binding of prymnesin to erythrocyte membranes during the prolytic period was investigated using tritium-labeled toxin, and it was found that about 10 % of the labeled material is loosely bound and about 30 % is more firmly bound.

INTRODUCTION

The euryhaline chrysomonad *Prymnesium parvum* (Carter) produced toxins that have hemolytic, ichthyotoxic, and cytotoxic activities[1-3]. Few studies have been concerned with the chemical nature of the hemolytic toxin (prymnesin)[2]. Useful purification schemes have been devised[1,2], and the properties of purified toxin are similar to those of acid polar lipids[2]. Recent work by ULITZUR AND SHILO[2] indicated the material to be a lipid–carbohydrate–protein complex, and presumably the chemical structure of prymnesin is being studied in more detail.

Comparatively little attention, however, has been given to characterizing the hemolytic activity of prymnesin, though this property is used as an assay of purity. Typically, the activity is expressed as hemolytic units, the amount of toxin needed to induce hemolysis of erythrocytes within a certain period under defined conditions. However, hemolysis is a complex set of processes. For example, previous studies by PADILLA[1] and RAUCKMAN AND PADILLA[3] of hemolysis of human erythrocytes by

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P. parvum toxin (prymnesin) indicated that toxin-induced hemolysis occurred in two stages (a prolytic period, followed by a lytic period), that maximum toxin activity was observed at pH 5.5, that the length of the prolytic period and the rate of the lysis was inversely related to the amount of toxin present, and that certain lipids (cephalin, cholesterol, and lecithin) inhibited the lysis of human erythrocytes.

The present study is concerned with the determination of the kinetics of prymnesin-induced lysis, particularly as these are revealed by kinetic data.

MATERIALS AND METHODS

Prymnesium parvum toxin

The toxin was isolated from *P. parvum* cultures in artifical sea water (10‰) maintained at 25° under constant illumination. The sea water was enriched with liver infusion (Oxoid, 300 mg/l), glycerol (0.25 M), and Vitamins B_1 and B_{12} (0.02 and 0.01 μg/l, respectively) (*cf.* ref. 1). Cells were collected after 5–7 days of growth at a density of 10^7 cells/ml by centrifugation (5000 × *g*, 10 min), ground in a tissue grinder, and the pigment was extracted with acetone. The insoluble residue was extracted several times with methanol to obtain the toxin. The methanolic extracts were combined, purified by gel filtration chromatography (see below), concentrated under reduced pressure, and a standard solution was kept at −20° for use in subsequent studies.

Labeled toxin

The toxin was obtained from cultures enriched with [^{3}H]-glycerol. A 500-ml sample of medium was inoculated with 100 ml of glycerol-free *P. parvum* stock culture. The inoculated medium was treated with 40 ml of 3.44 M glycerol containing 500 μC of [2-^{3}H]glycerol (Tracerlab, 200 mC/mmole). Cells were harvested after 7 days, and the toxin was isolated as usual. The toxin was purified further by gel filtration chromatography using Sephadex LH-20 in a 15 inch × 1 inch column and using methanol as eluant. Tubes 19–29 (Fig. 1) were used in subsequent studies.

Rabbit erythrocyte suspensions

Heparinized rabbit blood, stored at 4°, was suspended in isotonic blood buffer (10 mM phosphate-buffered saline solution (pH 5.5)). The erythrocytes were collected

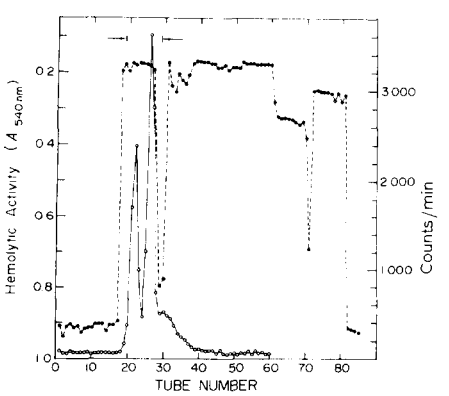


Fig. 1. Purification of tritium-labelled prymnesin by means of gel-filtration chromatography (Sephadex LH-20, methanol, about 50 min per tube, 4 ml per tube). The relationship between activity (counts/min, open circles, right-hand scale) and hemolytic activity[2] (closed circles, left-hand scale) is indicated. Arrows indicate the toxin fraction (Tubes 19–29) used in this study.

by centrifugation in a clinical centrifuge (2 min at 1800 rev./min), and washed twice with blood buffer. For kinetic studies, a standard erythrocyte suspension was prepared so as to have an absorbance of 1.0 at 540 nm. The electronic cell count of the standard suspension was about 10^7 cells/ml. When completely lysed, the standard suspension had an absorbance of about 0.19 at 540 nm.

Hemolytic units of toxin

A hemolytic unit (HD_{50}) was defined essentially as before[2] as that amount of toxin in 0.1 ml of methanol that caused 50 % hemolysis of 2.9 ml of standard erythrosuspension after 10 min at room temperature.

Lipids

Stock solutions (6 mM) of cholesterol (Aldrich Chemical Co.) and cephalin (Nutritional Biochemicals Corporation) in methanol were prepared. Additional solutions were prepared by serial dilution for the inhibition studies. In all such studies 0.05 ml of inhibitor solution and 0.05 ml of toxin solution were used with 2.9 ml of standard erythrocyte suspension.

Lysis kinetics (Tables I and II)

Rates of hemolysis were determined colorimetrically at 540 nm using a Beckman DB-G spectrophotometer, equipped with a thermostatted cell compartment and a recorder. In all determinations, 2.9 ml of standard erythrocyte suspension was allowed

TABLE I

SUMMARY OF KINETICS OF PRYMNESIN RABBIT ERYTHROCYTE REACTIONS AT 25.5°

Toxin concn.		*Prolytic period*	*Rate constants* $(min)^{-1}$*	
Relative	HD_{50} *units*	*(min)*	k'	k_ψ
1	1250	< 0.1		5.81 ± 0.18*
0.1	125	0.1		5.75 ± 0.28
0.02	25	0.1		5.73 ± 0.38
0.01	12.5	0.2	1.14 ± 0.01	5.22 ± 0.11
0.007	8.75	0.35	0.70 ± 0.04	3.84 ± 0.19
0.005	6.25	0.4	0.32 ± 0.03	2.80 ± 0.33
0.002	2.50	1.1	0.14 ± 0.04	1.62 ± 0.08
0.001	1.25	2		0.052
0.0001	0.125	approx. 5		0.0061

* ± S.D.

TABLE II

SPECIFIC RATE CONSTANTS AT SELECTED TEMPERATURES

Temperature	*Prolytic period (min)*	*Rate constants* (min^{-1})	
		k'	k_ψ
11.0°	0.5	0.15 ± 0.02	0.87 ± 0.10
15.0°	0.4	0.68 ± 0.02	1.07 ± 0.12
16.0°	0.3		1.43 ± 0.01
21.0°	0.2	0.73	1.73 ± 0.03
25.5°	0.2	1.14 ± 0.00	5.23 ± 0.11

to reach the reaction temperature before being mixed thoroughly with 0.1 ml of methanolic toxin solution. Kinetic data (absorbance as a function of time) were transcribed from the chart-strip recorder and replotted. Pseudo first-order rate constants, k_ψ, were calculated from the linear portions of the rate plot, $\log_e (A_t - A_\infty)$ as a function of time (min), using the conventional relationship $k = m$, where m is the slope, and A_t and A_∞ refer to the absorbance values at time t and at the completion of the reaction, respectively.

TABLE III

EFFECT OF INHIBITORS ON RATES OF HEMOLYSIS OF RABBIT ERYTHROCYTES BY TOXIN AT 25.5°

12.5 HD_{50} units of toxin were used.

Inhibitor	*Inhibitor concn. (μM)*	*Prolytic period (min)*	*Relative rate constant (k_{rel})*	*Inhibition parameter* *
		0.2–0.25	1.0	
Cholesterol	10	0.25–0.35	0.931 ± 0.017	0.074
Cholesterol	50	0.8–1.1	0.595 ± 0.051	0.68
Cholesterol	100	1.5–2.0	0.247 ± 0.024	3.049
Cephalin**	10	0.3–0.5	0.864 ± 0.004	0.157
Cephalin	25	0.6–0.7	0.626 ± 0.002	0.597
Cephalin	50	1.3–1.6	0.221 ± 0.07	3.525

* $(1 - k_{rel})/k_{rel}$.

** Mean molecular weight assumed to be 700.

TABLE IV

BINDING OF ^{3}H-LABELED PRYMNESIN BY ERYTHROCYTES AND GHOSTS

Prymnesin: about 6 HD_{50} units.

Million cells per 0.5 ml	*Toxin extraction method*	*Activity**		*Fraction of activity in pellet*
		Supernatant	*Pellet*	
Erythrocytes	A**			
48.5		466 ± 63***	36 ± 0	0.07
97		433	42	0.09
146		425	60 ± 5	0.12
Erythrocytes	B**			
9.3		155 ± 6	94 ± 3	0.38
93		159 ± 21	94 ± 11	0.37
930		175 ± 12	72 ± 6	0.29
Ghosts	B**			
2		266 ± 9	147 ± 20	0.36
		297 ± 26	174	0.37
10		323 ± 40	177 ± 5	0.35
		344 ± 4	155 ± 0	0.31
20		341 ± 72	324 ± 14	0.40
		316 ± 14	245 ± 15	0.44
		252 ± 2	265	0.43

* Counts/min, corrected for background.

** A, pellet washed with methanol to remove loosely bound prymnesin; B, pellet crushed and washed with methanol to give total extractable prymnesin

*** S.D.

Inhibition kinetics (Table III)

Essentially the same procedure was used except that a methanolic solution of the toxin (0.05 ml) was added to the inhibitor solution (0.05 ml) in the spectrophotometer cell, then as rapidly as possible, 2.9 ml of erythrocyte suspension were added to the mixture.

Swelling of erthyrocytes by prymnesin

Three erythrocyte samples were prepared: a 'standard sample' ($5.8 \cdot 10^6$ erythrocytes/ml), 2.9 ml *plus* 0.1 ml methanol; 'prolytic sample', 2.9 ml of erythrocyte suspension *plus* 0.1 ml of methanolic solution of toxin (12.5 HD_{50} units) were incubated, for 120 sec and quickly diluted 1:50 with buffer before lysis occurred; 'lysed sample', prepared similarly to the preceding sample except the incubation period was 15 min during which time about two-thirds of the cells lysed. The cells in each diluted sample were counted with a Model A Coulter Counter at 5 threshold-unit intervals (current 5, gain 5).

Prymnesin binding by red blood cells (Table IV)

Erythrocyte suspensions (2.9 ml) of known cell counts were treated with 0.1 ml ^3H-labeled toxin and cells were separated within 20 sec of mixing by centrifugation (36400 × *g*, 100 sec). The prolytic period was 0.4 min and the extrapolated reaction time was 3.5 min. The supernatant was separated and divided into four 0.5-ml portions. Two were used for controls, and two were treated with 0.3 ml 30 % H_2O_2 to bleach the hemoglobin. The pellet was treated with 1.5 ml of methanol (with and without crushing) to extract the toxin, and two 0.5-ml aliquots were saved for counting. The activity of each of the three duplicate samples in liquid-scintillation solution was determined. Raw counts were corrected for quenching using the percent efficiency calibration curve derived by the automatic external standardization procedure in the scintillation spectrometer (Nuclear-Chicago Mark II Series). Activities were redetermined at different times until efficiences of 50 % were obtained.

The experiment was repeated using suspensions of erythrocyte ghosts in blood buffer. Toxin (0.1 ml) was added to 0.4 ml of suspension and the ghosts were separated after 20 sec by centrifugation (36400 × *g*, 10 min). Replicate samples (100 μl) were removed from the supernatant. The pellet was treated with 0.5 ml of methanol, crushed, agitated, and centrifuged (36400 × *g*, 10 min) and replicate samples (100 μl) removed. The activities of all samples were determined as before.

RESULTS

Kinetics of hemolysis

The rates of hemolysis of rabbit erythrocyte suspensions were determined colorimetrically. A typical reaction curve (Fig. 2) shows the two phases that are observed. Each determination was characterized by a prolytic period during which no change in absorbance was observed. In a general way, the length of the prolytic period is related to the logarithm of the toxin concentration. The prolytic period was followed by the lytic period, the rate of the reaction being proportional to the slope (change of absorbance per unit time).

The two phases can be characterized in a more satisfactory manner by calculating the specific rate constants. Consecutive pseudo first-order rate constants k' and k_ψ are obtained from conventional first-order rate plots (Fig. 3). It is difficult to obtain

accurate values for the first rate constant which are associated with a process that evidently occurs during the prolytic period and just prior to the pronounced change in absorbance that is typical of the lysis period. The accuracy is limited by two factors.

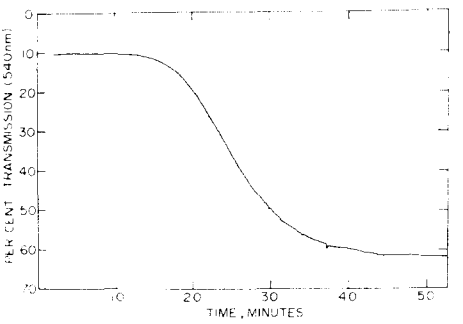


Fig. 2. Typical rate plot of hemolysis of rabbit erythrocytes, using 2.5 HD_{50} units of prymnesin.

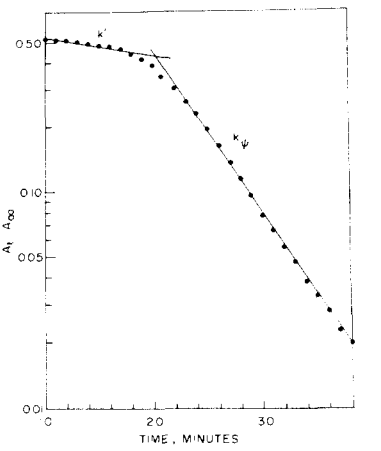


Fig. 3. First-order rate plot of prymnesin-induced hemolysis using data obtained from Fig. 2.

First, there are insufficient data for a satisfactory estimate except at lower temperatures. Second, enhanced accuracy would require an accurate value for A_∞, the infinity absorbance for the first process. It has not been possible to estimate the correct value because of the transient nature of the product of the first process, and a related constant value, the infinity absorbance value for the second process has been used instead. Nevertheless, the values for the first specific rate constant are reasonably constant for a given toxin concentration, and appear to be fairly consistent from one concentration to another.

The specific rate constant associated with a second major process, k_ψ, can be obtained with greater precision (Table I), and typically good first-order rate plots were obtained for better than 95 % of the reaction.

The variation of these constants with concentration of toxin, temperature, and inhibitor concentration has been determined.

Effect of temperature

Hemolysis rates were determined at several temperatures (Table II) in order to ascertain the nature of the two specific rate constants (k' and k_ψ) as well as to determine the activation energy. A conventional linear relationship was obtained from the Arrhenius plot for the first specific rate constant. The accuracy of the values is enhanced at lower temperatures as the rate constant decreases and additional data points can be obtained. An activation energy of 19 kcal/mole was calculated and this value corresponds to a Q_{10} value of 3.0 for the range of 15–25.5°.

In contrast, the activation energy could not be calculated for the second specific rate constant because the relationship was non-linear. The magnitude of the curvature is too great to be reduced to linearity by means of the modified Arrhenius plot ($\log_e (k/T^{1/2})$ *vs.* $1/T$; *cf.* ref. 4).

These results indicate a significant distinction between the two constants. The first constant is probably associated with a single process; the second constant must be a composite one that is associated with two or more processes that individually have different activation energies.

The nature of the two processes is further characterized by studies of the effect of concentration and inhibitors on the hemolysis rates.

Effect of concentration

Specific rate constant (k_ψ) were determined at 25.5° as a function of hemolysin concentration, expressed as hemolytic units (HD_{50}). A plot of k_ψ as a function of concentration, (expressed as $\log_e$ (HD_{50}) for the sake of convenience (Fig. 4)) indicates a sigmoidal relationship typical of a fragility curve. With the semi-logarithmic plot, three essentially linear portions are easily distinguished. At HD_{50} values of less than 1.25 (low range) and above 25 (high range), the rate constant is essentially independent of hemolysin concentration; with intermediate concentrations, the rate constant shows a marked concentration dependence.

Data from the intermediate and high concentration ranges were used in a Lineweaver–Burk plot (Fig. 5), and two parameters ($k_{\psi m}$ and K_m) were obtained. Here, $k_{\psi m}$ (7.69) refers to the maximum pseudo first-order rate constant, and K_m (9.53) has the usual meaning.

The two parameters were used to calculate values of k_ψ from the usual relationship (Eqn. 1), assuming that Michaelis–Menten kinetics adequately describe the lysis step.

$$k_\psi = \frac{k_{\psi m}(L,\ HD_{50})}{K_m + (L,\ HD_{50})} \qquad (1)$$

Here, (L, HD_{50}) refers to the concentration of hemolysin, expressed in hemolytic units.

The values calculated for the pseudo first-order rate constant (open circles, Fig. 4) are in excellent agreement with experimental values in the low and intermediate concentration ranges. The agreement is admittedly poor in the high ranges of hemolysin concentration.

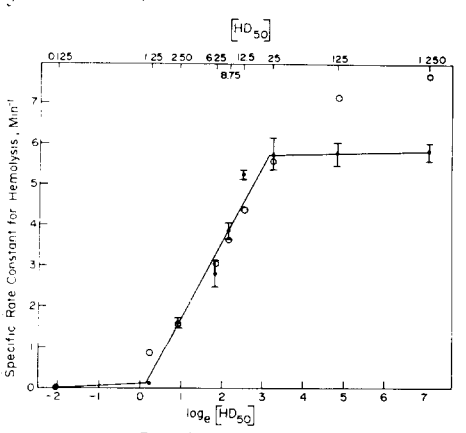


Fig. 4. Variation of specific-rate constant, k_ψ, for lysis with concentration of prymnesin. Observed (closed circles) values with ± S.D. are compared with calculated values (open circles, from Eqn. 1).

Two questions arise at this point. Is the agreement fortuitous? If it is not, why is the agreement between observed and calculated values of k_ψ poor in the high range?

The agreement does not seem to be fortuitous on the basis of two observations. First, good agreement was obtained in the low range ($HD_{50} = 0.125$, 1.25), though these data were not used in the Lineweaver–Burk plot (Fig. 5). Secondly, comparison of the data used for the two plots indicates no special distinction for those intermediate range values ($HD_{50} = 2.5$, 25) that exactly follow the Lineweaver–Burk relationship *versus* those that deviate ($HD_{50} = 6.25$, 8.75, 12.5) somewhat.

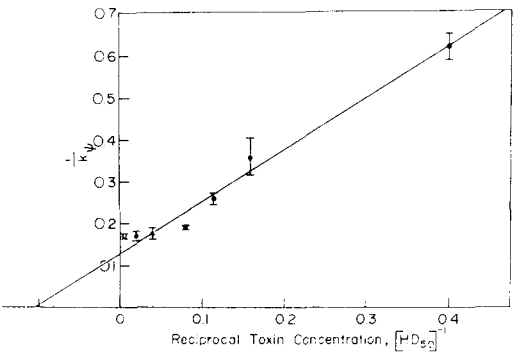


Fig. 5. Lineweaver–Burk plot showing the origin of the parameters used to calculate values of k_ψ. Vertical lines indicate ± S.D.

Poor agreement in the high concentration range could be ascribed to four possible causes: (1) experimental limitations, (2) incorrect Michaelis–Menten parameters, (3) inhibition by products, or (4) inhibition by hemolysin. The first two possibilities can be discounted. The value of $k_{\psi m}$ is probably less than 70 % of the experimental limitation of the instrumentation used in this study. Also, the parameters obtained (from Fig. 5) were conventionally obtained and are reasonably accurate. A drastic decrease (20 %) in the value of $k_{\psi m}$ would be needed to improve the agreement in the high range; then, the agreement in the intermediate range becomes poor.

By elimination, then, the poor agreement may be ascribed to inhibition either by hemolysin or byproducts. Both are plausible. The toxin, as released by *P. parvum* in natural blooms, is thought to form a micellar aggregate[5]. In the present study, the effect of such an aggregate would be most pronounced at the high range and would tend to limit the attack of other hemolysin units on the erythrocyte membrane. We do not suggest that such inhibition is exclusive; inhibition by lysis products, notably by erythrocyte lipids is a possibility that must be considered.

Inhibition by lipids

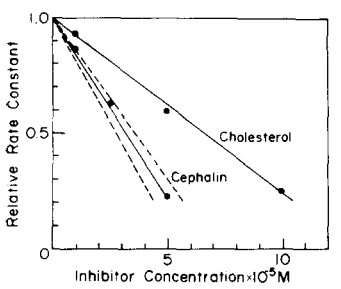
RAUCKMAN AND PADILLA[3] investigated the effect of three lipids on lysis of human erythrocytes, as indicated by the percent lysis following various periods of incubation of toxin, red blood cells, and inhibitor. Specific rate constants were not determined, but the effect of concentration on the extent of lysis was evaluated. With cephalin and lecithin, the same amount of lysis occurred whether toxin and lipid had reacted for 0 or 600 sec before blood was added. Cholesterol was different; it appeared

to be binding to the toxin as time progressed. On the basis of results with the method of SEEMAN[6] for distinguishing specific from non-specific hemolysins, RAUCKMAN AND PADILLA[3] concluded that prymnesin is a specific hemolysin and thus has an affinity for a specific component of the membrane, such as cholesterol. Cephalin, at concentrations higher than those used in this study, appeared to be a non-specific hemolysin in that it exerts a protective action by non-specific binding to the membrane, rather than binding directly.

In the present study, a different approach was used in an effort to gain an insight into the nature of inhibition by cholesterol and cephalin. Specific rate constants were determined as a function of concentration of added inhibitor, using a toxin concentration in the intermediate range (12.5 HD_{50}). In all instances, the prymnesin was added to the inhibitor solution, followed as quickly as possible by the addition of the erythrocyte suspension.

The inhibited rate data (Table III) are expressed in two ways: (1) as relative rate constants, k_{rel}; (the ratio of the inhibited rate constant to the uninhibited constant, k_ψ) and (2) as the inhibition parameter, $(1 - k_{rel})/k_{rel}$. The first parameter corresponds to fractional residual activity (r') and the second corresponds to $i/(1 - i)$, where i is fractional inhibition and is equal to $1 - r'$ (*cf.* ref. 7).

Examination of the inhibition data (Table III, Fig. 6), indicates two significant features. First in the range studied (approx. 0.01–0.1 mM) cephalin is not a hemolysin and it is a more effective inhibitor than cholesterol. For example, at $k_{rel} = 0.5$ (50 % inhibition), the inhibitor concentration required is 30 and 55 μM for cephalin and cholesterol, respectively. It should be noted that a mean molecular weight was assumed for cephalin. The reported[8] ranges were used to define the envelope of values (Fig. 6) that still attests to the greater effectiveness of cephalin.



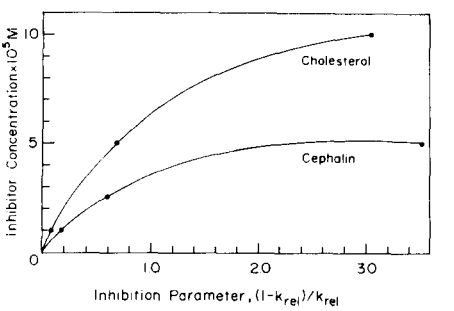


Fig. 6. Effect of lipids on the rate of prymnesin-induced lysis (HD_{50}, 12.5) at 25.5°.

Fig. 7. Diagnostic plot, inhibitor concentration as a function of the inhibitor parameter, showing the parabolic relationship indicative of the formation of inhibitor–prymnesin complex.

Secondly, the nature of the interaction of hemolysin (L), erythrocytes (E), and inhibitor (I) (Eqn. 2) is indicated by a diagnostic plot (Fig. 7), inhibitor concentration as a function of the inhibitor parameter. In analogy with enzyme processes, we assume that inhibition of lysis can occur *via* at least three pathways: (1) decrease in effective concentration of hemolysin by formation of a hemolysin–inhibitor complex (L_i) (2) formation of a complex (L_i) that reacts with erythrocytes in competition with hemolysin; (3) competitive hemolysin displacement by inhibitor.

$$E + L \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} C; \qquad C \underset{(k_{-2})}{\overset{k_2}{\rightleftharpoons}} E + P; \qquad L + I \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} L_i; \qquad E + L_i \underset{k_{-4}}{\overset{k_4}{\rightleftharpoons}} C_i \qquad (2)$$

Using the diagnostic plot (Fig. 7), we should expect three different relationships: for Pathway 1, a hyperbola (concave downward) should result; for Pathway 2, a linear relationship should result; for Pathway 3, a curve (concave upward) is expected[7]. Pathway 1 seems to be indicated for both inhibitors in the concentration range studied.

DISCUSSION

The kinetics of events preceding and accompanying lysis are of considerable interest, though they are complex and difficult to study. A review of thorough treatment of the problems by PONDER[9] indicate that they have a 3-fold basis. First, hemolysis is not subject to a simple delineation of kinetic parameters as is possible with simple chemical systems. Second, the utilization of lysin (amount in solution *versus* amount hemolytically active *versus* amount associated with erythrocytes) is not well understood. Finally, the effect of inhibitors, endogenous or added, is also not well understood.

All problems arise because of theoretical and methodological limitations, though the two are not truly separable. For example, PONDER[9] noted in several instances that a theoretical treatment had failed because some parameter could not be determined directly.

These difficulties have been mitigated in the present study. A spectrophotometric method has permitted a more adequate characterization of the rates of binding and hemolysis, and a radiochemical study has indicated the extent of binding of prymnesin to erythrocytes and ghost membranes.

A casual examination of these results might suggest that the kinetic patterns of binding and hemolysis can be reduced to a simple model that shows a good analogy with enzyme kinetics, particularly with respect to Michaelis–Menten or Henri equations. This is undoubtedly an oversimplification in view of the known complexity of prymnesin and the cell membrane. Thus, it may be useful to consider the basis of the apparent similarity and the evident simplicity of the kinetic pattern. Several reasons may be advanced.

First, there are some actual similarities, for example, the binding of substrate (L, Eqn. 2) to suitable sites (E). However, the assumption of only one active complex (C) is a mechanistic simplification, and the rate data do not permit a distinction between this possibility and a more likely one, *i.e.*, several active complexes (C, C', C'', *etc.*).

Secondly, the observation that the k_ψ–prymnesin relationship (Eqn. 1, Fig. 4) follows a common pattern might be puzzling at first glance if it is assumed that the total concentration of erythrocytes is critical to the validity of the relationship (Eqn. 1). The critical factor is the total concentration of reactive sites of the erythrocyte membranes (E_0), and this value appears to be significantly lower for erythrocytes than for ghosts (Table IV). Probably, the formation of the reactive complex(es) requires a precise interaction between a cumbersome lysin (L) and a limited number of reactive sites, so that the value of k_1 (or k') is probably much less than that of k_2, but once formed a complex is probably stable. Thus, k_{-1} should be much less than k_2, and a steady-state condition applies resulting in the validity of Eqn. 1, and the evident similarity with enzyme patterns.

Thirdly, the evident simplicity of the kinetic pattern, *i.e.*, two first-order rate constants, is valid in a gross sense, but it does not reflect the range of rate constants that must be involved, assuming a reasonable population of cells that have individual tendencies to lyse (*cf.* ref. 9). This simplicity is undoubtedly a consequence of the spectrophotometric method and a degree of insensitivity to subtle changes. For example, the kinetic parameters obtained are probably based upon only three extinction coefficients, one each for erythrocytes, active complex(es) and hemoglobin. Subtle changes might be expected during the course of binding and lysis, *e.g.*, an increase in the number of sites being occupied by one prymnesin molecule, or deformation caused by forces exerted by prymnesin bound to the membrane. Such changes could have profound effects (*e.g.*, release of various structural components), but these changes would not be reflected in the spectrophotometric rates. To detect these subtle changes other techniques would be needed, possibly ion-flux rates or changes in the rotatory dispersion curves.

With these limitations in mind, it is appropriate to consider the application of kinetic parameters and binding data to understanding the hemolytic action of prymnesin, particularly in comparison with lysins that have been studied previously. YARIV AND HESTRIN[11] suggested that prymnesin behaves like one such lysin, saponin.

The accumulation of lysin at the erythrocyte surface during the prolytic period is a central feature of lysin-induced hemolysis. With such lysins as saponin, sodium taurocholate and glycocholate, three concentrations must be considered[9]. These are: (1) the amount orginally added; (2) the amount determined, say, colorimetrically during the prolytic period; and (3) the amount determined at the same time by hemolytical assay. The first two quantities evidently are about the same; the third is about 60 % of the first[9]. The difference between the second and third amounts was ascribed[9] to lysin material that is chromogenic, but not hemolytic material. PONDER[9] offered two explanations: either the lysin had reacted with a component and the product diffused into the solution or the lysin molecules were rendered inert upon contact with the cell surface.

In the present study, it was possible to decide between the two possibilities and to demonstrate a significant difference between prymnesin and saponin. When ^{3}H-labeled prymnesin was used, about 40 % of the activity was associated with erythrocytes during the prolytic period. In short, the difference between the first two concentrations is significantly different, in contrast to the situation with saponin and other lysins. Though about 10 % of the material was loosely bound, as evidenced by its ease of removal with simple methanolic washing (Table IV), the rest was bound more firmly. The fraction bound appeared to be independent of the number of cells added. Moreover, the fraction of toxin associated with cells, either erythrocytes or ghosts, is quite similar. This suggests there are no fundamental differences in prymnesin-binding sites due to the deformation of membrane structure. Further, it seems to eliminate the first of the two suggestions of PONDER of where prymnesin is involved.

Some information bearing on the first and third lysin concentrations (total and hemolytically active during the prolytic period) is also available in the studies of RAUCKMAN AND PADILLA[3]. They found that during the prolytic period (100 sec for about 2 HD_{50} units of prymnesin) the toxin was bound to the erythrocytes in lineartime course and was completely removed from the solution during this interval. In a 0.5-min exposure, approx. 40 % of the toxin appeared to be removed

from the supernatantfluid, in agreement with our findings in the present study.

In addition to the two problems concerned with basic kinetic parameters and utilization of toxin, a third problem (effect of inhibitors) must be considered in an analysis of prymnesin-induced hemolysis. The rate parameters considered here are useful in several ways.

First, the rate parameters are sensitive to the presence of lipids (cholesterol, cephalin, and probably lecithin). A consideration of the inhibitor data (Table III, Figs. 6 and 7) and comparison of these data with a typical rate plot (Fig. 3) would indicate that prymnesin is not interacting with cholesterol or cephalin that may be released and accumulate at middle or late stages of the lytic period. If, during the course of lysis, loosely bound lipids are released, the value of k_ψ should continuously decrease and the rate plot (*cf.* Fig. 3) should change from a linear relation to a curve concave upward. Lipid release might be expected for ox erythrocytes, which are reported[10] to have about 10 % of the lipids loosely bound; these are lost to the suspension medium when the cells are osmotically hemolyzed.

Secondly, it appears that the magnitude of the first rate parameter, k', can be

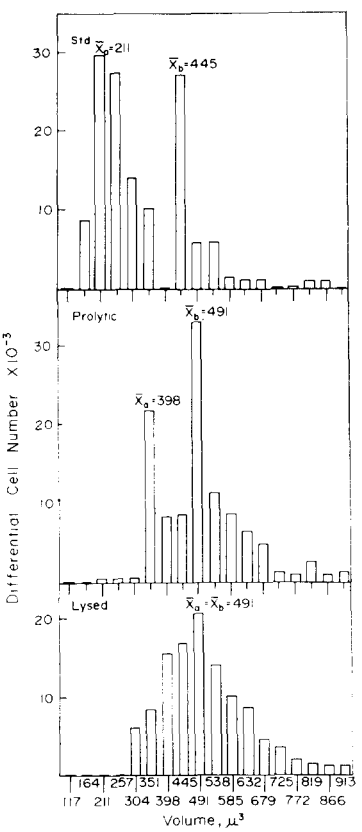


Fig. 8. Differential cell number–volume relationship for rabbit erythrocytes showing prymnesin-induced changes as observed for the standard suspension (before treatment, top), during the prolytic period (middle), and during the lytic period (bottom). The instrument was calibrated using latex spheres of known diameter (Dow Chemical Co., Bioproducts Department, 1.97–35 μm). Results are given as spherical equivalents.

used in two ways: to help characterize the binding step and to indicate the presence of inhibitors. The length of the prolytic period increases and k' is reduced.

In addition, it is instructive to consider the volume changes that occur during the prolytic and lytic phases. These have been reviewed by PONDER[9] and others. Little attention has been given to changes in cell volume distribution that occur, particularly as these are indicated by electronic counting (Fig. 8). For rabbit erythrocytes in counting solution, two cell size populations are noted. The values given are spherical equivalents, the volume cells would have if spherical. For example, the mean cell volume of rabbit erythrocytes (a non-swollen biconcave disc) is 57 μm^3. The value calculated using the maximum diameter (7.3 μm)[9] and assuming a sphere is 207 μm^3 (spherical equivalents). The observed value (Fig. 8), 211 μm^3, is remarkably close to the spherical value. This suggests that the cells are swollen in counting solution or that there is an artifact in the electronic counting technique and that the cells behave as spheres. In any case, we compare spherical equivalents for a given suspension under comparable conditions and are concerned with the changes. The populations shift during the prolytic period of toxin binding and coalesce during the lytic phase. These changes indicate that, at least for the system studied, changes in the cell-volume distribution may be as significant as the changes that occur in the average cell volume.

The importance of prymnesin as a membrane-modifying agent, as suggested by the observations in the present study is evident, and will warrant further investigation.

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