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HEMOLYSIS INDUCED BY *PRYMNESIUM PARVUM* TOXIN EFFECT OF PRIMAQUINE TREATMENT

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SUMMARY

The effect of primaquine (1 mM) incubation on rabbit erythrocytes was studied at 25° using the hemolytic toxin (prymnesin) of the chrysomonad *Prymnesium parvum*. One notable effect is the alteration of rate of prymnesin-induced hemolysis. The hemolysis rate constant, k_ψ, showed a biphasic dependence on length of primaquine incubation: a gradual increase in k_ψ was observed (0–4 h), followed by a more pronounced increase (4–6 h). Incubation with primaquine (an antimalarial) is known to cause invaginations and loss of cell surface by subsequent internalization. No biphasic effect of primaquine incubation was noted in the tendency of prymnesin to be bound (using ^{3}H-labeled toxin). Median cell volume, however, does show a biphasic relationship, and it appears that the biphasic effect depends upon changes in one out of two or more populations of cells.

INTRODUCTION

The kinetics of events preceding and accompanying hemolysis are of considerable interest, particularly as they can provide an insight into the nature of processes affecting the integrity of cell membranes. Although the nature of such processes may not be fully elucidated they may underly the destruction of erythrocytes in pathological conditions or irreversible physiological processes[1,2]. What is certain is that membrane alterations accompanying hemolysis can be associated with changes in active transport or reduction in surface area.

The last process, induced by chemicals, can occur by two mechanisms: "fragmentation", involving loss of membrane lipid accompanied by reduction in surface area with an increase in spheroidicity and decrease in deformability of the cell[2] and "internalization" involving invaginations of cell membranes, followed by closure and sealing processes leading to intracellular vacuoles[3]. The latter process is reminiscent of pinocytosis. It occurs when normal human erythrocytes are incubated with primaquine[3] (an 8-aminoquinoline derivative), which was found to be an effective antimalarial compound that produced hemolytic anemia in certain susceptible patients[4]. GINN *et al.*[3] suggested that the internalization process may be a biologically significant one leading to erythrocyte destruction.

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It seemed useful to have some means of determining how membrane surface loss accompanies internalization. Presently, it is necessary to assume that the vesicles formed do not unfold and do not communicate with the surface. (Such connections, admittedly, have not been observed, and the vesicles seem to persist even in lysed ghosts[3].) Moreover, it was of interest to learn if other alterations in the nature of surface membranes accompany internalization.

An approach towards clarifying these points was to study the kinetics of lysis induced by toxin from *Prymnesium parvum* (prymnesin). Previously PADILLA[5], RAUCKMAN AND PADILLA[6], and MARTIN AND PADILLA[7] demonstrated that toxin-induced hemolysis of human and rabbit erythrocytes occurred in two stages (a prolytic period is followed by a lytic period), which can be characterized by two first-order rate constants. The initial stage is associated with the binding of the toxin to the erythrocyte while the second one encompasses the lytic process[7]. Since both are sensitive to the presence of exogenous free lipids[6,7], it was felt that both rates should be sensitive to lipids released by primaquine treatment. Any alteration in the nature of the surface should alter the binding rate constant and any significant changes in deformability should be reflected in changes in prymnesin-induced lysis.

MATERIALS AND METHODS

Prymnesium parvum toxin

The toxin was isolated from cultures of the euryhaline flagellate *Prymnesium parvum* and purified as previously described[7].

Primaquine

Primaquine, 8-(4-amino-1-methylbutylamine)-6-methoxyquinoline, was obtained as the diphosphate salt from Sigma Chemical Co., and was used without additional purification.

Lysis kinetics

In all determinations 2.9 ml of standard erythrocyte suspension[7] and 0.05 ml of 0.0602 M primaquine were incubated in test tubes at 25°. At appropriate intervals 0.05 ml of methanolic prymnesin ($HD_{50} = 8.75$) were added and lysis kinetics were determined[7]. For control runs, 0.05 ml of distilled water was substituted for the primaquine solution.

Effect of primaquine incubation on binding

Two concentrated erythrocyte suspensions (2.9 ml, $150 \cdot 10^6$ cells/ml) were treated with 0.1 ml of distilled water ("control") and 0.0606 M primaquine solution ("test") and incubated at 25°. At various intervals from 0 to 6 h, samples were removed and diluted (approx. 1:10) for binding studies. The extent of binding was determined using ^{3}H-labeled prymnesin. Duplicate samples (0.4 ml) of diluted erythrocyte suspensions were treated with 0.1 ml of toxin, agitated, and centrifuged. Centrifugation was initiated before the end of the prolytic period and was continued for 5 min ($36400 \times g$). Three aliquot portions (100–150 μl) of supernatant were removed for counting. Toxin bound to the pellet was extracted by mixing and crushing it with 0.5 ml of methanol. After centrifugation ($36400 \times g$ for 10 min) three aliquots of

supernatant were removed for counting by liquid scintillation (Nuclear-Chicago, Mark II Spectrometer), with appropriate corrections for quenching and background.

Effect of sonication and primaquine incubation

Concentrated erythrocyte suspensions (2.9 ml, $150 \cdot 10^6$ cells/ml) were treated with 0.1 ml of distilled water ("control") and 0.1 ml primaquine solution (0.0606 M) ("test") and incubated 1 h at 25°. Samples were then diluted (1:10) and divided. One sample each (control and test) was sonified for 15 sec using the No. 6 setting of the Branson Sonifier (following the procedure of MARCHESI AND PALADE[8]). The extent of binding was determined by treating a 0.4-ml sample of sonified and non-sonified suspension with 0.1 ml of ^{3}H-labeled prymnesin. The mixtures were shaken for 20 sec, then diluted with 9 ml of blood buffer. Samples were subjected to centrifugation (96600 $\times$ *g* for 30 min). Duplicate 1-ml aliquot portions of the supernatant were removed for isotopic counting. The rest of the supernatant was discarded, and the pellet suspended in 0.5 ml of methanol for counting by usual procedure.

Effect of primaquine on erythrocyte volumes

Four erythrocyte suspensions were prepared (2.9 ml, approx. $3 \cdot 10^6$ cells/ml, $A_{540\ nm} = 1.0$): "standard": sample was treated with 0.05 ml of water mixed 1:50 with counting solution (0.9% NaCl, 0.1% KCl); "control": sample was treated with 0.05 ml of distilled water and incubated at 25° for 1 h, then diluted 1:50 with counting solution; and "lysed": samples were treated with 0.05 ml of primaquine solution (0.0602 M), incubated for 1 h, then treated with 0.05 ml of prymnesin (HD_{50}, 8.75), mixed, and allowed to react for 1 min and 2.5 min, respectively, before being diluted 1:50 with counting solution. All diluted solutions were counted electronically at 5 threshold-unit intervals, counts (cells/ml) were corrected for background, and differential counts were calculated. The instrument (Coulter Counter, Model A, 100 μm aperture) was calibrated in units of μm^3 using latex spheres (Dow Chemical Co., Bioproducts Division) of known volume.

Changes in total volume of packed cells were also studied for quadruplicate samples, using hematocrit capillary tubes centrifuged for 14 min. Hematocrits are expressed as the percentage of packed cells.

Finally, changes were determined for standard erythrocyte suspensions incubated with distilled water and primaquine. Aliquot portions were removed from samples in a 25° gyrorotatory water bath (New Brunswick, Model G76) at appropriate intervals, and median cell volumes were determined electronically for samples diluted with buffer, instead of counting solution. The instrument was recalibrated for volume determination in buffer solution using standard spheres as before.

Measurement of K^+ influx of rabbit erythrocytes

An aliquot of packed rabbit erythrocytes was washed twice with cold Mg^{2+} buffer (190 mM $MgCl_2$, pH 7, after adjustment with $MgCO_3$) and centrifuged in a refrigerated Sorvall RC-2B centrifuge (8000 rev./min, 10400 $\times$ *g* for 5 min). The washed cells were suspended in normal Ringer buffer[9]. Treated samples (9 ml; hematocrit, approx. 0.02) were incubated in 50-ml erlenmeyer flasks in a shaker bath at selected temperatures. After the incubation period, ^{42}KCl in Ringer buffer was added to a final concentration of 3 μC/ml. 2-ml aliquots were withdrawn within 15 sec

after isotope addition and at appropriate intervals thereafter. Samples were immediately pipetted into 5 ml of cold (0–5°) Mg^{2+} buffer and centrifuged within 5 min (10400 × *g* for 4 min). Supernatant fluid was collected and saved for counting. The pellets were resuspended twice with cold Mg^{2+} buffer and recentrifuged as before. Washed pellets were treated with 10 ml of hemolyzing solution (20 ml 15 M ammonia, 0.500 ml Acationox, 10.78 g CsCl in 16 l of distilled water). The absorbance of the hemolyzed solutions at 540 nm was determined. They were then assayed for activity in a γ-scintillation counter. All counts were corrected to time of initial counting.

Fluxes (Table I) were calculated (Eqn. 1) using a program developed by Robert Gunn of this Department, the background of which is given by HOFFMAN[10].

$$\text{Flux (mM/l original packed cells per h)} = \frac{G}{\Delta t \cdot S}\left[\frac{B}{A_B} - \frac{A}{A_A}\right] \quad (1)$$

Here, A and B are the activities (counts/min − background for the first and second aliquots, respectively; A_A and A_B are the absorbance values for corresponding hemolyzed pellets, S is the activity of the supernatant (observed counts/min − background), Δt is the time interval (in h) between the two aliquots; and G is a conversion factor (Eqn. 2)

$$G = \frac{V_k \cdot HO \cdot K_0}{V_H} \quad (2)$$

Here, V_k is the volume of supernatant counted (0.002 l); K_0 is the concentration (mM) of K^+ in the supernatant, as determined with a Perkin–Elmer 303 atomic absorption spectrophotometer; V_H is the volume (0.010 l) used in the measurement of A_A and A_B; and $HO = (A_{pc} \cdot D.F.)/Hct$, where A_{pc} is the absorbance of 1.6 ml of original erythrocyte suspension (equivalent to 2 ml of aliquot) diluted to 10 ml with hemolyzing solution, D.F. is the dilution factor[5], and Hct is the hematocrit value (approx. 0.02) for the original erythrocyte suspension.

RESULTS

The effect of primaquine treatment on rabbit erythrocytes has been studied in four ways: (1) by the alteration of prymnesin-induced hemolysis, (2) effect on toxin binding, (3) erythrocyte volume changes, and (4) the effect on K^+ influx. In all experiments the concentration of primaquine (approx. 2 mM) used corresponded to that used previously[3] to permit better comparisons.

Hemolysis kinetics

The kinetic patterns of prymnesin-induced hemolysis of rabbit erythrocytes have been described in detail[7], but a brief review will be useful in order to establish the effect of primaquine treatment. When rates of hemolysis are measured colorimetrically, two first order rate plots are obtained from which two constants, k' and k_ψ. The first constant was associated with the binding step and the second with lytic processes. The second constant was found to be a composite one and to follow a Michaelis–Menten or Henri pattern (*cf.* REINER[11]).

Erythrocyte incubated at 25° with 1 mM primaquine became more susceptible to prymnesin-induced hemolysis. The effect appears to be biphasic (Fig. 1, Table I),

though cells incubated without primaquine show no notable variation in susceptibility during a six-hour period (Table I). The first phase, 0–3 h, was characterized by a gradual increase in the value of the hemolytic rate constant, k_ψ. The second phase occurring at 3–6 h of incubation is characterized by a more pronounced increase in k_ψ.

The variation of parameters other than k_ψ was examined. The prolytic period, during which binding of prymnesin occurs, decreased during the first phase (Table I) and remained essentially constant during the second. The binding rate constant, k', decreased during the first phase and could not be measured during the second phase owing to the short prolytic period. Initial and final absorbance values of the erythrocyte suspensions were decreased from values observed for initial stages of incubation (Table I). The absorbance values during the second phase were constant, however,

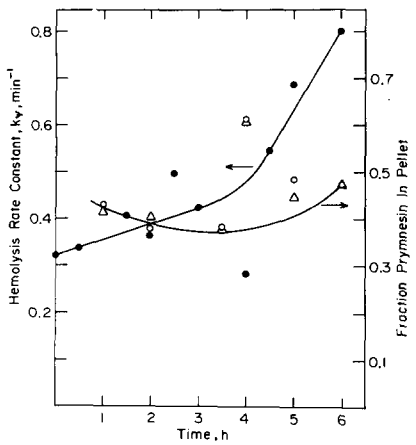

Fig. 1. Effect of primaquine incubation on prymnesin-induced hemolytic rate constant, k_ψ, (left ordinate, ●) in comparison with the fraction of ^{3}H-labeled prymnesin bound to rabbit erythrocytes during prolytic period (right ordinate: ○, control; △, test).

TABLE I

EFFECT OF INCUBATION WITH PRIMAQUINE ON PRYMNESIN-INDUCED LYSIS OF RABBIT ERYTHROCYTES AT 25°

Control, 0–6 h, with greater HD_{50} concentration. $k_\psi = 5.09 \pm 0.31$.

Incubation time (h)	*Absorbance*		*Prolytic period (min)*	*Rate constant (min^{-1})*	
	Initial	*Final*		*k'*	k_ψ
0	0.90	0.24	2	0.114	0.322
0.5	0.87	0.22	1.5	0.070	0.338
1.5	0.84	0.185	1.0	0.071	0.406
2.0	0.735	0.155	0.9	0.049	0.361
2.5	0.66	0.135	0.8		0.497
3.0	0.78	0.175	0.5		0.422
4.0	0.76	0.173	0.5	0.135	0.282
4.5	0.70	0.120	0.4		0.548
5.0	0.63	0.130	0.5		0.687
5.5	0.64	0.140	0.3		0.798
0 (no primaquine)	1.0	0.227	0.2	0.336	1.53

and cannot account for the increase in k_ψ noted. Finally, examining the rate plots, $\log_e (A_t - A_\infty)$ *vs.* time, revealed no substantial differences between these representatives of the first or second phase. Some deviation or retardation was noted at late stages of lysis in individual runs but no pattern appeared that would account for the biphasic relationship (Fig. 1).

Of the various parameters considered here the most significant were the alteration of prolytic period and binding rate constants. These were studied in more detail using ^{3}H-labeled prymnesin.

Toxin binding

Two different binding studies were made: (1) effect of primaquine incubation on fraction of ^{3}H-labeled prymnesin bound to erythrocytes during the prolytic period and (2) the effect of sonification and/or primaquine incubation on the fraction of labelled prymnesin bound.

TABLE II

BINDING OF ^{3}H-LABELED PRYMNESIN BY RABBIT ERYTHROCYTES INCUBATED WITH PRIMAQUINE

Incubation time (h)	*Sample*	*Cells/ml (× 10⁶)*	*Fraction of activity in pellet*
1.0	Control	14.2	0.431 ± 0.016
	Test	15.6	0.416 ± 0.020
2.0	Control	1856	0.289
	Test	1856	0.313
	Control	186	0.372
	Test	186	0.401
	Control	18.6	0.378
	Test	18.6	0.404
3.5	Control	*	0.384
	Test		0.377
4.0	Control	*	0.613 ± 0.012
	Test		0.608 ± 0.033
5.0	Control	*	0.485 ± 0.038
	Test		0.445 ± 0.0004
6.0	Control	*	0.464 ± 0.020
	Test		0.464 ± 0.002

* Approx. $18 \cdot 10^6$ cells in initial sample.

TABLE III

EFFECT OF SONICATION AND PRIMAQUINE INCUBATION ON ERYTHROCYTE BINDING OF PRYMNESIN

Sample	*Sonification*	*Fraction of activity in supernatant*
Control	+	0.917 ± 0.015
	+	0.960 ± 0.089
Control	−	0.898 ± 0.110
Primaquine incubated *	+	0.766 ± 0.089
	+	0.858 ± 0.104
Primaquine incubated *	−	0.779 ± 0.157
	−	0.642 ± 0.005

* 1 h at 25° with 2 mM aqueous primaquine.

Incubation with primaquine for varying periods has a very slight effect on the amount of prymnesin bound to erythrocytes (Fig. 1 and Table II), but the effect does not parallel the relationship observed with hemolytic rate constants (Fig. 1). A deflection is, however, noted at about 4 h for both the rate and reciprocal volume relationship (Figs. 1 and 4). Superimposed upon the binding–incubation relationship is the factor of cell numbers (Table II). Only at very high cell numbers did the fraction of toxin bound decrease, but in all binding experiments cell numbers were in the low range.

From the binding experiment, it appeared changes in binding fraction might be due to changes in surface fraction might be due to changes in surface area. This possibility was tested in the second binding study and erythrocyte suspensions with and without primaquine were sonified to increase the surface area, the sonification procedure was used by MARCHESI AND PALADE[8] to prepare ghosts which retain their original membrane orientation.

The results (Table III) were subjected to the *t* test (*cf.* CROXTON[12]) and the results show no significant differences among the four treatments. A possible exception is the distinction between the two extremes (control, sonified and primaquine incubated but not sonified).

Volume relationships

Two methods were used to study any changes in volumes as a result of primaquine-induced changes: hematocrit and electronic sizing.

Hematocrit values were measured for erythrocyte suspension treated with water (control) and primaquine (1 mM). Values (fraction of packed cells) for control and test, respectively, at various times were 0.37 ± 0.007 and 0.375 ± 0.004 (0 h), 0.371 ± 0.004 and 0.368 ± 0.002 (0.5 h), 0.365 ± 0.006 and 0.371 ± 0.004 (1 h), 0.368 ± 0.004 and 0.368 ± 0.001 (2 h), 0.362 ± 0.003 and 0.365 ± 0.001 (19 h). Within the limits of experimental error (as indicated by the standard deviations), no differences appeared that could be ascribed to primaquine treatment.

Electronic sizing was used to determine if changes occur within cell size distributions as a result of primaquine treatment. It appears that primaquine treatment

TABLE IV

PRIMARY AND SECONDARY VALUES OF MEDIAN CELL VOLUME FOLLOWING PRIMAQUINE INCUBATION AT 25° FOR 1 h AND TREATMENT WITH PRYMNESIN

Sample *	*Median cell volume* (μm^3)	
	Primary peak	*Secondary peak*
Standard (before incubation)	304	398
Control (after incubation)	304	211
Lysed samples (primaquine treated)		
(a) Control	304	445
(b) Prolytic phase**	351	257
(c) Lytic phase**	398	304

* See MATERIALS AND METHODS for details.

** Treated with prymnesin after incubation with primaquine.

does not affect the primary median cell volume but it does affect the secondary peak which appears upon osmotic stress using counting solution (*cf.* Table IV). Prymnesin treatment produces more dramatic changes in the median cell volume. The effect of primaquine treatment, however, does produce a notable change in the volume distribution, namely by shifting it to a narrow size range (Fig. 2). Prymnesin tends to shift the volume distribution in the other direction.

These results should be compared with the effect of primaquine incubation on median volume, determined using buffer solution, whereas the cell distributions were determined with conventional counting saline solution. As suggested earlier[7], counting solution appears to induce swelling, so that the values obtained represent volumes of spherical cells ("spherical equivalents"). In buffer, however (Fig. 3), the median volume was found to be 65–69 μm^3 which compared well with the mean cell volume values (57.5, 64.5) cited by PONDER[13]. Erythrocyte suspensions incubated without

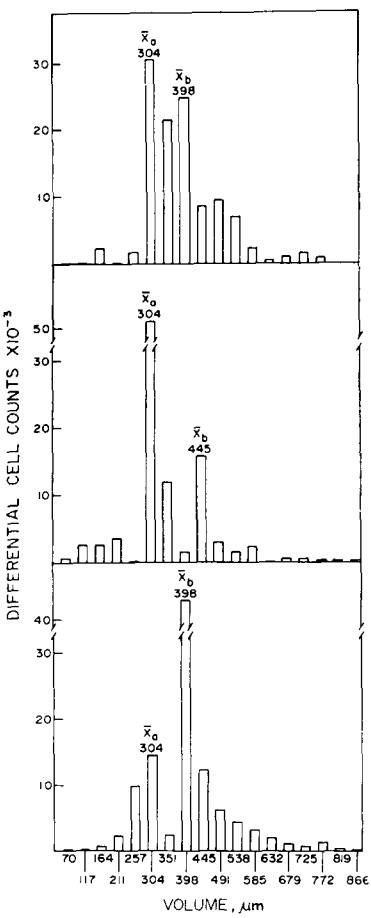

Fig. 2. Cell-volume distributions for rabbit erythrocytes exposed to primaquine and prymnesin in counting saline solution. Changes are shown for 3 samples taken from a standard suspension: before incubation (top); a sample incubated with 1 mM primaquine for 1 h at 25° (middle); and a sample treated with prymnesin to the lytic phase (bottom). Volume values correspond to spherical equivalents as discussed in text.

primaquine showed little change in median volume with time during a 6-h period (Fig. 4). Samples incubated with primaquine, however, show an interesting variation. The plot of reciprocal volume (as reciprocal threshold units or as reciprocal volume) as a function of time shows a biphasic relationship (Fig. 4) that parallels the hemolytic tendency (k_ψ *vs.* time of incubation with primaquine).

TABLE V

SUMMARY OF EFFECT OF PRIMAQUINE INCUBATION TIME ON K^+ FLUX VALUES AT 25°

Incubation time (h)	*Relative flux value**
0**	0.88
0.75	0.76
2	0.66
5	0.86

* Relative to control incubated for 10 min for which mean flux = 2.28 ± 0.26.
** 37°.

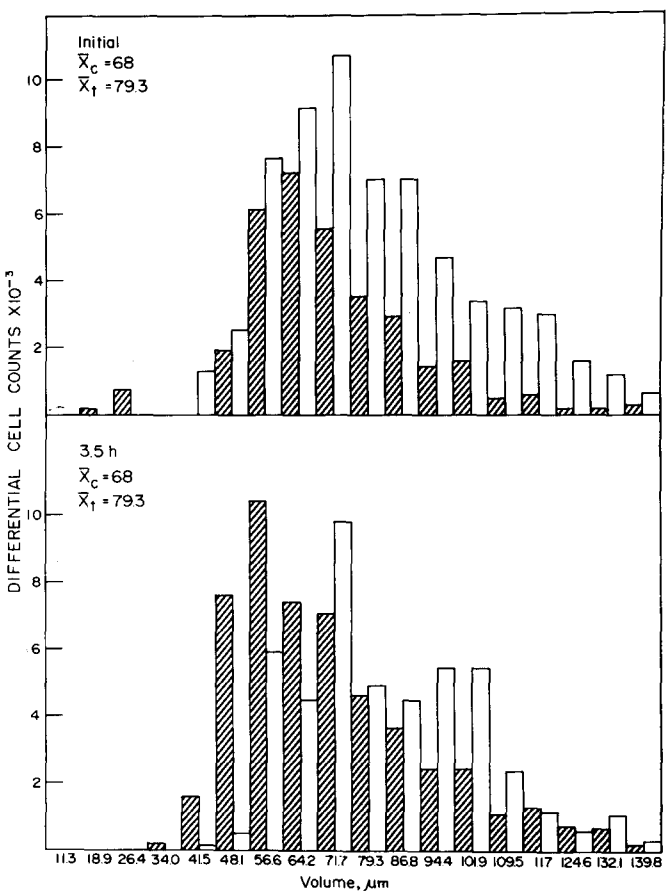

Fig. 3. Erythrocyte cell-volume relationships for rabbit erythrocytes in blood buffer showing effect of incubation with primaquine (1 mM) for about 45 min (top) and 3.5 h at 25° (bottom). Median cell volumes are given for control (dashed bars) and test samples.

K^+ flux measurements

Flux values for samples incubated with primaquine were compared with samples of freshly prepared erythrocyte suspensions that had been incubated for 10 min (Table V). In our hands, only a slight difference was noted between flux values at 25° and 37°. Control samples that had been incubated as long as the primaquine-treated samples tended to have erratic K^+ flux values and were not used in calculations.

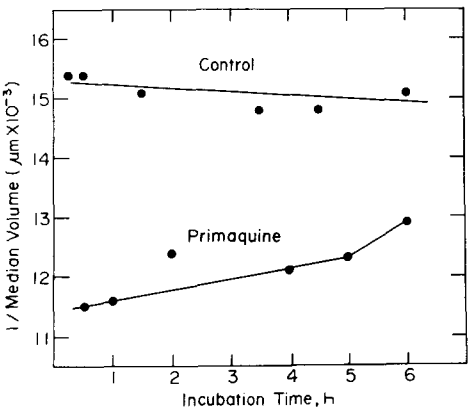

Fig. 4. Effect of primaquine incubation time on the reciprocal median cell volume, $1/x_j$. Values were obtained using normal blood buffer.

DISCUSSION

The results of this study show that prymnesin-induced hemolysis rate constants are a useful index of cellular function, particularly when used in conjunction with other methods of analysis such as volume changes. As a case in point, earlier workers[3] had suggested that "treatment with primaquine leads, eventually to deep invaginations and pinching off internally of portions of the surface membrane. This process effectively removes a part of the surface membrane, permitting the cell to reach critical hemolytic volume more rapidly".

Our results compare the effects of primaquine treatment on hemolytic tendency and cell volume changes, and it appears that the two are closely related. The effect is more complex than supposed, however. Primaquine incubation has a 2-fold effect. In early stages, the cell volume is decreased until some critical point is reached and in later stages of incubation time it increases. Hemolytic susceptibility should be inversely related to cell volume, and this would account for the parallelism between two representations of hemolytic tendency (Fig. 1) and reciprocal volume (Fig. 4).

The qualitative explanation fails to rationalize the biphasic relationship noted or the "break point" at about 3–4 h. These two problems can be considered in order.

First, the "break point" probably is not an invariant one and we suspect that it depends upon the age and treatment of the cells. We have not investigated this further, but have attempted to eliminate the variation by using fresh samples and by consistent procedures.

Second, the biphasic relationship is probably due to the existence of two (or more) cell populations of distinct volumes. These populations are not revealed by examining the median cell size, either by electronic counting or by hematocrit measure-

ments. They do appear, however, from differential cell volume plots particularly under osmotic stress of counting solution. Primaquine incubation causes a shift in the distribution to narrow size range, and even incubation for a short time causes a decrease in the secondary volume peak. These observations could be rationalized simply in terms of different ages of rabbit erythrocytes and the differential susceptibility of these cells with respect to osmotic stress or hemolysis. Though this is reasonable, additional verification would require a much more intensive investigation of the variation of the cell size distribution with time, together with parallel studies of hemolytic tendency and radiochromium or radioiron uptake to measure cell age, which may govern cell size distribution.

It may be noted that the effect of primaquine incubation involves a change in volume and not merely a change in surface area or in the nature of the surface. These conclusions are supported by labeled prymnesin binding studies. No biphasic change in the fraction of prymnesin bound to pellet during the prolytic period was noted (Fig. 1). Also, the change in surface area (by sonification using the procedure of MARCHESI AND PALADE[8]) did not alter the fraction of bound prymnesin. The sonification does not alter the nature of the membrane, though it does increase the surface area.

In addition, primaquine incubation does not involve a drastic chemical change. For example, relative K^+ flux values do not change substantially upon primaquine incubation (Table V). In fact, if constant flux values are indicative of membrane stability, the presence of primaquine solution appears to stabilize erythrocytes, relatively to the distilled water controls.

Utilization of prymnesin as a probe of erythrocyte membrane processes is indicated by the present study. Probably, complete and most effective utilization must await total chemical characterization of this toxin. Much is known about the chemical properties, and more investigation is evidently in progress (*cf.* ULITZUR AND SHILO[14]).

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REFERENCES

1 J. H. JANDL, *Blood*, 26 (1965) 367.
2 R. I. WEED AND C. F. REED, *Am. J. Med.*, 1 (1966) 681.
3 F. L. GINN, P. HOCHSTEIN AND B. F. TRUMP, *Science*, 164 (1969) 843.
4 E. BEUTLER, *Pharmacol. Rev.*, 21 (1969) 73.
5 G. M. PADILLA, *J. Protozool.*, 17 (1970) 456.
6 B. RAUCKMAN AND G. M. PADILLA, *14th Ann. Meeting Biophys. Soc., 1970, Baltimore, Md., Biophys. Soc. Abstr.*, New York, p. 73a.

7 D. F. MARTIN AND G. M. PADILLA, *Biochim. Biophys. Acta*, 241 (1971) 213.
8 V. T. MARCHESI AND G. E. PALADE, *J. Cell Biol.*, 35 (1967) 385.
9 M. F. VILLAMIL AND C. R. KLEEMAN, *J. Gen. Physiol.*, 54 (1969) 576.
10 P. G. HOFFMAN, JR., Doctoral Dissertation, Duke University, 1969.
11 J. M. REINER, *Behavior of Enzyme Systems*, Van Nostrand-Reinhold, 2nd ed., 1969, p. 190.
12 F. E. CROXTON, *Elementary Statistics*, Dover, New York, 1959, p. 238.
13 E. PONDER, *Hemolysis and Related Phenomena*, Grune and Stratton, New York, 1948, p. 80.
14 S. ULITZUR AND M. SHILO, *Biochim. Biophys. Acta*, 201 (1970) 350.