Biochimica et Biophysica Acta, 291 (1973) 156–164

BBA 76173

HEMOLYSIS INDUCED BY *PRYMNESIUM PARVUM* TOXIN

CALORIMETRIC STUDIES

JESSE S. BINFORD, JR, DEAN F. MARTIN* AND GEORGE M. PADILLA

Department of Chemistry, University of South Florida, Tampa, Fla. 33620 and Department of Physiology, Duke University Medical Center, Durham, N.C. 27710 (U.S.A.)

(Received July 10th, 1972)

SUMMARY

The relationship between binding of the hemolytic toxin (prymnesin) to bovine erythrocytes and the amount of heat liberated was examined as a function of pH using a flow microcalorimeter and ^{3}H-labelled toxin isolated from the euryhaline alga *Prymnesium parvum*. A high degree of correlation (correlation coefficient = 0.986) was found between the amount of heat generated and the quantity of toxin that was allowed to interact with the erythrocytes. No significant binding of toxin was observed at pH 7 but it increased linearly as the pH was reduced to 5.5. Maximum heat and binding occurred at a pH range 4.5–5.5. The same pattern was followed in terms of the amount of heat liberated and the hemolytic activity of the toxin. The differences in the maximum binding and heat production as a function of pH was independent of the average red cell volume which remained constant at pH 5.5 and 6.2 (102.4 and 102.6 μm^3, respectively).

INTRODUCTION

Previous studies in this series have been concerned with prymnesin, the toxin isolated from the euryhaline chrysomonad *Prymnesium parvum* (Carter) that produces toxins with hemolytic, ichthyotoxic, and cytotoxic activities[1,2]. Some studies have been concerned with the chemical nature of the hemolytic toxins[3] and useful purification schemes have been devised[1,3]. Ulitzur and Shilo[1] found the material to be a lipid–carbohydrate–protein complex, though the details of the chemical structure of prymnesin evidently are still under study. More recent studies[4,5] have been concerned with the kinetics and binding *P. parvum* toxin to red cells[4] the effect of primaquine on the prymnesin-induced hemolysis[6], and the effect of *Gymnodinium breve* toxin on the activities of prymnesin[5].

Some efforts have been directed toward describing the hemolytic activities of prymnesin in terms of kinetics of prymnesin-induced lysis[4]. Rates of hemolysis of rabbit erythrocytes, induced by prymnesin, were measured colorimetrically at 25 °C and two consecutive first-order rate processes associated with the prolytic and lytic periods were characterized. A critical factor in the hemolytic activity,

* To whom correspondence concerning this paper may be addressed.

obviously, is the binding of prymnesin to erythrocyte membranes. The binding during the prolytic period was investigated using ^{3}H-labelled toxins, and rabbit erythrocytes, and it appeared that about 10 % of the labelled material was loosely bound and 30 % is more firmly bound[5].

The present study is concerned with the nature of the binding of prymnesin, particularly as revealed by calorimetric data. Conventional calorimetry by means of batch methods has limitations as applied to studies of binding with erythrocytes because of large sample requirements and the consequent difficulties in maintaining a variety of stoichiometries. These difficulties, however, are mitigated by use of a flow microcalorimeter which permits variation of stoichiometry and measurement on small samples.

MATERIALS AND METHODS

P. parvum toxin (prymnesin)

The toxin was isolated from cultures of the euryhaline flagellate *P. parvum* and was purified by means of gel filtration chromatography as described previously[4]. Labelled (^{3}H) toxin was obtained from glycerol-free cultures that were enriched with [2-^{3}H]glycerol (Tracerlab, 200 Ci/mole) as described previously[4].

Erythrocyte suspensions

Heparinized bovine blood, stored at 4 °C, was suspended in isotonic buffer (10 mM phosphate-buffered saline solution, pH 7.2 or 6.2). Erythrocytes were collected by centrifugation in a clinical centrifuge and washed twice with buffer. For calorimetric studies, a suspension was prepared in buffer of defined pH so as to have a hematocrit value of approximately 40 %.

Microcalorimetry (Table I)

The calorimeter used in this work was a Beckman Model 190B Microcalorimeter, previously calibrated for batch measurements[7]. This instrument was modified for flow calorimeter using a design described by Sturtevant and Lyons[8]. Samples were introduced by syringe using a pair of Harvard Apparatus Company infusion pumps, Model 975. The thermopile of the calorimeter was maintained in a constant ambient temperature by inserting it in an insulated cylinder which was submerged in a constant temperature oil bath. Temperature was held at 24.987 °C by a TRONAC precision temperature controller Model PTC-1000 A.

Calibration as a flow calorimeter using both NaOH + HCl and Tris + HCl gave results within 4 % of the earlier electrical calibration. The two chemical measurements gave agreement within 0.1 %. A quantity ε was calculated at each flow rate which gives the heat production $\dot{q}$ when multiplied by the number of chart units deflection (C.U.) on range 25 (100 C.U. = 25 μV). The value of ε increases gradually with increasing total flow rate

$$\dot{q} = (\varepsilon)\,(\text{C.U.})\ \text{cal}\cdot\text{min}^{-1} \qquad (1)$$

Representative values of ε are shown in Table II.

Two heat measurements were made for each prymnesin–erythrocyte reactant ratio and were obtained in the following way. The heat for the control runs was

obtained by flowing erythrocyte suspension and methanolic buffer of the same pH together and measuring the heat rate as chart units on the potentiometric recorder. In a separate test run, prymnesin in methanolic buffer replaced the methanolic buffer in the second syringe and the same flow rate was repeated. At the highest

TABLE I

PRYMNESIN–BOVINE R&D CELL INTERACTIONS AT 25 °C IN PHOSPHATE BUFFER

pH	*Prymnesin concn (mg/ml)*	*Total flow rate (ml/min)*	*Corrected flow rates*		*mg prymnesin*	*Heat generated, C.U.**		
			Blood (cm^3 packed cells/min)	*Prymnesin (mg/min)*	*cm^3 packed cells*	*Control*	*Test*	*Net*
7.0**	0.90	0.380	0.075	0.0716	0.97	9.0	9.2	—
7.0	0.90	0.456		0.140	1.87	9.4	9.0	—
7.0	0.90	0.600		0.270	3.60	16.0	15.8	—
7.0	0.90	0.900		0.540	7.20	42	42	—
6.1	0.60	0.380	0.075	0.477	0.64	19.0	22.9	3.9
6.1	0.60	0.600		0.180	2.40	42.9	47.1	4.3
6.1	0.60	0.900		0.360	4.80	69.8	81.0	11.2
6.2	1.20	0.380	0.114	0.0954	0.837	32.8	41.5	8.7
6.2	1.20	0.456		0.187	1.64	44.9	53.9	9.0
6.2	1.20	0.600		0.360	3.16	68.4	76.6	8.2
6.2	1.20	0.900		0.72	6.32	122	124	2
6.2	0.90	0.196	0.0702	0.0355	0.506	15.8	16.0	0.2
6.2	0.90	0.236		0.0716	1.02	23.3	26.8	3.5
6.2	0.90	0.312		0.140	2.00	26.6	37.1	10.5
6.2	0.90	0.456		0.270	3.85	28.6	36.2	7.6
5.5	1.08	0.151	0.0319	0.0428	1.34	19.1	25.3	6.2
5.5	1.08	0.167		0.0599	1.88	22.0	32.3	10.3
5.5	1.08	0.189		0.0841	2.63	24.1	38.8	14.7
5.5	1.08	0.220		0.118	3.70	27.5	45.1	17.6
5.5	1.08	0.325		0.231	7.24	33.0	53.1	20.1
4.6	1.08	0.151	0.0389	0.0428	1.10	24.7	26.6	1.9
4.6	1.08	0.167		0.0599	1.54	29.5	33.3	3.8
4.6	1.08	0.189		0.0841	2.16	33.0	39.8	6.8
4.6	1.08	0.220		0.118	3.03	34.0	44.9	10.9
4.6	1.08	0.264		0.165	4.24	35.0	45.4	10.4
4.6	1.08	0.325		0.231	5.94	37.0	46.5	9.5

* Range 25, see Eqn 1 for conversion of chart units (C.U.) to heat.
** Temperature, 37 °C.

TABLE II

VARIATION OF ε VALUES WITH FLOW RATE ON RANGE 25

Total flow rate (ml/min)	$\varepsilon \times 10^5$ $(cal \cdot min^{-1} \cdot C.U.^{-1})$
0.1	5.47
0.2	5.52
0.3	5.56

flow rates of prymnesin and erythrocyte suspension, the residence time in the calorimeter was 33 s.

Size determinations

Solutions of erythrocytes in phosphate buffer (pH 6.2 or 5.5) were counted electronically at 4 threshold unit intervals. Counts (cells/ml) were corrected for background and differential counts and mean cell totals were calculated. The instrument (Coulter counter, Model B, 100μm aperture) was calibrated in units of μm^3 using latex spheres (Dow Chemical Company, Bioproducts Division) of known value.

Prymnesin (^{3}H-labelled) binding by red blood cells (Table III)

Erythrocyte suspensions in phosphate buffer (2.9 ml) of known concentrations were treated with 0.1 ml ^{3}H-labelled toxin. Reaction was stopped and cells were separated within 20 s of mixing by cooling in an ice–water bath and by centrifugation (36400 × *g*, 100 s). Supernatant was separated and pellets were treated with 1.5 ml of methanol to extract the toxin. Two 0.5-ml aliquots were used for counting, and the extractions were repeated twice. The activity of each sample in liquid scintillation solution was determined in a Packard Tri-Carb Model 3375 Liquid Scintillation Spectrometer. Total pellet activity was divided by total activity to give the fraction of bound ^{3}H-labelled toxin.

TABLE III

BINDING OF ^{3}H-LABELLED BY BOVINE ERYTHROCYTES

pH	*Cells/ml* ($\times 10^{-9}$)	*Samples*	*Fraction of activity*
7.0	1.93	2	0.110 ± 0.025*
6.2	1.94	3	0.186 ± 0.026
5.5	2.17	3	0.264 ± 0.033
4.6	2.70	3	0.263 ± 0.022

* ± 1 S.D.

RESULTS

Microcalorimetry

Before considering the microcalorimetry results it is useful to consider two basic requirements First, the solutions of prymnesin in buffer and methanolic buffer that were used in this study had to have the same pH, the same methanol concentration, and thus be microcalorimetrically equivalent. It was important to verify this because the heat of dilution of methanol could account for appreciable heat production, as could heat of neutralization assuming that the control- and test-run buffers had different pH values. Hence, there was a need to verify that the pH values were nearly the same, using a pH meter, and by testing that heat produced by mixing the two buffers was equal to that of viscous heating.

The second requirement was to correlate the heat produced with some useful parameter. The heat produced is a function of temperature, pH, concentration

of prymnesin, concentration of erythrocytes, *i.e.* hematocrit, and flow rate. We have combined several parameters into one useful one, the binding parameter which is defined (Eqn 2, Fig. 1)

$$\text{Binding parameter} = \frac{\text{prymnesin (mg)}}{\text{packed red cells (cm}^3\text{)}} = \frac{\text{flow of prymnesin solvent} \times \text{concn solution}}{\text{flow of red cell suspension} \times \text{hematocrit}} \qquad (2)$$

Referring to Table I, we compared the situation when the temperature is 25 °C, the pH was constant (6.2), but the prymnesin concentration was different (1.20, Line 9; 0.90, Line 14, Column 2) and the hematocrits were similar (0.38, Line 9; 0.45, Line 14). The net rate of heat produced (9.0 and 10.5, Column 9) and the binding parameters (1.64 and 2.00, Column 6) were both similar, and the trend was correct: the heat produced was a linear function of binding parameters for a constant flow of erythrocyte suspension (*cf.* Fig. 2). This is reasonable if the heat produced by binding should be related (at given pH and temperature) to the concentration of prymnesin and to the surface area, which is reflected in the concentration of packed cells.

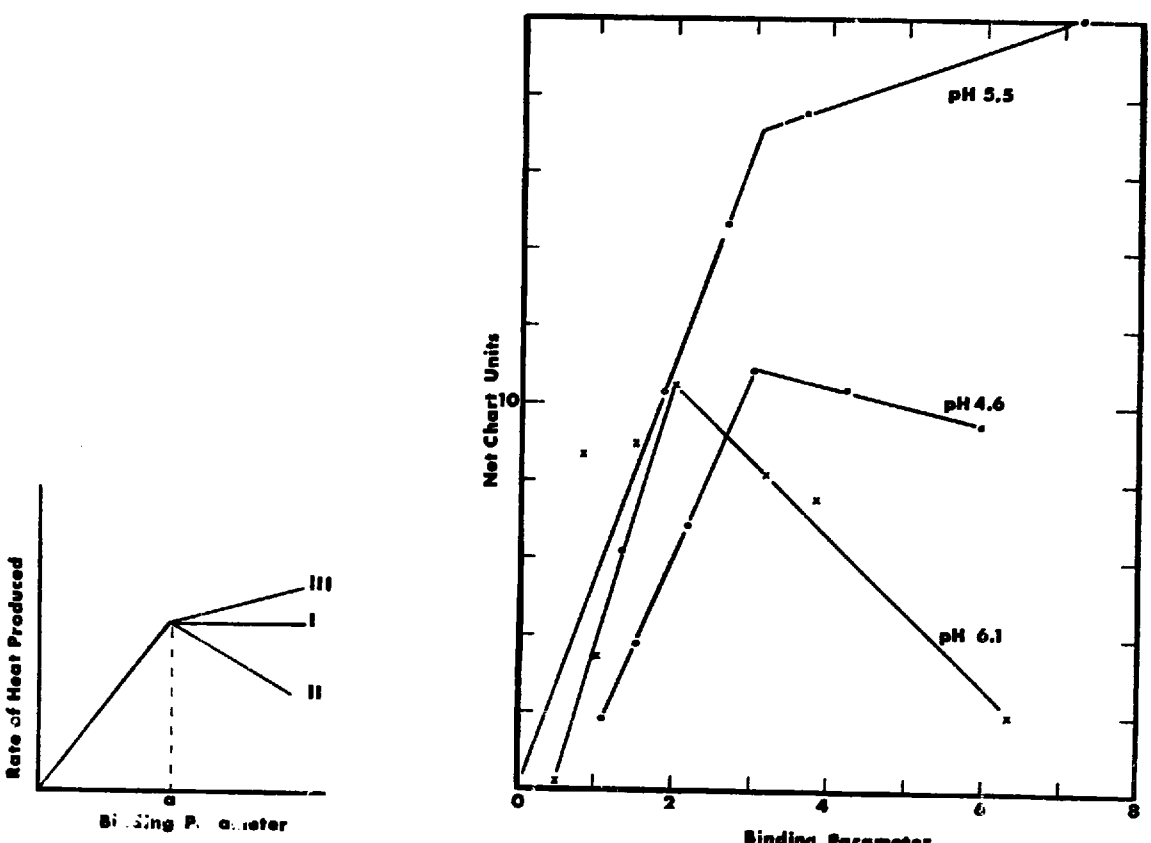

Fig. 1. Generalized representation of a flow microcalorimetry correlation plot, rate of heat produced (chart units) as a function of binding parameter (mg prymnesin/cm³ packed bovine erythrocytes). Here *a* represents the maximum binding parameter for three flow rates as described in the text.

Fig. 2. Rate of net heat production (ordinate: chart units) as a function of the binding parameter (abscissa: mg prymnesin per cm³ packed bovine erythrocytes) at 25 °C and indicated pH values

Three other factors are involved in magnitude of heat produced for a given value of the parameter: temperature, total flow rate, and pH and these can be considered in order.

The heat produced per unit flow rate of packed cells at 37 °C was greater than at 25 °C at a pH of 6.1–6.2 and a binding parameter of 0.5–0.6, but a temperature of 25 °C was selected to permit comparison with rate studies[4] and to mitigate effects of hemolysis.

The effect of total flow rate can be to diminish the apparent heat produced as seen in the correlation plot, heat produced as a function of binding parameters (Fig. 1). Given an optimal total flow rate (Case I) the amount of heat produced should be a linear function of the binding parameter until a maximum value for the parameter is attained. Beyond this point, a plateau should be observed, *i.e.* the heat produced is constant and independent of the binding parameter. At supraoptimal flow rates (Case II), *i.e.* those that are too fast, the residence time in the microcalorimeter reaction chamber may be less than the time needed for completion of the reaction, which would explain a negative slope. At suboptimal flow rates (Case III), two problems arise: (1) the heat output with time can become slightly erratic, and (2) the residence time may be excessively long and hemolysis can occur; and the slope beyond the value of the maximum binding parameter would be positive because of additional heat being liberated by hemolysis.

The third factor that affects the amount of heat liberated is the pH This is of special interest because the hemolytic activity of prymnesin was previously found to be maximal at pH 5 5 (ref. 3). In the present study, the pH had three different effects. First, the maximum binding parameter (Table I, Fig. 3) was sensitive to pH: no significant binding of toxin was observed at pH 7, but its increase to pH 5.5 was linear, and below this value to pH 4.6 there was no change in maximum binding parameter. Second, the heat associated with maximum binding parameter

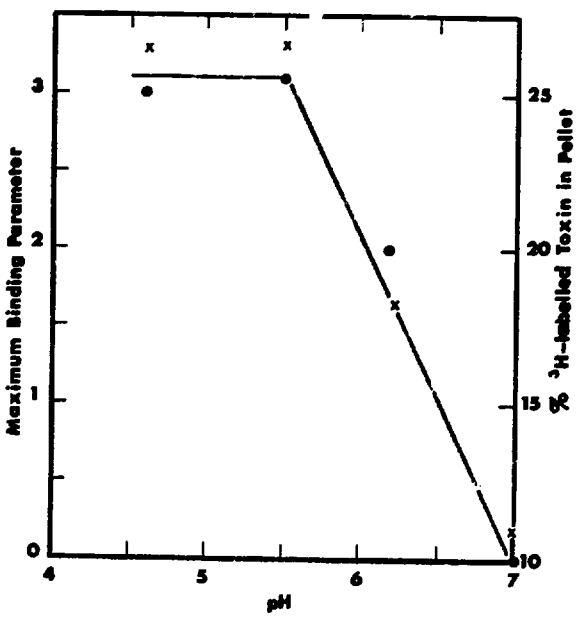

Fig. 3. Maximum binding parameter–pH profile for ^{3}H-labelled prymnesin and bovine erythrocytes at 25 °C. The left ordinate (●) shows the maximum binding parameter (Fig. 2) measured at each pH indicated. The right ordinate (*x*) indicates the fraction of the labelled toxin measured in the pellet. See text for details.

also varied with pH; a maximum was observed at pH 5.5 and the pattern followed that observed for hemolytic activity. Third, in the diagnostic plot (Fig. 2), changes of slope beyond the inflection point appeared to vary with pH, though many additional data would be needed to confirm this point.

Size determination

Mean cell values for bovine erythrocytes and latex spheres were determined in the phosphate buffers (pH 5.5 and 6.2) used in the microcalorimetry studies. The mean cell volumes were identical (102.4 and 102.6 μm^3 at pH 6.2 and 5.5, respectively). We conclude that the differences in maximum binding parameter and heat values at this parameter cannot be attributed to differences in mean cell volumes or total surfaces.

Prymnesin (^3H-labelled) by bovine cells

Binding values (fraction of ^3H-labelled toxin in pellet) showed two interesting results. First, the fraction bound at pH 5.5 for bovine cells (0.26 + 0.033) was similar to that reported[6] for rabbit cells (0.289 for $1.86 \cdot 10^9$ cells/ml). Second, the fraction of toxin bound to bovine cells showed a dependency (Fig. 3) on pH that is quite similar to that observed for the maximum binding parameter. The correlation coefficient (maximum binding parameter *vs* binding fraction) was 0.986 and the degree of association, R^2, was 0.972.

DISCUSSION

The results of this study show that microcalorimetry can be a useful means of characterizing the binding tendencies of a hemolysin such as prymnesin. We believe that the approach may be a generally useful one and that erythrocyte-binding tendencies of marine bioactive substances (and drugs in general) may be conveniently measured by flow microcalorimetry. Suitable instruments are commercially available and are becoming rather widely used.

Not only is the method convenient, but it offers advantages of directness and rapidity over some other procedures. The binding of certain hemolysin has been measured indirectly in previous studies. For example, the action of *G. breve* toxin in limiting hemolytic activity of prymnesin was measured as the former affected hemolytic rates[6] of the latter. Also, binding tendencies of prymnesin were measured as rate constants though direct binding of ^3H-labelled toxin was also determined[4]. Finally, Seeman's method (*cf.* Roth and Seeman[9]) has been used to measure the protective action of lysins using stressed red cells[2].

Our results compare the results with microcalorimetry (as maximum binding parameter) with binding of ^3H-labelled toxin, and we find that the two sets of values are closely related, as indicated by the high degree of association. This observation provides reason for confidence in the microcalorimetry procedure.

The maximum amount of toxin bound (pH 5.5, 4.6) was 26 $\pm$ 3 % for bovine cells (Table III) and 29 % for rabbit cells[6] (pH 5.5) using concentrated cell suspensions (approx. $1.9 \cdot 10^9$ cells/ml in both instances). This study also shows that prymnesin is maximally bound to rabbit cells at pH 5.5.

The results with microcalorimetry and with radiochemical studies are com-

plementary and dispel one possible concern that the ^{3}H-labelled toxin is profoundly different from the ordinary toxin. The hemolytic activity–fraction profile (using gel filtration chromatography) are similar for the two toxins. Microcalorimetry with ordinary toxin indicates the maximum binding parameter–pH profiles are similar to binding–pH profiles obtained for the ^{3}H-labelled toxin. The quality of agreement is emphasized by the high correlation coefficient (0.986) for the two profiles.

The reason for maximal binding in pH range of 4.6–5.5 is presently uncertain, but three reasonable possibilities can be considered. First, the critical pH value (approx. 5.5) could be related to the pK_D of prymnesin. Second, the value is related to the maximum rate of hemolysis. Third, the critical pH values could be related to the dissociation constant of sialic (nonulosaminic) acids (*cf.* Gottschalk[10]) present on the red cell membranes.

The first suggestion can be dismissed as being an unlikely cause. The critical pH would require a reactive carboxyl group associated with prymnesin with a pK_D equivalent to acetic acid, and current evidence does not support this possibility[11].

The second possibility is superficially an attractive one because the hemolytic tendency–pH profile (expressed as percent hemolysis for a 2% red blood cell suspension at various pHs) for rat blood[3] shows a similarity to the maximum binding parameter–pH profile for bovine blood. The tendency of bovine blood to lyse, however, shows a minimum, not a maximum, at pH 4.6–5.5 (0.0006 mg/ml red cell suspension). As noted previously, the kinetics of lysis are biphasic: (a prolytic phase is followed by a lytic phase) and are complex[4]. The length of the prolytic phase is inversely related in a general way to the rate of the lytic process, but the measurement of gross hemolysis (percent lysis after a defined incubation period) is not as revealing as rate data, and the lysis rate–pH profile has not been reported for red cell suspension.

The third suggestion seems more reasonable at first glance. Glaser[12] reported that the net charge on intact cells exists exclusively at the outer surface. On rat erythrocytes, the entire mobility–pH curve was consistent with a hypothesis that sialic acid is mainly responsible for the surface charge[12]. The mobility–pH profile (for human erythrocytes) also does not show any breaks in the pH range 4–6 or down to the isoelectric point (pH 2) of hemolysed cells[14]. The pK_D reported for sialic acid (2.8)[13] does not correspond to a change in the maximum binding parameter–pH change at pH 5.5, but it would be reasonable to expect that the pK_D of sialic acid incorporated on a membrane surface would be greater than that of the free acid. Thus, though the maximum binding parameter–pH profile cannot be related to mobility, it may be reasonable to try to relate a change in dissociation constant of bound sialic acid to changes in the properties of sialic acid on the erythrocyte membrane. This, however, must remain a tentative suggestion and a definitive solution would require much additional study.

ACKNOWLEDGEMENTS

We acknowledge the helpful technical assistance of James C. Foster and Priscilla Brown. This research was supported in part by a Public Health Service Research Career Development Award (1 K04 GM4259-03 to D.F.M.) from the

National Institute of General Medical Sciences and in part by grant 2 RO1 FD00120-06 from the Food and Drug Administration Consumer Protection and Environmental Health Service and Grant 20078-01 from the National Institute of Mental Health.

REFERENCES

1 S. Ulitzur and M. Shilo, *Biochim. Biophys. Acta*, 201 (1970) 350.
2 B. Rauckman and G. M. Padilla, *14th Annu. Meet. Biophys. Soc., February 27–29, 1970 Baltimore, Md.*, Biophys. Soc. Abstr., New York, p. 73a.
3 G. M. Padilla, *J. Protozool.*, 17 (1970) 456.
4 D. F. Martin and G. M. Padilla, *Biochim. Biophys. Acta*, 241 (1971) 213.
5 D. F. Martin, G. M. Padilla, M. G. Heyl and P. A. Brown, *Toxicon*, 10 (1972) 285.
6 D. F. Martin, G. M. Padilla and P. A. Brown, *Biochim. Biophys. Acta*, 249 (1971) 69.
7 J. S. Binford, Jr and D. M. Holloway, *J. Mol. Biol.*, 31 (1968) 91.
8 J. M. Sturtevant and P. A. Lyons, *J. Chem. Thermodyn.*, 1 (1969) 201.
9 S. Roth and P. Seeman, *Nature, New Biol.*, 231 (1971) 284.
10 A. Gottschalk, *The Chemistry and Biology of Sialic Acids and Related Substances*, Cambridge University Press, 1970, London.
11 M. Shilo, in S. Kadis, A. Ciegler and S. J. Ajl, *Microbial Toxins*, Academic Press, New York, 1971, p. 67.
12 R. M. Glaser, *Diss. Abstr.*, 25 (1964) 1502.
13 A. Gottschalk, *Glycoproteins*, Elsevier, 1966, Amsterdam.
14 E. Ponder and R. V. Ponder, *J. Exp. Biol.*, 32 (1955) 175.