EFFECT OF *pH* ON THE HAEMOLYSIS OF RABBIT ERYTHROCYTES BY STAPHYLOCOCCUS ALPHA-TOXIN

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LOMINSKI AND ARBUTHNOTT (1962) described the effect of staphylococcus α -toxin concentration on percentage haemolysis-time curves, for one temperature and one *pH* value. The curves were approximately sigmoid in character and indicated that the lag time, and the time to attain any particular percentage haemolysis, were decreased by increase in toxin concentration. When the slope of the approximately linear portion of the haemolysis-time curve was plotted against toxin concentration, a plateau was obtained.

The haemolytic reaction of this toxin has also been studied by Turpin *et al.* (1954), Marucci (1963) and Cooper, Madoff and Weinstein (1964) who reported similar results.

In the present work it was decided to investigate the kinetics of haemolysis over a wider variety of conditions in order to study the effects of variation of *pH*, concentration of erythrocytes, and purity of toxin, and to use a more sensitive physical method to follow the course of the reaction.

MATERIALS AND METHODS

Production and assay of crude staphylococcal α -toxin. Crude and partially purified α -toxin was supplied by Mr S. Gray, Department of Microbiology, The University, Glasgow. The crude α -toxin was prepared from cultures of *Staphylococcus aureus*, strain Wood 46 (NCTC7121), grown on semi-solid nutrient agar in the presence of 20 per cent. CO₂ (Lominski and Arbuthnott). The supernatant fluid obtained after centrifuging out the cells was lyophilised in 0.5-ml amounts, and stored at -20°C . Estimation of haemolytic activity by titration against washed rabbit erythrocytes was carried out as described by Lominski and Arbuthnott; such preparations contained *c.* 16,000 minimal haemolytic doses (MHD) per ml. Crude toxin was concentrated and partially purified to stage 2 of the method of Lominski, Arbuthnott and Spence (1963); such preparations contained *c.* 32,000 MHD per ml with a specific activity of 1500 MHD per mg protein.

Rabbit erythrocytes. Rabbit blood (approximately 6 ml) was obtained by bleeding from the ear vein into *c.* 4 ml of 3.8 per cent. trisodium citrate as anticoagulant. The same rabbit was used throughout as donor. The erythrocytes were washed at least four times in phosphate-buffered saline, and stored at 4°C for up to 5 days.

Phosphate-buffered saline (PBS) was prepared as described by Hendry (1948). The various solutions at the required *pH* values contained 66 per cent. of isotonic saline (0.85 per cent. NaCl) and 33 per cent. of the appropriate phosphate buffer. The phosphate buffers were prepared by mixing appropriate volumes of solutions of NaH₂PO₄ (26 g per litre) and Na₂HPO₄ (18 g per litre).

Calibrations. These were carried out by measuring both the light-scattering effect (extinction) and the number of red blood cells per unit volume for graded concentrations of

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erythrocytes. Various suspensions of rabbit erythrocytes were prepared from packed red blood cells by addition to isotonic buffered saline containing 0.6 per cent. (w/v) sodium chloride and 50 mM sodium phosphate buffer (Hendry). The optical densities of the various suspensions were measured at 650 nm in a Unicam spectrophotometer, model SP600, in cuvettes of 1-cm light path. The cell counts were made on an "improved" Neubauer counter using all 25 large squares in order to increase the number of cells counted. Calibrations were carried out at *pH* values differing by about 0.5 *pH* units over the range 5.5 to 8.5 for serially diluted suspensions of packed red cells in PBS. At *pH* values of 5.49, 6.84 and 8.54 calibrations were carried out with suspensions starting at a 1 in 300 dilution of packed red blood cells, but a starting dilution of about 1 in 3000 was used for tests at the other *pH* values.

Kinetic studies. A turbidimetric method similar to that of Turpin *et al.* was used to measure the rate of haemolysis of suspensions of rabbit erythrocytes by varying concentrations of toxin at $24.8 \pm 0.2^\circ\text{C}$. The decrease with time in extinction at 650 nm was followed spectrophotometrically. Reaction mixtures had a total volume of 3 ml and contained 2 ml of PBS at the desired *pH*, 0.5 ml of a dilution of α -toxin, and 0.5 ml of a 1 in 500 dilution of packed red blood cells. At 15-s intervals, beginning 45 s after addition of erythrocytes, readings of extinction were made against a blank containing PBS. Control runs were carried out in the absence of toxin. At least two kinetic runs were carried out for each toxin concentration, and all results quoted are mean values. Experiments with crude toxin were carried out for each of the toxin concentrations, 2.5, 5, 10, 20, 30, 40, 60 MHD per ml, at *pH* values of 5.50, 5.96, 6.30, 6.82, 7.30, 7.90 and 8.60 and with an initial dilution of 1 in 3000 of packed red blood cells in PBS. Experiments with the partially purified toxin were carried out with the same initial erythrocyte concentration and at approximately the same *pH* values, i.e., 5.49, 5.89, 6.38, 6.84, 7.42, 7.86, 8.54, but the range of toxin concentration was extended to include 80 and 120 MHD per ml.

To determine the order of the reaction with respect to erythrocytes, the kinetics were studied over the above range of *pH* for the crude toxin, and at *pH* 5.5, 7.0 and 8.5 for the partially purified toxin. The initial erythrocyte dilution ranged from 1 in 300 to 1 in 1500 of packed red blood cells in PBS.

Three criteria were used to assess the effect of toxin concentration on the rate of haemolysis: (1) the lag time, τ , i.e., the time during which no detectable haemolysis occurs; (2) the half-life, $t_{1/2}$, i.e., the time taken to reach 50 per cent. haemolysis; and (3) the quotient, R_m , which is the maximum rate of haemolysis divided by the maximum extinction. The maximum rate of haemolysis here refers to a maximum rate for any one run, i.e. the maximum absolute value taken by the slope of the plot of extinction against time, the slope being estimated by drawing tangents to the curve. The denominator corrects for any small changes in the concentration of initial erythrocyte suspensions easily apparent as small changes in the initial extinction, and for change in size or shape of the red blood cells before apparent lysis occurred. Such changes, apparent as an increase in extinction, were more marked at the lower *pH* values.

RESULTS

Calibrations

Over the range of erythrocyte suspensions normally used, i.e., for suspensions at dilutions of 1 in 3000 or more, plots of extinction against concentration were linear. For a given *pH* they were not normally superimposable from day to day owing to the difficulty of preparing, by volume, exactly identical red blood cell suspensions. At higher red cell concentrations, i.e., for dilutions from 1 in 3000 to 1 in 300, the plot fell off markedly with increasing concentration of erythrocytes, and consequently there was considerable loss in sensitivity of the calibrations for the lower dilutions.

TABLE

*Effect of toxin concentration on the haemolysis of rabbit erythrocytes
at 25°C at various pH values*

pH of reaction mixture*	Concentration of toxin (MHD per ml)	Pre-lytic lag time τ (min.)†	Time required for 50 per cent. haemolysis $t_{\frac{1}{2}}$ (min.)	Maximum rate factor R_m (min. ⁻¹)‡
5.49	120	1.25	2.25	0.705 ± 0.072
	80	1.75	3.63 ± 0.13	0.562 ± 0.037
	60	2.00	4.00 ± 0.25	0.492 ± 0.066
	40	2.50	4.25	0.437 ± 0.008
	30	2.00	4.25	0.399 ± 0.011
	20	2.50	4.63 ± 0.13	0.349 ± 0.019
	10	3.0	7.13 ± 0.13	0.218 ± 0.002
	5	4.13 ± 0.13	11.38 ± 1.13	0.117 ± 0.016
	2.5	6.0	30 ± 2	0.052 ± 0.007
5.89	120	1.50	2.50	1.100 ± 0.048
	80	1.75	3.0	0.952 ± 0.020
	60	1.50	2.75	0.925 ± 0.007
	40	1.88 ± 0.13	3.25	0.844 ± 0.042
	30	2.0	3.38 ± 0.13	0.723 ± 0.029
	20	2.13 ± 0.13	3.50 ± 0.25	0.641 ± 0.037
	10	3.0 ± 0.25	4.50 ± 0.25	0.466 ± 0.018
	5	3.75 ± 0.25	6.38 ± 0.13	0.362 ± 0.027
	2.5	6.25 ± 0.25	11.0 ± 0.5	0.172 ± 0.020
6.38	120	1.63 ± 0.13	3.13 ± 0.13	0.892 ± 0.031
	80	1.88 ± 0.13	3.38 ± 0.13	0.832 ± 0.088
	60	2.25	3.85 ± 0.13	0.820 ± 0.044
	40	2.00	3.38 ± 0.13	0.779 ± 0.034
	30	2.00	3.75 ± 0.25	0.700 ± 0.080
	20	2.50	4.50	0.561 ± 0.026
	10	2.75	5.00 ± 0.25	0.434 ± 0.026
	5	3.38	6.50 ± 0.5	0.315 ± 0.020
	2.5	5 ± 1	10.5 ± 1.5	0.220 ± 0.057
6.84	120	1.50	3.50	0.800 ± 0.015
	80	1.38 ± 0.13	3.63 ± 0.13	0.760 ± 0.046
	60	1.75	4.25	0.674 ± 0.005
	40	2.0	4.75 ± 0.25	0.568 ± 0.046
	30	2.25	4.50	0.541 ± 0.023
	20	2.50	5.13 ± 0.13	0.440 ± 0.040
	10	3.63 ± 0.13	6.88 ± 0.13	0.364 ± 0.020
	5	4.00 ± 0.50	8.13 ± 0.38	0.281 ± 0.008
	2.5	5.75 ± 0.25	11.88 ± 0.13	0.150 ± 0.012
7.42	120	1.75	4.0 ± 0.25	0.726 ± 0.028
	80	2.00 ± 0.25	4.25 ± 0.25	0.672 ± 0.078
	60	2.25 ± 0.25	4.50 ± 0.25	0.597 ± 0.027
	40	2.50 ± 0.25	4.75 ± 0.25	0.515 ± 0.090
	30	2.75	5.5	0.512 ± 0.049
	20	2.75	5.68 ± 0.13	0.434 ± 0.004
	10	3.75 ± 0.5	7.30 ± 0.4	0.355 ± 0.035
	5	4.00	8.38 ± 0.13	0.263 ± 0.004
	2.5	5.48 ± 0.31	12.65 ± 0.65	0.135 ± 0.006

TABLE—continued

pH of reaction mixture*	Concentration of toxin (MHD per ml)	Pre-lytic lag time τ (min.)†	Time required for 50 per cent. haemolysis $t_{\frac{1}{2}}$ (min.)	Maximum rate factor R_m (min. ⁻¹)‡
7.86	120	1.50	3.88±0.13	0.670±0.009
	80	1.50	4.38±0.13	0.567±0.019
	60	2.00	5.00±0.25	0.553±0.017
	40	2.25	5.13±0.13	0.490±0.030
	30	2.50	5.13±0.13	0.473±0.021
	20	3.25	5.88±0.13	0.455±0.004
	10	3.85±0.13	7.38±0.13	0.305±0.006
	5	4.0	7.68±0.13	0.281±0.012
	2.5	6.13	13.87±0.37	0.126±0.009
8.54	120	1.50±0.25	4.13±0.38	0.755±0.080
	80	2.0±0.25	4.88±0.13	0.610±0.040
	60	1.88±0.13	4.5±0.25	0.614±0.060
	40	2.50±0.25	5.25	0.513±0.020
	30	2.84±0.34	5.60±0.4	0.460±0.056
	20	2.68±0.13	5.81±0.4	0.441±0.053
	10	3.13±0.13	7.38±0.13	0.341±0.030
	5	5.25±0.25	10.13±0.13	0.207±0.003
	2.5	6.75±0.25	140±0.5	0.120±0.012

* The reaction mixture was as described in the *Methods* section.

† The pre-lytic lag is the initial period during which no visible reaction occurs.

‡ The maximum rate factor is the quotient of the maximum rate of haemolysis divided by the maximum extinction value obtained.

The calibrations were more reliable and reproducible when both the extinction and the number of red blood cells present per unit volume were measured for given suspensions. Calibrations for suspensions of packed red blood cells at concentrations of less than a 1 in 3000 dilution indicate that, within the experimental error (which is high for cell counts on such dilute suspensions), the calibration is independent of pH. Calibrations for higher concentrations up to a dilution of 1 in 300 again indicated that, although there was a considerable decrease in sensitivity, the extinction value for a given erythrocyte suspension is independent of pH.

Kinetics: order with respect to toxin

The results of these studies are summarised in the table.

Experiments with crude toxin. The kinetic curves were essentially similar to those reported earlier by Lominski and Arbuthnott. Approximately sigmoid haemolysis-time curves were obtained, and plateaux in R_m -toxin concentration, τ -toxin concentration, and $t_{\frac{1}{2}}$ -toxin concentration curves were found at all pH values (see fig. 1).

The effect of pH on haemolysis is very marked, and the shape of the profile changes as the toxin concentration is increased (fig. 2). This behaviour is reflected in similar plots for τ and $t_{\frac{1}{2}}$.

Experiments with partially purified toxin. Qualitatively the results are similar to those obtained with the crude toxin, but the plateaux obtained in the R_m -toxin concentration plots occur at higher toxin concentrations than with the crude toxin. Fig. 3 shows the pH profiles obtained from the smooth curves drawn through the R_m -toxin concentration plots as, for example, in fig. 1. A comparison of figs. 2 and 3 shows that for a given concentration of toxin

FIG. 1.—Effect of concentration of toxin on the maximum rate of haemolysis (R_m), time to 50 per cent. haemolysis ($t_{1/2}$), and lag period (τ). Reaction mixtures: to 2 ml of isotonic buffered saline (0.6 per cent. NaCl and 50 mM sodium phosphate buffer at pH 5.89) were added 0.5 ml of the test dilution of α -toxin and 0.5 ml of a 1 in 3000 dilution of packed rabbit erythrocytes. Control runs were carried out in the absence of toxin. The values of R_m (●), $t_{1/2}$ (□) and τ (△) were determined from the sigmoid haemolysis curves as described in the *Methods* section.

(in MHD per ml), the values of R_m for the crude toxin may be either greater or less than, or equal to those for the partially purified toxin, depending upon the pH of the test. With the partially purified toxin the effect of pH is marked, but the profile has now the same shape for all concentrations of toxin.

Kinetics: order with respect to erythrocytes

Within the range of dilutions of erythrocytes used for the great majority of experiments, i.e., 1 in 3000 to 1 in 15,000 dilutions of packed red blood cells, the lysis is strictly first order with respect to erythrocytes, irrespective of whether the crude or the partially purified toxin is used. At lower dilutions, however, the results were much less conclusive, and there may be a slight falling off in first-order behaviour at the lowest dilutions studied (especially at pH 7 and 8.5). This may well be a consequence of the inaccuracies resulting from the marked

FIG. 2.—Values of the maximum rate of haemolysis at various pH values for different concentrations of crude toxin. The experimental procedure was as detailed for fig. 1. The final test concentrations of toxin were 60 (○), 40 (△), 30 (□), 20 (●), 10 (▲), 5 (■) and 2.5 (◐) MHD per ml.

FIG. 3.—Values of the maximum rate of haemolysis at various pH values for different concentrations of partially purified toxin. The experimental procedure was as detailed for fig. 1. The final concentration of toxin was 120 (○), 80 (△), 60 (□), 40 (●), 30 (▲), 20 (■), 10 (◐), 5 (◑) and 2.5 (▼) MHD per ml.

flattening in the curve of extinction with increasing concentrations of erythrocyte suspensions.

DISCUSSION

pH profiles

It can be seen that, even with toxin at stage 2 of the purification procedure, purity has a marked effect on the kinetics of lysis, and this effect is greatest at the lower *pH* values of 5.5 and 6.0. For the partially purified toxin no great effect of *pH* is apparent above *pH* 7, whilst a quite definite maximum in rate occurs around *pH* 6.0, with a very rapid increase in rate from *pH* 5.5 to 6.0. The shape of the *pH* profile for the partially purified toxin is the same for all toxin concentrations studied: the slight deviations in the profiles are well within the range of experimental error. It would appear that the crude toxin contains a trace of a haemolytic impurity that is strongly lytic at *pH* 5.5 (note that the effect of the impurity increases with stoichiometric concentration of toxin) and is less markedly so at *pH* 8.5. The suspected impurity appears to have no great haemolytic effect between *pH* 6.5 and *pH* 8.0. My conclusion that there is a lytic impurity present in the crude toxin is supported by the results of recent work (Freer, Arbuthnott and Bernheimer, 1968) on the action of fractions of highly purified α -toxin on artificial lipid spherules.

Order with respect to erythrocytes

The reaction is first order with respect to erythrocytes. This is consistent with the effective toxin concentration being in excess over the actual substrate concentration, or with the toxin concentration remaining effectively constant, i.e., where the toxin is acting catalytically.

Order with respect to toxin concentration

The reaction is first order with respect to toxin over a very small range of toxin concentrations; at concentrations greater than 120 MHD per ml the maximum rate becomes independent of the toxin concentration. These results are much less easy to explain, especially in the absence of a quantitative estimate of the actual substrate concentration. This cannot be calculated from the number of red blood cells per ml, since the substrate in the intact erythrocyte membrane for staphylococcus α -toxin is not yet known.

SUMMARY

The effect of toxin concentration on the kinetics of haemolysis of rabbit erythrocytes has been studied at *pH* values differing by about 0.5 *pH* units over the range 5.5 to 8.5 for both crude and partially purified toxin at concentrations of 2.5, 5.0, 10, 20, 30, 40, 60 MHD per ml and at 80 and 120 MHD per ml for the partially purified toxin.

Increased concentration of toxin caused a decrease in the prelytic lag time (τ) of the sigmoid haemolysis curves, a decrease in the time to reach 50 per cent. haemolysis ($t_{\frac{1}{2}}$), and an increase in the maximum rate of haemolysis (R_m).

Plateaux in R_m -toxin concentration, τ -toxin concentration and t_4 -toxin concentration curves were found at all pH values: these were displaced to higher concentrations in plots drawn for the partially purified toxin.

The effect of pH on haemolysis is very marked, and pH profiles for the maximum rate of haemolysis were constructed: for the crude toxin, the shape of the profile changed as the toxin concentration was increased, while for the partially purified toxin the shape was independent of toxin concentration.

The pattern of the profiles indicates that the crude toxin contains a small concentration of impurity that is strongly lytic at pH 5.5 and less markedly so at 8.5; the impurity has no significant effect at intermediate pH values.

The reaction is first order with respect to toxin over a very small range of concentrations only, and the maximum rate of haemolysis is independent of toxin concentration at concentrations greater than 120 MHD per ml.

The reaction is first order with respect to erythrocytes at all pH values, and over all measurable concentrations.

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