Haemolysis Induced by Tyrosine Crystals

MODIFIERS AND INHIBITORS

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Tyrosine as a solid, but not in solution, caused human erythrocyte haemolysis. Haemolysis was increased with higher tyrosine concentrations and extended incubation times; it was greater at 37° than 4°C, and decreased by higher erythrocyte concentrations. Titration of phenolic groups on the surface of di-iodotyrosine crystals altered the extent of di-iodotyrosine-induced haemolysis. Haemolysis induced by tyrosine was inhibited by polyethylene glycol (mol.wt. 6000 or 20000) in a competitive fashion; polyoxyethylene/polyoxypropylene non-ionic detergents, polyvinylpyrrolidone (mol.wt. 40000 or 360000), 0.25–1.0M-NaCl, 0.25–1.0M-KCl and 0.25M-NaSCN also inhibited haemolysis. H⁺-ion donation from the phenolic groups of tyrosine is suggested as part of the mechanism of haemolysis. Non-ionic detergents may inhibit tyrosine-crystal-induced haemolysis by binding the phenolic groups at the surface of the crystal.

The erythrocyte membrane is a commonly used model for biological membranes. The interactions of soluble molecules with components of this membrane have been studied to define the mechanism of hormone action and enzyme, protein, carbohydrate or lipid localization. The interaction of solids with membranes have been studied less frequently; those studied include sodium urate (Wallingford & McCarty, 1971; Weissmann & Rita, 1972), calcium pyrophosphate dihydrate (Weissmann & Rita, 1972) and several minerals, including silicates (Nash *et al.*, 1966; Schnitzer *et al.*, 1971), chrysotiles (Schnitzer & Pundsack, 1970), asbestiform fibres (Schnitzer & Pundsack, 1970; Harington *et al.*, 1971) and magnesium hydroxides (Schnitzer *et al.*, 1971). The interactions between the cell membrane and a solid may be a useful model system for defining the characteristics of interactions between cell membranes and insoluble macromolecular complexes, such as other cells, viruses, bacteria and other micro-organisms.

A poorly soluble crystalline amino acid, tyrosine, and its structural analogues were used in these studies. As solids, but not in solution, they were able to lyse erythrocytes and lysosomal membranes as previously described in a preliminary communication (Goldsmith, 1975). I now report studies that elucidate further the interactions between tyrosine crystals and erythrocytes and the characteristics of the inhibitors of this interaction.

Experimental

Materials

L-Tyrosine, α -phenylglycine (α -aminophenylacetic acid), 3,5-di-iodotyrosine, lysophosphatidylcholine, retinal and polyvinylpyrrolidone (mol.wt. 40000 and 360000) were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Polyethylene glycol (mol.wt. 6000 and 20000) was purchased from Fisher Chemical (Raleigh, NC, U.S.A.). Several pluronic detergents (see Table 1) were from Wyandotte Chemicals (Wyandotte, MI, U.S.A.). [3,5-³H]Tyrosine (60.3 Ci/mmol) and Aquasol were purchased from New England Nuclear Corp. (Boston, MA, U.S.A.). [3,5-³H]Tyrosine was purified before use by the procedure of Miller & Thompson (1972). All other reagents and salts were of the highest grade available.

Human erythrocytes were collected with 16i.u. of heparin (Upjohn Co., Kalamazoo, MI, U.S.A.)/ml, and washed at least twice with 0.9% NaCl/0.2M-sodium phosphate, pH7.2 (buffered saline), before use. In some experiments the erythrocytes were obtained from Ficoll-Hypaque gradients (Boyle & Seegmiller, 1971), and identical results were obtained. Erythrocytes were used within 5 days. Frog, sheep and turkey erythrocytes were used in some experiments, as specified. The buffer for frog erythrocytes was 0.11M-NaCl/10mM-Tris/HCl, pH7.4.

Crystal size was controlled at 5–25 μ m in length by grinding with a porcelain mortar and pestle, and

microscopic examination. Unground tyrosine (Sigma) as well as ground crystals caused haemolysis. L-Tyrosine samples from Aldrich Chemical Co. (Milwaukee, WI, U.S.A.) and Nutritional Biochemicals (Cleveland, OH, U.S.A.) were slightly haemolytic before grinding; after grinding they were as haemolytic as L-tyrosine from Sigma, which was used as a routine. To produce finer particles, the crystals were placed in the sample capsule of a pulverizing freezer mill (Spex Industries, Metuchen, NJ, U.S.A.), and ground for 1–5 min at maximum impact frequency in a liquid N₂ bath.

Haemolytic assays

Haemolysis was assayed as a routine in disposable pyrex tubes (13 mm × 100 mm) by incubating 2.5 ml of a 1% (v/v) suspension of washed erythrocytes in buffered saline with tyrosine or other chemicals for 1 h at 37°C in a Dubnoff metabolic shaker at 60 rev./min or a rotating mixer at 20 rev./min, as specified. Preliminary experiments showed the same amount of haemolysis occurred in glass or polypropylene tubes. The mixture was centrifuged at 2000g for 10 min at 4°C, and 1 ml of the supernatant was added to 4 ml of aq. 0.6% NH₃ and the E_{540} or E_{410} measured. Blanks with erythrocytes in buffered saline were always measured and values subtracted from experimental readings. Complete haemolysis was obtained by suspending the same number of erythrocytes in 0.18% NaCl/4 mM-sodium phosphate buffer, pH 7.2.

Tests of the importance of physical contact

To determine the importance of physical contact between the tyrosine crystals and the erythrocytes, 2 ml of a 1% (v/v) suspension of fresh washed human erythrocytes were placed in washed dialysis bags and then suspended in 200 ml of buffered saline containing 5 mg of tyrosine/ml and 0.05 μ Ci of radioactive tyrosine/ml; also suspended in this mixture were dialysis bags containing only buffered saline. Similarly treated erythrocytes were suspended in 200 ml of buffered saline without tyrosine. The solutions were stirred at 4°C in a cold-room. At 20, 25 and 42 h, bags were removed from both the tyrosine-free and tyrosine-containing solutions, and haemolysis in the erythrocyte supernatants was determined. Radioactive tyrosine in the bags containing erythrocytes, buffer and tyrosine suspensions was determined by liquid-scintillation counting of samples of the supernatants dissolved in Aquasol with a Packard 3375 spectrometer.

Ionic strength

To study the effect of ionic strength, cells were washed with 0.02 M-sodium phosphate buffer, pH 7.2,

containing various concentrations of NaCl. After two washings, the cells were suspended in the same buffer and incubated for 1 h with tyrosine to observe the haemolytic reactions. Similar experiments were carried out with sodium phosphate buffer, pH 7.2, containing KCl or NaSCN.

Inhibitors of haemolysis

To determine the effects of various protective agents, a 1% (v/v) erythrocyte suspension was incubated with the agent being tested at 37°C for 10 min before the addition of tyrosine or phenylglycine.

To test the reversibility of polyethylene glycol (mol.wt. 20000) inhibition, cells were incubated at 37°C for 10 min with 1 mg of polyethylene glycol (mol.wt. 20000)/ml washed five times with a 15-fold excess of buffered saline and then tested in the standard haemolytic assay with tyrosine. Cells not exposed to polyethylene glycol were washed in parallel under the same conditions.

To test the effect of polyethylene glycol (mol.wt. 20000) on tyrosine solubility, tubes with 10 mg of tyrosine/ml in water were incubated with 2.5 ml of buffered saline containing 0, 100 or 1000 μ g of polyethylene glycol (mol.wt. 20000)/ml for 18 h at 37°C on a shaking-water bath, and the E_{280} of the supernatant was measured.

Results and Discussion

To analyse the nature of the tyrosine-erythrocyte interaction and its inhibition, several components of the system had to be considered: the necessity for solid tyrosine, potential reactive groups on the surface of tyrosine, the solvent, the erythrocyte surface including potential reactive sites for tyrosine, a multiple-step process of change in membrane permeability, and finally haemoglobin release. An attempt to separate this multicomponent process into its individual parts was begun.

Necessity for physical contact

Haemolysis did not occur without physical contact of cells and crystals. Erythrocytes suspended in dialysis bags in tyrosine suspensions were not haemolysed after suspension for 42 h in buffered saline. The concentration of radioactive tyrosine was the same inside and outside the dialysis bag, which suggests that tyrosine was at equilibrium concentrations. Haemolysis is not merely a consequence of physical and mechanical damage from the contact of erythrocytes and crystals. This has been shown by experiments in which amphibole asbestiform crystals (Schnitzer & Pundsack, 1970) and calcium pyrophosphate dihydrate crystals (Weissmann

Fig. 1. Effect of erythrocyte concentration on the extent of tyrosine-induced haemolysis

Erythrocyte suspensions at 0.5, 1, 2 and 5% (v/v) concentration were incubated at 37°C with four concentrations of tyrosine [32 (○), 16 (●), 8 (□) and 4 (■) mg/ml]. After 1 h haemolysis the E_{540} was measured as described in the Experimental section. The ratio, K (haemolysis produced after 1 h)/(total haemolysis produced in 0.18% saline), was determined.

& Rita, 1972) were not haemolytic, whereas urates (Wallingford & McCarty, 1971; Weissmann & Rita, 1972) and silicates (Nash *et al.*, 1966) were.

Particle size influenced the amount of haemolysis produced by the crystal-erythrocyte interaction. Large tyrosine crystals were poorly haemolytic before mortar-and-pestle grinding. If grinding was followed by a 5 min pulverization in a freezer mill, particles 1–3 μ m in size were produced. These particles were twice as efficient (on a weight basis) as mortar-and-pestle-ground crystals.

These results suggest the total available surface area of the crystal was an important variable, as is the case with silicates (Harley & Margolis, 1961; Staldor & Stoben, 1965). In the tyrosine studies, the surface areas of the crystals was not determined; it is possible that some differences in this respect affected some results in a quantitative fashion between individual experiments. However, within any single experiment, crystal size was constant.

Dependence of haemolysis on erythrocyte and tyrosine concentration and on temperature

It is not expected that procedures for kinetic analysis conventionally applied with solutes (e.g. as for enzyme catalysis) would be completely applicable to the present study of haemolysis involving the interaction of cells and crystalline particles. It was possible, however, to characterise empirically the dependence of haemolysis on erythrocyte and

tyrosine concentration (Fig. 1). The extent of haemolysis at 1 h was greater at lower erythrocyte concentrations. At the lowest tyrosine concentration there is a decrease in the absolute amount of haemolysis with increasing erythrocyte concentration. It would seem reasonable to suggest that as erythrocyte concentration increases, cell-to-cell interactions may decrease the extent of cell-tyrosine interaction.

Tyrosine-induced haemolysis increased as the tyrosine concentration was increased in the reaction mixture. After incubating a 1% erythrocyte suspension at 37°C with a tyrosine concentration of 16, 32 or 48 mg/ml, tyrosine-induced haemolysis (compared with hypo-osmotic haemolysis) was 36.2 ± 6.7 (S.E.M., $n = 5$), 63.2 ± 6.6 and $82.8 \pm 6.5\%$ respectively. At 4°C the haemolysis with 32 or 48 mg of tyrosine/ml was 25.4 ± 2.3 (S.E.M., $n = 5$) and $29.4 \pm 2.4\%$ respectively ($P < 0.0005$, Student's t test) for both tyrosine and temperature variation. Since the liquid phase was saturated even at the lowest amounts of tyrosine studied, it was considered that an increased crystal density produced more haemolysis. A similar dose-response relationship is observed with other haemolytic crystals (Goldsmith, 1975).

The increase in haemolysis observed with more tyrosine in the reaction mixture was less marked at 4° than at 37°C. This might have been related to differences in the cell membrane or solvent. The viscosity of the entire erythrocyte suspension would be almost doubled at 4° compared with 37°C (Reemtsma & Creech, 1962). Mechanical fragility of erythrocytes is less at 5° than at 37°C (Po-tun & Schubothe, 1960).

pH effects on haemolysis

Tyrosine haemolysis was unchanged in the pH range 6.0–9.5 in three experiments. Higher pH values caused haemolysis and prevented further titration studies with tyrosine. The tyrosine pK values for $-\text{NH}_2$ and the phenolic hydroxyl group are 9.11 and 10.13 respectively. The amino and phenolic pK values of another poorly soluble compound, di-iodotyrosine, were more suitable for titration; the pK values for $-\text{NH}_2$ and phenolic hydroxyl group are 7.82 and 6.48 respectively. There was a decrease in the haemolysis from pH 6.0 to 7.5 (Fig. 2). Di-iodotyrosine was still haemolytic at pH 9.5 (results not shown) to about the same extent as at pH 7.5. Only a small fraction of the di-iodotyrosine was solubilized at higher pH values, and this difference was not enough to be responsible for the decrease in haemolysis (Goldsmith, 1975). Haemolysis was decreased by half by an ionizable group in the vicinity of the pK value of the hydroxyl (6.48) group of

Fig. 2. Effect of pH on di-iodotyrosine-induced haemolysis

Erythrocytes were washed twice with a 15-fold excess of normal saline buffered to pH values from 6.5 to 7.5 with 0.02M-sodium phosphate buffer or with 0.2M-glycine/NaOH buffers from pH 7.5 to 9.95. The haemolytic reactions were then performed in the standard manner with 1% erythrocyte suspensions, and the extent of haemolysis measured by determining the E_{410} (●). Insoluble di-iodotyrosine was determined by incubating 2.5ml of 0.9% NaCl/0.02M-sodium phosphate buffer at various pH values with 32mg of di-iodotyrosine/ml. The amount of di-iodotyrosine solubilized was determined by the method of Lowry *et al.* (1951). Di-iodotyrosine standards showed a linear relationship between E_{750} and concentration. The amount of insoluble di-iodotyrosine (○) was determined by difference.

di-iodotyrosine. Over this pH range, there was no change in the haemolysis caused by tyrosine or phenylglycine, which suggested a pH effect on the di-iodotyrosine and not on an erythrocyte component. A tentative interpretation and extension of the titration data is that phenolic groups on the tyrosine crystal are involved with the erythrocyte interactions. Crystallographic studies of tyrosine show phenolic groups on the crystal surface (Gurskaya, 1968) that would be available for such interactions. With silicates (Harrington *et al.*, 1971) and urates (Weissmann & Rita, 1972) the mechanism of haemolytic action has been related to the capacity for hydrogen-bond formation involving their weakly acidic (—OH) groups. There are two possibilities: the —OH hydrogen may act as an electron acceptor or the oxygen may act as a donor of electrons to suitable hydrogen atoms on the membrane.

Salt concentration and haemolysis

Increasing concentrations of NaCl decreased haemolysis at four concentrations of tyrosine studied.

Fig. 3. Inhibition of tyrosine- or α -phenylglycine-induced haemolysis by polyethylene glycols

A suspension of erythrocytes (1%, v/v) was incubated with various amounts of polyethylene glycol (mol.wt. 20000 or 6000) before testing for haemolysis with various amounts of tyrosine. Control experiments without polyethylene glycols were carried out simultaneously. α -Phenylglycine was tested under similar conditions with polyethyleneglycol (mol.wt. 20000). At 32mg of α -phenylglycine/ml, and with 50 or 100μg of polyethylene glycol/ml, haemolysis was inhibited 45 and 70% respectively. At those polyethylene glycol concentrations with 32mg of tyrosine/ml, 100% inhibition was observed. Polyethylene glycol (mol.wt. 20000, —) was tested with tyrosine at 32mg/ml (●), 16mg/ml (○), and 8mg/ml (□). Polyethylene glycol (mol.wt. 6000, ----) was tested with tyrosine at 32mg/ml (△).

NaCl (5.0% w/v) decreased haemolysis 69, 68, 65 and 61% with 4, 8, 16 and 32mg of tyrosine/ml respectively. There were smaller, but proportionate, decreases with 1.8 and 3.6% NaCl compared with the 0.9% NaCl control. 0.2M-KCl inhibited 65% with 64mg of tyrosine/ml, and 0.5M-KCl inhibited by 74 and 83% with 64 and 32mg of tyrosine/ml respectively; 0.2M-NaSCN inhibited by 94 and 100% with 64 and 32mg of tyrosine/ml; 0.5M-NaSCN was haemolytic. The salts may have shielded electrostatic interactions of crystals and cells or directly decreased haemolysis by shrinking the cells or altering binding sites.

Non-ionic detergents as inhibitors of haemolysis

Polyethylene glycols (mol.wt. 6000 and 20000) were very effective inhibitors of tyrosine-induced haemolysis. The protection by polyethylene glycols was reversible, suggesting that polyethylene glycol itself was the inhibitor and that it did not permanently alter the erythrocytes. The inhibition was competitive, since increased amounts of tyrosine decreased the extent of the inhibition (Fig. 3). Polyethylene glycol of mol.wt. 20000 was a more effective inhibitor than polyethylene glycol of mol.wt. 6000 on both a weight and molar basis. Concentrations of polyethylene glycol (mol.wt. 20000) that completely

Table 1. *Inhibition of tyrosine-induced haemolysis by pluronic acids at 4° and 37°C*

Details of the molecular weights, subunit compositions and critical micellar concentrations for the pluronics used are given by Schmolka & Raymond (1965).

Pluronic	Mol.wt.	[Tyrosine] (mg/ml)	Inhibition at 37°C with 100 µg of pluronic acid/ml (%)	Inhibition at 37°C (%) Inhibition at 4°C (%) Amount of pluronic acid/ml (µg)	
				25	100
P123	5650	16	100	1.38	1.39
		32	100	1.71	1.92
		64	92	1.42	2.14
P85	4600	16	81	1.0	1.1
		32	88	1.74	1.06
		64	87	4.0	1.38
F38	5000	16	42	0.32	0.42
		32	46	∞	0.73
		64	20	0.19	0.42
F68	8350	16	77	2.79	1.97
		32	82	1.21	2.10
		64	46	5.25	2.09
F127	11 500	16	96	2.43	1.57
		32	96	2.37	1.71
		64	94	1.79	2.04

inhibited tyrosine haemolysis, decreased phenylglycine haemolysis by only 50–70% (see the legend to Fig. 3). Polyethylene glycol (mol.wt. 20000) was not protective against several other haemolytic agents studied. Tested at 1 mg/ml, 100 µg/ml and 330 µg/ml it gave no protection against haemolysis from lyso-phosphatidylcholine (50 µg/ml); retinal (vitamin A aldehyde, 10 µg/ml) or thymol (2 mg/ml) respectively; nor, at 17 µg/ml, against complement-mediated haemolysis of antibody-sensitized sheep erythrocytes. Under the conditions studied, polyethylene glycol (mol.wt. 20000) did not increase the solubility of tyrosine. Polyethylene glycols (mol.wt. 6000 and 20000) were effective at concentrations at which they were not osmotic protectants. They may have prevented haemolysis by specific interaction with tyrosine or a tyrosine-binding site. Phenolic hydroxyl groups bind to the ether oxygen atoms of polyoxyethylene chains by hydrogen-bonding [see reviews by Elworthy & MacFarlane (1965) and Jacobs *et al.* (1971)]. But, since polyethylene glycol was more effective than one-hydroxyl-group-containing polyoxyethylene derivatives, other mechanisms may play a role in this protection.

Moreover, the inhibition of phenylglycine-induced haemolysis makes it necessary to consider other modes of interaction. Polyethylene glycol may have been a more effective inhibitor of tyrosine-induced haemolysis, because of better binding of the whole tyrosine molecule to polyethylene glycol. Studying an analogous system, the binding of aromatic compounds to polyvinylpyrrolidone, Molyneux & Frank (1961)

suggested that there was increased binding of aromatic molecules with polar groups, compared with the aromatic compounds themselves. Alternatively, the haemolytic activities of phenylglycine and tyrosine may have been inhibited by completely different mechanisms.

To define the mechanism of inhibition of tyrosine-induced haemolysis by non-ionic detergents, several members of the polyoxyethylene/polyoxypropylene non-ionic detergents termed 'pluronic' were studied. These detergents are block polymers with different total molecular weights, different percentages of hydrophilic and hydrophobic groups, and different physical properties (Schmolka & Raymond, 1965). There was no simple relationship between any of these parameters and inhibition (Table 1). The best inhibitors were present in concentrations above their critical micelle concentrations; they had various percentages of hydrophobic and hydrophilic groups, but tended to have high-molecular-weight hydrophilic and hydrophobic subunits. Four of the five inhibitory pluronics inhibited more effectively at 37° than at 4°C; this may have been related to micelle size increasing with temperature (Elworthy & MacFarlane, 1965), and a decrease in critical micellar concentration or possibly a different mechanism of inhibition at the different temperatures. Probably their method of inhibition is related to their ability to bind reactive groups on the crystals; they did not change crystal size as determined by direct examination.

Polyvinylpyrrolidone (mol.wt. 40000 and 360000)

Table 2. *Inhibition of tyrosine-induced haemolysis by two polyvinylpyrrolidone polymers*

The experiments were performed at 37°C in a rotomixer at 20 rev./min. I_{50} is the concentration that inhibited tyrosine-induced haemolysis by 50%. Polyvinylpyrrolidone was tested at several concentrations, and the I_{50} determined by extrapolation.

[Tyrosine] (mg/ml)	Polyvinylpyrrolidone mol.wt. ...	I_{50}			
		40000		360000	
		(nmol)	(nmol/mg)	(nmol)	(nmol/mg)
16		6.9	0.45	7.2	0.43
32		8.3	0.35	11.1	0.26
48		22.2	0.30	14.4	0.46
64		29.2	0.52	33.3	0.46

is an acceptor for hydrogen-bond formation that inhibits haemolysis caused by urates and silicates (Wallingford & McCarty, 1971; Weissmann & Rita, 1972). Both molecular-weight species significantly inhibited haemolysis with tyrosine at concentrations where they were not osmotic protectants (Table 2). As the amount of tyrosine per reaction mixture increased, the concentrations necessary for inhibition increased. When results were expressed as nmol of polyvinylpyrrolidone/mg of tyrosine necessary to inhibit haemolysis by 50%, the results fell into a narrow range (mean 0.40, range 0.26–0.52). This stoichiometry suggests that an interaction between polyvinylpyrrolidone and the crystals is responsible for the inhibition rather than interaction between polyvinylpyrrolidone and the erythrocyte.

Non-human erythrocytes

Turkey, frog and sheep erythrocytes were tested in a manner similar to that used for human cells. Tyrosine was haemolytic to all cells tested. At 32 mg/ml and 37°C, 67% of turkey cells were haemolysed, 65% of frog cells and 52% of sheep cells, compared with 50% of human cells for that amount of tyrosine. Since sheep erythrocytes lack phosphatidylcholine (Nelson, 1967) it would appear that this constituent is not necessary for the crystal-erythrocyte interaction.

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