HEMOLYSIS AND LYSOSOMAL ACTIVATION BY SOLID STATE TYROSINE

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Received March 25,1975

SUMMARY

L-Tyrosine crystals hemolyzed human erythrocytes. Erythrocytes within dialysis bags suspended in tyrosine suspensions were not significantly hemolyzed. Ortho and meta tyrosine caused less hemolysis than para tyrosine. L-Tyrosine crystals labilized lysosomal β-glucuronidase from rat liver lysosomal preparations. Sucrose, glucose, propylene glycol, Me_2SO, polyethylene glycols, serum, albumin, globulins and cyclic AMP protected against the effect of tyrosine. Tyrosine analogues with a lower pK of the phenolic hydroxyl group than tyrosine, or with steric blockage of the phenolic hydroxyl group, had less hemolytic activity than tyrosine. It is proposed that tyrosine crystals in vitro possess membraneolytic effects by donation of phenolic hydrogens to membrane components.

Tyrosinemia and increased tissue tyrosine concentrations occur in humans because of a genetically determined deficiency of tyrosine amino transferase (1), or in rats after feeding a high tyrosine-low protein diet (2). In both cases, affected individuals have extensive exudative lesions of the corneal epithelium and plantar and palmar epidermis. Electron microscopic studies of these lesions (1) showed residual bodies and suggested lysosomal activation in the sites of pathology. These findings suggested studies to determine the effect of tyrosine and tyrosine analogues on human erythrocytes and rat lysosomes.

MATERIALS AND METHODS

L-Tyrosine, L-phenylalanine, α-phenylglycine, L-DOPA, tyramine, 3,5-diiodo-L-tyrosine, 3,5 dibromo-L-tyrosine, L-thyroxine, and N^6, O^2-dibutyryl cyclic monophosphoric acid, Triton X-100, bovine serum albumin, and gamma globulins were purchased from Sigma Chemical Company. D-p-Tyrosine, DL-m-tyrosine, DL-o-tyrosine were purchased from Aldrich Chemical Company, enzyme grade sucrose from Schwarz-Mann, and polyethylene glycols from Fisher. All preparations of tyrosine and its analogues were used as fine crystalline powders and were checked microscopically for crystal size. All other reagents and salts were the highest grades available. Human erythrocytes were collected with 500 units heparin per 30 mls and washed three times with 0.9% NaCl-0.02M phosphate buffer pH 7.2 (buffered saline) before use. Red blood cells could be used for five days; they were washed twice immediately before use each day.

Hemolysis was assayed by incubating a 2.5 ml volume of a 1% (v/v) suspension of washed erythrocytes in phosphate buffered saline with various chemicals for one hour, centrifuging the suspension at 2,000 x g for 10 minutes at 4°C, adding 1 ml of the supernatant to 4 ml of 0.6% NH_4OH and measuring the A_{540}. Blanks with erythrocytes in buffered saline were always measured. Hemolysis was compared to the A_{540} obtained after hemolyzing in water the same number of erythrocytes as were in the experimental tubes ("water hemolysis").

To determine if the tyrosine crystals themselves were hemolytic, 1% solutions of erythrocytes in washed dialysis bags were suspended in 50 ml of either buffered NaCl, water, or buffered NaCl containing 20 mg/ml, 40 mg/ml and 80 mg/ml L-tyrosine. The suspensions were stirred on magnetic stirrers with the temperature maintained at 27±2°C in the suspensions. After 6 hours, the bags were removed, the contents carefully removed, and the A_{540} on the supernatants determined as above.

In experiments to determine the effects of various protective agents, the agent was preincubated with erythrocytes in buffer at 37°C for 10 minutes before addition of L-tyrosine. Assays at 37°C were performed in a constant temperature room on a rotary shaker at 30 rpm or in a metabolic shaker; at 4° on a rotary shaker in a cold room; or at 24-25°C with a rotary shaker.

Rat liver lysosomes from male rats were prepared with slight modification of the techniques of Sawant (3). The liver was homogenized as a 10% suspension in 0.25M sucrose with 12 strokes of a glass-Teflon Duall homogenizer and centrifuged for 5 minutes at 600 x g. The pellet was discarded and the supernatant centrifuged for 5 minutes at 3,640 x g at 4°C. The 3,640 x g supernatant was centrifuged at 4°C for 30 minutes at 16,000 x g. The 16,000 x g pellet was used as a 10% suspension in 0.5M sucrose or was washed twice with 0.9% saline buffered with 0.02M phosphate pH 7.2 before being suspended in that buffer before use. Aliquots of the suspension containing additions of tyrosine, buffer alone, and with Triton X-100 to a final concentration of 0.1 mg/ml were incubated for 1 hour at 37°C. The mixtures were then centrifuged at 20,000 x g at 4°C for 20 minutes. 0.5 ml of the supernatant mixture was assayed for β-glucuronidase with phenolphthalein glucuronide as a substrate at pH 4.5 (4).

RESULTS

The extent of hemolysis was related to the amount of crystalline tyrosine in the reaction mixture (Figure 1). When erythrocytes were suspended in dialysis bags within tyrosine suspensions, although the incubation times were six times longer than the usual incubation time, the hemolysis which occurred was much less that observed when there was direct contact between tyrosine crystals and red cells. In these dialysis experiments 20 mg/ml tyrosine caused no hemolysis; 40 mg/ml, 12% hemolysis; and 80 mg/ml 7% hemolysis. This compared with 32 mg/ml tyrosine causing 64% hemolysis when there was direct contact between tyrosine crystals and erythrocytes for one hour.

The extent of hemolysis was related to the reaction temperatures, being greater at the higher temperatures. The hemolysis obtained with tyrosine was usually 10-20% less than that seen with water hemolysis. Tyrosine crystals

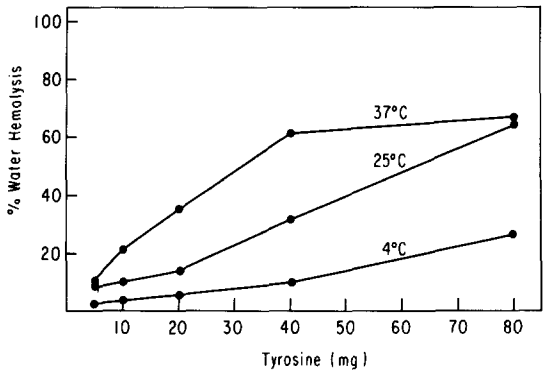

Figure 1. Hemolysis of 1% human erythrocyte suspensions in the presence of varying amounts of L-p-tyrosine crystals. The tube was rotated at 30 rpm for one hour at the different indicated temperatures. The hemolysis was quantitated as discussed in materials and methods.

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were equally hemolytic when phosphate buffered saline which contained in addition to saline and phosphate buffer 100 mg/l calcium chloride, 100 mg/l $MgCl_2 \cdot 6H_2O$ and 200 mg/l KCl was used instead of the 0.9% NaCl-0.02M pH 7.2 phosphate buffer which was standardly used.

D-Tyrosine and L-tyrosine were both hemolytic (Table 1); however, DL-o-tyrosine and DL-m-tyrosine were not. The tyrosine analogues, L-dihydroxy-phenylalanine, 3,5-diiodo-L-tyrosine, 3,5-dibromo-L-tyrosine, and L-throxine caused significantly less hemolysis than L-tyrosine. The analogues were as poorly soluble in the buffer used as tyrosine itself.

L-Phenylalanine, tyramine and α-phenylglycine, compounds without phenolic hydrogens, also caused hemolysis; α-phenyglycine was as hemolytic as L-tyrosine. In the concentrations used these compounds were not completely soluble.

Hemolysis with 32 mg/ml tyrosine was completely inhibited by preincubation of the erythrocytes for ten minutes with 0.8 mg/ml of bovine serum albumin or 0.8 mg/ml bovine γ-globulin, 0.8% serum from two patients with α_1-antitrypsin deficiency, 0.8% normal human serum and 0.8% human serum heated to 56°C for one hour. Human serum diluted to a final concentration of 1:100,000 inhibited tyrosine induced hemolysis by 30%. At 0.001 mg/ml bovine serum albumin did not inhibit

TABLE 1

Hemolysis by Tyrosine and Structural Analogues

Compound	Concentration (mg/ml)	% H_2O Hemolysis	Phenolic Hydroxygen pK_a.
L-tyrosine	0.4	0	10.13
	4	2	
	16	47	
	32	64	
D-p-tyrosine*	32	79	10.13
DL-m-tyrosine*	32	4	10.11
DL-o-tyrosine*	32	0	10.66
L-dihydroxy phenylalanine	16	5	9.88
	32	6	
3,5-diiodo-L-tyrosine*	8	2	7.82
	16	7	
	32	21	
3,5-dibromo-L-tyrosine*	8	0	7.60
	16	1	
	32	3.2	
L-thyroxine*	16	12	10.1
L-Phenylalanine	16	3	—
	32	15	
	64	30	
	80	25	
α-Phenylglycine	2	0	—
	4	3	
	8	18	
	16	26	
	32	62	
Tyramine*	16	10	—
	32	14	

Hemolysis was tested at 25° or 37° (*) in triplicate for all compounds at the stated concentrations except for L-thyroxine which was studied in duplicate; hemolysis was quantitated as described in materials and methods; pK data from (5,6).

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hemolysis. Microscopic examination confirmed that this inhibition was not due to agglutination of erythrocytes by these proteins.

Sucrose, glucose, propylene glycol and threonine all decreased hemolysis to

TABLE 2

Inhibitors of Tyrosine Induced Hemolysis

Inhibitor	Inhibitor Concentration (M)	% Inhibition of Tyrosine Induced Hemolysis Amount Tyrosine Present 8mg/ml	16mg/ml	32mg/ml
Sucrose	0.25	70	63	58
Glucose	0.25	66	0	-
Propylene Glycol	2.2	-	-	53
Dimethylsulfoxide	3.2	-	95	82
Threonine	0.5	34	13	-
N, $^{6}O^{2}$dibutyryl	3.8×10^{-3}	-	-	53
Adenosine 3':5'-cyclic monophosphoric acid	0.75×10^{-3}	-	-	15
Polyethylene Glycol 20,000	1.0×10^{-4}	-	-	90
	0.2×10^{-4}	-	-	95
Polyethylene Glycol 6,000	3.3×10^{-4}	-	-	80
	0.6×10^{-4}	-	-	73

Inhibition of hemolysis was tested by incubating a 1% suspension of human erythrocytes in 0.9% saline buffered saline with 0.02M phosphate pH 7.2 with the above agents for 10 minutes at 37°C and then incubating them with the above amounts of L-p-tyrosine for 1 hour at 37°C and then determining hemolysis as described in materials and methods.

varying degrees (Table 2). Millimolar concentrations of dibutyryl cyclic AMP were also inhibitory. Dimethylsulfoxide at a concentration that was not hemolytic, inhibited tyrosine hemolysis; the solubility of tyrosine was not enhanced by Me_2SO

TABLE 3

β-glucuronidase Release from Liver Lysosomal Preparations by Tyrosine

Compound	Amount (mg/ml)	β-glucuronidase release (as % of Triton X-100 Release) Exp. 1 $NaCl/PO_4$	Exp. 2 $NaCl/PO_4$	Exp. 3 .5M Sucrose
No addition		27.8 (2)	20.9 (2)	16.1 (3)
L-tyrosine	20	24.6 (3)	-	9.5 (3)
	40	28.3 (3)	-	5.2 (3)
	80	43.9 (3)	47.9 (2)	8.5 (3)
D-tyrosine	80	-	36.1 (2)	-
DL-o-tyrosine	80	-	13.7 (3)	-
DL-m-tyrosine	80	-	9.9 (3)	-

Number of experimental tubes in parenthesis. The experiments were performed as described in Materials and Methods.

as determined by direct analysis. Polyethylene glycol (10^{-4}M), significantly decreased tyrosine induced hemolysis. On a molar basis, the higher molecular weight polymer was more protective than the lower molecular weight. On a weight basis polyethylene glycol inhibited tyrosine hemolysis as effectively as albumin or gamma globulin. It did not prevent water hemolysis.

Rat liver lysosome preparations incubated with 80 mg/ml L-tyrosine released more β-glucuronidase than lysosomal preparations incubated in buffered saline (Table 3). In 0.5M sucrose there was no increased β-glucuronidase release compared with controls. Similar experiments were obtained with lysosomal preparations from

mouse liver. In another experiment, D- and L-tyrosine caused definite β-glucuronidase release from liver lysosomes suspended in buffered saline; the ortho and meta tyrosines did not (Table 3).

DISCUSSION

Solid tyrosine crystals were necessary for significant hemolysis to occur under the experimental conditions used. The extent of hemolysis was related to the amount of tyrosine present and the temperature.

It is possible that phenolic hydrogen donation to membrane components is the mechanism for hemolysis and lysosomal activation by crystalline p-tyrosine. This mechanism has been proposed to explain the hemolysis and lysosomal activation caused by silicates (7,8) and sodium urates (9). Tyrosine crystals have phenolic groups at their surface (10) which could participate in this reaction. The crystal structures of ortho and meta tyrosines are not published and although the pK_as of their phenolic hydroxyls are very similar to that of p-tyrosine the groups may not be at a surface and available for hydrogen donation. In the other tyrosine analogues studied, the phenolic group may be unavailable for steric reasons, have a pK which is much lower than that of the tyrosine hydroxyl group, or the phenolic group may not be at the crystal surface.

The hemolysis induced by crystalline L-phenylalanine, α-phenylglycine and tyramine requires an explanation other than phenolic hydrogen donation.

Although a variety of agents decreased or prevented tyrosine-induced hemolysis these agents may have prevented hemolysis through a variety of mechanisms. The protection by both sucrose and glucose suggested that the protection with sucrose was not purely osmotic. Cyclic AMP, at the concentration used, may have worked by direct membrane stabilization, since both lysosomes (11) and mast cells (12) have been protected against osmotic lysis with cyclic AMP. Me_2SO may have functioned as a protective agent through several mechanisms including its role as a proton acceptor decreasing proton transfer to membranes, by extraction of membrane phospholipids which may have been hydrogen acceptors, by direct association with phenolic hydrogens (13),

or by altering the pKa of the phenolic hydrogens (14). Polyethylene glycols may have decreased hemolysis by direct association or binding to tyrosine. They are reported to bind to other phenolic compounds such as m-cresol (15). Polyethylene glycols and the protective proteins may have decreased hemolysis by additional mechanisms including functioning as hydrogen acceptors, or by direct addition or incorporation into the erythrocyte or lysosome membrane stabilizing the membrane.

The interaction of crystalline tyrosine and similar solids with membranes may be generally useful for defining the mechanism of other membrane active agents. The *in vitro* data that tyrosine can activate lysosomal membranes may be predictive of the *in vivo* mechanism of tyrosine related eye and skin toxicity.

ACKNOWLEDGEMENTS

The work was supported by grant AM-17253 from the U.S. Public Health Service. The author is the recipient of a research career and development award #AM-1K04-00008 from the U.S. Public Health Service.

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