Effect of pH on Erythrocyte Stabilization by Anti-Inflammatory Drugs (35212)

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Previous studies (1, 2) concerned with the stabilization of erythrocytes to thermal labilization by anti-inflammatory drugs employed canine erythrocytes (RBC). Lysergic acid diethylamide (LSD-25) and the steroidal or nonsteroidal anti-arthritic agents stabilized the RBC membrane while very few compounds representing other classes of pharmacologic agents were effective. The purpose of the present study was to determine, in part, the mechanism of stabilization and whether RBC from species other than dog can be employed. A secondary purpose was to define the effectiveness of methysergide, an LSD-25 analog, and cyproheptadine, in order to ascertain whether antiserotonin agents, in general, can be detected in this system.

Methods. Human blood (ACD) was collected from male volunteers. Rat (Sprague-Dawley) and guinea pig blood was obtained by decapitation; horse, goat, and rabbit blood was collected by venipuncture. Heparin was used as an anticoagulant except in human blood. All blood was used on the day of collection. Until use it was stored at 2 to 5° in either an ice bath or refrigerator. Other procedures were similar to those previously described (1, 2). Anesthetized (sodium pentobarbital, 30 mg/kg iv) mongrel dogs of either sex weighing 10–14 kg were used. Whole blood was collected by catheterization of a carotid artery using heparin to prevent clotting. The blood was centrifuged at 650*g* for 15 min between 0–5°. The volume of erythrocytes (RBC) was measured and reconstituted as a 40% (v/v) suspension with cold (0–5°) 0.067 *M* sodium phosphate buffer, pH 7.4, containing 0.9% saline. Test agents were dissolved in 0.9% saline, using 0.10 *N* NaOH to dissolve insoluble compounds. The pH of all solutions was adjusted to 7.4. Three-ml portions of the drug solutions, cooled to 0–5° in an ice bath, were added to 3-ml samples of RBC suspension. The solutions were mixed gently by inversion. Suspensions of RBC were incubated at 53° for exactly 20 min *without* agitation in plastic or glass tubes, placed immediately in an ice bath, cooled, and then centrifuged (14,000*g*, 15 min, 2 to 4°). This yielded solutions containing released hemoglobin. All solutions were kept at 2° at all times except for the released hemoglobin solution which was allowed to attain room temperature prior to determination of its absorbance at 540 mμ. When absorbance values greater than 2.00 were encountered, dilution to readable levels of the control and sample tubes with RBC suspending medium (0.067 *M* sodium phosphate buffer, pH 7.4, containing isotonic saline) or saline eliminated loss of such samples. When RBC are initially resuspended in 0.067 *M* sodium phosphate buffer containing isotonic saline, control absorbance values are seven to tenfold higher than those obtained when RBC are resuspened in phosphate buffer only (1). This increased hemolysis is due to an osmotic effect upon addition of 3 ml of 0.9% saline, after the RBC have equilibrated in hyperosmolar solution. Inhibition of hemolysis was calculated using the previously reported formula (1).

Results. Methysergide (Table I), an analog of LSD-25, stabilizes canine RBC to an extent slightly less than that reported for LSD. That the assay cannot be generally employed for antiserotonin agents, however, is shown by the inactivity of cyproheptadine, a potent antiserotonin agent, and the previ-

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TABLE I. Stabilization of Canine Erythrocytes by Methysergide.

Drug	% Inhibition (±SE)[b] of hemolysis at 5×10^{-4} M	5×10^{-5} M
LSD-25[a]	84.2	55.6
Methysergide	80.0 ± 3.6	25.7 ± 8.3
Cyproheptadine • HCl	−0.4 ± 1.3[c]	2.3 ± 1.7
Indomethacin	60.8 ± 3.2	19.3 ± 4.6
Phenylbutazone	74.3 ± 2.0	33.0 ± 7.7

[a] Data from a previous report (1).

[b] Values are based on 4–14 experiments employing four different samples of canine blood.

[c] Negative sign indicates labilization.

ously reported (1) inactivity of compounds such as methdilazine and promethazine which also manifest *in vivo* antiserotonin activity (3). The stabilization of canine RBC by phenylbutazone or indomethacin (Table I) is similar to that found in previous studies (1). Results were similar using human RBC (Table II) except that indomethacin was much less active.

The resistance of RBC from various species to thermal labilization was found to be, in order of increasing resistance: dog<rabbit <rat<human<guinea pig = horse<goat. The relative hemolysis of RBC in % was, in order of increasing resistance: dog, 100; rabbit, 43; rat, 33; human, 21; horse, 13; guinea pig, 13; and goat, 9. Anti-inflammatory drugs were effective in stabilizing only canine (1, 2) and human RBC. Human RBC from outdated blood (22 days old), however, were not stabilized. Control absorbance values were 50% higher than those of fresh human RBC.

Attempts to alter the surface charge of canine RBC membranes by employing buffers or variable pH indicate that phenylbutazone is effective when RBC suspensions are prepared in buffers of pH 5.0–10.0, Table III. A marked loss in stabilization by phenylbutazone occurs at lower or higher pH. At pH 4.0 cells were completely destroyed as indicated by their brownish-black, "charred" appearance and large increase in volume. The latter phenomena were not noted at pH as high as 11.0. Almost identical results were obtained when studying the effect of pH on the stabilizing property of phenylbutazone[2] using RBC of human blood types AB, B, and O (all positive).

TABLE II. Stabilization of Human Erythrocytes by Methysergide.

Drug	% Inhibition (±SE)[a] of hemolysis at 5×10^{-4} M	5×10^{-5} M
Methysergide	62.2 ± 1.0	20.7 ± 1.8
Indomethacin	28.0 ± 0.8	5.4 ± 1.5
Phenylbutazone	74.9 ± 2.3	18.8 ± 5.8

[a] Based on 4 to 8 experiments using 1-day-old blood from two different donors, both A pos.

Discussion. Why stored (22 days) human RBC are not stabilized by the anti-inflammatory drugs is unknown. A large amount of RBC potassium, however, leaks into the plasma after blood has been stored for this period of time (5) suggesting an increase in membrane permeability. *Fresh* human RBC are clearly stabilized by phenylbutazone over a pH range of 5.0 to 10.0. Devenuto *et al.* (6) using blood from male or female donors have recently shown that proteins from solubilized RBC membranes undergo electrophoretic changes after 42 days of storage. The latter may relate to membrane defects associated with potassium leakage and loss in stabilization by phenylbutazone.

At pH 4.0 human or canine RBC completely disrupt, turn brownish black, and appear "charred." At pH values of 10 to 11 marked hemolysis of canine or human RBC is again noted. It thus appears that certain chemical groups which ionize at or near pH 4.0 and/or 11 are required for the maintenance of RBC stability and the integrity of the proteolipid membrane. Since neuraminic acid or possibly sialic acid is responsible for the surface charge of the RBC, and since it contains carboxyl groups (pK near 4.0), these groups or those of the protein side chains within the membrane could be involved in maintaining RBC membrane integrity. In the carboxylate form (pH>4.0) the

[2] Less stabilization of canine RBC at low pH by phenylbutazone was reported while this paper was being prepared (4).

TABLE III. Effect of pH on the Activity of Phenylbutazone.

pH	Human RBC; % inhibition (±SE)[a] of hemolysis at		Canine RBC; % inhibition (±SE) of hemolysis at	
	5×10^{-4} *M*	5×10^{-5} *M*	5×10^{-4} *M*	5×10^{-5} *M*
5.0[b]	60.2 ± 2.8	11.3 ± 2.5	70.0 ± 7.9	−3.9 ± 3.2[e]
6.0[c]	77.6 ± 1.7	12.4 ± 4.9	58.7 ± 6.6	2.7 ± 4.8
7.0[c]	79.9 ± 1.1	31.3 ± 5.6	68.3 ± 7.1	25.1 ± 4.9
7.4[c]	74.9 ± 2.3	18.8 ± 5.8	73.8 ± 3.0	12.0 ±13.7
8.0[c]	76.0 ± 2.1	36.0 ± 4.3	91.2 ± 0.4	27.9 ± 1.9
9.0[d]	68.0 ± 3.0	26.4 ± 8.0	64.5 ± 4.5	13.5 ± 6.2
10.0[d]	67.0 ± 3.9	17.9 ± 7.6	66.5 ± 1.1	7.1 ± 1.6
11.0[d]	29.7 ± 6.4	18.1 ± 8.5	6.6 ± 2.7	8.6 ± 0.7

[a] Based on 4 to 8 experiments using 2 different samples of human blood (A pos) and 2 samples of canine blood.

[b] Erythrocytes reconstituted as a 40% (v/v) suspension in 0.067 *M* sodium acetate buffer containing 0.9% saline.

[c] As above (*b*) in 0.067 *M* sodium phosphate buffer.

[d] As above (*b*) in 0.067 *M* glycine buffer.

[e] Negative sign indicates labilization.

latter groups could contribute to stability by forming hydrogen or ionic bonds with the hydrogens of nonionized thiol or phenolic hydroxyl groups, or the positive charge of imidazolium, *epsilon*-ammonium or guanidino groups of amino acid side chains of other proteins. If these latter groups, with pK values of approximately 10 to 11, except for imidazolium (p*K* 7) and guanidino (p*K* 12) groups, were involved in stabilizing RBC membranes by interaction with carboxylate groups, then the cells should become unstable at or near pH 4.0 or 10.0; they do.

In agreement with this hypothesis, Ham *et al.* (7) have shown that heating human RBC at 49° for 60 min (pH 7.4) results in increased osmotic and mechanical fragilities, formation of microspherocytes due to budding or outcroppings of the RBC, increased vicosity of RBC and decreased filterability through microfilters (8.0 to 8.7 μ). The formation of microspherocytes due to budding yields a 30% increase in the number of cells. Increases in viscosity can also be obtained with heated hemolysis (sonic, freeze–thawing) products. Thus this latter phenomenon may relate to the conformational changes in RBC membrane proteins noted by Glaser *et al.* (8). In the latter experiments membrane proteins were shown to be converted from 43 to 23% α-helix when the temperature was increased from 26 to 85°, *i.e.*, random coiling occurs.

On the basis of the experiments reported herein the following mechanism of action of phenylbutazone is suggested. Phenylbutazone (pK_a *ca.* 4.5) penetrates and accummulates in the RBC membranes. Its enolate group, or carboxylate groups in the case of other anti-inflammatory agents (1, 2), interacts with membrane proteins and thus stabilizes the membrane by the same type of interactions discussed above for carboxylate groups of normal membranes. That anti-inflammatory drugs can stabilize proteins is well known (9). Salicylic acid, acetylsalicylic acid, mefenamic acid, flufenamic acid, indomethacin, oxyphenbutazone or phenylbutazone (the latter two compounds contribute phenolic hydroxyl equivalent groups or acidic enolate functions), and namoxyrate (1) may stabilize the RBC membrane by a similar mechanism.

In addition to the required chemical properties of antiarthritic compounds implied in this hypothesis, certain solubility characteristics and dimensions (to fit into the membrane) are needed. A compound must be sufficiently lipid soluble to prevent complete passage through the lipid layer of the mem-

brane, but not completely insoluble in water so that solution of the drug cannot be attained. Salicylate is relatively water soluble, passes through the membrane very rapidly (10, 11) and is active only at more than 10 times the highest concentration of phenylbutazone studied (1).

Since RBC of rabbit, rat, horse, guinea pig, or goat are not stabilized by the anti-inflammatory agents, a mechanism of action as proposed above for the interaction of anti-inflammatory drugs with proteolipid membranes is not entirely tenable. Thus unaffected RBC of the species noted above also have proteolipid membranes. Some specific compounds which react with the drugs may be present in canine or human RBC but may be absent in the unaffected RBC. Another explanation for the lack of stabilization is that the proteins in the membranes of these RBC are conformationally dissimilar to those of human or canine RBC. Thus they may contain more carboxylate groups and increasing the number of such groups due to anti-inflammatory drug binding cannot increase their stabilizing, membrane interactions further.

Summary. Phenylbutazone inhibits hemolysis of canine or human erythrocytes (RBC) when suspensions (40%) of RBC are incubated at 53° for 20 min in 0.067 *M* sodium phosphate buffer containing isotonic saline. Methysergide and indomethacin are also effective but indomethacin is much less effective in stabilizing human than canine RBC. Stabilization of RBC by phenylbutazone was observed when RBC were incubated at pH 5.0 through 10.0. Much less stabilization was noted at lower or higher pH; at pH 4.0, complete RBC destruction occurs and phenylbutazone is ineffective. Guinea pig, rat, rabbit, horse, and goat RBC were *not* stabilized by the anti-inflammatory agents.

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