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Further Studies on the Erythrocyte Anti-Inflammatory Assay (33051)

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Similarities between the action of agents and procedures on red cells and lysosomes suggested that membranes bounding erythrocytes and lysosomes have common properties. Work based on this assumption has shown that nonsteroidal anti-inflammatory agents such as phenylbutazone and indomethacin protect erythrocytes from heat-induced hemolysis (1).

In our search for a suitable solvent to test steroids in this system, the effect of various concentrations of dimethylsulfoxide (DMSO) and ethyl alcohol on heat-induced hemolysis of erythrocytes was determined. The results of these studies are summarized in this report.

Materials and Methods. The *in vitro* system for studying the action of nonsteroidal anti-inflammatory agents has been described previously (1). The same methods were utilized in these studies except for solubilization of steroids or other drugs in 0.067 *M* sodium phosphate buffer, pH 7.4, containing 0.9% saline and either 1.0% DMSO or 5.0% ethanol.

The following steroids, listed in subsequent tables by generic or common names, were employed in studies of the present report: cholanic acid; cholesterol, 5-cholesten-3β-ol; cholic acid, 5β-cholanic acid-3α,7α,12α-triol; cortisone, 4-pregnen-17α,21-diol-3,11,20-trione; dexamethasone,1,4-pregnadien-9-fluoro-16α-methyl-11β,17α,21-triol-3,20-dione; diethylstilbestrol, [3,4-bis-(-4-hydroxyphenyl)-3-hexane]; dimethisterone, 6α-methyl-17α-propynylandrost-4-en-17β-ol-3-one monohydrate; ergosterol, 5,7,22-cholestatrien-24β-methyl-3β-ol; estradiol, 1,3,5(10)-estratrien-3,17β-diol; 17α-ethinylestradiol, 1,3,5(10)-estratrien-17α-ethinyl-3,17β-diol; etiocholanolone, 5β-androstan-3α-ol-17-one; hydrocortisone, 4-pregnen-11β,17α,21-triol-3,20-dione; medroxyprogesterone, 17α-hydoxy-6α-methyl-4-pregnen-3,20-dione acetate; methyltestosterone, 4-androsten-17α-methyl-17β-ol-3-one; paramethasone acetate, 6α-fluoro-11β,17,21-trihydroxy-16α-methyl-pregna-1,4-diene-3,20-

TABLE I. Action of DMSO and Ethanol on Heat-Induced Hemolysis.

DMSO (%) in buffered saline, pH 7.4[a]	Inhibition (%)
1	7.8
5	51.6
10	70.8
20	46.6
Above 20	Discoloration and precipitation occurred
Ethanol (%) in buffered saline, pH 7.4[a]	
1	No apparent effect
5	No apparent effect
10	Hemolysis enhanced with discoloration

[a] Assays were in duplicate.

TABLE II. Inhibition of Heat-Induced Hemolysis by Nonsteroidal Anti-inflammatory Compounds in 1% DMSO.

Compound	Inhibition (%) at $5 \times 10^{-4} M$	$5 \times 10^{-5} M$	$5 \times 10^{-6} M$
Phenylbutazone	83.8[a] [6][d] (60.4)[b]	51.8 [4] (45.7)	31.7 [4] (25.1)
Indomethacin (33 ×)[c]	83.7 [4] (51.4)	74.2 [2] (28.3)	38.0 [2] (7.5)
Mefenamic acid (13 ×)[c]	89.4 [4] (51.5)	62.4 [2] (34.8)	30.0 [2] (12.6)
Flufenamic acid (4 ×)[c]	83.9 [6] (62.0)	64.2 [4] (39.9)	25.0 [2] (19.1)
Oxyphenbutazone	77.5 [6] (52.3)	39.1 [2] (25.6)	11.8 [2] (8.1)
Acetylsalicylic acid	30.8 [4] (40.0)	16.0 [2] (16.3)	9.1 [2] (4.7)
Sodium salicylate	14.1 [2] (17.2)	0 [2] (19.9)	0 [2] (0)

[a] Compounds dissolved in 1% DMSO (v/v) in sodium phosphate buffered saline solution, pH 7.4.

[b] Compounds dissolved in 0.9% saline pH 7.4; results of previous work (1).

[c] Potentiation observed in 1% DMSO based on parallel shift in log dose–response curve and ED_{42} values.

[d] Numbers in brackets represent number of replicates.

dione-21-acetate; prednisolone, 1,4-pregnadien-11β-17α,21-triol-3,20-dione; prednisone, 1,4-pregnadien-17α,21-diol-3,11,20-trione; pregnenolone, 3β-hydroxy-5-pregnen-20-one; progesterone, 4-pregnen-3,20-dione; triamcinolone, 9α-fluoro-16α-hydroxy-prednisolone.

Results. As shown in Table I, 1% DMSO solutions did not modify heat-induced hemolysis of canine erythrocytes (RBC). However, concentrations of 5–20% stabilized RBC, while higher concentrations induced further hemolysis. Ethanol had little effect on hemolysis at concentrations of 1 and 5% but 10% enhanced hemolysis.

Various nonsteroidal anti-inflammatory agents were retested in these solvent systems and compared with previously reported results (Table II). DMSO enhanced the effectiveness of some agents in their ability to protect erythrocytes from heat-induced hemolysis. Acetylsalicylic acid, phenylbutazone, oxyphenbutazone, and sodium salicylate were exceptions.

Tables III and IV show that many steroids, most of which are unrelated to the treatment of inflammation, will protect erythrocytes from hemolysis when dissolved in 1% DMSO; the two most active steroids are estrongenic. Thus steroids with a 17-hydroxy group and a 3-keto or alcohol group, lacking the characteristic adrenocortical side chain at C-17, were most active. Addition of a C-17 side chain decreases activity as seen in paramethasone, prednisone, prednisolone, triamcinolone, cortisone, dexamethasone, etc. However, part of this decrease in activity could be due to the extra 11-keto or 11-hydroxy group in this series of compounds.

The activity of several of these steroids has also been tested in buffered saline containing 5% ethanol. In Table V, results clearly demonstrated that these agents were apparently as soluble in this solvent system but had much less protective effect, except with ergosterol, which was apparently as active in 5% ethanol as 1% DMSO; acetylsalicylic acid was less effective in 5% ethanol than either saline or 1% DMSO.

TABLE III. Inhibition of Heat-Induced Hemolysis by Steroids and Related Compounds in 1% DMSO.

Compound[a]	Inhibition (%) at 5×10^{-4} M	5×10^{-5} M	5×10^{-6} M
Cholanic acid	69.3 [2][b]	57.2 (Cly.)[c] [2]	13.4 [2]
Cholesterol	35.1 [4]	12.9 (Cly.) [2]	8.3 [2]
Cholic acid	49.7 [4]	29.7 (Clear) [2]	14.6 [2]
Cortisone	64.8 [8]	25.4 (Clear) [6]	9.7 [4]
Dexamethasone	66.4 [4]	35.2 (Clear) [4]	0 [2]
Diethylstilbestrol	68.3 [4]	56.3 (Cly.) [4]	21.7 [2]
Dimethisterone	82.9 [4]	60.1 (Cly.) [2]	36.5 [2]
Ergosterol	46.6 [2]	23.2 (Cly.) [2]	16.5 [2]
Estradiol	84.2 [4]	53.7 (Cly.) [2]	9.5 [2]
17-α-Ethinylestradiol	72.8 [4]	64.0 (Cly.) [2]	11.9 [2]
Etiocholanolone	76.6 [2]	37.7 (Clear) [2]	22.2 [2]
Hydrocortisone	56.5 [4]	12.3 (Clear) [2]	12.3 [2]
Medroxyprogesterone	16.2 [4]	3.9 (Cly.) [2]	0 [2]
Methyltestosterone	84.7 [4]	48.8 (Cly.) [2]	13.1 [2]
Paramethasone acetate	39.8 [2]	30.1 (Sl. cyl.) [2]	0 [2]
Prednisolone	53.2 [4]	21.6 (Clear) [2]	0 [2]
Prednisone	46.3 [4]	19.1 (Clear) [2]	0 [2]
Pregnenolone	53.9 [2]	31.4 (Sl. cly.) [2]	1.0 [2]
Progesterone	63.8 [4]	31.0 (Cly.) [4]	1.2 [2]
Triamcinolone	34.6 [4]	22.8 (Sl. cly.) [2]	0 [2]

[a] All compounds dissolved or suspended in solutions of 1% DMSO (v/v) buffered saline, pH 7.4.

[b] Numbers in brackets represent number of replicates.

[c] Cly. = cloudy solution; Sl. cly. = slightly cloudy solution; 5×10^{-6} M concentrations gave clear solutions in all cases.

Discussion. Weissman *et al.* (2) have shown that the concentration of cortisone, cortisol, their acetates, or prednisone, necessary to stabilize lysosomes, is reduced 10–1000-fold when these substances are dissolved or suspended in DMSO (1% v/v); however, *steroids other than these were* not *tested*, leaving the question of specificity unanswered. The possibility now arises that steroids other than those investigated by Weissman *et al.* (2) might also stabilize lysosomal membranes when 1% DMSO is used in the solvent system. Results in this report certainly cast doubt on the presumption that specific adrenocortical steroid analogues actually exert their prime pharmacologic action in inflammatory states by stabilizing cellular or subcellular organelle membranes.

The mechanism by which steroidal or non-steroidal drugs stabilize erythrocytes is, as yet, undefined. However, the following points are relevant. DMSO is a suitable lipophilic solvent and the RBC membrane is, in large part, composed of lipoid substances(3–5). Organic cations or anions penetrate human RBC and this is approximately related to their lipid-to-water partition coefficients at pH 7.4 (6,7). The dominant characteristic which determines penetration appears to be lipid solubility. Since for most organic bases, the undissociated form is lipid soluble and the ionized form lipid insoluble, the degree of ionization is important indirectly in that it affects the proportion of compound in the lipophilic form (7).

Steroids are also lipophilic compounds. Thus a solvent such as 1% DMSO, which is known to increase penetration of large molecules through living biologic membranes (4), can increase the chance of steroids becoming attached to or penetrating RBC,

TABLE IV. Inhibition of Hemolysis by Steroids in 1% DMSO.

Compound	AED_{42}[a]	Relative potency (rank)
Cholanic acid	2.4×10^{-5}	5
Cholesterol	$> 10^{-3}$	19
Cholic acid	2.1×10^{-4}	12
Cortisone	2.7×10^{-4}	13
Dexamethasone	9.0×10^{-5}	9
Diethylstilbestrol	2.0×10^{-5}	4
Dimethisterone	2.0×10^{-5}	3
Ergosterol	4.0×10^{-4}	15
Estradiol	8.6×10^{-6}	1
17-α-Ethinylestradiol	1.3×10^{-5}	2
Etiocholanolone	7.0×10^{-5}	8
Hydrocortisone	2.4×10^{-5}	6
Medroxyprogesterone	Inactive	20
Methyltestosterone	2.7×10^{-5}	7
Paramethasone acetate	5.1×10^{-4}	17
Prednisolone	2.9×10^{-4}	14
Prednisone	5.0×10^{-4}	16
Pregnenolone	1.4×10^{-4}	11
Progesterone	1.1×10^{-4}	10
Triamcinolone	1.1×10^{-3}	18

[a] Interpolated graphically from data in Table III.

thereby causing the first step in the process of stabilization. Ethanol (5%), a solvent in which the steroids were observed to be apparently as soluble as in 1% DMSO, causes no effect on RBC hemolysis per se and, when steroids are dissolved in it, much less effect on stabilization by these compounds is seen than when they are dissolved in 1% DMSO. One may conclude that lipophilicity, although an important factor, is not the only one involved in the action of these steroids. This is implied by the structural requirements for maximal activity, i.e., estrogenic steroids with 17-hydroxy *and* 3-keto or hydroxy groups demonstrated the strongest stabilizing effects.

It is interesting to note that 5% ethanol, a presumably more lipophilic solvent than buffered saline, altered the action of nonsteroidal drugs to only a minor extent. However, 1% DMSO did cause considerable enhancement in the activity of indomethacin and mephenamic acid. All of the nonsteroidal drugs at pH 7.4 must surely exist, in large part, in anionic forms and could have penetrated the RBC (6). Salicylate (14 mg/100 ml), which is 99.6% ionized at pH 7.4, penetrates the RBC so rapidly that its rate can hardly be measured (6). However, other drugs in both anionic and cationic forms have been studied in our system, some stabilizing, others not (1). Since hemolysis must ultimately involve changes in the RBC membrane (8), we assume that anti-inflammatory drugs which actively stabilize RBC in our system do so by one of the following mechanisms: (i) drugs attach themselves to the membrane and prevent water uptake with subsequent hemolysis; (ii) drugs penetrate into the RBC and prevent detachment of hemoglobin from its internal binding sites; (iii) drugs prevent formation of membrane holes sufficiently large for hemoglobin leakage; or (iv) drugs prevent extrusion of hemoglobin through the lipoprotein membrane by a combined action of the first three mechanisms above.

However, since hemolysis can considerably be retarded by 2.5 or 5.0% saline being added to our standard medium, causing 14.5 and 33.9% inhibition, respectively, the first mechanism probably prevails. Addition of 10% saline to the medium enhanced hemolysis. It is pertinent that the membrane of RBC is freely permeable to estradiol, hemoglobin is largely responsible for its binding, and its affinity for RBC ghosts is practically nonexistent. Other studies have shown that binding to RBC was strongest for estradiol and estrone, least for cortisol and corticosterone, while progesterone and testosterone had intermediate affinities (9). Apparently only negligible amounts of steroid hormones complex with lipoproteins and in fact, many steroids bind very strongly to serum albumin. However, a corticosteroid-binding protein probably exists that binds corticosteroids specifically (9).

In regard to corticosteroids being less active in stabilizing RBC in 1% DMSO than estrogenic steroids or steroids containing both a 17-hydroxy group and a 3-keto or hydroxy group, it is relevant that introduction of electronegative groups (e.g., hydroxy) into the steroid molecule decreases the interaction of steroids with proteins (9). Another possibility, which cannot be easily tested, is that

TABLE V. Inhibition of Heat-Induced Hemolysis by Drugs in 5% Ethanol.

Compound	Inhibition (%) at 5×10^{-4} M[a]			5×10^{-5} M		
Cholanic acid	—	[2][c]	(Susp.)[b,d]	0	[2]	(Sl. susp.)
Cholesterol	11.3	[2]	(Susp.)	8.6	[2]	(Cly.)
Cortisone	4.1	[2]	(Clear)	1.4	[2]	(Clear)
Dexamethasone	25.8	[2]	(Sl. cly.)	2.2	[2]	(Clear)
Diethylstilbestrol	40.7	[2]	(Susp.)	40.1	[2]	(Sl. susp.)
Dimethisterone	41.2	[4]	(Susp.)	32.4	[2]	(Cly.)
Ergosterol	47.4	[4]	(Cly.)	21.4	[4]	(Sl. cly.)
Estradiol	19.6	[2]	(Susp.)	3.9	[2]	(Clear)
17-α-Ethinylestradiol	—	[2]	(Susp.)[d]	27.1	[2]	(Clear)
Etiocholanolone	—	[4]	(Susp.)[d]	2.3	[4]	(Clear)
	(39.4)	[4]	(Susp.)[e]	(7.3)	[4]	(Clear)
Hydrocortisone	21.4	[2]	(Cly.)	0	[2]	(Clear)
Methyltestosterone	30.8	[2]	(Susp.)	18.5	[2]	(Clear)
Prednisolone	11.8	[2]	(Clear)	3.3	[2]	(Clear)
Progesterone	26.5	[2]	(Susp.)	21.0	[2]	(Clear)
Triamcinolone	14.4	[2]	(Susp.)	0	[2]	(Sl. susp.)
Acetylsalicylic acid	13.9	[6]	(Clear)	6.4	[6]	(Clear)
Indomethacin	50.3	[4]	(Clear)	11.4	[4]	(Clear)
Mephenamic acid	45.0	[4]	(Clear)	27.7	[4]	(Clear)
Phenylbutazone	64.6	[2]	(Clear)	26.3	[2]	(Clear)

[a] Drugs were dissolved in buffered saline as in Table III but solution also contained 5% ethanol.

[b] Susp. = suspension; Sl. cly. = slightly cloudy; Cly. = cloudy solution.

[c] Numbers in brackets represent number of replicates.

[d] Enhanced hemolysis.

[e] Etiocholanolone suspended or dissolved in buffered saline, pH 7.4.

steroids and some nonsteroidal anti-inflammatory drugs potentiate the protective effect of DMSO, rendering 1% DMSO protective. The order of binding of steroids to whole blood or human serum albumin parallels the rank of potency in stabilizing RBC. However, it should be emphasized that even 5×10^{-6} M concentrations of steroids far exceed physiologic levels in plasma. Moreover, the decreasing order of solubility of steroids in saline at 5°C is cortisol > testosterone > progesterone > estradiol, which is opposite to their rank of potency in the RBC assay (10). All of these above-mentioned data are consistent with results obtained on relative effectiveness of steroids in stabilizing RBC (Table IV) and probably relate to the first two mechanisms previously discussed.

Addition of DMSO to a final concentration of 1% and either steroids or nonsteroids described in this report may have a beneficial effect on blood storage, certain hemolytic anemias, or sublethal damage to erythrocytes by mechanical pumping.

Summary. Several steroids, including estrogens, corticosteroids and analogues, have been shown to protect erythrocytes from heat-induced hemolysis *in vitro*. When dissolved or suspended in 0.067 M sodium phosphate buffered, isotonic (0.9%) saline, pH 7.4, containing 1% dimethylsulfoxide (DMSO), greater protective effect was observed than in the presence of 5% ethanol in the same medium without DMSO, even though the compounds are apparently as soluble in the solvent containing 5% ethanol. The protective effects of nonsteroidal anti-inflammatory agents dissolved in these two solvents were compared with their activity in the absence of 1% DMSO or 5% ethanol; DMSO (1%) enhanced protection afforded by indomethacin, mephenamic acid, and flu-

fenamic acid while acetylsalicylic acid, sodium salicylate, phenylbutazone, and oxypenbutazone were unaffected. In the presence of 5% ethanol, the protective action of acetylsalicylic acid was attenuated, while that of indomethacin, mephenamic acid, or phenylbutazone was unaltered. Although 1% DMSO or 5% ethanol did not stabilize erythrocytes to heat-induced hemolysis per se, 5, 10, and 20% DMSO did so, while 10% ethanol enhanced hemolysis.

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Mechanism of Norepinephrine Depletion by 5-Hydroxytryptophan (33052)

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DL-5-Hydroxytryptophan (5-HTP) at high doses produces overt stimulation in the rat characterized by piloerection, panting, and increased motor activity including circling and backing up. Brodie *et al.*, have suggested that excitation produced by 5-HTP is associated with release of brain norepinephrine (NE) (1). Their data demonstrated that a large dose of 5-HTP reduced rat brain NE by 50%.

The decarboxylation of 5-HTP to serotonin (5-HT) is catalyzed by the same enzyme, L-aromatic amino acid decarboxylase, which converts dihydroxyphenylalanine (DOPA) to dopamine (DA) in an intermediate step in the synthesis of NE (2). This large dose of 5-HTP together with its efficiency for penetrating the CNS might be expected to produce substrate inhibition of the decarboxylase and thereby inhibit decarboxylation of DOPA.

This report confirms the effect of 5-HTP in lowering rat brain NE. However, data presented here indicate that inhibition of NE synthesis rather than a 5-HTP (or 5-HT) mediated release is the cause of NE depletion.

Methods and Materials. L-α-methyltyrosine (L-α-MT) was obtained from Merck Institute for Therapeutic Research, DL-α-methyltyrosine methyl ester (H 44/68) from Dr. H. Corrodi, Hassle Laboratories, and pargyline from Abbott Laboratories. All other compounds are commercially available.

Upjohn Sprague-Dawley male rats (125–150 gm) were used in this investigation. Where fasted conditions are noted rats were deprived of food overnight (16–20 hours). Fasted, weanling rats (23 days old) were used in the incorporation experiment.

The i.v. administration of H 44/68 was facilitated by dissolving the drug in Merlis solution (3). Except for a solubilized preparation of reserpine, all other compounds were suspended in 0.25% aqueous methylcellulose and administered i.p. In each experiment control rats also received the appropriate diluent.

In brain amine studies rats were sacrificed by decapitation, brains were removed and