was demonstrated 5 days after operation. The specific activity of the acid mucopolysaccharides was lowered during the first 11 days. This indicated that the increased content of acid mucopolysaccharides in this period was predominantly due to non-sulfated mucopolysaccharides.

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A Novel *in vitro* Assay for Anti-Inflammatory Agents Based on Stabilization of Erythrocytes. (32219)

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In vitro studies by de Duve *et al* suggested that several compounds including cortisone might act to regulate the release of enzymes from lysosomes(1). Subsequent work confirmed their prediction and further suggested that glucocorticoids may exert pharmacologic effects by preventing the disruption of the enveloping membranes of lysosomes (2-6).

Only indirect evidence supports the hypothesis that certain diseases of connective tissues may be due to an abnormal fragility of lysosomes(6). Investigators suggested that gold salts (used in the treatment of rheumatoid arthritis) may act in inflammatory states by inhibition of lysosomal enzymes of phagocytic cells in the inflamed synovial tissue(7). Furthermore, acute and chronic arthritis can be produced in rabbits by repeated injections of streptolysin S, a lysosome-disruptive agent(8). However, the role of lysosomes in the etiology of rheumatic diseases remains to be established.

Several agents capable of releasing hydrolytic enzymes from lysosomes also injure erythrocytes. *In vivo* exposure to detergents (9), lecithinase(10), excess Vitamin A (11), and ultraviolet irradiation(12) can disrupt red cell membranes. These agents and procedures also disrupt lysosome membranes (2,13-15). Similarities between the action of streptolysin S and streptolysin O on red cells and lysosomes also suggest that the membranes bounding erythrocytes and lysosomes have common properties(5).

While this paper was in preparation, Miller and Smith(16) demonstrated that acetylsalicylic acid stabilizes lysosomal membranes *in vitro*. When this report was written, we could find no evidence to show that nonsteroidal anti-inflammatory agents might stabilize red cell membranes.

A new *in vitro* technique for the rapid screening of potential anti-inflammatory compounds, based on their ability to inhibit heat-induced hemolysis of red blood cells, forms the basis of this report.

Methods. Anesthetized (sodium pentobarbital, 30 mg/kg i.v.) mongrel dogs of either sex weighing 10-14 kg were used. Whole blood (usually 100-150 ml per dog) was collected by catheterization of an external carotid artery using heparin to prevent clotting. The blood was centrifuged at 650 $\times$ *g* for 15 minutes between 0-5°C. The volume of erythrocytes (RBC) was measured and reconstituted as a 40% (v/v) suspension with cold (0-5°C) M/15 sodium phosphate buffer, pH 7.4. The

suspension was maintained at 0-5°C and used on collection day. Tested agents were dissolved in 0.9% saline, using 0.10 N HCl or 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°C in an ice bath, were added to two duplicate pairs of centrifuge tubes. Then three ml samples of RBC suspension from one dog were added to each tube. The solutions were mixed gently by inversion. One pair of tubes was incubated for 20 minutes at 53°C in a water bath using a mercury-in-glass type thermoregulator. The other pair was maintained at 0-5°C in an ice bath. After incubation both pairs were centrifuged at 14000 $\times$ g for 15 minutes between 0-5°C. The supernatant fluid was aspirated and absorbance determined at 540 mμ as soon as possible. All tubes were kept at 0-5°C until absorbance determinations were made. Two duplicate pairs of tubes each containing 3.0 ml of 0.9% saline, pH 7.4, and 3.0 ml RBC suspension were included with each set of tubes as controls. Absorbance was determined using M/15 sodium phosphate buffer as a blank.

Per cent inhibition of heat-induced RBC hemolysis was calculated as shown in Eq. (1).

$$100 - \left[\left(\frac{\text{O.D. test sample, heated} - \text{O.D. test sample, unheated}}{\text{O.D. control sample, heated} - \text{O.D. control sample, unheated}} \right) \times 100 \right] = \% \text{ inhibition.} \quad (1)$$

Suspensions of RBC prepared from several animals by this method were essentially free of hemolysis. Supernatant fluids of the unheated saline controls gave absorbance readings of 0.030-0.080. Preliminary trials indicated that desired absorbance readings for heated controls could be obtained by heating samples for 20 minutes at 53°C. After subtraction of the absorbance of the corresponding unheated samples, fifty-nine duplicate sets (118 tubes) of saline controls gave a mean absorbance value $\pm$ S.E. of 0.911 $\pm$ 0.047 (95% confidence limits, 0.818-1.004). Use of blood from animals in poor nutritive condition or those with low hematocrit readings (<0.35) was avoided. Several drug solutions could thus be tested on any given sample of each dog's blood.

Results. Table I compares the ability of several compounds to inhibit the hemolysis of RBC. Active agents readily inhibit by 40 to 60% the heat-induced hemolysis at concentrations of 5×10^{-4} M or in some cases less. Aminopyrine and sodium salicylate showed only slight activity at equimolar concentrations of 5×10^{-3} M. Isoxsuprine at similar concentrations shows a potency comparable to that of phenylbutazone and other active compounds. The slopes of regression lines of log dose versus % inhibition did not differ from each other at the 95% confidence level (Table II). This indicates a similar mechanism of action for these compounds, but each slope did differ significantly from zero.

A 28% inhibition was found to be significant at the 95% confidence level with a minimum of 9 determinations (aspirin, 9 dogs, 18 duplicates). However, phenylbutazone was significantly active at 18% inhibition. With

TABLE I. Inhibition of Heat-Induced Erythrocyte Hemolysis.

Compound	Per cent inhibition ± S.E.									
	5.0×10^{-4} M		1.75×10^{-4} M		5.0×10^{-5} M		1.75×10^{-5} M		5.0×10^{-6} M	
Phenylbutazone	60.4 ± 4.7	(10)*	46.4 ± 4.1	(5)	45.7 ± 6.6	(5)	24.5 ± 3.7	(5)	25.1 ± 6.8	(5)
Indomethacin	51.4 ± 5.4	(10)	31.0 ± 7.1	(6)	28.3 ± 7.3	(5)	16.8 ± 7.3	(6)	7.5 ± 5.1	(5)
Oxyphenbutazone	52.3 ± 5.6	(8)	38.3 ± 7.7	(5)	25.6 ± 6.7	(5)	19.7 ± 4.5	(5)	8.1 ± 4.3	(5)
Mefenamic acid	51.5 ± 5.3	(8)	56.2 ± 6.2	(5)	34.8 ± 6.8	(5)	24.6 ± 2.4	(5)	12.6 ± 6.9	(5)
Flufenamic acid	62.0 ± 4.0	(8)	54.1 ± 5.4	(5)	39.9 ± 6.8	(5)	25.8 ± 5.3	(5)	19.1 ± 10.2	(5)
Acetylsalicylic acid	40.0	(3)	—	(—)	16.3	(3)	—	(—)	4.7	(3)
Aminopyrine	21.4	(2)	—	(—)	12.0	(2)	—	(—)	0	(2)
Sodium salicylate	17.2	(2)	—	(—)	19.9	(2)	—	(—)	0	(2)
Isoxsuprine	52.6	(2)	—	(—)	21.4	(2)	—	(—)	8.6	(2)
2-(4-Biphenyl) butyric acid (Namoxyrate)	68.5	(3)	—	(—)	23.6	(2)	—	(—)	0	(2)

* No. of animals used.

TABLE II. Statistical Data for Calculated Regression Lines.

Compound	Regression coefficient (slope ± S.E. regression line)	Standard deviation of the regression line	Slope (95% confidence limits)	Slope significance $P <$
Phenylbutazone	18.81 ± 1.57	± 8.58	(14.49–23.13)	.001
Indomethacin	21.23 ± 2.85	± 16.11	(13.27–29.19)	.001
Oxyphenbutazone	21.80 ± 2.56	± 13.52	(14.65–28.95)	.001
Mefenamic acid	20.90 ± 2.65	± 14.02	(13.48–28.32)	.001
Flufenamic acid	22.63 ± 2.67	± 14.13	(15.15–30.11)	.001
Aspirin	16.31 ± 5.20	± 12.74	(2.29–30.33)	.025

TABLE III. Inhibition of Heat-Induced Erythrocyte Hemolysis.

Compound	% Inhibition at 5.0×10^{-4} M	5.0×10^{-5} M	5.0×10^{-6} M
LSD-25	84.2 (3)*	55.6 (2)	29.6 (2)
Chlorpromazine	33.2 (3)	17.2 (1)	—
Methotrimeprazine (levomepromazine)	37.5 (2)	11.0 (2)	—
Triflupromazine	†	40.6 (2)	0 (2)
Trifluoperazine	‡	23.3 (2)	3.4 (2)
Imipramine	25.6 (2)	—	—
Amitriptyline	27.2 (2)	—	—
Chlordiazepoxide	45.9 (3)	7.1 (1)	5.4 (1)
Pentobarbital, Na	47.1 (3)	21.8 (2)	2.5 (2)
Hexobarbital, Na	33.1 (1)	16.9 (1)	—

* Indicates No. of animals used.

† Chlorpromazine and triflupromazine were also tested at concentrations of 5×10^{-3} M. Hemolysis was enhanced in both instances.

‡ Trifluoperazine was insoluble at this concentration.

the exception of flufenamic acid *vs* aspirin, the inhibitory actions of 5×10^{-4} M concentrations of the first 6 compounds listed in Table I did not differ significantly ($P>0.05$). At lower concentrations however, these agents are different in relative potencies. Using 42% inhibition, which is half the maximal inhibition obtained with the drugs that were tested, as a criterion of potency, lysergic acid diethylamide (LSD-25, Table III) was the most active. By assigning a potency rating of 1.00 to LSD-25, the order of decreasing potency becomes: LSD-25 = 1.00, phenylbutazone = 0.25, flufenamic acid = 0.24, mefenamic acid = 0.15, oxyphenbutazone = 0.08, indomethacin = 0.06, and acetlysalicylic acid = 0.02. Thus, it appears that LSD-25 is 50 times more potent than acetylsalicylic acid. Phenylbutazone is almost 13 times more potent than acetylsalicylic acid. The ratio of clinical dosages of acetylsalicylic acid to phenylbutazone is approximately 15 to 1. The clinical ratio of dosages of flufenamic acid versus phenylbutazone is approximately 2 to 1. We arbitrarily classed as very active those drugs showing significant inhibition between 10^{-5} and 10^{-4} M; moderately active, those exhibiting significant inhibition between 10^{-4} and 10^{-3} M; and slightly active, those showing significant inhibition between 10^{-3} and 10^{-2} M.

Complete log dose-response relations to 100% inhibition were not determined for all drugs because mefenamic acid and trifluoperazine were insoluble at 5×10^{-3} M, and indomethacin, triflupromazine, and chlorpromazine caused hemolysis at 5×10^{-3} M. Most known anti-inflammatories tested also yielded reasonably high levels of inhibition at 5×10^{-4} M, therefore we considered this as a practical endpoint. However Table III shows that values close to 100% inhibition can be attained in this test, *e.g.*, LSD-25. Log dose-response relations obtained with a sample of blood from any one dog were linear and almost every point fell on the line. Variation in the sensitivity of RBC samples from different animals was therefore probably the greatest

TABLE IV. Compounds Inactive at 4×10^{-4} M Concentration.

Xylocaine hydrochloride	MJ 1999*
Procaine hydrochloride	α-Methyldopa
l-Epinephrine bitartrate	Serotonin creatinine sulfate
Norepinephrine bitartrate	Penicillin G
l-Ephedrine hydrochloride	N-Acetyl-l-cysteine
Methdilazine	Histamine diphosphate
Promethazine hydrochloride	Iproniazid phosphate
Amphetamine sulfate	Thiamine hydrochloride
Phentolamine methanesulfonate	Pyridoxal-5′-phosphate
Morphine sulfate	Pronethalol
Meprobamate	Tyramine hydrochloride
Cocaine	α-Chloralose
Chloroquine phosphate	Atropine sulfate
Aminophylline	l-DOPA
Dopamine	Mescaline sulfate
Tranylcypromine sulfate	Caffeine alkaloid
Succinylcholine chloride	Codeine sulfate
Hydroxyzine hydrochloride	Caramiphen hydrochloride
Dextromethorphan hydrobromide	

* 4-(2-Isopropylamino-1-hydroxyethyl) methane sulfonanilide • hydrochloride.

source of error. The variance encountered with duplicate RBC determinations from the same animal was slight. The difference in % inhibition of hemolysis between 60 replicates (30 dogs) for phenylbutazone and 64 replicates (32 dogs) for indomethacin reported in Table I was 2.96 ± 0.57 (S.E.) and 3.33 ± 0.51 (S.E.) respectively. Variation between duplicates for the other drugs was similar.

In an effort to determine the specificity of the test, 48 nonanti-inflammatory agents were tested at concentrations up to 5×10^{-4} M (Table 3 and 4). Table III shows that the phenothiazine-like compounds, and the two barbiturates tested, were active. All drugs listed in Table IV were inactive at concentrations of 5×10^{-4} M.

Discussion. Nonsteroidal anti-inflammatory agents actively protected erythrocytes over a wide range of concentations from heat-induced hemolysis (Table I). In this test, good correlation exists between the relative potencies of anti-inflammatory drugs and those found with the protein denaturation technique of Mizushima(17) (Table V).

Even though isoxsuprine possesses antiedemic properties in dextran-induced inflammation in the rat's paw(18,19), McKinney and Lish(18) suggested that this was a nonspecific anti-inflammatory effect, possibly due to a hemodynamic action. In our system isoxsuprine stabilized membranes of erythrocytes, indicating a mechanism of action similar to phenylbutazone. Other investigators(20-22) described some of the analgesic and anti-inflammatory properties of Namoxyrate, the 2-dimethyl-aminoethanol salt of 2-[4-biphenyl]butyric acid. Our studies with the free acid of this compound indicate that it actively stabilizes erythrocytes.

The preservation of erythrocytes by phenothiazines received the attention of other

TABLE V. Correlation of Activity in Various Tests for Anti-Inflammatory Agents.

Compound	Formalin edema (rat's paw)	Carrageenin edema (rat's paw)	UV-erythema (guinea pig)	Protein denaturation	RBC hemolysis
Phenylbutazone	Inactive*	Active	Active	Very active	Very active
Flufenamic acid	Inactive[35]	" [35]	" [36]	" "	" "
Mefenamic acid	n.t.	n.t.	"	" "	" "
Oxyphenbutazone	Inactive	n.t.	"	Moderately active	Moderately active
Indomethacin	"	Active	"	Moderately active	Moderately active
Acetylsalicylic acid	Active[35] (>100 mg/kg)	Active[35] (≥100 mg/kg)	"	Slightly active	Moderately active
Aminopyrine	Inactive	n.t.	" [37]	Inactive	Slightly active
Salicylic acid	"	n.t.	Active[38] (>100 mg/kg)	Slightly active	" "
2(4-Biphenyl) butyric acid (namoxyrate)	"	Active	Active	Moderately active	Moderately active

* Inactive at doses <100 mg/kg. n.t. = not tested.

investigators who demonstrated their stabilizing effects on membranes(23,24). In protecting human red blood cells against hypotonic hemolysis, chlorpromazine and trifluoperazine were active at concentrations as low as 7.5×10^{-6} M and 1.0×10^{-6} M, respectively(23). We sought conditions, however, which would be more specific for anti-inflammatory agents and less so for phenothiazine derivatives. In view of the various actions of phenothiazines as tranquilizers, local anesthetics, and antihistamines, it would appear that some common mechanism such as a membrane phenomenon might be operating. When one considers the variation in lipid composition of biological membranes, and, therefore, their two-dimensional states(25), possible explanations for drug specificity or potency are obvious. Lipid-protein interfaces could be quite specific for a particular drug under given conditions. The results shown in Table III with chlorpromazine and 3 analogs (levomepromazine, trifluoperazine, triflupromazine) further validate the stabilizing effects of these compounds on membranes. The results might explain the weak effects of amitriptyline and imipramine, which are somewhat related structurally to phenothiazines.

The inactive drugs, epinephrine, norepinephrine, and morphine listed in Table IV are active in other tests for detecting anti-inflammatory agents(26,27). Epinephrine and norepinephrine block pedal edemas induced by egg white or dextran(18,26,28) and carrageenin.* They probably exert a vasodilator action since β-blockers reverse the inhibition seen with these drugs and α-blockers do not (18). These drugs are inactive in our system, probably because they are not anti-inflammatory agents *per se*. They do not antagonize various mediators of inflammation. On the contrary, Rocha e Silva(29) proposed that these catecholamines play some role in the production of inflammation under certain conditions. Furthermore, admixing increasing quantities of serotonin with a constant amount of epinephrine decreases the inhibiting potential of epinephrine(30), as an anti-inflammatory agent in pedal edemas. LSD-25, one of the most potent drugs tested, shows inherent anti-inflammatory activity when serotonin is the agonist(31).

There is an interesting correlation between the stabilizing effects of the drugs tested on RBC, blockade by the same drugs of endogenous substances such as SRS-A and bradykinin in guinea pig lung(32) and anti-erythema activity(33). Antagonism by these drugs of SRS-A and bradykinin in guinea pig lung *in vivo* in decreasing order of effectiveness is: LSD-25 $\geqq$ indomethacin $>$ mefenamic acid $=$ flufenamic acid $>$ phenylbutazone $>$ acetylsalicylic acid $>$ aminopyrine $>$ salicylic acid. Chloroquine, morphine, aminophylline, cincophen, etc., are all much less active. The order of decreasing antagonism of hemolysis using our classification for activity is: LSD-25 $>$ flufenamic acid $=$ mefenamic acid $=$ phenylbutazone $>$ indomethacin $=$ oxyphenbutazone $=$ acetylsalicylic acid $>$ aminopyrine $=$ salicylic acid. Phenylbutazone, aspirin and salicylic acid were shown to be inhibitors of the vasodepressor and capillary permeability enhancing action of human salivary kallikrein in the dog and the rabbit(34). In both instances the order of decreasing antagonism is: phenylbutazone $>$ aspirin $>$ salicylate.

Drugs tested in our system cannot be related to a local anesthetic action since xylocaine, procaine, and cocaine were all inactive. The antihistamines, methdilazine and promethazine, which also possess local anesthetic action, were inactive. Other inactive antihistaminics, not included in Table IV, were: dimethindene, pyrilamine, tripelennamine, diphenhydramine, promazine, and chlorpheniramine. These compounds enhanced hemolysis using concentrations of 5×10^{-3} M or in some cases 5×10^{-4} M. The antiphlogistic drugs, aspirin and phenylbutazone, are devoid of antihistaminic activity at effective antibradykinin dose levels(31). Thus the stabilizing action of the drugs tested on RBC does not appear to be related to antihistaminic activity *per se*.

In the RBC model of inflammation, antiserotonin activity may play some role since LSD-25 appears to stabilize RBC. Phenylbutazone produces only marginal and erratic

* Unpublished results.

inhibitory responses in serotonin edema, and aspirin is totally ineffective(31). The phenothiazines, chlorpromazine and methotrimeprazine, which are active in stabilizing RBC, clearly inhibit the local edemic response of serotonin in rats(26). It thus appears that in the RBC model of inflammation, anti-bradykinin, anti-SRS-A, and, possibly, anti-serotonin actions could be operative factors.

The present study offers no evidence to support a mechanism of action for the apparent protection of RBC membranes from heat-induced hemolysis by anti-inflammatory drugs. However, it might be similar to that suggested by Freeman and Spirtes. They concluded that the preservative effect of chlorpromazine on erythrocytes was largely due to the inhibition of passive water movement by the drug and to the prevention of K^+ leakage and Na^+ entrance(23).

Summary. An *in vitro* technique for screening nonsteroidal anti-inflammatory compounds based on their ability to inhibit heat-induced hemolysis of canine erythrocytes has been demonstrated by incubating red blood cells (3.0 ml) as a 40% (v/v) suspension in M/15 sodium phosphate, pH 7.4, for 20 minutes at 53°C with nonsteroidal anti-inflammatory agents (3.0 ml) dissolved in 0.9% saline, pH 7.4. Lysergic acid diethylamide, phenylbutazone, flufenamic and mefenamic acids, oxyphenbutazone, indomethacin, and aspirin inhibit hemolysis by 40 to 60% at concentrations of 5×10^{-4} M or less. An inhibition of hemolysis of 28% was significantly different from zero at the 95% confidence level. To determine the specificity of the test, more than 48 nonanti-inflammatory agents were tested.

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Interferon and Cytomegalovirus *in vivo* and *in vitro*.* (32220)

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Cytomegalovirus (CMV) is one of several viruses that have been associated with long-term, chronic infection in humans, and with the capacity to cross the placenta and initiate chronic infection in the fetus(1). The nature of the cytomegalovirus-host interaction which may result in such persistent virus infection in the presence of an otherwise normal host defense mechanism has never been fully defined. Children chronically infected with this virus have a normal immunological response and neutralizing antibody may be demonstrated in their sera(1). Current evidence suggests that interferon may be one determinant of host resistance to viral infections(2-4). The present study was initiated to examine the capacity of cytomegalovirus to induce the production of interferon in human cells *in vitro*, as well as to determine the sensitivity of this virus to the inhibitory activity of interferon. The investigation was then extended to elucidate the effect of endogenous interferon on the rate of excretion of cytomegalovirus in the urine of children who are chronic carriers of this virus.

Materials and methods. Sindbis virus, originally an isolate from a Malayan mosquito pool, was obtained from Dr. Philip K. Russell at the Walter Reed Arbovirus Unit and was grown and assayed in chick embryo fibroblasts (CEF). Chikungunya virus (CV), a standard reference strain, was obtained from the Walter Reed Arbovirus Unit and prepared from the brains of infected suckling mice and assayed in rat embryo fibroblasts (REF). Adenovirus type 2 was obtained from Dr. Harold S. Ginsberg. Stock pools were prepared and assayed in WI-38 or human foreskin cultures. Newcastle Disease virus (NDV), Hertz strain, was provided by Dr. Samuel Baron. Stock preparations were grown in embryonated hens' eggs and assayed by the hemadsorption plaque technique in chicken embryo fibroblasts. A calf lymph strain of vaccinia virus, obtained from Dr. Karl Habel at the National Institutes of Health was prepared and assayed in HeLa cells. Vesicular stomatitis virus (VSV), Indiana strain, was obtained from the American Type Culture Collection. Stock supplies were prepared and assayed in L cells. Herpesvirus (*Herpesvirus hominis*) was a recent isolate that has been maintained in this laboratory. Stocks were prepared and assayed in WI-38 cells. ECHO 11 virus was obtained from Dr. A. D. Heggie and was prepared and assayed in monkey kidney cells. Cytomegalovirus, strain AD 169, was obtained from Dr. Wallace P. Rowe, National Institutes of Health. Stock virus pools were prepared and assayed in WI-38 cells and contained approximately $10^{3.5}$ tissue culture infective doses ($TCID_{50}$) per ml.

Stock human interferon was prepared by infecting WI-38 cultures with 5×10^6 plaque forming units (pfu) of CV. Supernatant fluids were harvested at 24 hours and processed as previously described(5). Materials to be assayed for cytomegalovirus interferon were harvested from CMV-infected cultures of WI-38 or human foreskin cells (HFS) and similarly processed. Interferon preparations were assayed by the plaque reduction tech-

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