Aggregation of Red Cells and Effects of Nonsteroidal Anti-inflammatory Drugs (35869)

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The erythrocyte sedimentation rate (ESR) is a reliable index of red cell adhesion, rouleaux formation, inflammatory processes, changes in electrostatic charge on the membrane and a variety of other experimental and clinical situations (1).

Nonsteroidal anti-inflammatory drugs (NAIFD) inhibit red cell hemolysis caused by heat, hypotonicity, and heterologous antiserum to red cell membranes (2–4). They inhibit the release of acid phosphatase and lactic dehydrogenase from platelets treated with thrombin and calcium and platelet antiserum (5). Phenylbutazone and other NAIFD have been shown recently to inhibit red cell settling in response to high molecular weight dextran (250,000), fibrinogen, and gelatin (6, 7). The present report summarizes an extension of this work and shows the interrelationship between isolated observations in the test tube and those occurring in animals with tissue damage.

Methods. Blood is collected in heparinized syringes from the dorsal aorta of anesthetized Carworth male rats, weighing more than 300 g. Bloods are centrifuged at high speeds, plasmas are discarded, and red cells are washed once with equal volumes of saline, after which they are diluted to 50% (v/v) with saline. One milliliter of dextran or some other accelerator of red cell settling is added to 1 ml of suspension, making a final red cell concentration of 25%.

Nonsteroidal anti-inflammatory drugs, as well as other types of drug, are made up in isosmotic NA_2PO_4 buffer (pH 7.0) to which desired amounts of dextran, carrageenan, or other accelerator is added. Tubes, in duplicate or triplicate, are allowed to stand at room temperature for 30 min; then contents are mixed and transferred by capillary pipettes to Wintrobe Dispo-Type hematocrit tubes. The ESR is recorded at 60 min. For routine assays, and whenever possible, the rate of red cell settling in control hematocrit tubes is adjusted to attain a rate of 60–70 mm/hr. Washed rat red cells alone do not display a tendency to fall when allowed to stand in hematocrit tubes for 24–28 hr.

Studies in intact animals are in male Carworth rats, weighing 225–250 g. Local inoculations of various agents are made into the right hindpaws of rats in 0.1 ml of sterile saline. Six hours later, rats are killed by chloroform inhalation, both paws removed at the tibiotarsal joint with a Harvard guillotine, weighed and the difference recorded as milligrams of edema/100 g of final body weight. Untreated, vehicle-treated and standard anti-inflammatory drug-treated rats are included in each assay.

Antisera to rat red cells are raised in rabbits (5) and antisera to rat serum proteins are obtained from Pentex.

Polyions, enzymes, and most other reagents are obtained from Sigma, phenylbutazone from Geigy, indomethacin from Merck, Sharp and Dohme, mefenamic and flufenamic acids from Parke-Davis, steroids from The Upjohn Company, isobutyl phenylacetic acid (ibufenac) and isobutyl phenylpropionic acid (Motrin, The Upjohn Company) from Boots Pure Drug Company, and phytohemagglutinin-P from Difco.

Results. Carrageenan and dextran, for example, amplify the ESR when added to washed red cells. The rate is related to concentrations. Carrageenan (0.15%) is more efficacious than dextran, producing a rate of fall of 60–70 mm/hr compared to 1% dex-

TABLE I. Standard Nonsteroidal Drug Effects on the ESR.

Anti-inflammatory drugs	Final conc (mg/ml)	Percentage inhibition[a]		Hindpaw potency[b]
		Dextran	Carrageenan	
Acetylsalicyclic acid	1.0, 2.0, 3.0	0, 5, 0	0, 0, 0	0.3
Gentisic acid	1.0, 2.0, 3.0	0, 22, 30	0, 25, 33	0
Aminophylline	0.5, 1.0, 2.0	0, 0, 0	0, 0, 0	0
Salicylic acid	0.5, 1.0, 2.0	13, 27, 84	15, 30, 75	0.3
Oxyphenbutazone	0.25, 0.5, 1.0	25, 58, 80	27, 59, 80	0.9
Phenylbutazone	0.25, 0.5, 1.0	75, 95, 99	88, 93, 96	1.0 (standard)
Indomethacin	0.25, 0.5, 1.0	13, 35, 50[c]	12, 33, 41[c]	19.0
Isobutyl-phenylpropionic acid	0.25, 0.5, 1.0	55, 77, 80	59, 80, 88	1.5
Isobutyl-phenylacetic acid	0.25, 0.5, 1.0	70, 87, 92	88, 90, 92	0.1
Flufenamic acid	0.25, 0.5, 1.0	77, 90, 48[c]	65, 79, 0[c]	2.0
Mefenamic acid	0.25, 0.5, 1.0	55, 74, 77	60, 70, 79	1.0

[a] The above numbers represent those obtained from duplicate hematocrit tubes. The rate of fall in the dextran (1%) tubes was 70 mm/hr; that in the carrageenan (0.15%) tubes was 71 mm/hr.

[b] Oral multidose potencies in rats for carrageenan-induced edema assays.

[c] Hemolysis.

tran. At equally effective concentrations, both responses are inhibited by the addition of NAIFD (Table I). Gentisic acid and aminophylline, used as negative controls routinely in all our *in vitro* assay systems (3), do not inhibit the ESR significantly, while indomethacin, unlike other NAIFD, has a shallow dose–response and elicits red cell lysis at the higher concentrations shown here. Aspirin, unlike salicylic acid, is inactive in this system.

More than 150 selected chemicals have been added to washed cells in varying and increasing amounts. Agents producing a ESR of 50–70 mm/hr are: acid phosphatase, 0.1%; alpha-amylase, 0.1–1.0%; polygalacturonic acid, 5 mg/ml; polycytidylic acid, 5 mg/ml; polyadenylic acid, 5 mg/ml; poly-L-glutamic acid, 5 mg/ml; poly-L-proline, 5 mg/ml; polyadenylic-cytidylic acid, 3 mg/ml; polyadenylic-inosinic acid, 3 mg/ml; and phyothemagglutinin-P, 10 mg/ml; among others. Although more than 30 enzymes and 12 polyions of different types have been added to washed rat red cells, most fail to elicit increases in the ESR. All accelerators of the ESR are less effective in whole blood than in washed cells. It requires 1.5% dextran to produce the same rate of fall in whole blood as 1% in washed red cell preparations. Discernible differences in inhibitory effects of the NAIFD are not seen if drugs are added 30 min before, at the same time, or at least 2 hr after, the addition of dextran and reactions are allowed to go to completion. Potassium chloride substitutes readily for NaCl, with no discernible effect on the ESR. The dextran-induced ESR is inhibited completely by resuspending cells in rat plasma. The ESR cannot be made to reappear when dextran is added again, following recentrifugation and resuspension of the plasma-washed cells.

The NAIFD, shown in Table I, inhibit the chemically-induced ESR in a dose-related manner, except that induced by acid phosphatase and alpha-amylase. Metabolic inhibitors, like iodoacetic acid, *p*-chloromercuribenzoate, 2,4-dinitrophenol, sodium fluoride, potassium cyanide, and sodium isothiocyanate have been added to red cells at 10^{-5}–10^{-2} *M*. 2,4-Dinitrophenol alone inhibits the dextran-induced ESR, producing the same inhibitory effects as phenylbutazone at 10^{-5}, 10^{-4}, 10^{-3}, 10^{-2} *M* (20, 60, 85, and 100%, respectively). Bubbling nitrogen through washed rat red cells has no effect on the dextran- and carrageenan-induced ESR.

Different classes of drugs, including 5 tranquilizers, 4 steroidal anti-inflammatory drugs,

TABLE II. Induction of Acute Inflammation with Various Drugs which Promote the ESR.[a]

Chemical substance	(mg/ml)	ESR (mm/hr)	Mg/paw in 0.1 ml NaCl[b]	Edema[c] (mg/100 g)
Vehicle	—	0	—	0
Dextran	10.0	69	10.0	235 ± 9
Carrageenan	0.5	70	0.5	260 ± 25
Polyadenylic acid	2.5	34	5.0	369 ± 19
Poly-L-glutamic acid	2.0	73	10.0	252 ± 6
Poly-L-proline	4.0	50	7.5	238 ± 16
Poly-L-tyrosine	5.0	0	10.0	12 ± 2
Poly-L-phenylalanine	5.0	0	10.0	28 ± 8
Acid phosphatase	2.0	66	10.0	467 ± 5
Alpha-amylase	4.0	60	10.0	408 ± 14
Phytohemagglutinin-P	5.0	40	10.0	396 ± 44

[a] ESR in duplicate hematocrit tubes; hindpaw edema: 5 rats/group.

[b] Right paws injected at 0 time and removed 6 hr later.

[c] Edema is expressed as difference in weights (± SEM) between injected —uninjected paws, expressed on basis of final body weights.

1 uricosuric agent, 4 CNS depressants, 6 immunosuppressants ("anticancer" drugs), 6 vitamins, 4 antiallergy drugs, 2 oral antidiabetics, 2 muscle relaxants, 4 bariturate analogues, 3 diuretics, 2 cardiotonic agents, 8 antibiotics, 1 antithyroid drug, 6 "hypotensives," 3 adrenergic agents, 2 adrenergic blocking agents, 1 anticonvulsant, 4 prostaglandins, and 1 ganglionic blocking agent, and others, like warfarin, diphenadione, and sodium citrate, have been tested against the dextran-induced ESR. Probenecid (uricurosuric), disodium cromoglycate (antiallergy), chlorothiazide ("hypotensive") and ethacrynic acid (diuretic) are the only active drugs tested thus far. Various metals like $MgCl_2$, $MnCl_2$, LiCl and $CaCl_2$ have been added to the dextran-induced ESR, without effect.

Probenecid, chlorothiazide, disodium cromoglycate, and ethacrynic acid are all inactive in carrageenan-induced, acute anti-inflammatory assays (8) when given orally at high dosages (up to 100 mg/kg) and compared to multidoses of phenylbutazone and aspirin.

Available accelerators of the ESR are

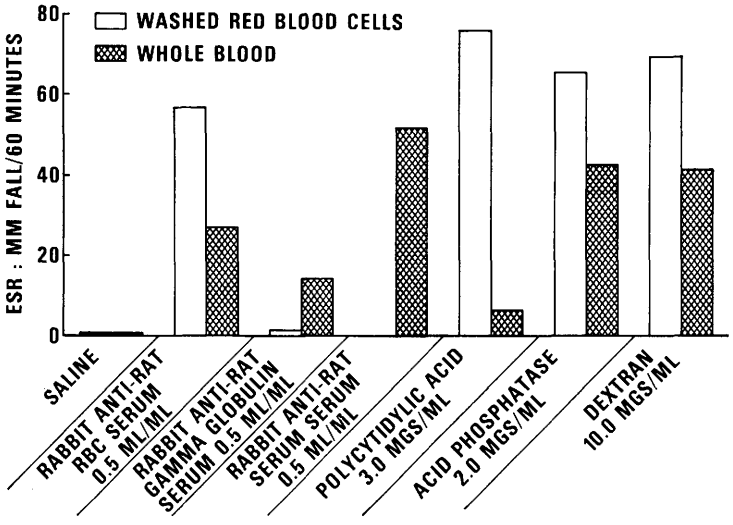

FIG. 1.

TABLE III. Duration of Action of Various Drugs Following A Single Oral Dose.[a]

Drug	(Mg/kg)	Time (hr)	Edema (mg/100 g of body wt)	Percentage inhibition
Vehicle	—	—	261 ± 12	0
Salicylic acid	300	6	202 ± 20	22
		30	219 ± 12	16
		54	245 ± 12	6
		78	310 ± 15	0
Isobutyl phenyl propionic acid	100	6	159 ± 21	39
		30	195 ± 17	25
		54	239 ± 18	8
		78	238 ± 10	9
Phenylbutazone	50	6	160 ± 20	39
		30	197 ± 15	24
		54	240 ± 14	8
		78	260 ± 11	0
Vehicle[b]		—	311 ± 13	0
Indomethacin[b]	10	6	179 ± 5	42
		30	107 ± 8	65
		54	169 ± 14	46
		78	228 ± 12	27

[a] Ten rats per group for the carrageenan-induced hindpaw edema. ± = SD. Drugs administered once orally at 0 time and carrageenan (0.5 mg/rt paw) injected 6 hr before removal at indicated times shown.

[b] Experiments were done at a different time from those above.

inflammatory when inoculated into the right hindpaw of rats (Table II). These inflammatory reactions are inhibited to varying degrees by the administration of increasing amounts of isobutyl phenylpropionic acid and phenylbutazone (5–100 mg/kg, po).

Rabbit antiserum to rat red cells and serum elicit dose-related increases in the *in vitro* ESR. Antiserum to rat serum is effective only when added to whole blood (Fig. 1). These two antigen–antibody reactions are inhibited also by the same NAIFD shown in Table I.

Different NAIFD were added at equivalent inhibitory concentrations to triplicate tubes of dextran-treated red cells and the reactions were allowed to go to completion (2 hr). Cells were centrifuged subsequently af-

TABLE IV. Effect of Arthritogenic Amounts of *M. butyricum* on the ESR of Rats.[a]

After inoculation (days)	ESR(mm/hr)	Arthritic score
0	1.3 ± 0.1	0
2	20.7 ± 2.4	0
4	22.3 ± 2.4	0
7	17.3 ± 2.1	0
9	12.6 ± 1.5	0
11	13.4 ± 2.5	0.9 ± 0.6 (3/10)
14	22.6 ± 2.7	16.0 ± 0 (10/10)
16	22.7 ± 3.3	14.0 ± 1.6 (10/10)
18	28.1 ± 3.3	15.4 ± 0.2 (10/10)
18 (no inoculations)	0	0

[a] Ten rats per group. Inoculated with *M. butyricum* (0.5 mg/0.1 ml of mineral oil in tail) on the first day. Rats anesthetized on indicated days; bloods heparinized and removed from dorsal aorta at indicated times; and ESR determined within 1 hr in Wintrobe Dispo tubes.

TABLE V. Cardiotoxic and ESR Effects in Rats Injected with Isoproterenol.[a]

Dose (mg/rat, sc)	Heart (mg/100 g of body wt)	ESR
0	298 ± 8	0
10	386 ± 17	41.0 ± 0.6
20	402 ± 16	41.2 ± 0.4
40	449 ± 19 (aneurysms)	38.0 ± 0.3
80	462 ± 20 (aneurysms)	35.0 ± 0.6
160	402 ± 7.9 (aneurysms and 4/10 dead)	35.0 ± 1.0

[a] Ten rats per group; injected subcutaneously at 0 time; hearts and bloods were removed 24 hr later; values ± SEM.

ter repeated washing with equal volumes of saline. Four washes are required to remove completely the inhibitory effects of 400 μg/ml of phenylbutazone and 800 μg/ml of isobutyl phenylpropionic acid. One wash removes completely the inhibitory effects of salicylic acid (2000 μg/ml) and 8 washes the effects of 200 μg/ml of indomethacin. These results compare favorably with the duration of action of these particular drugs in rats (Table III). Indomethacin has the longest duration of action. These results have been reconfirmed with a number of other long-acting drugs on other occasions.

Local inoculations of arthritogenic amounts of *M. butyricum* into the tail of rats (0.5 mg/0.1 ml of heavy mineral oil) elicit rapid and long-lasting increases in the ESR (Table IV). These results confirm those obtained by others (9). Similar rapid increases are obtained in isoproterenol-injected rats (Table V). Both heart weights and the ESR increase. When given at effective anti-inflammatory dosages (and rats exsanguinated 3 hr later), phenylbutazone alone lowered the ESR within this relatively short time in both arthritic and isoproterenol-treated rats (Table VI). These same drugs, given chronically at lower dosages to rats with developing arthritis (twice each day for 14 days), inhibit both the arthritis and the ESR. The effects are dose related, as we have shown many times.

Summary and Discussion. Carrageenan, dextran, phytohemagglutinin-P, alpha-amylase, acid phosphatase, polycytidylic acid, polyadenylic acid, polygalacturonic acid, poly-L-proline, poly-L-glutamic acid and others promote red cell sedimentation rates (ESR) when added to washed red cells. Rabbit antisera to red cells and serum promote the ESR when added to either whole blood or washed red cells. All of these various accelerator responses, with the exception of those

TABLE VI. Single-Dose Effects of Drugs on the ESR of Arthritic and Isoproterenol-Injected Rats.[a]

Drug	(Mg/kg)	ESR mm/hr		Percentage inhibition	
		Arthritic	Isoproterenol	Arthritic	Isoproterenol
Vehicle		12.3 ± 0.8	8.6 ± 2.1	0	0
Indomethacin	80	10.3 ± 1.4	7.9 ± 2.1	0	0
Isobutyl phenyl-propionic acid	300	24.0 ± 1.8	9.9 ± 0.8	0	0
Salicylic acid	600	24.0 ± 2.3	10.7 ± 1.1	0	0
Phenylbutazone	160	2.1 ± 0.5	3.9 ± 0.2	83	55
No treatment; no injections		0	0	0	0

[a] Arthritic rats had severe disease (4+ in all 4 appendages) and normal rats were injected sc with 80 mg of isoproterenol. Arthritic rats were given drugs on day 16 after *M. butyricum* and 24 hr after isoproterenol injections. Bloods were removed 3 hr after giving drugs.

induced by enzymes, are inhibited by nonsteroidal anti-inflammatory drugs (NAIFD). Many other pharmacologic classes, with the exception of ethacrynic acid, probenecid, sodium cromoglycate and chlorothiazide, fail to display similar inhibitory effects. The elevated ESR is inhibited when drugs are added before, during, or after the addition of dextran. The ESR is inhibited by 2, 4-dinitrophenol, but not other metabolic inhibitors. Heavy metal ions and anerobiosis have no effect on dextran-induced ESR.

Agents accelerating the ESR induce acute inflammatory responses when inoculated locally into the hindpaws of rats.

Drugs whose inhibitory effects are less readily removed from dextran-treated red cells by repeated saline washings, *e.g.*, indomethacin, have longer durations of action when tested in acute inflammatory reactions of intact animals against salicylic acid whose effects are removed more rapidly.

Mechanisms of action of accelerators or inhibitors of the ESR are unknown. The NAIFD are, for the most part, acidic. They may adhere to available positive charges on damaged or altered cell membranes and prevent their tendency to "stick," adhere, aggregate, clump or pull together.

Although red cell aggregation is among the many convenient models for the assessment *in vitro* of well-known NAIFD, it fails, like many other isolated methods (3–5), to have predictive value for unknown agents. Many undisclosed chemical agents have been discovered to be extremely potent in the isolated ESR system. When given subsequently at varying dosages and routes of administration to animals, however, they fail (in our hands, at least) to display "anti-inflammatory" activity.

The dextran- and carrageenan-induced ESR appears to be a rather specific method for screening NAIFD discovered by other methods and for the study of their probable mechanisms of action.

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Received April 13, 1971. P.S.E.B.M., 1971, Vol. 138.