In Vitro Effects of Non-steroidal Anti-inflammatory Drugs (NAIFD) (33785)

E. Myles Glenn and Barbara J. Bowman

The Upjohn Company, Research Division, Kalamazoo, Michigan 49000

Whitehouse (1) has summarized the effects of NAIFD *in vitro*. They bind to various serum proteins (2), inhibit hyperthermic and hypotonic-induced erythrocyte lysis (3, 4), bind the epsilon amino groups of lysine (5), accelerate the binding of 5,5′-dithiobis(2-nitrobenzoic acid) to S–S groups on serum proteins (6) and prevent the aggregation of platelets in response to collagen and other substances (7).

Methods are described for the prevention of lactic dehydrogenase (LDH) and acid phosphatase (ACPase) release from thrombin-aggregated platelets by NAIFD and other drugs. Their inhibitory effects on hypotonic-hyperthermic erythrocyte lysis *in vitro* are described also. Comparisons were made between some of these *in vitro* effects (3, 5, 6).

Methods. With the exception of hypotonic-hyperthermic lysis of erythrocytes and release of LDH and ACPase from platelets, all methods have been described (1, 3, 5, 6). Hyperthermic-hypotonic lysis of rat erythrocytes was produced by centrifuging freshly drawn heparinized rat blood and making a 10% erythrocyte suspension by the addition of 0.15 *M* phosphate buffer (pH 7.4). To 3.0 ml of erythrocyte suspension was added 3.0 ml of 0.015 *M* phosphate buffer containing drug at the desired final concentration. The mixture was heated at 53° in a water bath for 20 min and cooled in an ice bath 5 min. The Technicon AutoAnalyzer was used to determine the amount of hemolysis. This was done by appropriate dilution daily of a hemolyzed solution of 5% rat erythrocytes (100% hemolysis) used in the original reaction mixture. Assays were done twice each day in duplicate on the same drugs. When testing unknown drugs, phenylbutazone or indomethacin were assayed simultaneously at 3 different concentrations.

For the investigation of LDH-ACPase (lactic dehydrogenase-acid phosphatase) release from platelets, the appropriate amounts of the NAIFD, in 0.5 ml of 0.15 *M* PO_4 buffer, were added to 2.0 ml of rat platelet-rich plasma (PRP) containing an excess of 25 units of thrombin (Upjohn) added in 0.5 ml of 0.15 *M* NaCl and 0.108 *M* $CaCl_2$. Mixtures were heated at 37° for 1 hr, centrifuged, and ACPase was determined immediately (8); LDH the following day (9). Assays were done in duplicate, twice daily, along with the appropriate controls and standard drugs (either indomethacin or phenylbutazone).

TABLE I. Hypotonic-Hyperthermic Lysis of Erythrocytes and Inhibitory Effects of NAIFD.[a]

Drug	Type of erythrocyte lysis (% inhibition)		
	Hypotonic	Hyperthermic	Hyperthermic-hypotonic (H + H)
Indomethacin	25	60	73
Aspirin	17	0	27
Aminophylline	18	0	24
Gentisic acid	7	10	20
Phenylbutazone	22	25	42
Flufenamic acid	33	62	78
Oxyphenbutazone	5	12	20
Ibuprofen	35	20	55
Ibufenac	18	30	41
Mefenamic acid	22	25	50

[a] All drugs were assayed at least 2 times in duplicate at 2.5×10^{-4} *M*; see Ref. (12) for dose-response relationships.

Carrageenin-induced hindpaw edema of the rat was used to assess the acute anti-inflammatory potencies of various NAIFD (10). Drugs were suspended and given orally in water (5.0 ml/kg).

Results. Hyperthermic-hypotonic erythrocyte lysis is more sensitive to the effects of the NAIFD than either process alone (Table I).

TABLE II. Effect of Thrombin and Calcium on LDH and ACPase Release from Platelet-Rich Plasma (PRP).[a]

Addition	Concentration	LDH release (units/ml × 10^3)	ACPase release (units/ml)
None	—	0.65	0.25
Calcium	0.036 *M*	0.92	0.55
Thrombin	8.5 units/ml	0.64	0.24
Calcium + thrombin	—	1.55	1.73

[a] Each value is the mean of four assays.

In the presence of thrombin, calcium is required for maximal release of LDH and ACPase from platelets (Table II). The time required for maximal LDH and ACPase release is 30 min (Table III). Indomethacin and phenylbutazone inhibit the release of LDH and ACPase from platelets. The effects are related to drug concentrations (Table IV). These and all other drugs were tested to determine their effects on lactic dehydrogenase and acid phosphatase released from PRP in the presence of the appropriate substrates. The NAIFD and other drugs do not exert inhibitory effects on the two enzymes released from platelets.

The effects of various standard NAIFD on epsilon amino group binding to lysine on serum albumin, acceleration of S–S group reactions on serum albumin, various types of erythrocyte lysis and prevention of LDH and ACPase release from thrombin-calcium-induced platelet aggregation were compared (Table V). The numerical order of effectiveness in the various systems and oral potencies in the carrageenin edema assay do not coincide.

TABLE III. LDH and ACPase Release from Platelets as a Function of Time.[a]

Time (min) at 37°	LDH release (units/ml × 10^3)	ACPase release (units/ml)
0	0.125	0.591
15	0.423	1.150
30	0.923	1.230
45	1.002	1.241
60	1.100	1.198
90	1.156	1.200

[a] Assays in duplicate; calcium (0.036 *M*) and thrombin (8.5 units/ml) in reaction mixture. Filipin (32 μg/ml) induced the additional release of 0.5 × 10^3 units/ml of LDH and 1.12 units/ml of ACPase above control values in the absence of thrombin, and following removal of the thrombin-induced clot from PRP, no additional release was obtained upon the subsequent addition of filimarisin (Filipin).

TABLE IV. Effect of Indomethacin and Phenylbutazone on LDH and ACPase Release from PRP.[a]

Drug	Concentration (× 10^{-3} *M*)	LDH (units/ml × 10^3)	ACPase (units/ml)
None	—	1.230	1.125
Phenylbutazone	0.5	1.001	0.999
	1.0	0.550	0.519
	2.0	0	0.050
Indomethacin	0.5	1.124	1.000
	1.0	0.233	0.432
	2.0	0	0.212

[a] The values represent the means of 8 assays with phenylbutazone and 24 with indomethacin.

Sixty pharmacologic agents were tested in the various systems *in vitro*. Some of these were divided arbitrarily, for want of a better method, into "active" and "inactive" drugs (Tables VI and VII). A few drugs of both artificial categories are inhibitory in the hind-paw edema assay.

Discussion. If a clear-cut relationship exists between "effective anti-inflammatory" drugs *in vivo* and *in vitro*, it has not been proved or disproved by the data here. It is likely, as Whitehouse (11) generalizes, that NAIFD are "polyvalent." These conclusions as well as those we have summarized, do little to further our understanding of the so-called mechanism of action of NAIFD; rather, all the data taken together show the diversity of drug action and the difficulties of

TABLE V. Standard Drugs and Effects on Various Systems.[a]

Compound	RBC ($2.5 \times 10^{-4} M$; % inhibition)		S–S group acceleration ($2.0 \times 10^{-3} M$) Increase E_{412} (—log T)	Epsilon amino lysine binding ($2.0 \times 10^{-3} M$) % inhibition	Platelet protection (% inhibition)				Numerical order of increasing *in vitro* activity	Biological activity hindpaw × phenylbutazone
					Acid phosphatase release		Lactic dehydrogenase release			
	Heat only	Hypotonic and heat			$1 \times 10^{-3} M$	$2 \times 10^{-3} M$	$1 \times 10^{-3} M$	$2 \times 10^{-3} M$		
Indomethacin	61	71	0.832	80	35	85	38	99	8	19
Acetylsalicylic acid	0	3	0.018	12	24	43	53	73	1	0.3
Aminophylline	0	0	0.018	13	52	68	73	79	2	0
Gentisic acid	7	8	0.072	21	13	18	25	27	3	0
Phenylbutazone	22	39	0.178	41	42	82	37	92	4	1
Flufenamic acid	64	76	0.673	54	75	78	91	93	10	2
Oxyphenbutazone	11	16	0.060	43	72	86	89	95	5	1
Ibuprofen	23	49	0.373	44	72	76	95	100	7	1.5
Ibufenac	27	38	0.211	38	67	79	84	94	6	0.1
Mefenamic acid	—	53	0.179	63	80	86	95	98	9	1

[a] *In vitro* assays in duplicate; *in vivo* assays multidose (4–5 dosage levels for each drug); 10 rats/group. Separate assays conducted with 4–5 dosages of phenylbutazone as standard in each assay.

TABLE VI. Effect of Various Drugs on *in Vitro* Assay Systems.

Drug	S–S group acceleration (2×10^{-3} *M*) (—log T)	(% inhibition) Epsilon amino group binding (2×10^{-3} *M*)	RBC hemolysis (H + H) (2.5×10^{-4} *M*)	Acid phosphatase release (1×10^{-3} *M*)	Lactic dehydrogenase release (1×10^{-3} *M*)	Hindpaw edema potency phenylbutazone = 1.0
Bishydroxycoumarin	0.757	88	32	64	100	0.8
Bithionol	0.368	ND[b]	INC	65	100	0
Chloroquine phosphate	0	0	INC	60	88	0
Cycloheptolamide sodium	0.683	52	29	17	17	1.2
Dibenzylamine, *N*-(2-chlorethyl)–hydrochloride	0	ND	0	32	67	0
Diphenadione	ND	28	INC	30	51	0
Disulfiram	0.011	0	12	71	0	0
Erythromycin	0	0	INC	58	48	0[a]
Folic acid	ND	26	14	0	52	0.1[a]
Hexachlorophene	0.687	83	INC	78	57	0.14
Indomethacin (standard)	0.832	80	83	62	95	19
Methandrostenolone	0.132	4	INC	55	66	0
Novobiocin sodium	0.596	58	62	41	34	0.2
Probenecid	0.218	16	20	36	60	0
Sulfadiazine	0.038	26	7	46	39	0.3[a]
Tolbutamide	0.439	54	21	36	39	0.5[c]
Zoxazolamine	0.090	0	INC	60	86	0.2

[a] Single oral dose assay (50 mg/kg); all others assayed at multidoses *in vivo*.

[b] ND = not done; INC = increase in hemolysis.

[c] Inactive in adrenalectomized rats.

TABLE VII. "Inactive" *in Vitro* Drugs in the Hindpaw Assays *in Vivo*.[a]

Drug	HP potency	Drug	HP potency	Drug	HP potency
Acetazolamide	0.4[b]	Chlorpropamide	0[b]	Perphenazine	>1.0[b]
Acetohexamide	0[b]	Chlorothiazide	0	Psicofuranine	0[b]
Adenine	0.2[b]	Colchicine, *N*-desacetyl-*N*-methyl-	>2.0	Pyrazinamide	0[b]
Adenosine	0.2[b]			Reserpine	<1.0
Alloxan hydrate	0[b]	Cycloheximide	1.6 × Indocin	Rhodanine	0
Aminopyrine	0.3			Salicylic acid, 4-amino	0
Atropine sulfate	0	Cysteine	0	Sarcine	0.5[b]
Benzoic acid, *p*-amino	0[b]	Diphenhydramine • HCl	0	Sarcosine • HCl	0[b]
Benzphetamine • HCl	0[b]	DL-Methionine	0[b]	Streptomycin sulfate	0[b]
Bufotenine	1.0	DL-Penicillamine	0	Sulfaguanidine	0.3[b]
Caffeine	0[b]	Indomethacin (standard)	19	Sulfisoxazole	0[b]
Carbutamide	0.2[b]	Inosine	0[b]	Thiouracil	0[b]
Celesticetin	0[b]	Inositol	0[b]	Tomatidine	0[b]
Centrophenoxine	0	Methoxsalen	1.0	Triparanol	0.2
Chlorambucil	0[b]	Nicotinic acid	0.8[b]	Tubercidin	6.0
Chlorphenesin carbamate	0	Penicillin G potassium	0	Vanillin	0[b]

[a] Except where indicated, drugs were compared to phenylbutazone.

[b] One to 2 dose assays; other 4–5 dosages using 10 rats/group. HP = hindpaw. Concentrations of drugs for *in vitro* tests same as those depicted in Table VI.

in vitro–in vivo correlations.

Glenn (12), for example, assumed erroneously, perhaps, that the "anti-inflammatory" action of the NAIFD is related directly to their membrane-protective effects. The available data, although attractive, do not allow these interpretations.

If all of the currently available NAIFD act on the various *in vitro* systems similarly, and/or qualitatively the same, as we and Whitehouse (11) have shown, the question one must ask is: "Are these drugs *really* different?" It appears that, aside from toxicity, tolerance and side effects *in vivo*, they may all act similarly—if one can rely on evidence obtained *in vitro* for the assessment of drug effects *in vivo*.

Some of the NAIFD possess inhibitory effects on the clotting mechanism also (13). The *in vitro* effects reported here may be more closely connected with the clotting phenomenon than to inflammation. But insufficient data exist to make positive correlations. There are many reasons for doing experiments of the type detailed here, among which are: (i) The search for more appropriate *in vitro–in vivo* assay methods, and (ii) The search for the exact mechanism(s) of action of a pharmacologic class of drugs. Our knowledge of the "inflammatory" process itself is so limited that it appears premature to attempt correlative studies of this type. The many different methods for assessing so-called "anti-inflammatory" drugs in animals lends credence also to the belief that these, too, are deficient and not entirely satisfactory. Our attention in the future may be more appropriately diverted, perhaps, to intensive efforts at understanding the relationships, if any, between various animal models of disease and their human counterparts. Although our studies have been directed to rats, rat erythrocytes, and rat platelets, our major interests center around the treatment of human "inflammatory" diseases like rheumatoid arthritis, lupus erythematosus, and related connective tissue disorders. Studies of the type outlined in this report may take us further afield, rather than increase our understanding, of the actual problems in human connective tissue diseases.

Summary. The NAIFD inhibit the release of LDH and ACPase from thrombin-aggregated platelets *in vitro*. They inhibit hypotonic-hyperthermic lysis of erythrocytes. The effects of NAIFD *in vitro* have been

compared to their "anti-inflammatory" effects when given orally. A direct relationship does not exist. Of a large number of "active" and "inactive" drugs found in these and various other *in vitro* systems, active "anti-inflammatory" drugs appeared in both classes.

Indomethacin was obtained from Merck, Sharpe and Dohme, phenylbutazone from Geigy Pharmaceutical Company, and ibuprofen from Boots Limited of England. Oxyphenybutazone, ibufenac, mefenamic acid, chloroquine phosphate, probenecid, zoxazolamine, acetazolamide, and perphenazine were obtained from Geigy, Boots, Parke-Davis Co., Sterling-Winthrop Research Inst., Merck, Sharpe and Dohme, McNeil Laboratories, Lederle Laboratories, and the Schering Corporation, respectively.

1. Whitehouse, M. W., Drug Res. **8**, 321 (1965).

2. Mizushima, Y. and Kobayashi, M., J. Pharm. Pharmacol. **20**, 169 (1968).

3. Brown, J. H., Mackey, H. K., and Riggilo, D. A., Proc. Soc. Exptl. Biol. Med. **125**, 837 (1967).

4. Inglot, A. D. and Wolna, E., Biochem. Pharmacol. **17**, 269 (1968).

5. Skidmore, I. F. and Whitehouse, M. W., J. Pharm. Pharmacol. **17**, 668 (1965).

6. Gerber, D. A., Cohen, N., and Giustra, R., Biochem. Pharmacol. **16**, 115 (1967).

7. Packham, M. A., Warrior, E. S., Glynn, M. F., Senyi, A. S., and Mustard, J. F., J. Exptl. Med. **126**, 171 (1967).

8. Sigma Bulletin #104.

9. Sigma Bulletin #500.

10. Winter, C. A., Risley, E. A., and Nuss, G. W., Proc. Soc. Exptl. Biol. Med. **111**, 544 (1962).

11. Whitehouse, M. W., Biochem. Pharmacol. Spec. Suppl. March, 293 (1968).

12. Glenn, E. M., Bowman, B. J., and Koslowske, T. C., Biochem. Pharmacol. Spec. Suppl. March, 27 (1968).

13. Packham, M. A., Nishizawa, E. E., and Mustard, J. F., Biochem. Pharmacol. Spec. Suppl. March, 171 (1968).

Received Dec. 9, 1968. P.S.E.B.M., 1969, Vol. 130.