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Anti-Inflammatory and Liver Sulfhydryl Content-Altering Effects of Certain Nonsteroids in the Rat

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Abstract □ Selected agents were evaluated orally in the male rat for their capacity to prevent carrageenin-induced pedal edema and to alter liver sulfhydryl levels. Indomethacin was the most potent anti-inflammatory agent, followed by tetrabenazine and chlorpromazine. Cryogenine and phenylbutazone were equieffective, with cyproheptadine, aspirin, and sparteine being least potent. Carrageenin-induced pedal edema alone did not lower liver sulfhydryl content. Aspirin elevated the sulfhydryl concentration both in the presence and absence of carrageenin-induced edema, while chlorpromazine, indomethacin, phenylbutazone, cryogenine, tetrabenazine, and sparteine were without significant effect at anti-inflammatory dosages. Cyproheptadine lowered liver sulfhydryl levels, but this was considered to be a nonspecific or toxic effect resulting from the high dosages employed.

Keyphrases □ Anti-inflammatory action—nonsteroids □ Sulfhydryl content alteration, liver—nonsteroids □ Carrageenin-induced pedal edema, rats—anti-inflammatory testing

The work of Marozzi and Malone (1) indicated that a correlation might exist between a compound's ability to protect against stress-induced hepatic sulfhydryl depletion and its potential in inhibiting carrageenin-induced acute pedal edema in the rat. The present study is concerned with a comparison of such activities, using both clinically useful antirheumatic agents (aspirin, phenylbutazone, and indomethacin) and compounds that are potent inhibitors of several models of inflammation in animals but which are not used clinically as anti-inflammatory agents (chlorpromazine and cyproheptadine). Cryogenine (vertine), an alkaloid shown to be equipotent to phenylbutazone in both carrageenin-induced acute inflammation and in adjuvant-induced (*Mycobacterium butyricum*) chronic inflammation (2), as well as sparteine and tetrabenazine, was also investigated. The latter two agents bear some structural resemblances to possible degradation products of cryogenine.

EXPERIMENTAL

Carrageenin-Induced Pedal Edema—Adult male rats of the Wistar strain[1] were allowed to equilibrate in air-conditioned quarters for at least 1 week after receipt of shipment. Animals were allowed free access to Purina laboratory chow and tap water at all times. Experimental procedures used were similar to those described by Van Arman *et al.* (3). On the day of the experiment, rats were removed from food at −1 hr. and dosed orally to avoid the parenteral "counterirritant" effect. The test drugs were administered either dissolved or suspended in 0.25% agar solution at a constant dosage volume of 10 ml./kg. Control animals received the agar vehicle alone. Plantar injections of 0.1 ml. of aged, 1% carrageenin were made into the rat hind paw at 0 hr. The paw volume of all animals was recorded plethysmographically immediately afterward and again at +3 hr. (peak edematous response) just prior to sacrifice and removal of liver samples. On the basis of preliminary testing, a predictable high and a predictable low effective oral anti-inflammatory dose were selected for each experimental compound[2].

Each experimental run consisted of 60 animals divided into six interrelated groups: Group I, agar control (10 ml./kg. of 0.25% aqueous agar orally at −1 hr.); Group II, carrageenin control (agar solution at −1 hr. followed by plantar injection of carrageenin at 0 hr.); Group III, low drug control (at −1 hr. the lower dosage of the test drug shown by preliminary experimentation to be effective orally in reducing carrageenin-induced acute inflammation); Group IV, high drug control (the higher dosage of the test drug); Group V, low drug experimental (the lower oral dose of the drug being evaluated at −1 hr., followed by plantar injection of carrageenin at 0 hr.); and Group VI, high drug experimental (the higher dosage of the test drug followed by carrageenin injection).

[1] Obtained from E. G. Steinhilber Co., Oshkosh, Wis.

[2] Sources of the test agents were: aspirin, Merck and Co., Inc., Rahway, N. J.; indomethacin and cyproheptadine HCl, Merck Sharp & Dohme Research Institute, West Point, Pa.; phenylbutazone, Geigy Pharmaceuticals, Ardsley, N. Y.; chlorpromazine HCl, Smith Kline & French Labs., Philadelphia, Pa.; tetrabenazine, Hoffmann–La Roche, Inc., Rahway, N. J.; sparteine SO_4, K and K Labs., Inc., Plainview, N. Y.; and cryogenine, Dr. A. E. Schwarting, Division of Pharmacognosy, University of Connecticut, Storrs, Conn. The cryogenine (mol. wt. = 435.53) used in this study is an alkaloid isolated from *Heimia salicifolia* Link and Otto, and not the trade name product Cryogénine (phenylsemicarbazide, mol. wt. = 151.2), a specialty of Lumière of Lyons, France, and distributed by Laboratoires Sarbach of Châtillon, France.

Table I—Relative Anti-Inflammatory Potency against Carrageenin-Induced Acute Pedal Edema in the Rat

	Oral Dosage, mg./kg.	Mean Increase in Paw Volume, ml.	Inhibition, %	Relative Potency	Molar Potency
Control	—	1.32(0.95–1.63)[a]	—		
Aspirin	160.0	1.11(0.86–1.30)	16.0	1.0	1.0
Aspirin	400.0	0.83(0.49–1.26)	37.2		
Control	—	1.31(1.23–1.42)	—		
Cyproheptadine HCl	125.0	1.01(0.87–1.24)	23.2	1.33(0.71–2.51)[b]	2.41(1.28–4.53)[b]
Cyproheptadine HCl	312.5	0.88(0.54–1.19)	32.7		
Control	—	1.27(1.06–1.48)	—		
Indomethacin	5.0	1.00(0.62–1.18)	21.0	34.43(19.26–61.53)	67.95(38.02–121.45)
Indomethacin	12.5	0.83(0.67–0.99)	34.6		
Control	—	1.29(1.15–1.49)	—		
Phenylbutazone	50.0	1.14(0.91–1.33)	11.6	1.62(0.79–3.28)	2.76(1.36–5.59)
Phenylbutazone	125.0	1.02(0.83–1.20)	21.6		
Control	—	1.10(0.97–1.41)	—		
Chlorpromazine HCl	40.0	0.74(0.52–0.99)	32.7	7.90(3.35–18.64)	15.74(6.67–37.12)
Chlorpromazine HCl	100.0	0.53(0.10–0.92)	51.8		
Control	—	1.28(0.77–1.61)	—		
Cryogenine	100.0	1.04(0.73–1.35)	18.8	1.59(0.99–2.56)	3.93(2.44–6.34)
Cryogenine	250.0	0.86(0.61–1.40)	32.9		
Control	—	1.13(0.75–1.43)	—		
Tetrabenazine	15.0	0.87(0.73–1.11)	23.0	8.94(5.72–14.05)	17.64(11.24–27.71)
Tetrabenazine	37.5	0.72(0.53–0.81)	36.3		
Control	—	1.22(1.01–1.37)	—		
Sparteine SO_4	80.0	1.13(0.48–1.47)	7.4	0.81(0.45–1.45)	1.49(0.83–2.67)
Sparteine SO_4	200.0	0.99(0.92–1.75)	18.9		

[a] Figures in parentheses in this column represent the observed range of experimental values. [b] Figures in parentheses represent the 95% confidence limits of the potency.

Liver Sulfhydryl Determinations—The amperometric titration of hepatic sulfhydryl groups (expressed as milligrams percent glutathione), requiring a platinum rotating electrode in neutral buffered media, followed essentially the method of Benesch and Benesch (4). All rats were sacrificed by craniovertebral dislocation immediately after the +3-hr. paw volume measurements. Sacrifice was timed to occur between 1800 and 1900 hr. to avoid diurnal variation, unless otherwise specified. Portions (about 3 g.) of the central lobe of the liver were rapidly removed and frozen with a dry ice–acetone mixture in marked, capped, individual vials. All metal surfaces contacting the tissue were silicone coated. The livers were kept frozen with dry ice until the time for glutathione extraction, at which time 9.2 ml. of a 2.5% solution of sulfosalicylic acid was added to each gram (wet weight) of liver tissue and the combination was homogenized with a motor-driven glass homogenizer in an ice jacket. The homogenate was then centrifuged for 1.5 min. at maximum speed in a Safeguard centrifuge[3], and the supernatant was filtered through hardened filter paper (Whatman No. 50). Filtrates were collected in individual tubes, corked, and maintained in a frozen state until time of amperometric titration. This was done in a 150-ml. beaker containing the following: 4 ml. of 1.0 *M* tris-(hydroxymethyl)aminomethane (Fisher certified primary standard), 3.4 ml. of 1.0 *M* HNO_3, 0.3 ml. of 1.0 *M* KCl, 1.0 ml. of 9×10^{-4} *M* calcium disodium versenate (Riker), 1.0 ml. of liver filtrate, and water to make a total volume of 30 ml. (pH = 7.4).

All solutions concerned with the titration procedure were made with deionized, distilled water. Because of the instability of glutathione in alkaline solutions, this neutral, aqueous, and buffered solution was preferable to the alcoholic, ammoniacal system commonly used (5). The titrant (0.002 *M* $AgNO_3$) was made fresh daily from a 0.1 *M* stock solution. Titrant was delivered by means of a 2.0-ml. micrometer syringe (0.002 ml./division)[4]. The end-point was determined graphically by plotting microammeter deflections *versus* the volume of $AgNO_3$ added. The point of intersection of the two resulting straight lines was considered the end-point of the titration. The accuracy of the titration system was checked periodically throughout the day when known amounts of standard glutathione were recovered from identical aliquots of liver filtrates. The average recovery of standard glutathione over the range of 0.005–0.3 mg. was 98%. All data were analyzed according to the analysis of variance methods of Bliss (6).

[3] Clay-Adams, Inc.
[4] Roger Gilmont Instruments, Inc.

RESULTS AND DISCUSSION

That stress is a real phenomenon is accepted generally; however, the study of stress is difficult. The assessment of stress, aside from those noxious stimuli that cause actual cellular damage, often involves a certain degree of subjective and emotional interpretation which becomes increasingly complex and important as one ascends the evolutionary ladder. Within a species, the ability to withstand stress, like pain, varies. The ability of a noxious stimulus to act as a stressor also depends on both the intensity and duration of such a stimulus. Moreover, Hudak and Buckley (7) demonstrated a sex variation in that stress-induced hypertension in rats peaks after 22 weeks of exposure in the male and 28 weeks in the female. Usually, the more general the effect of a stressor on the body, the more efficient it is in eliciting the stress response.

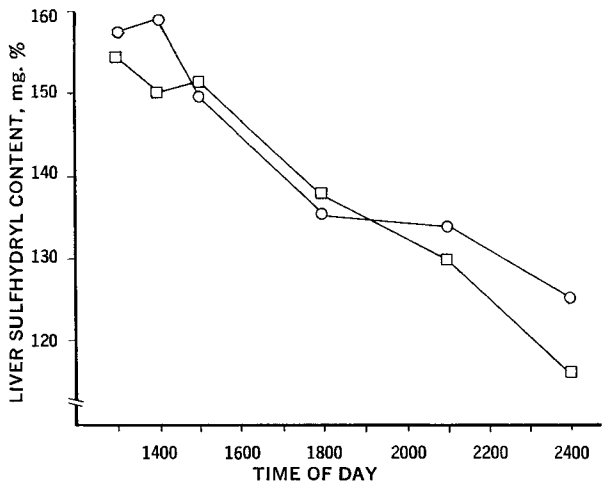

Figure 1—*Variation with time of male rat hepatic sulfhydryl concentration (calculated as glutathione) in the presence and absence of carrageenin-induced hind paw edema. Key:* O, *control; and* □, *carrageenin-treated. At each time period, levels in carrageenin-treated rats were not significantly different* ($p > 0.05$) *from the controls (7–10 animals/group). Corresponding levels at 1800 hr. and following were significantly lower* ($p < 0.05$) *than at 1300 hr. Pooled* SE *for controls = 6.3; pooled* SE *for carrageenin-treated = 6.4.*

Table II—Relative Drug Effects on Rat Liver Sulfhydryl Content at 1800 Hours

	Oral Dosage, mg./kg.	Mean Liver Glutathione[a] — No Carrageenin: mg. % (SE)	p	With Carrageenin: mg. % (SE)	p	Significance, p
Control	—	127.2(5.0)	—	125.9(4.6)	—	>0.20
Aspirin	160.0	144.7(4.7)	<0.01	137.1(5.6)	<0.01	>0.20
Aspirin	400.0	146.3(10.9)	<0.01	150.6(4.4)	<0.01	>0.20
Control	—	133.9(5.8)		127.8(2.6)	—	>0.20
Cyproheptadine HCl	125.0	109.4(4.3)	<0.01	126.6(4.2)	>0.20	<0.10
Cyproheptadine HCl	312.5	104.5(4.0)	<0.01	115.5(4.0)	<0.05	>0.05
Control	—	133.5(5.1)	—	141.0(4.6)	—	>0.20
Indomethacin	5.0	138.3(6.6)	>0.20	147.0(6.1)	>0.20	>0.10
Indomethacin	12.5	140.0(7.4)	>0.20	155.3(3.8)	>0.05	>0.05
Control	—	135.9(7.2)	—	125.7(7.4)	—	>0.20
Phenylbutazone	50.0	118.8(10.5)	>0.10	113.8(6.7)	>0.20	>0.20
Phenylbutazone	125.0	126.3(4.9)	>0.10	125.5(6.7)	>0.20	>0.20
Control	—	121.7(6.5)	—	121.1(7.9)	—	>0.20
Chlorpromazine HCl	40.0	131.5(5.5)	>0.20	118.4(3.0)	>0.05	<0.05
Chlorpromazine HCl	100.0	129.1(6.8)	>0.20	106.3(7.1)	>0.10	<0.05
Control	—	140.1(4.8)	—	128.4(4.0)	—	>0.05
Cryogenine	100.0	130.9(2.5)	>0.10	143.8(4.7)	<0.05	<0.05
Cryogenine	250.0	133.4(4.2)	>0.10	133.4(2.9)	>0.20	>0.20
Control	—	128.2(7.6)	—	129.6(9.7)	—	>0.20
Tetrabenazine	15.0	126.3(6.8)	>0.20	131.4(4.2)	>0.20	>0.05
Tetrabenazine	37.5	128.7(5.6)	>0.20	139.0(6.7)	>0.20	>0.20
Control	—	124.5(4.4)	—	137.6(5.8)	—	>0.05
Sparteine SO_4	80.0	124.5(6.2)	>0.20	133.5(6.7)	>0.20	>0.20
Sparteine SO_4	200.0	121.3(5.5)	>0.20	122.7(4.2)	<0.05	>0.20

[a] Liver glutathione is expressed as glutathione equivalents. Drugs were administered orally at −1 hr., carrageenin at 0 hr., and sacrifice with liver sampling just after the +3-hr. plethysmographic recordings.

Concomitant with physiological changes, stress-induced biochemical alterations such as increased lipolysis and glycogenolysis as well as adrenal ascorbic acid and cholesterol depletion take place. Bartlett and Register (8) showed that a reduction in liver sulfhydryl content occurs in response to cold or restraint. This has been shown to happen in various other tissues such as brain, kidney, and blood and in response to other types of stress such as heat, pain, hemorrhage, and various drugs (9–11). While the mechanism of stress-induced glutathione depletion is not well understood, it generally is thought to be the result either of activation of the pituitary–adrenocortical axis with the release of corticosteroids (during which process glutathione is rapidly oxidized and finally hydrolyzed to glycine and glutamylcysteine) or of stimulation of the sympathoadrenal system with the release of epinephrine and subsequent glycogenolysis (the reduction is thought to be the result of glutathione acting as a tightly bound prosthetic group of glyceraldehyde-3-phosphate dehydrogenase) (12).

Marozzi and Malone (1) indicated that a possible correlation exists between a compound's ability to protect against stress-induced (hind leg tourniquets) hepatic glutathione depletion and its potential in inhibiting carrageenin-induced acute pedal inflammation. However, in the present study, carrageenin-induced pedal edema in itself did not deplete liver sulfhydryl content (Fig. 1), possibly because of the localization of the swelling and the relative mildness of the phlogiston. While the lack of effect on liver sulfhydryl content was somewhat unexpected, it was still felt that measurement of this parameter might be useful in defining and separating antistress and anti-inflammatory activity during drug screening.

All of the oral anti-inflammatory potencies in Table I were calculated in terms of aspirin since it is still the most widely used nonsteroidal anti-inflammatory agent. Because the compounds used in this study differ widely in their molecular mass as well as in their structures, relative anti-inflammatory potency based on moles per kilogram was also calculated for each. The λ value (s/b) is generally regarded as the best single mathematical characteristic for a specific assay procedure. The mean λ value for the seven assays in Table I was 0.32 (SE = 0.018).

In support of the results depicted in Fig. 1, the glutathione levels of control animals receiving carrageenin (Table II) were never significantly different from the levels detected in those controls without the carrageenin challenge.

Histamine, serotonin, and bradykinin have been suggested as possible mediators of the inflammatory response. Cyproheptadine, a potent antagonist of these agents, has been shown to be active in several experimental models of inflammation but inactive in the carrageenin-induced acute rat hind paw edema at 3 mg./kg. orally (13) and at 0.5 mg./kg. s.c. (3). During the preliminary work of the present study, cyproheptadine was found to be inactive against such edema at 20 and 50 mg./kg. orally. The antiphlogistic activity observed in Table I with high dosages (125 and 312.5 mg./kg. orally) is probably the result of a secondary nonspecific effect. It is well known that diverse pharmacologic agents can inhibit this inflammatory response if sufficiently high dosages are employed. This toxic effect concept is supported by the significantly lowered hepatic glutathione levels reported in Table II.

Aspirin was unique among the drugs studied, since it caused a significant elevation of hepatic glutathione content both in the presence and absence of carrageenin-induced pedal edema. Like aspirin, indomethacin and phenylbutazone possess analgesic and antipyretic activity and are capable of uncoupling oxidative phosphorylation (14). More recently, Gerber *et al.* (15) showed that these agents are also capable of accelerating a disulfide interchange reaction between human serum sulfhydryl groups and 5,5′-dithiobis(2-nitrobenzoic acid). The relative ability of these agents to facilitate this reaction is of the same order as their anti-inflammatory potency determined by other laboratory tests. The exact mechanism by which indomethacin and phenylbutazone exert their anti-inflammatory activity is not known, although it is generally thought to be independent of pituitary involvement. Unlike aspirin, both of these clinically useful compounds had no detectable effect on rat liver glutathione content at effective oral anti-inflammatory dosages.

The Mexican plant, *Heimia salicifolia*, has been reputed to possess sudorific, antipyretic, diuretic, hemostatic, and vulnerary activities (16). One of its alkaloids, cryogenine, has been shown to possess hypothermic and selective CNS depressant activity (17), as well as anti-inflammatory activity against both acute carrageenin-induced pedal edema and chronic adjuvant-induced edema (2). No deaths were reported in rats treated with cryogenine orally at 100 mg./kg./day for 13 days; histopathologic examinations showed no lesions of the myocardium, liver, lung, kidney, or spleen (2). In a comprehensive recent study (18), chronic dosage of 100 mg./kg./day orally in rats for 21 days did not produce any significant histopathologic lesions. Since no alterations were noted in regard to thymus, adrenals, liver, and blood, these authors felt that cryogenine's anti-inflammatory activity was probably not mediated by the endogenous release of corticosteroids. Jiu (19) independently reported that extracts of *Heimia salicifolia* were effective in two additional models of inflammation (yeast-induced foot edema and cotton pellet granuloma). Trottier and Malone (20) showed that cryogenine can antagonize the effects of furtrethonium, histamine, and serotonin on

various isolated tissues, although with less potency and specificity than diphenhydramine and cyproheptadine. In the isolated guinea pig uterus, cryogenine evidenced some specificity for the histaminic receptor (20).

The present study confirms the previous findings on the anti-inflammatory efficacy of orally administered cryogenine and reports that cryogenine by itself was without effect on liver glutathione. While there was an apparent elevation of glutathione levels in animals receiving 100 mg./kg. of cryogenine plus carrageenin, no such effect was seen at the 250-mg./kg. dosage level, and the agar control (without carrageenin) had a statistically comparable high value. Therefore, the "elevation" can be dismissed as an artifact arising out of the experimental design. In a similar fashion, the statistically significant lowering of glutathione by 200 mg./kg. of sparteine in the presence of carrageenin also seems to be without real significance.

Tetrabenazine was consistently without effect on liver glutathione. Although tetrabenazine has been used as an edemogen in the rat (inducing local inflammation with a marked depletion of local histamine) (21), the present study is the first where tetrabenazine has been shown to possess oral antiphlogistic activity. Its potency was second to that of indomethacin in the series of agents studied.

Chlorpromazine, although not classified therapeutically as an anti-inflammatory agent, has been shown to be effective in suppressing yeast-, serotonin-, and formalin-induced edema and 45° thermic- and granuloma-edema, in addition to carrageenin-induced edema. The mechanism of chlorpromazine's inhibitory activity is not well understood. Results obtained in this study indicate that its activity appears not to be related to its antihistamine, antiserotonin, or antibradykinin action, since cyproheptadine is a more potent antagonist of these suspected mediators of inflammation and this agent showed a relatively low order of inhibitory activity. Although chlorpromazine is known to possess peripheral adrenergic blocking activity, the effects seen with tetrabenazine (negligible peripheral effect) suggest that such activity cannot account for the potent anti-inflammatory effect seen with chlorpromazine.

The present findings of antiphlogistic activity without significant changes in liver glutathione content would be consistent, therefore, with those of Brown *et al.* (22) which support the CNS as a possible site of action for some clinically useful anti-inflammatories. Bhattacharya and Marks (23) recently noted that a single large dose of 25 mg./kg. i.p. of chlorpromazine would cause a prompt rise in plasma corticosteroid levels 30 min. after drug treatment and that it would last for 48 hr. after injection. Hypothermia seemed not to be involved in this effect, since no significant differences were found between animals kept at room temperature and those maintained at an ambient temperature of 31°. Unlike those of tetrabenazine and cryogenine, the chlorpromazine dosages employed in the present study caused marked CNS depression. Such depression would be an important limiting factor for its use in a clinical situation. Therefore, the results of this study suggest that chemical modification of tetrabenazine (and possibly, cryogenine) could result in a new class of potent, nonsteroidal, anti-inflammatory drugs.

Within the limitations of the experimental design of this study (all glutathione determinations made at equivalently effective anti-inflammatory dosages), there appeared to be no correlation between anti-inflammatory capacity and effects on liver sulfhydryl content.

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