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Research Articles

Anti-Inflammatory Evaluation of Cryogenine

By HARVEY R. KAPLAN*, RICHARD E. WOLKE†, and MARVIN H. MALONE

Cryogenine was effective in limiting the development of artificially induced inflammatory responses in acute and chronic experiments in rats. Cryogenine altered the acute edematous reaction to plantar injection of carrageenin and inhibited development of increased foot thickness induced by plantar injection of nonviable mycobacterial adjuvant in chronic studies. Histopathologic examinations did not suggest actions that would interfere with the anti-inflammatory evaluations. Cryogenine demonstrated a low order of analgesic (rat tail flick) and antipyretic (peptone fever) activity, and was capable of partially reversing edema and pain produced by silver nitrate injections in the ankle joints of rats. Cryogenine blocked serotonin, bradykinin, and histamine responses in guinea pig ileum; and pretreatment limited serotonin-induced pedal edema in rats and blocked histamine-induced intradermal wheals in the rabbit. Cryogenine was ineffective as a fibrinolytic agent. Prototype nonsteroidal anti-inflammatory compounds as well as agents with structural similarities to cryogenine were also investigated. Cryogenine may, like aspirin, manifest its anti-inflammatory activity through a combination of selective central and nonspecific peripheral mechanisms.

CRYOGENINE (vertine) was first isolated from *Heimia salicifolia* Link and Otto in 1963 by Blomster *et al.* (1), and its chemical structure has been proposed (2) and confirmed (3). The folklore concerning this Mexican plant is interesting and includes mention of its abilities to induce bizarre central nervous system reactions (4–6). The initial pharmacologic studies of Robichaud *et al.* (7) on both plant extracts and purified cryogenine alkaloid revealed qualitative and quantitative similarities between the two with the most interesting activity being the production of a selective central nervous system depression in unanesthetized animals. The general pharmacodynamics of cryogenine and other of the naturally occurring alkaloids have been reported in the literature (8, 9). Preliminary evaluation in this laboratory also indicated that cryogenine possessed activity in an acute anti-inflammatory screen in rats. These initial data suggested that cryogenine could be a new chemical prototype with nonsteroidal anti-inflammatory activity. Most recently Jiu has reported (10) that ethanol extractives from the plant showed depressant central nervous system activity, anti-atherogenic potential, and anti-inflammatory ability.

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Cryogenine base was supplied by the Division of Pharmacognosy, University of Connecticut, while carrageenin (Sea-Kem type I) was courtesy of Seaplant Chemical Corp., New Bedford, Mass., and synthetic bradykinin (BRS-640) from Sandoz Pharmaceuticals, Hanover, N. J. The pharmacological and histopathological studies were partially supported by grants AM-11861 and TIGM 985-05, respectively, from the U. S. Public Health Service, Bethesda, Md.
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EXPERIMENTAL

General—During the investigations cryogenine was employed either as an aqueous suspension of

free base in 0.25% agar or as a solution of the acetate salt prepared by dissolving the base in 0.5% v/v of glacial acetic acid. As is often the case with the pharmacologic evaluation of natural products, the relatively limited availability of test alkaloid restricted extensive replication of the experimental procedures. The young adult albino rats used in this study were of the Wistar strain and obtained from the E. G. Steinhilber Co., Oshkosh, Wis. Unless otherwise specified here, the animals were allowed continuous access to Purina laboratory chow and tap water.

Acute Anti-Inflammatory Evaluation Using Carrageenin-Induced Edema—Nonfasted male and female rats were employed and the experimental procedures were similar to those described by Winter *et al.* (11). Test drugs were administered orally (10 ml./Kg.) in order to minimize counter-irritation phenomena often seen after parenteral injection. To insure a uniform degree of hydration among the individual rats, each animal received a supplementary amount of water so that the total volume administered to each rat was 5.0 ml. One hour following the oral administration, 0.1 ml. of the phlogistic agent (1% suspension of carrageenin in sterile 0.9% saline) was injected aseptically into the plantar aponeurosis of the left hind paw using a 27 gauge 0.5 in. needle attached to a 0.25 ml. capacity syringe. The width or volume of the injected paws was determined immediately after the carrageenin injection and 3 hr. later. For width determinations, a vernier caliper was used with medial foot thickness measured to the nearest 0.1 mm. When volume measurements were obtained, a modified plethysmographic apparatus similar to that described by Harris and Spencer (12) was employed by which foot volume was determined to the nearest 0.01 ml. Rectal temperatures were recorded throughout the test period using a precalibrated thermistor-type thermometer.

Assay of Cryogenine Versus Phenylbutazone—Plethysmographic measurements were made following the administration of 0.05 ml. of 1% carrageenin. A randomized complete block experimental design was utilized with a low (50 mg./Kg.) and a high (100 mg./Kg.) level for both cryogenine acetate and sodium phenylbutazone. Each treatment consisted of three experimental units per day with the assay conducted on 3 separate days resulting in a final number of 9 units for each of the four treatments. On any one day, the rats were equally but randomly assigned among the four test treatments. The statistical evaluation was patterned after the methods of Bliss and Calhoun (13).

Multiple Drug Comparisons—The plethysmographic technique was employed with 0.05 ml. of 1% carrageenin injected into the paw as the phlogistic substance. Young unfasted adult rats (2 male and 2 female) were assigned to the treatment groups in a random fashion. Drugs were administered orally as suspensions in 0.25% agar. The treatment effects were analyzed employing the multiple range test of Duncan (14).

Chronic Anti-Inflammatory Evaluation—Nonfasted male rats were randomly caged in groups of 5 and allowed to equilibrate with their environment for 1 week prior to experimentation. Daily determinations of body weight, medial foot thickness (caliper method), and average food and water consumption were made. Commencing on day 0 animals were dosed daily for 2 weeks (day 0 through 13) with the test agents. These drugs were administered orally as suspensions in 0.25% agar (10 ml./Kg.). On day 1 all animals received an injection of a suspension of *Mycobacterium butyricum* (Difco Laboratories 0640-33) in light liquid petrolatum prepared according to the method of Nuss (15). Twenty milliliters of the petrolatum was added to 100 mg. of the desiccated organisms in a Kontes hand homogenizer. After fine subdivision the resultant suspension was autoclaved at 15 lb. pressure for 20 min., allowed to cool, and placed into a refrigerator overnight. Prior to injection the suspension was allowed to equilibrate to room temperature and resuspended by shaking by hand. Each rat was injected with 0.05 ml. into the right plantar aponeurosis using a 27 gauge 0.5 in. needle directed medially following its insertion under the skin in order to avoid back seepage and to minimize local vascular involvement. Medication was discontinued on days 14 through 30; however, body weight, foot thickness, and food and water consumption measurements were continued throughout the duration of the experiment. The percentage inhibition of increased foot thickness for the injected foot was calculated according to the methods of Newbould (16) on days 13 and 30. Gross skeletal specimens of the "arthritic" paws were prepared on day 40 using the antiformin method (17).

Tissue taken for histopathologic examination included sagittal sections of both femorotibial articulations and transverse sections of metatarsal areas, myocardium, liver, spleen, lung, kidney, and abdominal skin. Following euthanasia, the tissue was fixed in 10% formalin. Osseous sections were decalcified by means of a thermo-agitating bone decalcifier employing an 18% solution of formic and hydrochloric acids. After decalcification, sections were placed into a saturated solution of lithium carbonate for a period of 24 hr., rinsed, and replaced in formalin. Tissue blocks were embedded in paraffin, sectioned at 10 μ, and stained with hematoxylin and eosin.

Antipyretic Evaluation Using Peptone-Induced Fever—Male rats were acclimatized overnight in a temperature controlled room (23°). Control rectal temperatures of the animals were recorded using a thermistor-type thermometer with the flexible probe inserted to a depth of 5 cm. and held in position for 35 sec. The rats were rendered febrile by the injection of 0.6 ml. of a 5% aqueous solution of peptone tryptic digest of casein (N-Z amine type E tryptic digest, Sheffield Chemical Co. 02-537) which had been previously filtered clear and incubated in a Dubnof shaker at 37.5° for 12 hr. The site of the subcutaneous injection was in the dorsal region of the rat's neck. Four hours after injection of the pyrogen, core temperatures were recorded again. Any animal showing a temperature elevation of less than 1° was not considered adequately febile and was discarded from the test. At this time 5 rats were assigned randomly to each of the treatment groups and the drugs administered orally. At +1 and 2 hr. following drug administration, the rectal temperatures were determined. During the experiment food was withheld, although water was freely accessible.

Analgesic Evaluation—A modified conduction

dolorimeter (Metro Scientific, Inc. ME-5410) was utilized for measuring the rat tail flick response to a standard thermal stimulus. Only male rats possessing healthy nonkeratinized tails were employed. In order to record accurately the reaction times to the thermal stimuli, a photocell and an electric timer were adapted to the instrument so that as the rat's tail was placed over the photocell opening, a circuit was closed activating both the heater and the electric timer. Subsequent removal of the tail opened the circuit and stopped the timer. Control reaction times for each animal were determined just prior to drug administration and at +15, 30, 45, 60, and 120 min. after injection. The per cent analgesia was calculated by considering a reaction time of 20 sec. minus the control reaction time as the equivalent of 100% analgesia. Exposure to the thermal stimulus for longer than 20 sec. is generally regarded as undesirable since irreversible damage to the sensory receptors in the tail may occur.

Simultaneous Analgesic and Anti-Inflammatory Testing—The method employed was a modification of the technique initially developed by LaBelle and Tislow (18). Freshly prepared aqueous 1% silver nitrate was injected (0.2 ml.) into the right paw between the Achilles tendon, ankle, and heel bones *via* the plantar surface of the paw using a 27 gauge needle. This technique allows only insignificant amounts of silver nitrate to penetrate the foot areas subsequently measured for edema. It has been suggested that a local release of endogenous mediators acting in a humoral manner are responsible for the inflammation (19). Eighteen hours after injection of silver nitrate, the animals' reaction to pain in the injected paw was evaluated. This required the abrupt manual flexion of the ankle joint with scoring of the vocalization (squeal) and struggling using an arbitrary scale of 0 to +3 (0 = no squealing and struggling, +1 = audible squeals but no struggling, +2 = definite squealing and some struggling, +3 = loud squeals and violent struggling). Any animal either failing to produce a pain response of +2 or 3 or not showing a measurable edema (caliper technique) was eliminated from the experiment. Rats meeting criteria were subdivided randomly into the treatment groups and the drugs then administered orally as suspensions. Animals were scored for pain responses at hourly intervals for 5 hr. and at the end of this time foot widths were determined again. At the time of the silver nitrate injection, the animals were taken off food and at the time of the drug administration taken off water for the balance of the experiment.

Antiphlogistic Evaluation Using a Histamine-Induced Intradermal Wheal—Unanesthetized albino rabbits of either sex obtained from local suppliers were securely fastened to rabbit boards in a supine position. Abdominal hair was carefully removed 2 hr. prior to the experiment using electric clippers. Each shaved abdomen was then marked with 8–10 circular regions (approximately 30 mm. in diameter) using a felt marking pen. The test drugs were injected intraperitoneally 30 min. prior to intradermal injection of 0.3 ml. of 0.02 mg./ml. (as base) of histamine diphosphate into the center of each of the marked regions. Fifteen minutes after the intradermal injections, trypan blue was administered intravenously (1 ml./Kg. of a 1% aqueous solution) and the colors of the histamine-induced wheals rated at +30, 60, and 180 min. later using the scoring scale of Hoppe *et al.* (20).

Serotonin-, Bradykinin-, and Histamine-Induced Pedal Edema—Serotonin creatinine sulfate (0.01 mg. as the base), synthetic bradykinin (0.05 mg.), or histamine diphosphate (0.15 mg. as base) contained in an injection volume of 0.05 ml. were administered into the plantar aponeurosis of rats. Inflammation was measured (caliper technique) at 30-min. intervals for 180 min. following injection of the phlogiston. Cryogenine base was administered orally as a suspension 1 hr. prior to the pedal injections.

Antagonism Studies on Isolated Guinea Pig Ileum—Nonfasted virgin female guinea pigs obtained from local suppliers were sacrificed by cranio-vertebral dislocation and 2–3 cm. segments of ileum immediately excised and placed into calibrated 50 ml. baths containing fresh Tyrode's solution (37.5°) constantly oxygenated with a 95% O_2 and 5% CO_2 gas mixture. Isotonic frontal recording levers were used to record longitudinal contractions of the tissues. Bath concentrations of 1×10^{-6} *M* for serotonin (creatinine sulfate), histamine (diphosphate), and synthetic bradykinin were used to induce reproducible submaximal contractions of the tissue. After thoroughly washing out the agonists, various log molar concentrations of cryogenine (acetate) were incubated (1×10^{-9} to 1×10^{-7} *M*) with the ileum for 5 min. prior to addition of the agonists in order to ascertain its effects on the reference contractions. A fresh individual ileum segment was used to study the effects of cryogenine in regard to each agonist.

Fibrinolytic Evaluation—The method of von Kaulla (21) was employed with the reference clots formed from human plasma by the addition of 0.25 ml. of 5% calcium chloride to each 5.0 ml. of plasma used. The preformed clots were suspended in various molar concentrations of the drugs studied and then incubated at 37° for 24 hr. Evaluation of fibrinolytic activity was made at the end of this time and expressed as per cent lysis of clot. Any cloudiness of the solution, shrinking of the clot, or sediment formation was also noted.

RESULTS

Acute Anti-Inflammatory Evaluation Using Carrageenin-Induced Edema—The results as summarized in Table I show cryogenine to possess significant antiphlogistic activity at an oral dosage of 100 mg./Kg. when compared to the acetic acid vehicle

TABLE I—ANTI-INFLAMMATORY ACTIVITY OF 100 mg./Kg. OF CRYOGENINE ORALLY IN RATS WITH ACUTE CARRAGEENIN-INDUCED PEDAL EDEMA

Treatment	Mean % Increase Paw Width	Paw Vol.	Level of Significance Observed *P*
Cryogenine	13.4 (2–24)[a]	...	0.01–0.05
Acetic acid control	26.8 (18–36)[b]	...	...
Cryogenine	...	27 (2–61)[b]	<0.001
Acetic acid control	...	118 (51–197)[b]	...

[a] Observed range of 5 observations 3 hr. after carrageenin injection. [b] Observed range of 6 observations 3 hr. after carrageenin injection.

controls. The inflammatory state induced by carrageenin did not elevate core temperature and treatment with either cryogenine alkaloid or the vehicle control did not cause significant changes in rectal temperature.

Assay of Cryogenine Versus Phenylbutazone—Calculation of the 2 × 2 assay revealed that cryogenine has 0.86 times the anti-inflammatory potency of phenylbutazone in this acute test procedure. The 95% confidence limits for the potency are 0.61–1.11. The λ of the assay (calculated as either s/b or as $s_M\sqrt{N/2}$) equaled 0.22. Cryogenine and phenylbutazone may be considered equipotent orally since there was no significant difference between drugs (P = 0.25–0.50) and no significant departure from parallelism (P = >0.50).

Multiple Drug Comparisons—The results of this one level experiment as treated by Duncan's multiple range test (14) are summarized in Table II

TABLE II—MULTIPLE DRUG COMPARISIONS OF ANTI-INFLAMMATORY ACTIVITY IN RATS WITH ACUTE CARRAGEENIN-INDUCED PEDAL EDEMA

Treatment	Oral Dosage, mg./Kg.	Mean Increase in Paw Vol., ml.	Significant Subsets[a]
Control	...	0.45(0.37–0.56)[b]	A
Cinnamic acid	100	0.44(0.32–0.66)	A
Control	...	0.44(0.39–0.54)	A
Papain	400	0.35(0.29–0.40)	B
Sparteine	100	0.34(0.30–0.38)	B
Cryogenine	100	0.27(0.19–0.36)	B
Indomethacin	5	0.22(0.18–0.31)	B
Chloropromazine	100	0.21(0.05–0.33)	B

[a] The treatment effects represented by A and B differ significantly from each other (P = 0.05); however, the treatments within these subsets do not differ significantly (P = 0.05) from one another. [b] The figures within the parentheses represent the observed range of the 4 experimental values.

and show a division of the various treatment effects into two major subsets of means. The anti-inflammatory properties of cryogenine were confirmed. Although both cinnamic acid and sparteine have some structural chemical relationships to cryogenine, only sparteine could be considered as having some potential for anti-inflammatory activity. It should be noted here that in the two previous experiments cryogenine was administered as the soluble acetate salt, whereas in this experiment the base itself was administered orally as a suspension.

Chronic Anti-Inflammatory Evaluation—The effects of cryogenine and phenylbutazone therapy on the progression of the artificially-induced, chronic "arthritic" state in rats is presented graphically in Fig. 1 and 2. Table III also summarizes the results for several other selected reference compounds tested concurrently by this procedure. These compounds can categorically be classified as either well documented reference anti-inflammatory agents (phenylbutazone, indomethacin, DMSO, chloroquine, hydrocortisone), structurally related moieties of cryogenine (cytisine, sparteine, cinnamic acid), or pharmacologically related agents (chlorpromazine, papaverine, atropine) (7–9). The dosages employed are either those suggested in the literature or previously determined in these laboratories to be effective and apparently nontoxic for the rat after acute administration. The protection afforded by cryogenine and by phenylbutazone when compared with a nontreated control is very apparent in Fig. 3 which pictures representative gross specimens 40 days following the *M. butyricum* adjuvant administration. Secondary lesions in other tissues similar to those described in the literature by Newbould (16) were observed; however, the variability of lesions as to frequency, size, and site did not allow them to be used as a test metameter.

Two of the three control animals selected for histopathologic evaluation had no lesions of the

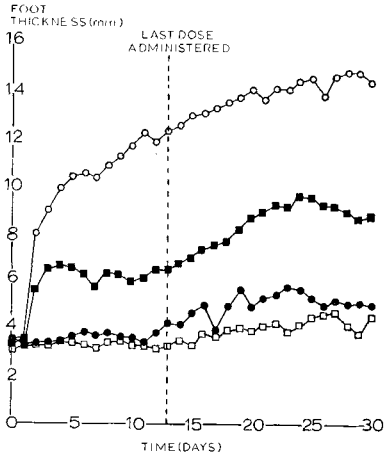

Fig. 1—Effect of 2 weeks of daily administration of 100 mg./Kg. of cryogenine orally on the development of adjuvant-induced polyarthritis. Key: ○, mean width of injected foot (controls); ●, mean width of contralateral foot (controls); ■, mean width of injected foot (cryogenine);. □, mean width of contralateral foot (cryogenine).

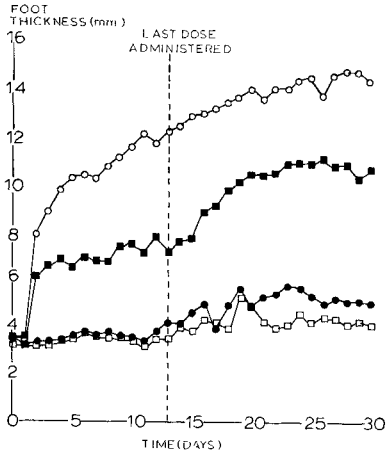

Fig. 2—Effect of 2 weeks of daily administration of 100 mg./Kg. of phenylbutazone orally on the development of adjuvant-induced polyarthritis. Key: ○, mean width of injected foot (controls); ●, mean width of contralateral foot (controls); ■, mean width of injected foot (phenylbutazone); □, mean width of contralateral foot (phenylbutazone).

TABLE III—ANTI-INFLAMMATORY EVALUATION UTILIZING ADJUVANT-INDUCED CHRONIC POLYARTHRITIS IN RATS

Treatment	Daily Oral Dosage, mg./Kg.[a]	Quantal Survival Day 13	Quantal Survival Day 30	Inhibition of Foot Thickness[b] Day 13	Inhibition of Foot Thickness[b] Day 30	Mean Body Wt., Gm. Day 0	Mean Change in Body Wt., Gm. Day 13	Mean Change in Body Wt., Gm. Day 30	Mean Consumption: Food, Gm./Water, ml. Day 0	Mean Consumption: Food, Gm./Water, ml. Day 13	Mean Consumption: Food, Gm./Water, ml. Day 30
Cryogenine	100	5/5	5/5	63	55	166	+26	+71	14/24	17/27	16/30
Phenylbutazone	100	5/5	5/5	58	41	201	+32	+65	18/33	20/33	17/32
Indomethacin	0.5	5/5	5/5	50	30	190	+32	+74	15/23	20/24	18/35
Dimethylsulfoxide	...[c]	5/5	5/5	20	28	236	+22	+50	18/27	20/29	21/34
Chloroquine	100	4/5	3/5	76	35	246	−70	−6	17/26	5/12	36/20
Hydrocortisone	100	5/5	5/5	66	43	249	−20	+18	18/25	16/23	18/29
Aspirin	100	5/5	5/5	33	29	216	+15	+46	20/31	15/24	16/31
Sparteine	100	5/5	4/5	16	4	242	+24	+56	23/35	17/26	16/26
Cytisine	25	5/5	5/5	42	1	213	+13	+70	22/31	20/27	17/38
Cinnamic acid	100	5/5	5/5	8	2	209	+35	+83	20/32	20/29	16/39
Papaverine	100	5/5	5/5	−6	10	166	+37	+91	...[d]	...	...
Atropine	100	4/5	4/5	41	4	162	+35	+85	...[d]	...	...
Chlorpromazine	25	4/5	4/5	15	0	150	+26	+71	...[d]	...	...
Controls	...	30/30	28/30	(10)[e]	(8)[e]	191	+27	+81	19/29	16/22	17/28

[a] Administered as suspensions in 0.25% agar from day 0 through day 13, with the mycobacterium adjuvant injected into the right foot on day 1. [b] Percentage inhibition = $100[1 - (a - x)/(b - y)]$ where y = mean thickness right feet of controls before adjuvant, b = mean thickness right feet of controls on specified day, x = mean thickness right feet of treated rats before adjuvant, and a = mean thickness right feet of treated rats on specified day. [c] The adjuvant-injected paw was soaked for 1 min. daily. [d] Values not determined quantitatively. [e] Absolute differences between the larger and smaller values.

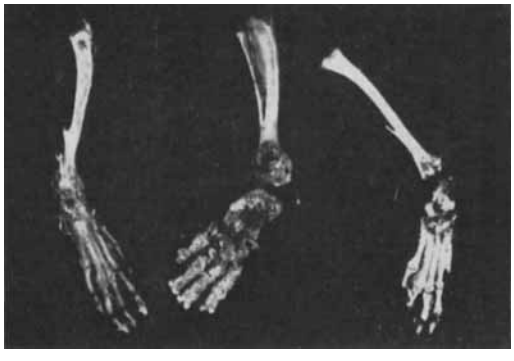

Fig. 3—Typical gross skeletal specimens of adjuvant-injected rat paws. Cryogenine-treated (left), non-treated controls (center), and phenylbutazone-treated (right).

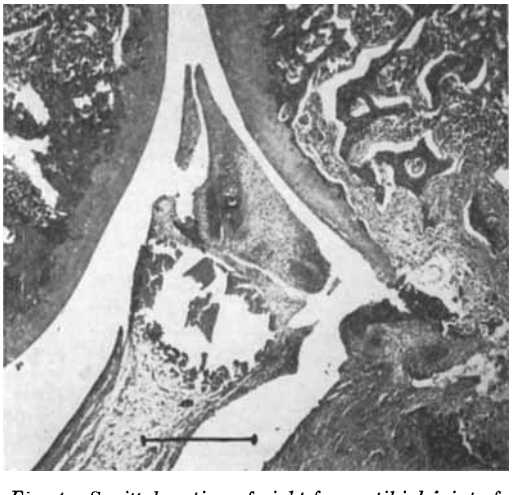

Fig. 4—Sagittal section of right femorotibial joint of a control animal with granulomatous reaction and destruction of the tibial cartilage seen at right. Marked distance = 0.5 mm.

femorotibial articulations, while all three revealed changes in the metatarsal areas. Lesions present in the most severely affected control animal were similar to those described by Glenn and Grey (22) with suppurative granulomas and fibrosis in zones of primary and secondary ossification bordering the femorotibial joint. Destruction of articular cartilage, an occasional finding, appeared secondary to the progressing subchondral granulomatous response. Within the joint proper, the synovial membrane was swollen, edematous, hyperplastic, and invaded by lymphocytes, mast cells, and mononuclear cells (Fig. 4). Progressive involvement resulted in fibrosis of the synovial membrane with an occasional pannus-like proliferation completely filling the joint cavity between the articular surfaces. Transverse sections of metatarsal areas had multiple subdermal granulomas and foci of mononuclear cells. A striking periosteal new bone proliferation was present about the periphery of all metatarsal bones. New bone was highly cellular, coarsely woven, and easily differentiated from mature cortical lamellar bone from which it was separated by a distinct basophilic cement line (Fig. 5). A decreased inflammatory response was observed in femorotibial sections of the cryogenine-treated rats (Fig. 6). Suppurative granulomatous reaction was seen in one instance in the tibial metaphysis and, with the exception of edema of the synovial membrane, pathologic changes were not present within the joint cavity. Metatarsal sections from cryogenine-treated animals revealed lesions as severe as those seen in the control rats (Fig. 7).

One rat received 100 mg./Kg. of cryogenine orally daily from day 0 through day 13 but did not receive an injection of the mycobacterium adjuvant. Examination of the soft tissues revealed no lesions of the myocardium, liver, lung, kidney, spleen, or abdominal skin.

The possibility of spurious anti-inflammatory activity can be detected by the adjuvant-induced polyarthritis procedure when chronic toxic mani-

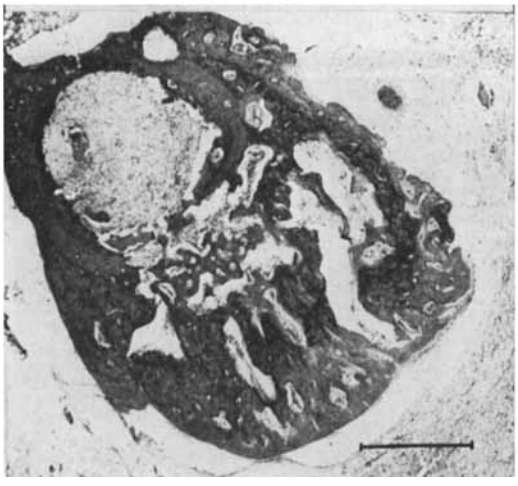

Fig. 5—Transverse section of right metatarsus of a control animal with extensive periosteal new bone proliferation. Marked distance = 0.5 mm.

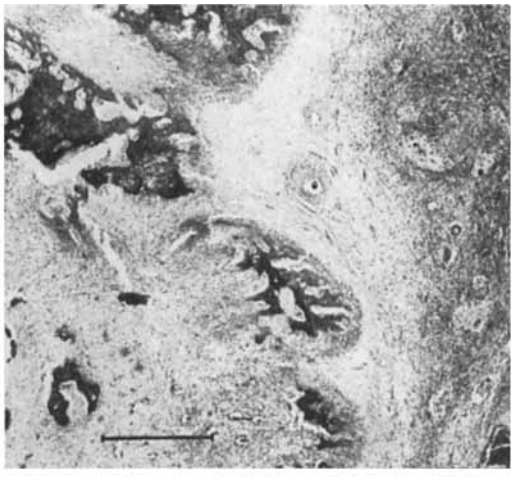

Fig. 7—Transverse section of right metatarsus of a cryogenine-treated rat with multiple granulomas and severe destruction of osseous tissue with periosteal new bone proliferation. Marked distance = 0.5 mm.

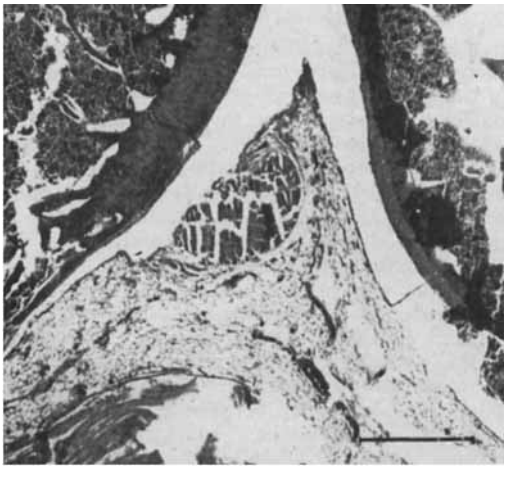

Fig. 6—Right femorotibial joint of a cryogenine-treated rat with absence of both articular cartilage destruction and inflammation of synovial membrane. Marked distance = 0.5 mm.

festations are noted. In Table III this is illustrated by decreased quantal survival rates (chloroquine, atropine, chlorpromazine), changes in the total body weight (chloroquine, hydrocortisone), reduced food and water consumption (chloroquine), and gross changes in general appearance and reactivity (chloroquine, atropine, chlorpromazine). Sparteine and cinnamic acid did not show any cryogenine-like activity. Cytisine may have some potential, although this activity appears to be short-lived and correlated with a decreased rate of body weight gain. In this experiment cryogenine, phenylbutazone, and indomethacin all appear as desirable anti-inflammatory agents, with indomethacin being considerably more potent than the other two.

Antipyretic Evaluation Using Peptone-Induced Fever—Figure 8 represents the results of the antipyretic evaluation. At +4 hr. and just prior to drug administration there was no significant difference between the respective means and variances of the three randomly assembled, experimental groups ($P > 0.50$). At +5 hr. there was no significant difference between the controls and the cryogenine-treated rats ($P = 0.25$–0.50), while there was a highly significant difference between the controls and aspirin-treated ($P = <0.001$) and the cryogenine-treated and aspirin-treated groups ($P = <0.001$). Subsequently, at +6 hr. a highly significant difference was still present between the untreated controls and the aspirin treated ($P = <0.001$) and the cryogenine-treated and aspirin-treated ($P = <0.001$). Moreover, there was a significant difference between the controls and the cryogenine-medicated animals ($P = 0.01$–0.025). Aspirin was most effective in that an oral dosage of 300 mg./Kg. almost totally abolished the pyretic response (69 and 91% reduction at 1 and 2 hr. after dosage, respectively). Cryogenine base at an oral dosage of 100 mg./Kg. was much less effective, although a 41% reduction of the peptone-induced fever was observed 2 hr. after administration.

Analgesic Evaluation—Table IV presents a summary of the analgesic evaluation using the rat tail flick technique which is considered generally to be most valid in the detection of narcotic analgesics. Cryogenine had an apparent low order of activity using this procedure; however, this may be

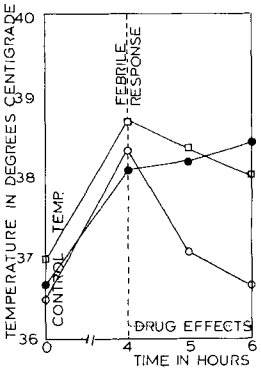

Fig. 8—Effects of orally administered cryogenine and aspirin on peptone-induced fever in the rat. Key: □, 100 mg./Kg. cryogenine; ○, 300 mg./Kg. aspirin; ●, untreated controls.

TABLE IV—ANALGESIC SCREENING OF CRYOGENINE AND REFERENCE AGENTS

Treatment	Dosage, mg./Kg.[a]	Mean % Analgesia[b] +15	+30	+45	+60	+120 min.
Control	...[c]	−2	−2	−6	−4	+2
Cryogenine	100	−10 (0)[d]	+17 (+)	+1 (±)	+3 (+)	−2 (±)
Phenylbutazone	100	+1 (±)	−2 (±)	−2 (±)	−2 (±)	−1 (±)
Morphine	10	+68 (+)	+86 (+)	+88 (+)	+80 (+)	+87 (+)

[a] Cryogenine was administered orally, continuing the mode of administration established in the earlier experiments where it was felt necessary to minimize possible counter-irritating influences of parenteral administration on the anti-inflammatory testing. However, all of the other agents and the control group were injected intraperitoneally as is usual with analgesic testing. Five rats per test group. [b] Per cent analgesia = 100 $[(R_x - R_0)/(20 - R_0)]$ where R_0 = pretreatment reaction time in seconds and R_x = post-treatment reaction time in seconds at the time specified. [c] The mean reaction times in seconds (observed range) for the 25 control animals are—preinjection: 4.58 (2.7–10.2); +15 min.: 4.46 (2.9–9.0); +30 min.; 4.41 (2.5–7.6); +45 min.: 3.94 (2.7–6.5); +60 min.: 4.22 (2.4–5.7); and +120 min.: 3.81 (2.7–6.0). [d] Key: 0, no significant analgesia in that the mean per cent analgesia is lower than −2 standard errors of controls; ±, mean per cent analgesia is included within ±2 standard errors of controls; +, significant analgesia in that the mean per cent analgesia is greater than +2 standard errors of controls.

due to its capacity to block conditioned responses (8) rather than to true analgesic ability. Without doubt cryogenine at effective anti-inflammatory dosage levels lacks morphine-like analgesic potency.

Simultaneous Analgesic and Anti-Inflammatory Testing—Both cryogenine and phenylbutazone demonstrated analgesic activity (Fig. 9), but it was significantly less both as to degree and duration than the response produced by indomethacin. None of the agents produced the degree of analgesia produced by morphine. By +5 hr. cryogenine, phenylbutazone, and indomethacin had caused reductions of the developed pedal edemas (10, 11, and 8%, respectively, 4 rats per treatment), whereas the control and morphine-treated animals demonstrated slight increases in the edematous response (0.4 and 2%, respectively). Using this technique it is quite apparent that the morphine-like narcotic analgesics can be distinguished readily from the nonsteroidal anti-inflammatory compounds which have an analgesic component.

Antiphlogistic Evaluation Using a Histamine-Induced Intradermal Wheal—Histamine diphosphate uniformly elicited the classic blue wheal in nontreated animals with the maximal score of 16 noted at all time intervals. Premedication with diphenhydramine HCl (20 mg./Kg. intraperitoneally) partially blocked the inflammatory response with mean scores of 0, 0.8, and 2.0 noted at +30, 60, and 180 min. after the histamine injection. Cryogenine (10 mg./Kg. intraperitoneally) completely blocked the action of histamine producing mean scores of 0, 0, and 0.2 at +30, 60, and 180 min., respectively. In addition, cryogenine produced a noticeable sedative action on the secured rabbit.

Serotonin-, Bradykinin-, and Histamine-Induced Pedal Edema—The progression of the edematous responses in the control and cryogenine-treated animals is illustrated in Fig. 10. The edemas produced by all of the phlogistic agents approached maximal response within 30 min. after injection. The histamine inflammation appeared much less pronounced in comparison to that produced by serotonin and synthetic bradykinin. The total areas under the respective curves were determined with the aid of a compensating polar planimeter. Cryogenine pretreatment afforded statistically significant protection ($P < 0.05$) against serotonin-induced edema. No significant protection ($P > 0.05$) was seen against bradykinin or histamine.

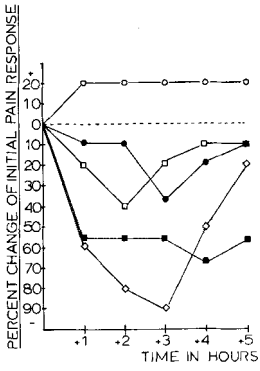

Fig. 9—Analgesic evaluation of orally administered cryogenine and other reference agents against silver nitrate-induced pain. Key: □, 100 mg./Kg. of cryogenine; ■, 100 mg./Kg. of indomethacin; ●, 100 mg./Kg. of phenylbutazone; ◇, 25 mg./Kg. of morphine; ○, untreated controls.

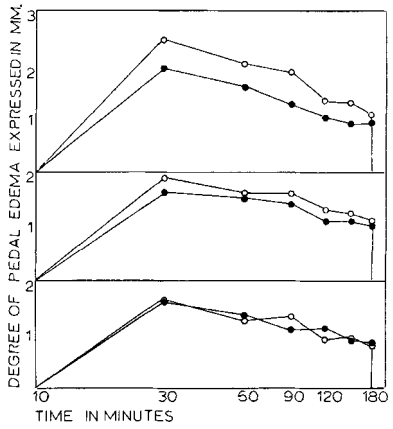

Fig. 10—Effect of oral cryogenine premedication on serotonin (top), bradykinin (middle), and histamine (bottom) induced pedal edema. Key: ●, 100 mg./Kg. of cryogenine; ○, nonmedicated controls. Five rats per each test group.

Antagonism Studies on Isolated Guinea Pig Ileum—At final bath concentrations of $10^{-7}M$, cryogenine almost totally blocked contractions induced by the reference agonists: serotonin, histamine, and bradykinin. In the cases of bradykinin and serotonin, the inhibition could not be reversed by increasing the concentration of the agonist; however, a partial reversal was possible in dealing with histamine.

Fibrinolytic Evaluation—A summary of the results is given in Table V. The various concentra-

TABLE V—FIBRINOLYTIC SCREENING OF CRYOGENINE AND REFERENCE AGENTS

	% Clot Lysis at *M* Concn.					
	0.04[a]	0.03	0.02	0.01	0.008	0.005
Phenylbutazone	10	100	>95	0	0	0
Indomethacin	10	90	...[b]	0	0	0
Cryogenine	...[b]	...[c]	...[c]	...[c]	...[c]	0
Sparteine sulfate	...[b]	...[d]	...[d]	...[d]	...[d]	...[d]
Chloroquine diphosphate	0	0	0	0	0	0

[a] Sufficient amount of the drug was weighed to produce 0.05 *M* stock solution upon dilution to 10 ml. Either NaOH or HCl was used to facilitate solution and/or adjust the final pH to 7.4. Cryogenine, phenylbutazone, and indomethacin were not totally soluble at a pH of 7.4; therefore, their saturated solutions (37°) were filtered through glass wool until clear. Appropriate dilutions were made with triple distilled water to make the final bath volume 2.5 ml. [b] Concentration was not tested. [c] No detectable lysis; some cloudiness of perfusion liquid noted. [d] No detectable lysis; some sediment formation noted.

tions of cryogenine, sparteine, and chloroquine did not produce visible lysis of the preformed clots. In the incubated test solutions of cryogenine, some degree of cloudiness was observed and in solutions of sparteine a white sediment was apparent. These phenomena may be expected considering the possible interactions between the soluble drug and the macromolecular matrix of the clot. Both phenylbutazone and indomethacin produced varying degrees of clot lysis.

DISCUSSION AND CONCLUSIONS

Cryogenine was shown to be partially effective in preventing the development of artificially induced inflammatory responses in rats using both acute (carrageenin) and chronic (*M. butyricum* adjuvant) experimental procedures. The possibility of interference due to "counter-irritation" was minimized by using the oral route of administration. In both acute and chronic evaluations, cryogenine appeared to be equipotent to phenylbutazone. Histopathological studies indicated that cryogenine may have some anti-inflammatory activity at the femorotibial joint. It is important to emphasize that the efficacy of cryogenine in both acute and chronic procedures appeared to be unrelated to toxic phenomena. After treatment(s) with cryogenine there were no indications of malaise and no deaths were recorded. The histopathological evaluation of a cryogenine-treated rat (100 mg./Kg. orally for 14 days) revealed no lesions of the organs examined.

Although cryogenine has been demonstrated to induce hypothermia in normothermic acclimatized animals upon intraperitoneal injection (7), it possessed only slight antipyretic activity in rats rendered febrile by peptone administration and then it was clearly inferior to aspirin in this respect. Cryogenine produced only equivocal analgesic activity utilizing the rat tail flick thermal technique. This low order of activity may be due to cryogenine's ability to alter conditioning phenomena. Untreated control animals usually show negative mean per cents of analgesia since they become conditioned to the heat stimuli and actually remove their tails from the area prior to actually receiving their threshold painful stimulus. Robichaud *et al.* (8) have shown cryogenine to be active after intraperitoneal administration in blocking conditioned responses in both nondiscriminated (continuous) avoidance and conditioned discrete avoidance escape situations. It is not uncommon to find anti-inflammatory, antipyretic, and analgesic activity for a single chemical entity. Rosenkilde (23) points out that the two central actions (antipyretic and analgesic) are apparently linked to a peripheral activity (anti-inflammatory). Since cryogenine did show some analgesic and antipyretic potential and was capable of partially reversing both silver nitrate-induced edema and pain responses, it may have both central and peripheral mechanisms involved in its anti-inflammatory activity.

Cryogenine inhibited responses both *in vivo* (histamine in the rabbit and serotonin in the rat) and *in vitro* (histamine, serotonin, and bradykinin in isolated guinea pig ileum) by certain suspected mediators of the inflammatory process. However, its action appeared to be relatively nonspecific since the results did not implicate any one mediator.

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