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Effects of cryogenine and selected anti-inflammatory and immuno-suppressive agents on developing and established mycobacterium-adjuvant polyarthritis in the rat

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EFFECTS OF CRYOGENINE AND SELECTED ANTI-INFLAMMATORY AND IMMUNO-SUPPRESSIVE AGENTS ON DEVELOPING AND ESTABLISHED MYCOBACTERIUM-ADJUVANT POLYARTHRITIS IN THE RAT

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INTRODUCTION

In 1956, Pearson (1) observed the development of both generalized and local arthritis, synovitis, periostitis, and tendonitis in rats approximately two weeks after an intracutaneous inoculation of tubercle bacilli in mineral oil. The inoculation was made in the posterior cervical region. Subsequent studies described the occurrence of subcutaneous nodules, ocular, and genital tract lesions, temporally correlated with the arthritis and periarthritis (2,3). The development of these delayed lesions following the inoculation of a Freund-type adjuvant in rats constitutes an experimental disease syndrome known as adjuvant arthritis (AA)[1].

In 1960, Waksman et al. (4) produced AA by inoculating rats intradermally in the hindpaw with the same adjuvant used in Pearson's study. An immediate swelling occurred in the inoculated extremity which peaked in about four days and began to recede thereafter. Ward and Jones (5) have reported a similar acute reaction following the hindpaw injection of tubercle bacilli in saline but these authors also noted that the delayed AA lesions failed to develop. Glenn and Gray (6) confirmed that the hindpaw

[1] For a listing of abbreviations used in the text and their respective definitions see Appendix A.

inoculation with the mineral oil adjuvant produced a local, severe and immediate response. Pearson (7) reported that erythema and nodularity developed at the height of the swelling. This led to loosening and then sloughing of the skin overlying the nodules by the seventh to eighth post-inoculation day. In some animals, a shallow, purulent ulcer with a red, granulating base occurred. Sokoloff (8) reported suppuration about oil vacuoles seen in the inoculated hindpaw. Newbould (9) reported that the peak in hindpaw swelling co-incided with a leukocytosis. It was noted that an increase in the total leukocyte count was attributed to increases in the polymorphonuclear fraction. Glenn and Gray(10) reported a similar finding for rats inoculated with adjuvant in the tail. These authors found depressed albumin/globulin ratios co-incident with a decrease in the albumin fraction and an increase in glycoproteins, mucoproteins, alpha-1, alpha-2, and beta globulins. The erythrocyte sedimentation rate (ESR) was similarly increased. These changes occurred between the second and sixth days. Gamma globulin plasma concentrations decreased between the sixth and eighth days.

Waksman et al. (4) recorded that on or about the tenth day, the inoculated hindpaw became swollen again. Swelling developed in the contralateral hindpaw followed by forepaw and tail swelling. Waksman continued his observations through the 40th post-inoculation day noting that the

extent and magnitude of the swelling in the inoculated hindpaw was always greater than that of the other extremities. Disseminated "arthritis" peaked approximately between the 18th and 25th day. A gradual recession occurred towards the 30th day.

Between the 11-16th day, Pearson (7) described the arthritis/periarthritis as pink, edematous swellings along the tarsal or carpal bones, on the heel or over one or several of the small digital joints. A predilection existed for joints of the hindpaws. It was noted that a fusiform swelling of the interphalangeal joints was common.

These clinical signs co-incided with an early hypocellular, edematous response in the connective tissue planes. Para-articular subsynovial tissues, as well as tendon sheaths and areas between muscle planes were involved. Synovial fluid volume and protein content increased. This edematous phase gave way to a cellular phase involving several inflammatory cell types: monocytes, small, round mononuclear cells which were either undifferentiated connective tissue cells, histiocytes, and lymphocytes. Neutrophils were only sparingly found. The appearance of fibroblasts was noted.

An acute synovitis in conjunction with synoviocyte proliferation, moderate joint effusion and pleocytosis occurred next. This appeared to be a manifestation of the previous inflammatory cell infiltration. Pearson did not

record purulent effusions. The subsynovial tissues remained edematous and perfused by a few polymorphonuclear leukocytes (PMNs) and many mononuclear cells: histiocytes, lymphocytes and plasma cells. This condition became coupled with an intense proliferation of fibroblasts. Peritendonitis and bursitis were recorded.

Glenn and Gray (10) observed an increase in muco- and glycoproteins and the total leukocyte count during this time period (11-16th day). Newbould (9) and Piliero et al. (11) confirmed the leukocytosis and indicated that it was associated with a relative lymphopenia and neutrophilia. Piliero further observed a significant decrease in the albumin/globulin ratio co-incident with depression of the albumin fraction and elevation of the alpha-2 and beta globulin levels. A statistically insignificant rise occurred in the gamma globulin level. Plasma fibrinogen levels and the ESR were elevated. Piliero in a separate study documented increases in serum lysozyme levels on day 14 (12).

The clinical morphology of the articular/periarticular lesions reached a plateau between the 18th and 25th day (13). The tail developed segmental radial swellings or ridgings which encircled the tail adjacent to the intervertebral discs of the caudal vertebrae. The aforementioned fibroblastic proliferation gave rise to an invasive connective tissue pannus in the tendons, subchondral bone and articular

cartilage. Osteoblastic proliferation resulted in osteoid formation on bones adjacent to the joints. The connective tissue reactions contributed to a clinical ankylosis. In some animals a spondylitis developed which was detectable only by radiologic or pathologic analysis. The histology was like that already described for the limbs and tail regions.

Pearson and Wood (13) observed that while 90% of the adjuvant-inoculated animals (range: 75-100%) developed arthritis, the incidence of non-articular lesions was about 25%. These authors stated that the non-articular lesions occurred in conjunction with the arthritis and rarely in its absence. Indurated erythematous nodules developed on the ears, tail surface, prepuce and exposed surfaces of the feet. The nodules presented a focal edema, vascular engorgement and a poorly demarcated infiltration of histiocytes, lymphocytes and a few PMNs.

Waksman and Bullington (2) reported conjunctivitis, episcleritis, iridocyclitis and keratitis characterized histologically by a relatively non-specific mononuclear infiltration accompanied by varying degrees of fibrinous and polymorphonuclear exudation.

Pearson et al. (3) reported that the genitalia presented balanitis, periurethritis, frequent inflammation of the periurethral glands and connective tissue proliferation in the vascular corpora cavernosa. Female rats

developed leukorrhea and occasionally urethritis. As in the other lesions, varying numbers of fibroblasts, plasma cells and neutrophils were present. Other non-articular lesions observed during this interval (18-25th) included an acute dermatitis and diarrhea.

Newbould (9) indicated that on the 21st day total blood leukocyte count was declining, but still elevated relative to the 14th day. Glenn and Gray (10) noted that the ESR, glycoproteins and mucoproteins were still elevated, while the albumin/globulin ratio appeared to remain depressed co-incident with a decrease in the albumin fraction.

Pearson, Waksman and Sharpe (3) observed that the non-articular symptoms, nodular, ocular, genital, etc., subsided towards the 25th day, while the arthritic/peri-arthritic symptoms involuted more slowly, beginning on the 30th day.

Pearson (7) charted a fluctuating course in about 25% of the animals beyond the 30th day. Fibrous adhesions between articular surfaces and thickening of joint capsular structures, proliferation and reduplication of synovial villi, ossification of osteoid and occasional bridging across joint margins especially in the tail and bony ankylosis across articular surfaces were observed during this late period. Additionally, a low grade inflammatory reaction in and about some joints occurred. The predominate cell type found was the lymphocyte, with occasional

collections of plasma cells. These underlying events intensified the earlier ankylotic condition seen between the 18th and 25th day and left the limbs and tail permanently deformed.

Pearson examined some animals nearly 365 days after the adjuvant inoculation (7). A local proliferation of connective tissue elements, tissue edema, fibrin deposition into the joint space or edematous tissue and some foci of acute and subacute inflammatory cells were recorded. Some authors (6) have failed to observe any evidence of a cyclic regression/exacerbation pattern beyond the 30th post-inoculation day. It will be helpful to restate these observations into a more coherent sequence of biologic events:

> A biphasic response has been described which follows the intradermal inoculation of a Freund-type adjuvant into the rat hindpaw. The first phase is represented by an immediate swelling in the hindpaw, which peaks in four days and recedes between the seventh and eighth day. Clinical features of this phase include erythema, edema and (less often) ulceration and a purulent discharge at the site of inoculation. Polymorphonuclear-induced leukocytosis, depressed albumin/globulin ratios, elevated serum glyco- and mucoproteins, alpha-1, alpha-2, beta globulins and erythrocyte sedimentation rates tend to coincide with the peak in primary hindpaw swelling.
>
> The second phase is delayed, beginning around the tenth post-inoculation day and tapering off by the 30th day. This second phase can be roughly divided into early (11-16th day) and late (18-30th day) stages based upon histological changes. The early stage involves the development of articular/periarticular lesions -- clinically apparent as erythema and swelling. These appearances are temporally correlated with: (1) exudation of plasma fluid and proteins into

the synovial and subsynovial regions, (ii) localization of inflammatory cells, including lymphocytes, plasma cells, monocytes, histiocytes and some neutrophils, and (iii) an acute synovitis, periarthritis, and peritendonitis.

The late stage of the second phase is marked by: (i) continuance of the early stage inflammatory reaction, (ii) a proliferative connective tissue reaction, and (iii) the development of non-articular lesions. The connective tissue reaction includes: (i) proliferation of fibroblasts and osteoblasts, (ii) pannus tissue invasion of tendons, subchondral bone and articular cartilage, and (iii) an ankylosis of both the limbs and tail.

The non-articular lesions involve mucocutaneous tissues of the tail, ears, eyes and genitalia. The lesions are coupled with histological features common to the articular lesions, with perhaps a greater neutrophil contribution and fibrinous deposition.

The hematological profile for both stages of the second phase approximates: (i) a lymphopenia/neutrophilia-related leukocytosis, (ii) a hypoalbuminemia/hyperglobulinemia-related depression of the albumin/globulin ratio, excluding participation by the alpha-1 and gamma globulin fractions, and (iii) elevated fibrinogen, ESR, and lysosome levels. It was noted that the leukocytosis appeared to be declining towards the end of the late stage.

In general, the non-articular lesions tend to be more transient than their articular counterparts, usually fading off on the 25th day. Pearson (3) did record a chronic dermatitis emerging between the 30th and 40th day. These non-articular lesions tend to occur in about 25% of the population and are rarely present in the absence of the articular lesions.

Subsidence of the articular lesions by the 30th day is co-incident with recession of the underlying inflammatory and connective tissue reactions. When the syndrome progresses beyond the 30th day, fibrous and bony ankylosis are predictable outcomes.

Earlier studies indicated that the first phase (day one to day eight) could be categorized as a non-specific acute inflammatory reaction mediated by the intradermal intrusion of the

tubercle bacilli adjuvant (4,8). This deduction can be based upon: (i) the rapid onset and short duration, (ii) the clinical signs of a transient erythema/edematous response, (iii) a hematological profile characteristic of general inflammatory processes (14), (iv) an apparent suppurative-related neutrophil reaction, and (v) the absence of any true functional disability following the inflammatory reaction.

The second phase presents both erythema/edema and general inflammatory hematology, but differs in several respects: (i) the response begins in 10-14 days and lasts about 20 days and sometimes several months, (ii) while the first phase involves the inoculated area only, the second phase involves all four paws, the tail, spinal column and includes the development of various mucocutaneous lesions of the tail, eyes, ears, genitals and skin, (iii) the histological features include primarily a mononuclear inflammatory cell reaction in contrast to the greater granulocytic involvement in the first phase, (iv) the intense connective tissue reaction (fibro- and osteoplasia) produces a severe and disabling ankylotic condition, and (v) a general decrease in total body weight gain occurs during the course of the second phase (7).

This analysis indicates that while the second phase includes an acute inflammatory component, the overall picture suggests a chronic inflammatory classification.

Pathogenic inquiries have focused on both immune and non-immune mechanisms. The evidence appears to favor an immune mechanism, presumably delayed hypersensitivity (DHS).

Jones and Ward (15) intradermally inoculated rats in the

tail with a mineral oil suspension of Mycobacterium butyricum, biosynthetically labeled with ^{14}C. Noteworthy was the localization of radioactivity in regional and mesenteric lymph nodes as well as perisynovial tissues of the feet.

Newbould (16) inoculated rats in the hindpaw with tubercle bacilli adjuvant and measured lymph node weight on days 1-15. Popliteal, inguinal, and para-aortic nodes increased rapidly in weight during the first five days. The weight of the internal axillary node, which is more remote from the inoculation site, increased gradually during the experimental period. In another experiment, Newbould removed all major lymph nodes draining the right side of test rats: popliteal, inguinal, para-aortic, renal and internal axillary. These animals then received an inoculation of adjuvant in the right hindpaw and were observed for the presence of secondary lesions. Two out of three animals failed to present secondary lesions. When secondary lesions did develop, it was noted that they were of a mild type and that the left para-aortic node weighed 120 mg in contrast to 30-40 mg for those of refractory animals; thus, the occurrence of secondary lesions was apparently associated with dissemination of adjuvant to the contralateral lymph node.

Removal of the draining lymph nodes up to the fifth post-inoculation day prevented the delayed, secondary lesions (16). However, removal on the seventh day did not impair the formation of secondary lesions. Turk and Stone (17) noted

that the development of hypersensitivity to chemical sensitizing agents in guinea pigs was inhibited when draining lymph nodes were removed up to the fourth day, while removal after four days had no effect. These authors recorded the development of large pyroninophilic cells in the draining node four days post-inoculation. They believed these cells to be precursors of the immunologically competent lymphocytes induced in the skin reaction. Newbould speculated that the increase in lymph node weight in rat during AA may have been a reflection of increased activity within the nodes.

Waksman and Wennersten (18) removed living lymph node cells from adjuvant-inoculated, inbred rats seven to ten days, post-inoculation. These cells were then transferred into normal unsensitized recipients of the same inbred strain. In four to eight days, AA developed in the recipients. The pathology was qualitatively identical to that of adjuvant arthritic controls, although of a somewhat less severe character. Transfer of killed cells (i.e.: heated for 10 minutes at 48°C) or cells from control donors (sensitized by injection of oil alone) failed to produce arthritis. These results were corroborated by Pearson and Wood (19). It was noted in this latter study that: (i) transfer of "debris" composed of a few viable and many non-viable cells and stroma from either nodes, spleen or thymus failed to induce AA, (ii) while spleen cells were

capable of transferring the disease, thymocytes were not, (iii) lymph node and spleen cells were not competent to transfer the disease before the eighth nor after the 14th post-inoculation day, and (iv) the lymph nodes removed for cell harvesting 8-11 days after inoculation were enlarged and often quite firm, while towards the 14th day, the nodes became small and flabby.

Waksman, Pearson and Sharpe (4) discovered earlier that serum from AA donors failed to transfer the disease to normal recipient rats.

In defining the DHS reaction, Movat (20) states the following:

> "... an immunologically determined inflammatory response characterized by the relatively slow development of a grossly and histologically typical lesion following local injection of antigen into an appropriately sensitized animal. The reaction is further defined as one which can be transferred to normal recipients by mononuclear cells from sensitized donors, but not by serum. The reaction is usually elicited by intradermal injection, but can also be produced in a variety of other tissues, such as cornea and joint spaces."

It was concluded that AA constitutes an immunologic reaction of the delayed or cellular type (18,19).

Additional evidence in support of this conclusion includes: (i) administration of Freund adjuvant is a potent sensitizing and antibody-promoting technique (21), (ii) an anamnestic response occurs to a second injection of adjuvant (4,22), (iii) young rats are refractory to AA (6,22), (iv) AA lesions are intensified following the application of non-

specific stimuli on or after the seventh post-inoculation day (22), (v) adjuvant inoculation of immunologically immature rats prevents later induction of the disease (4), (vi) Mycobacterium admixed with saline given to adult rats prevents later induction of the disease (23), (vii) seven week post-treatment protection from AA is provided for rats given heterologous anti-lymphocyte serum administered one day prior and 21 days following injection with tubercle bacilli adjuvant and this protection is not associated with increases in corticosterone serum levels (24).

While the delayed inflammatory syndrome appears amenable to a DHS classification, the antigen responsible for triggering this immune response has not been fully characterized. Waksman, Pearson and Sharpe (4) proposed an exogenous antigen, i.e. some constituent of the tubercle bacilli. These workers claimed that the suppression of arthritis by pretreatment with tubercle bacillus constituents in rats which, nevertheless, retained their ability to develop allergic encephalomyelitis (an autoimmune disease) was strong evidence in favor of a specific relationship between the arthritis and tubercle bacillus constituents. However, evidence exists which supports an autoimmune mechanism involving some constituent of the rat tissue.

AA has been produced repeatedly by a Wax D fraction of the tubercle bacilli (19). The Wax D fraction is

capable of producing tolerance to AA in both immunologically immature rats (4) and in adult rats (25). The Wax D fraction is classified as a peptidoglycolipid containing minimal amounts of protein (26). A preparation of the Wax D fraction has been reported to induce AA, while failing to elicit a tuberculin reaction in guinea pigs injected three weeks previously (19). It has been shown that the Wax D fraction in suspension has an affinity for proteins (27). Pearson and Wood (28) found a dose-related inhibition of AA associated with the in vitro admixture of hen egg albumin (HEA) with adjuvant prior to inoculation in rats. The addition of 10 mg of HEA reduced the incidence of AA from 95% to 18%, while 5 mg reduced it to 38%. This suggested that the HEA protein was adsorbed by the Wax D fraction thus pre-empting combining sites otherwise available for combination with an arthritogenic moiety supplied by the rat. Analogous results for bovine gamma globulin and rat gamma globulin were obtained.

Gery and Waksman (29) produced evidence which lends a different interpretation to these results. These workers pretreated rats with soluble bovine gamma globulin and later inoculated them with an admixture of bovine gamma globulin and adjuvant. An inverse relationship was found between the development of arthritis and sensitization to bovine gamma globulin. Where pretreatment with bovine gamma globulin inhibited later sensitization to bovine gamma

globulin, AA was well expressed, despite the admixture with this protein. Where sensitization to the admixed protein occurred, the arthritis was inhibited. This relationship was confirmed in another study in which bovine serum albumin (BSA) sensitization was abolished by neonatal thymectomy. As before, arthritis developed despite admixture with this protein (30). It was concluded that inhibition of AA was due to competition of antigens. This would not necessitate that the protein admixed with adjuvant pre-empt combining sites on the tubercle bacilli or the Wax D fraction, thereby preventing adsorption of some arthritogenic moiety supplied by the rat. This evidence tends to refute an autoimmune mechanism involving adsorption of tissue constituents at sites of inoculation. Further negation of this theory is provided by studies in which arthritis developed in rats inoculated in the tail after amputation of the tail, proximal to the inoculation site within two hours (15).

Rheumatoid arthritis (RA) generally is regarded as an autoimmune disease (31). Supporting evidence includes the presence of circulating antibodies directed against altered gamma globulin and a variety of macromolecular elements, nuclear components and connective tissue breakdown products, large polypeptide fragments and denatured protein (31,32). Bland and Phillips (32) hypothesized the following etiology and pathogenesis for RA: (1) an

endogenous or exogenous antigen (e.g., virus, bacteria, foreign protein, altered natural protein, or physical agent) locates acutely or chronically in extravascular connective tissue spaces, (ii) antibody is produced, (iii) antigen combines with antibody activating the complement sequence, leading to precipitation of the antigen-complement-antibody immune complex and initiation of an inflammatory response, (iv) phagocytosis of immune complexes results in the intra- and extra-leukocytic release of lysosomal enzymes (strong hydrolytic enzymes, proteases, collagenase, and depolymerases), and (v) lysosomal enzymes denature or otherwise alter a spectrum of cells and extracellular tissue components which become antigenic foci leading to the production of autoantibodies.

Weissmann (31) first proposed the concept that degradative enzymes released from lysosomes might denature the native constituents of cells of connective tissue and thereby effect the production of autoantibodies. This author emphasized that this autoimmune response should be considered as part of the normal immune response and that the autoantibodies would be directed not only against denatured constituents, but also against antigenically related normal tissues as well. Kaplan (33) studied an interesting autoimmune phenomenon which suggested an antigenic relation between components of hemolytic streptococci and human cardiac tissue. Antibodies were

found to cross-react with the invading bacteria and the host cardiac tissue to induce tissue damage. Katz (34) has reported finding a circulating auto-antibody in the serum of adjuvant-injected rats. It was stated earlier that serum lysozyme levels increase in adjuvant arthritic animals. Pearson et al. (3) compared the peripheral joint lesions in AA against those of rheumatoid arthritic patients and found a striking similarity in most subclinical features. The close association between the intensity of the early acute inflammatory reaction at the site of inoculation and the later induction of the immune-mediated, delayed inflammatory syndrome in AA tend to further support an autoimmune mechanism involving some lysozyme-induced altered constituent of the rat tissue (12). However, the evidence presented by Ward and Jones (15) also detracts from this autoimmune alternative. As mentioned before, these workers observed the development of arthritis in animals inoculated in the tail followed in two hours by amputation of the tail proximal to the site of inoculation. Perhaps an autoimmune mechanism plays a role in augmenting the established, delayed inflammatory syndrome and perhaps explains the occasional continuation of arthritis beyond the usual 30 day period.

Definitive identification of the antigen must await further studies. The concept of DHS as the mechanism which underlies the delayed response is now generally accepted.

However, non-immune mechanisms have been proposed in the past. Sokoloff (8) submitted that the delayed articular/periarticular lesions were manifestations of disseminated adjuvant into the subcutaneous tissues of the paws. A diffuse inflammatory reaction characterized as a cellulitis was observed in conjunction with granulomatous lesions similar to those found at the site of adjuvant inoculation. It was suggested that the pathogenesis of the so-called arthritis was the same as that involved in the depot site, i.e. a non-specific inflammatory reaction to the tubercle bacilli. Sokoloff explained the invariable 10-14 day latency period as a reflection of a protracted transport time rather than a delayed tissue responsiveness. Pearson (35) responded that this theory failed to explain that: (i) an inoculation of adjuvant in the posterior cervical region leads to a much more severe inflammatory reaction in the hindpaws, and (ii) a hindpaw inoculation leads to a later flare-up of inflammation in all four paws at the same time.

Pearson (72) and Wood (13) confirmed the presence of granulomatous foci in the subcutaneous tissues of the paws, in lungs and liver in about 10, 50, and 5%, respectively, of AA animals. Waksman and Bullington (2) reported granulomatous lesions in the ocular regions in about 25% of AA animals. However, Pearson found that it was not possible to correlate the frequency of appearance of granulomas with

the severity of the arthritis. The previously mentioned successful transfer of AA using viable sensitized lymphoid cells seems to confirm the immunological character of AA and precludes the suggestion that the lymphoid cells contained disseminated adjuvant (6) -- especially since non-viable sensitized lymphoid cells were incapable of transferring AA.

Streptobacillus moniliformis (36) and certain strains of Mycoplasmataceae (pleuropneumonia-like organisms, PPLO) commonly found in rats (37) can produce a form of arthritis. It has been suggested that the accidental transfer of a similar endogenous infection or the activation of a dormant infection might account for the production of disease in rats subjected to the stress of inoculation with adjuvant (4). Pearson and Wood (22) have reported finding PPLO-like organisms in a small proportion of AA animals. Waksman and Bullington (2) have summarized the evidence against this infective hypothesis: (i) the disease is readily produced with freshly autoclaved adjuvant, (ii) while PPLO were observed in a small number of AA animals, it has been impossible in a large number of AA rats to culture either bacteria or PPLO-like organisms from the blood, eyes, joints, or from the inoculation site itself, either during the latent period or during the acute phase of the disease, (iii) the injection of blood or involved joint tissue even as a suspension in oil into normal rats failed to produce

any arthritis, (iv) AA is characterized by nonpurulent lesions, unlike those caused by arthritis-producing strains of PPLO-like organisms, and (v) the disease is not suppressed by penicillin, streptomycin or tetracycline when given in high dosage throughout the latent period.

A compromise between a strictly immune and a non-immune pathogenesis has been suggested by Kapusta, et al. (38). These workers studied the effect of pyran copolymer, a synthetic polyanionic interferon inducer in AA. Noteworthy was that, (i) pyran suppressed arthritis in both Sprague-Dawley and CDF (trade name for a highly inbred rat species) rats, (ii) single injections of pyran effectively inhibited arthritis before, but not after the onset of clinical inflammation, and (iii) pyran induced interferon in both species of rats. Since interferon can inhibit the intracellular replication of virus (39,40), these authors proposed that the pathogenesis of AA involves an immune response to mycobacterial antigen(s) and activation of a latent virus by this immune response. Such a mechanism exists for the activation of latent herpes simplex virus in the brain or cornea following an immune reaction (41,42). They indicated that this pathogenic mechanism would explain several of the immunological findings for AA. The immune phase would account for the inhibiting effect of tolerance to mycobacterial antigen(s), the effectiveness of anti-lymphocyte serum and, in part, the characteristic latent

period. Antilymphocyte serum does not inhibit AA if administered during the entire second post-induction week (43), whereas pyran was highly effective if given on the seventh post-induction day. The polyoma virus system in adults requires the use of intact tumor cells for successfully transferring the disease to other animals (44). Assuming that the viral agent is fully pathogenic during the second post-induction week and is located within lymphoid cells, then the transfer of AA via intact lymphoid cells only during the second post-induction week would be reconcilable with a viral hypothesis. These authors noted that these data only raise the question of the role of a viral agent in what is presumed to be an immune disease. Substantiation of this viral theory must await studies demonstrating that purified rat interferon directly inhibits adjuvant disease.

Borrowing from Movat's description of the pathogenesis of the delayed reaction (20), the apparent events in the delayed AA reaction will now be summarized.

> "The first exposure to antigen stimulates the proliferation of immunologically competent cells in lymphoid tissue, some of which enter the circulation."

An intradermal inoculation of the tubercle bacilli is made in the rat hindpaw which rapidly disseminates to regional lymph nodes as evidenced by ^{14}C studies. This causes an apparent proliferation of immunologically

competent cells as suggested by an increase in lymph node weight and strongly supported by transfer studies with viable lymph node cells. These cells seem to be released into circulation 8-14 days post-inoculation as evidenced by the failure to transfer AA with lymph node cells before the eighth and after the 14th day.

> "Following the challenging injection, a few of these circulating cells come in contact with antigen, which results somehow in an inflammatory reaction characterized by accumulation of cells which are not immunologically competent. Most of these infiltrating cells are mononuclear cells derived from rapidly dividing precursors in the bone marrow."

Presumably, the release of immunologically competent lymph node cells between the eighth and 14th day are attracted to residual adjuvant in articular/non-articular areas. Ward and Jones (15) indicated that while the highest concentration of adjuvant was found at the site of injection, dissemination into other tissues occurred. The sensitized lymph node cells then react with the disseminated antigen in the articular/non-articular regions beginning sometime between the tenth and 14th day. This event then initiates the chronic inflammatory syndrome, characterized by a primary mononuclear cell response.

> "The delayed reaction has two major components: (i) the contact of a few specifically sensitized cells with antigen, and (ii) the ensuing immunologically non-specific inflammatory reaction."

AA has been of interest to pathologists and pharmacologists as a possible animal model for the rheumatoid variants, most notably Reiter's syndrome and

rheumatoid arthritis (13). Based upon this possible link, AA has been widely employed as a pre-clinical anti-inflammatory screen in conjunction with a non-immune acute inflammatory model, the carrageenin pedal edema assay in rats (45, 46). Traditionally, anti-inflammatory drugs are administered for two to three weeks and evaluations of effectiveness made at or near termination of therapy. In this way, AA has been shown to detect clinically effective anti-inflammatory (AI) steroids, non-steroids as well as immunosuppressive (IS) agents (47,48,49,50,11,52,12,53,54,55,56,57). Winder et al. (53) have reported that the use of AA for pharmacological screening yields valid bioassays for clinically prescribed drugs. Using paw volume and body growth as experimental parameters the fenamates and phenylbutazone gave dose-graded effects in both prophylactic and therapeutic treatment regimens and at dose levels comparable to those used in the clinic. Since the swelling in the feet of AA animals reflects the final result of a variety of histological and hematological parameters, suppression of this outward manifestation should correlate with suppression of these underlying processes. Piliero et al. (11) found significant inhibition of the development of adjuvant-induced inflammation and concomitant total or partial normalization of the blood picture, fibrinogen levels and various protein fractions in rats treated with paramethasone, phenylbutazone, oxyphenbutazone, indomethacin,

amethopterin and 6-mercaptopurine. In another study, AA rats presenting increased serum lysozyme levels and decreased serum turbidity measurements as determined by the stability of serum protein against heat denaturation were normalized by paramethasone, phenylbutazone and indomethacin (12). However, orally administered aspirin at 200 mg/kg/day was found to be ineffective against these underlying parameters and against the inflammatory response. This agrees with the observations of Ward and Cloud (49). However, Graeme (58) using the same dosage did observe some inhibition of both the acute paw swelling and the delayed lesions. Bogden et al. (59) observed suppression of the delayed lesions with 82 and 166 mg/kg of aspirin administered orally twice daily; thus, failure to effect an anti-inflammatory response may be related to dosage.

Ward and Cloud (49) noted that the average human dose of aspirin is 3,600-6,000 mg/day. Thus, 200 mg/kg/day in rats would be equivalent to 14,000 mg/day for a 70 kg human. This discrepancy may reflect a difference in metabolic handling of the drug in the two species, rather than a difference in pathophysiological mechanisms. Such a precedent exists for indomethacin (60) which is known to be metabolized differently in the rat than in man. As another example of species variation, phenylbutazone's biologic half-life is six hours in the rat in contrast to three days in man (61).

Chloroquin represents a notable example of a pharmacologic false negative in AA. Most workers agree that this clinically effective antirheumatic drug is not active in AA except when significant body weight loss is also noted (49, 51,58).

Waltz et al. (56) have explored the pharmacological specificity of AA using agents from a variety of pharmacological classes: anti-cholinesterase, anti-cholinergic, CNS depressant, hypotensive, hypoglycemic, tissue irritant (phenol, formalin, croton oil), hepatotoxic (carbon tetrachloride), antihistamine, antihistamine-antiserotonin, analgesic, nephrotoxic, CNS stimulant, sympathomimetic, monoamine oxidase inhibitor, antidepressant, tranquilizer and diuretic. Agents were administered orally for 17 days. Evaluations of "anti-inflammatory" activity were made either three or 16 days after the adjuvant inoculation. Agents from all classes could be detected as false positives by evaluating hindpaw volume of both the inoculated and non-inoculated extremity and the change in body weight. These workers stated that the pharmacologic specificity of AA appeared to be somewhat greater than that of other anti-inflammatory assays.

Recently, pharmacological studies conducted using AA have attempted to discriminate between conventional AI and IS drugs (55,56).

Standard IS agents, azathioprine (5 and 30 mg/kg) and

cyclophosphamide (10 mg/kg), orally administered starting on the day of adjuvant inoculation and continued until the 12th post-inoculation day, totally inhibited the secondary lesion in the non-inoculated hindpaw on day 21 (55). Using the same dosing regimen, standard AI agents, indomethacin (0.5 mg/kg) and phenylbutazone (100 mg/kg), were found to be ineffective in preventing the disease in the non-inoculated hindpaw. Paramethasone, a compound with both AI and IS activity also inhibited swelling of both hindpaws at 0.5 mg/kg. Thus, treatment of developing AA appeared to permit the selective detection of IS activity.

If animals were allowed to develop AA and then treated from the 18th until the 29th post-inoculation day, the same IS drugs were found to be inactive. However, indomethacin given at 0.05, 0.025, 1.25 and 3.0 mg/kg yielded dose-related reduction in swelling in the inoculated hindpaw. While reduction in the non-inoculated hindpaw occurred, this effect was not dose-related. Phenylbutazone was active against both hindpaw swellings as was paramethasone. Thus, treatment of the established disease permitted detection of standard AI agents.

Waltz et al. (56) confirmed this work in a later study. Daily oral administration of cyclophosphamide (5 mg/kg) between either 10-14th or 14-18th post-inoculation day yielded negative results. This was in contrast to phenylbutazone (50 mg/kg), indomethacin (1 mg/kg) and

prednisolone (20mg/kg) -- all of which significantly reduced the delayed arthritis. In another experiment, all agents were administered through the induction period and into the established period. Treatments were stopped on the 17th post-inoculation day and evaluations of various parameters were made on the third and the 16th day. Noteworthy was the absence of any anti-inflammatory action on the inoculated hindpaw on the third day in rats given 5 mg/kg of cyclophosphamide. However, standard AI drugs, indomethacin and prednisolone, were effective against the early acute reaction. Waltz (56) noted that this selectivity facilitates the pharmacologic differentiation between AI and IS activity.

When evaluations were made on day 16, all agents were found to suppress significantly both inoculated and non-inoculated hindpaw swelling. Thus, a standard IS agent such as cyclophosphamide could be observed to be "anti-inflammatory" while a standard AI agent such as indomethacin might be suspicioned to possess IS potential.

Since 1964, reports in the literature have indicated that Heimia salicifolia Link & Otto (Lythraceae) possesses ten structurally related alkaloids (62,63,64,65). All but one of the compounds possesses a diphenylquinolizidine lactone. Abresoline, a minor alkaloid has been recently characterized as a diphenylquinolizidine ester. The major alkaloid, cryogenine, is structurally depicted in Fig. 1.

Robichaud *et al.* (66) have briefly reviewed the folk literature concerning the pharmacological properties associated with the oral consumption of hydroalcoholic extracts of *Heimia salicifolia*. Such concoctions have been reported to possess emetic, antisyphilitic, hemostatic, febrifuge, diuretic, laxative, vulnerary, sudorific, tonic, as well as hallucinogenic-like properties.

A preliminary study of the powdered crude material and extracts containing the total alkaloids of *Heimia salicifolia*, indicated that the plant did possess pharmacological potential, thus supporting certain of these folklore accounts. These workers indicated that cryogenine mimicked qualitatively and semi-quantitatively the action of the total alkaloid extracts of *Heimia salicifolia*.

Subsequent investigations indicated that this alkaloid possessed some selective CNS depressant activity, but unlike that seen with reserpine and chlorpromazine (67,68). Hypotension, skeletal muscle relaxation and extrapyramidal side effects were not observed thus indicating that cryogenine might be pharmacologically unique and perhaps therapeutically advantageous. However, cryogenine's neuroleptic activity has only been documented upon parenteral administration. Therapeutic index calculations (IP LD_{50}/IP Neuroleptic $Dose_{50}$) indicate a value of only 3.9 for cryogenine (chlorpromazine = 60) thus suggesting that the central effect can be regarded as insignificant

(66,68,69).

In 1966, Jiu (70) reported that lyophilized extracts of Heimia salicifolia were anti-inflammatory against yeast-induced foot edema and cotton-wad granuloma in rats. An extensive anti-inflammatory evaluation published in 1967 by Kaplan et al. (71) indicated that cryogenine was equipotent to phenylbutazone in preventing experimentally induced carrageenin and AA inflammatory responses. Further publications (72,73,74) corroborated the anti-acute inflammatory property of cryogenine in the carrageenin model.

DeCato (74) indicated that the initial edematous response seen in the carrageenin-injected foot is more susceptible to the effects of autonomic stimulation and autonomically acting drugs. Cryogenine significantly inhibited this early phase by 31%. However, a 63% suppression of the later non-autonomic stage was also recorded. Early autonomic and cardiovascular studies of cryogenine have indicated that its activity is not mediated via sympathetic or parasympathetic-like receptors (75). DeCato has indicated that cryogenine's anti-inflammatory activity cannot be associated with depletion of whole brain or heart monoamines (76).

In related experiments, DeCato (74) has reported that adrenalectomy almost totally reduced cryogenine's effects against the early-stage swelling in the carrageenin model, while late-stage swelling was reduced by 50%.

Adrenalectomy was also found to reduce markedly the alkaloid's activity against a proliferative model of inflammation -- cotton pellet granuloma in the rat.

Kocialski et al. (73) have reported that cryogenine accentuated blood glucose levels in rats with carrageenin-induced inflammation, and in normal and alloxan-diabetic rats. These effects were seen upon oral administration using the standard anti-inflammatory dose of 100 mg/kg. Kellett (77) has reported that alloxan-diabetic rats are significantly more resistant to the immediate and delayed inflammatory responses in AA. DeCato has concluded that part of cryogenine's anti-inflammatory activity may be mediated by stimulation of the pituitary-adrenal axis (74). This theory would not appear to be in agreement with histopathological studies of rats orally receiving 100 mg/kg of cryogenine for 21 days (76). Noteworthy in this study was the absence of any alterations in adrenal and thymus glands.

Winder et al. (78) have reported that ultraviolet-induced erythema in guinea pigs will detect clinically effective non-steroidal AI drugs, but is insensitive to AI steroids. Kosersky et al. (79) found cryogenine to be effective in this selective anti-inflammatory screen, whereas hydrocortisone and 6-mercaptopurine were ineffective. Winder et al. (78) have noted that this model detects only agents affecting biochemical and physiological events

occurring early in the multi-phasic inflammatory process. *In vitro* studies indicate cryogenine to be a competitive antagonist of both histamine and bradykinin, two suspected mediators of the inflammatory response (80,81).

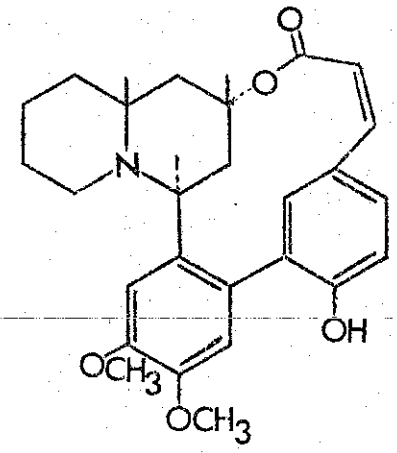
In a recent review, Whitehouse (82) noted that the distinction between AI agents and IS drugs has become blurred, as a result of: (*i*) a clearer realization that certain chronic inflammatory studies in man and experimental animals may be initiated or sustained by malregulated immunocompetent cells, and (*ii*) the increasing recognition that some of the immunoregulatory drugs may confer great clinical benefit in both human disease and experimental animal models characterized by progressive debilitation with chronic inflammation (arthritis, demyelinating disease, myositis, etc.). Preliminary reports have indicated that cryogenine was equipotent to phenylbutazone in reducing the inflammatory symptomology of AA (71). In a more thorough investigation (83), cryogenine's activity in AA was again confirmed. Cryogenine was orally administered each day in 100 mg/kg doses beginning one day prior to adjuvant inoculation. Dosing was continued through the 20th post-inoculation day. It was observed that cryogenine: (*i*) achieved an early arrest of swelling in the inoculated hindpaw, (*ii*) inhibited the delayed lesions in the non-inoculated hindpaw and non-articular areas (ear and tail) during treatment, and (*iii*) provided a relatively long lasting

anti-inflammatory effect after termination of treatment. This activity was equivalent to that obtained by paramethasone, 6-mercaptopurine and phenylbutazone. The early protection against the acute inflammatory reaction in the inoculated hindpaw is consistent with previous studies utilizing non-immune models of inflammation, as is the inhibition achieved during treatment. Since cryogenine was administered throughout the induction period and into the established period of disease, the possibility arises that part of cryogenine's activity may have been mediated by immune suppression. This might explain partially the long lasting post-treatment protection afforded by the compound. In conjunction with this same study, several animals with established arthritis were treated for a week with cryogenine. The drug appeared to inhibit the already established lesions -- this could have been predicted from the earlier studies documenting cryogenine's non-immune, anti-inflammatory properties (71,72,73,74).

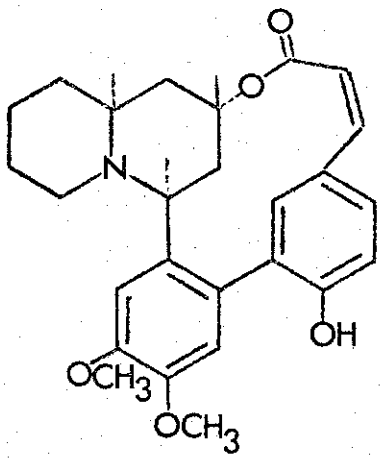
However, up to the present time no studies have been conducted to establish unequivocally whether cryogenine possesses: (i) only AI activity, or (ii) mixed AI-IS activities. Since Perper et al. (55) were able to discriminate between standard AI and IS drugs by administering them during either developing (1-12 days) or established periods (18-29 days) of AA disease, it seemed feasible to subject cryogenine to this differentiated

model to define its relative anti-inflammatory/immuno-suppressive activity. In addition, standard AI drugs (phenylbutazone and hydrocortisone) and an IS agent (cyclophosphamide) in non-toxic, effective doses will be studied for reference purposes.

Wiebelhaus (84) recently has claimed that lythrine, (Fig. 1), one of the Lythraceae alkaloids found in Heimia salicifolia, may have potent diuretic activity. Since cryogenine and lythrine appear to be structurally related, daily water consumption and urine volumes for cryogenine-treated animals and controls will be recorded during the present study.



CRYOGENINE



LYTHRINE

Figure 1.-- Two Major Alkaloids of Heimia salicifolia.

MATERIALS AND METHODS

I. Adjuvant Arthritis

Animals.-- Approximately seven-week-old, male, albino, Sprague-Dawley rats[1] weighing between 120 and 170 gm were allowed at least six days in our rat quarters after shipment to acclimate with the environment before experimentation. Daily body weights were checked to document whether or not normal growth patterns were present. Rat quarters consisted of an 8.5 meter x 5.2 meter room kept at constant temperature (70°F) and humidity and supplied with natural illumination from a frosted window 3.4 meter x 0.8 meter. Cages were positioned to approximate uniform lighting. Artificial lights were kept off, except during actual experimentation. Block lab chow[2] (placed on the cage floor) and tap water (bottles attached to cages) were supplied ad libitum. Animals were either paired in double cages or housed in

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[1] Horton Laboratories Inc., 411 Pendleton Way, Oakland, California.

[2] Purina Laboratory Chow. Crude protein not less than 23.0%; crude fat not less than 4.5%; crude fiber not more than 6.0%; and ash not more than 9.0%.

single cages[1] depending upon experimental conditions described under the Treatment section. Double cages were 24.1 cm x 17.8 cm x 17.8 cm with galvanized sheet metal sides and wire-mesh floors. Single housing was of much the same design but with the following modifications: (i) stainless-steel construction, (ii) a 20.3 cm x 11.4 cm x 11.4 cm main cage, (iii) a 12.1 cm x 5.1 cm x 5.1 cm one-way corridor leading from the back of the cage to a food reservoir, opening from the floor at the end of the corridor, and (iv) a urine-collecting funnel attached underneath the cage, opening into a removable glass vial. The food reservoir was not used. Lab chow was placed on the cage floor.

Adjuvant Preparation and Inoculation.-- The methods used were essentially those described by Kosersky (83). The contents of an ampule labeled as containing 100 mg of heat-killed, desiccated Mycobacterium butyricum[2] was triturated in a glass mortar with Light Mineral Oil, N.F. to a final concentration of 5 mg/ml. This suspension was then more finely divided and uniformly suspended with a hand tissue homogenizer[3] and pestle[3] fitted to an electric motor[4] and

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1 Acme Research Products, 5500 Muddy Creek Rd., Cincinnati, Ohio.
2 Difco Laboratories, Detroit, Michigan. Catalog number 0640-25.
3 Kontes Glass Co., Vineland, New Jersey. Kontes glass jacketed tissue homogenizer, mortar #71, pestle #52.
4 Talboys Engineering Corp., Emerson, New Jersey, model 106.

spun at approximately 1500 R.P.M. The pestle was moved up and down about 10 times. The adjuvant was poured into 10 ml parenteral vials, covered with rubber serum caps and autoclaved at 120°F, 20 lbs of pressure for 20 minutes. The freshly sterilized adjuvant was stored at 4°C no more than one week prior to use.

To make pedal injections, rats were pre-dosed with sodium pentobarbital[1] 30 mg/5 ml/kg IP and allowed to develop marginal loss of the righting reflex. A 25 gauge, 3/4 inch needle[2] attached to a 2 ml capacity micrometer syringe[3] was introduced underneath the subplantar surface of the right hindpaw, distal to the metatarsal region and directed medially to the metatarsal region and .05 ml injected of either mineral oil vehicle or adjuvant. Care was taken to prevent backward seepage of the injected material and injection into apparent blood vessels. The day of injection was designated as Day 0.

II. Treatment of Adjuvant Arthritis

Drugs.-- All drugs were suspended in 0.25% agar[4] and orally administered using a constant 10 ml/kg dosage volume. Preparation of drug suspensions and storage conditions were

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1 Robinson Laboratory Inc., San Francisco, California, Sodium Pentobarbital Powder, U.S.P. Control SC8603.
2 Becton, Dickinson and Co., Rutherford, New Jersey. Hyperchrome stainless hypodermic needle with regular point.
3 RGI Inc., Vineland, New Jersey.
4 Difco Laboratories, Detroit, Michigan. Catalogue number 0140-01.

as follows: cryogenine base[1], 100 mg/kg, was made up daily just prior to use; phenylbutazone[2], (Butazolidin), 100 mg/kg, and hydrocortisone alcohol[3], 10 mg/kg, were prepared every four to five days and stored at 4°C; and cyclophosphamide[4], (Cytoxan), 6 mg/kg, was reconstituted in doubly distilled water and stored as a stock solution at 4°C for not more than six days. At the time of preparation, a measured volume was removed and suspended with agar.

Developing Adjuvant Arthritis.-- Animals were randomly divided into negative controls (pedal injection of mineral oil) and positive controls (pedal inoculation of adjuvant) receiving the agar vehicle and adjuvant-inoculated animals receiving either cryogenine, phenylbutazone, hydrocortisone or cyclophosphamide. Four negative control rats were housed in single cages and 12 negative controls in double cages. Ten positive controls and ten cryogenine-treated animals were housed in single cages and all other drug-treated animals (ten animals/treatment) were housed in double cages. On Day -1, rats were dosed with either the agar vehicle or one of the drug treatments. Dosing continued

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[1] Cryogenine was isolated from Heimia salicifolia, Link & Otto according to the revised procedure used by Omaye (85). Extraction and purification details are summarized in Appendix B.

[2] Ciba Pharmaceutical Co., Div. Ciba-Geigy Corp., Summit, New Jersey. Control SN52731.

[3] Nutritional Biochemicals Corp., Cleveland, Ohio. Control 2912.

[4] Mead Johnson Laboratories, Evansville, Indiana. Each vial contains 100 mg cyclophosphamide and 45 mg sodium chloride, U.S.P. Control MFE 50 B.

daily through the 12th post-inoculation day. Urine volumes and water bottle weights were recorded every 24 hours for all rats housed in the single cages.

Established Adjuvant Arthritis.-- Upon arrival, ten animals were randomly selected, designated as negative controls, and housed in double cages. The 74 remaining animals were designated positive controls and placed as pairs in double cages. On the 18th post-inoculation day, these animals were screened for degree of AA involvement. Animals with a left hindpaw volume (as determined under the Evaluation section) of less than 2.09 ml or more than 4.35 ml were eliminated from further study (but kept with their cage mates). This left a population of 50 AA animals. Graph paper was marked off on the x-co-ordinate in 0.16 ml intervals starting from the left side. The left hindpaw volume for each animal was then plotted individually. A table of random numbers (86) was obtained. The last digit of each number less than one or greater than five was lined out. Since five experimental groups were needed, numbers one, two, three, four and five were assigned, respectively, to the positive control group receiving the agar vehicle, and the cryogenine, phenylbutazone, hydrocortisone and cyclophosphamide groups. Starting from the top of the column of random numbers and the left side of the graph paper, the first dot (or rat) was assigned to the group whose number appeared first in the column. This procedure

was continued until one animal had been assigned to each of the five groups. The selection process was repeated placing a second animal in each of the five groups, while moving down the column of random numbers and across the x-co-ordinate. Since 50 dots (or rats) were plotted, this selection procedure produced five groups of ten randomly selected animals/group -- each group composed of rats representing the complete range of left hindpaw volumes. Animals were dosed with either the agar vehicle or a test drug beginning on the 18th post-inoculation day and continued daily through the 29th post-inoculation day.

III. Evaluation of Adjuvant Arthritis

Experimental measurements began just prior to the adjuvant inoculation on Day 0 and continued generally every third post-inoculation day.

Body Weight.-- Animal weights were recorded for purposes of assessing drug effects on body growth and for establishing drug dosages.

Inflammogram Scoring.-- In order to evaluate drug effects on the extent and severity of the generalized inflammatory manifestations of AA, a subjective scoring system was devised, based upon those used by other workers (58,83,87,88). It has been the author's experience that the inflammatory expressions of AA are difficult both to qualitate and quantitate, except in the fore- and hindpaws,

tail and ears. In general terms, inflammogram scoring was as follows: (i) the forepaws were "divided" into carpal, metacarpal and phalanx regions and graded with respect to severity of swelling, (ii) the hindpaws were "divided" into tarsal, metatarsal and phalanx regions and graded with respect to severity of swelling, (iii) erythema of the paws was normally generalized and, therefore, all three regions of each paw were graded together in terms of severity of redness, (iv) the tibiotarsal joint of the hindpaws was graded in terms of the severity of ankylosis, (v) segmental radial swellings of the tail were graded in terms of extent and severity of swelling, (vi) round, firm nodules seen on the tail were graded in terms of number present, (vii) erythema of the ears was graded in terms of extent and severity of redness, and (viii) nodules of the ears were graded in terms of the number present. The original inflammogram scoring form is shown in Fig. 2. Anatomical regions and parameters not considered in the present study are lined out. Figures 3 through 7 depict photographic examples of each anatomical region evaluated and the range of possible scores. Using this system, each animal on a given day could develop a score ranging from 0 to 50.

Plethysmographic Determination of Hindpaw Volume.-- The method was similar to that described by Winter and Nuss (51). A line was drawn across the top edge of the lateral malleolus of both hindpaws using an indelible pencil lightly

wetted with water. Hindpaw volumes were then determined to this mark using the apparatus shown in Fig. 8 . The opposite page defines the working procedure for calibration of the plethysmograph. Once the machine was calibrated, each hindpaw was dipped twice, from which an average could be calculated. Drug effects were translated in terms of percentage change of increased hindpaw volume as described by Newbould (89):

$$\text{percentage change} = 100 \left\{1 - \left[(a-x)/(b-y)\right]\right\}$$

where y = mean hindpaw volume of positive control rats immediately prior to adjuvant inoculation, b = mean hindpaw volume of positive control rats on a particular day, x = mean hindpaw volume of drug-treated rats immediately prior to adjuvant inoculation, and a = mean hindpaw volume of drug-treated rats on a particular day.

Figure 2.-- Original Xerox Inflammogram Scoring Form Used During Experimentation.

Date: ____________

Continued From Page: ________ Page: ________

Data: RE: INFLAMMOGRAM = Chronic Evaluation of Adjuvant Arthritic, Male, Sprague-Dawley Rats. Adjuvant = Mycobacterium butyricum(heat,killed) 5mg/ml In Light Mineral Oil, N.F.

Date of 0.05ml Right Hindpaw Subplantar Adjuvant Injection: ____________

Data Book: ____________ pp.: ____________ ; ____________ pp.: ____________

Key:r=right;l=left;fp=forepaw:carpal,metacarpal&phalanx area;hp=hindpaw: tarsal,metatarsal&phalanx area;t=tail;pe=penis;a=ear's auricle;e=eye;n=nose; ERY=erythema:0=absent,1=light-dull redness,2=bright redness;SWE=swelling: 0=absent,1=mild-moderate,2=severe;ANK=ankylosis:0=150degree movement,1=>60-<150degree movement,2=30-60degree movement,3=<30degree movement;NOD=nodule: 0=absent,1=1-2,2=>2;OTH OBS=other observations.

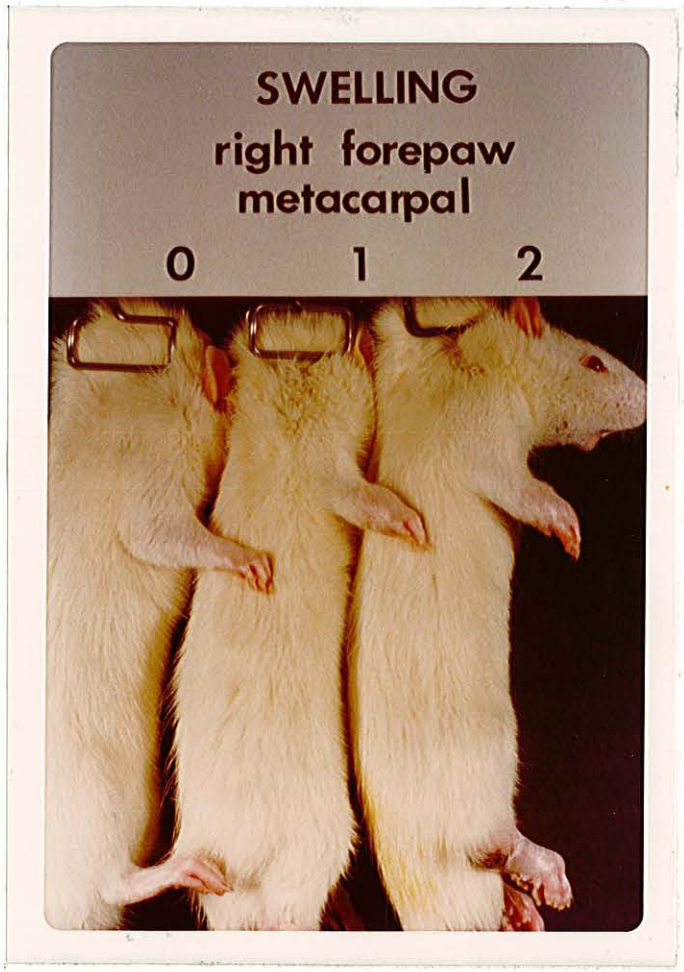
	rfp / rhp	lfp / lhp	pe	t	ra / la	re / le	n		rfp / rhp	lfp / lhp	pe	t	ra / la	re / le	n		rfp / rhp	lfp / lhp	pe	t	ra / la	re / le	n
ERY 0-2								ERY 0-2								ERY 0-2							
SWE 0-2								SWE 0-2								SWE 0-2							
ANK 0-3								ANK 0-3								ANK 0-3							
NOD 0-2								NOD 0-2								NOD 0-2							
OTH OBS								OTH OBS								OTH OBS							

	rfp / rhp	lfp / lhp	pe	t	ra / la	re / le	n		rfp / rhp	lfp / lhp	pe	t	ra / la	re / le	n		rfp / rhp	lfp / lhp	pe	t	ra / la	re / le	n
ERY 0-2								ERY 0-2								ERY 0-2							
SWE 0-2								SWE 0-2								SWE 0-2							
ANK 0-3								ANK 0-3								ANK 0-3							
NOD 0-2								NOD 0-2								NOD 0-2							
OTH OBS								OTH OBS								OTH OBS							

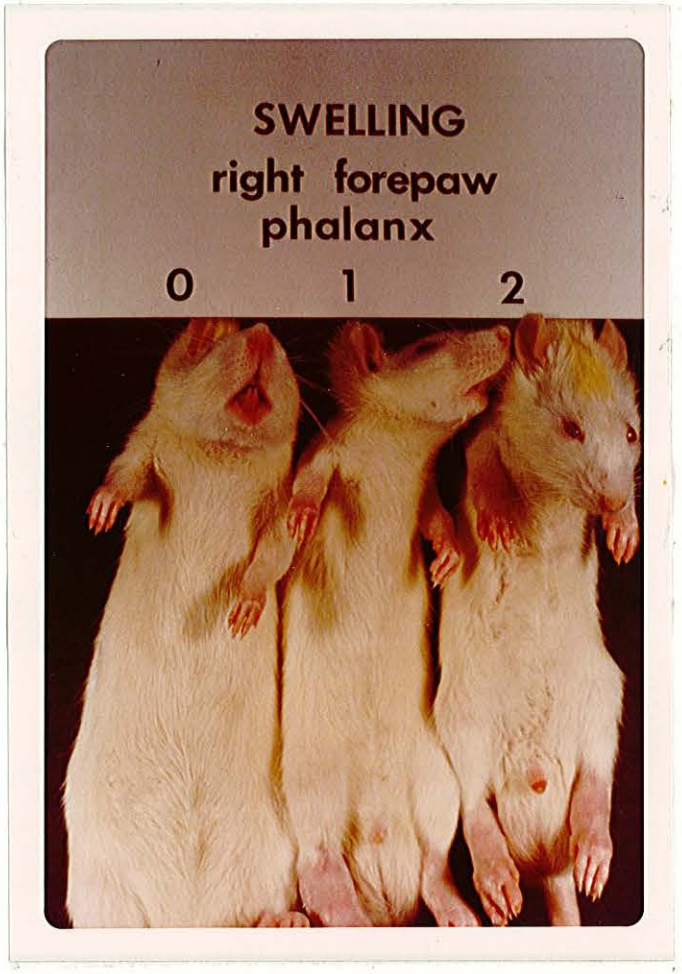
Evaluator: ____________

a.-- Erythema: 0 = absent, 1 = light-dull red, 2 = bright red.

b.-- Swelling: 0 = absent, 1 = mild-moderate, 2 = severe.

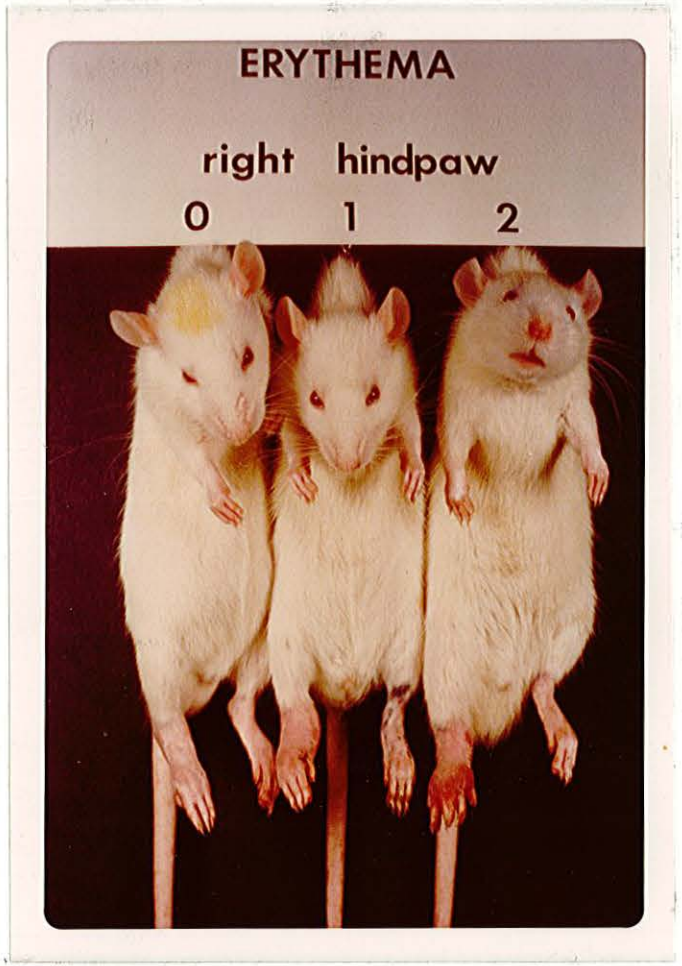


c.-- Swelling: 0 = absent, 1 = mild-moderate, 2 = severe.

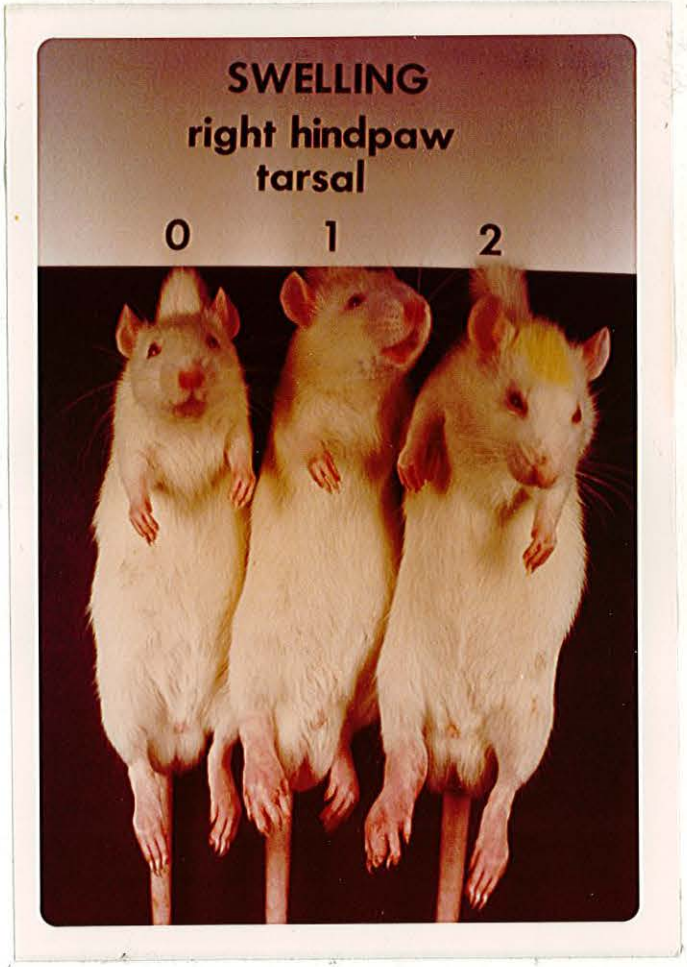


d.-- Swelling: 0 = absent, 1 = mild-moderate, 2 = severe.

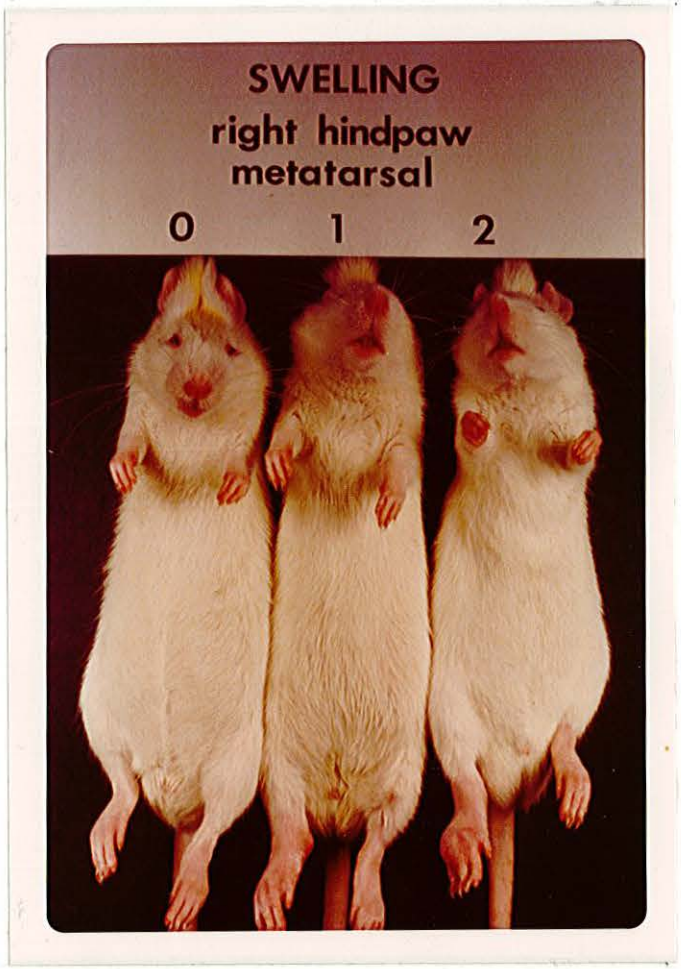
Figure 3.-- Inflammogram Scoring of Forepaws.



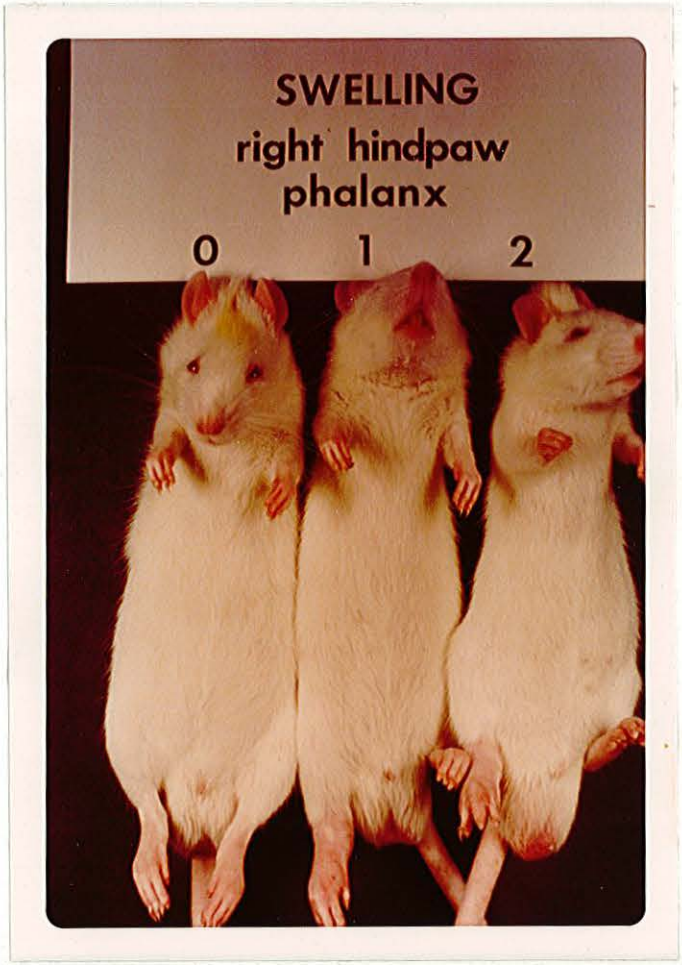
a.-- Erythema: 0 = absent, 1 = light-dull red, 2 = bright red.



b.-- Swelling: 0 = absent, 1 = light-dull red, 2 = bright red.



c.-- Swelling: 0 = absent, 1 = light-dull red, 2 = bright red.



d.-- Swelling: 0 = absent, 1 = light-dull red, 2 = bright red.

Figure 4.-- Inflammogram Scoring of Hindpaws.

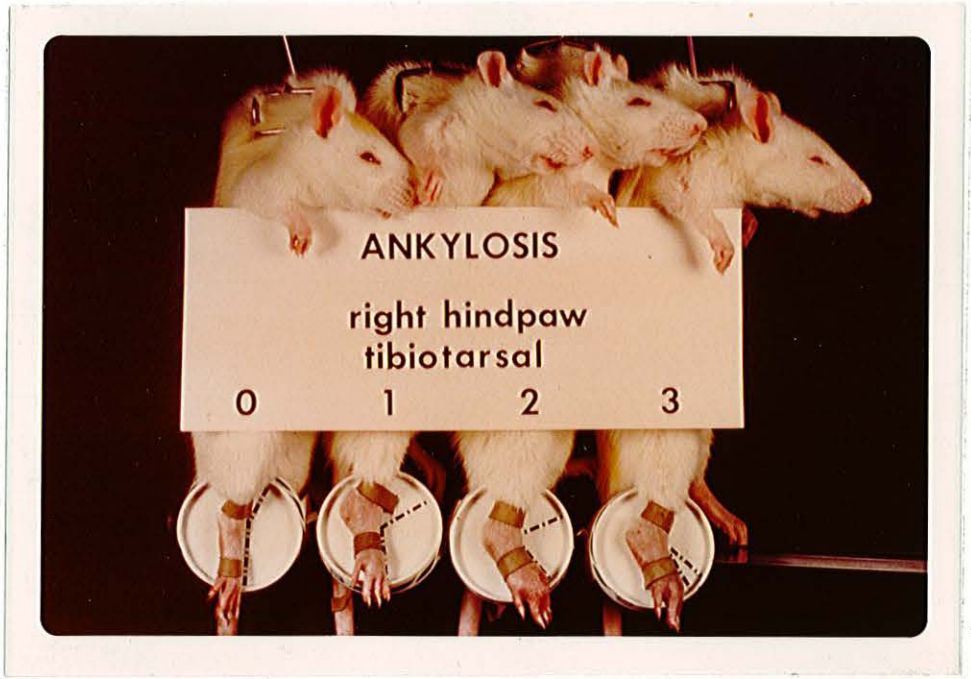
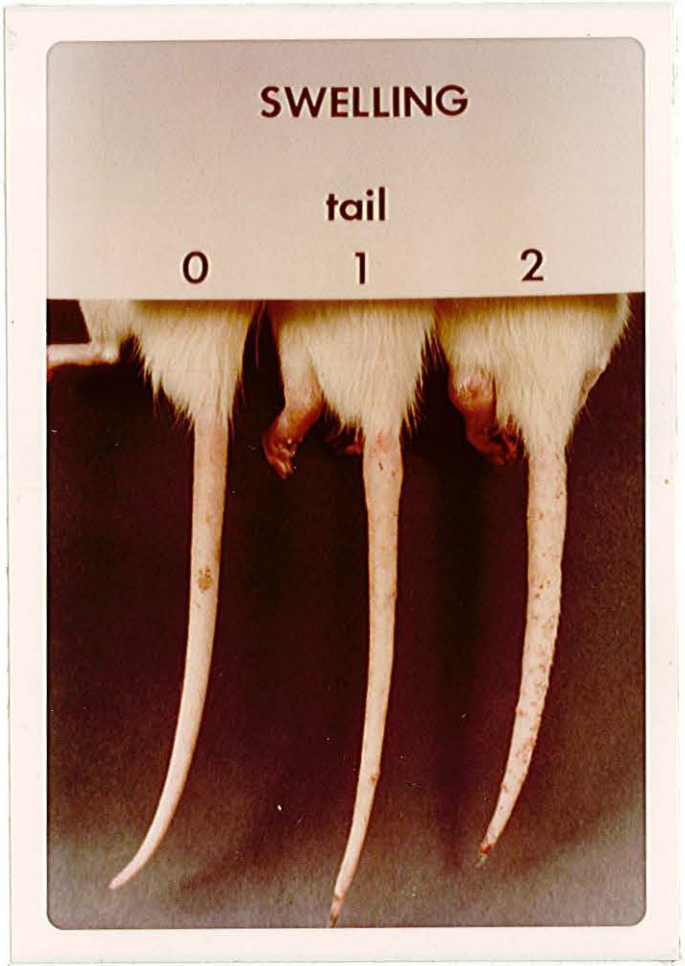
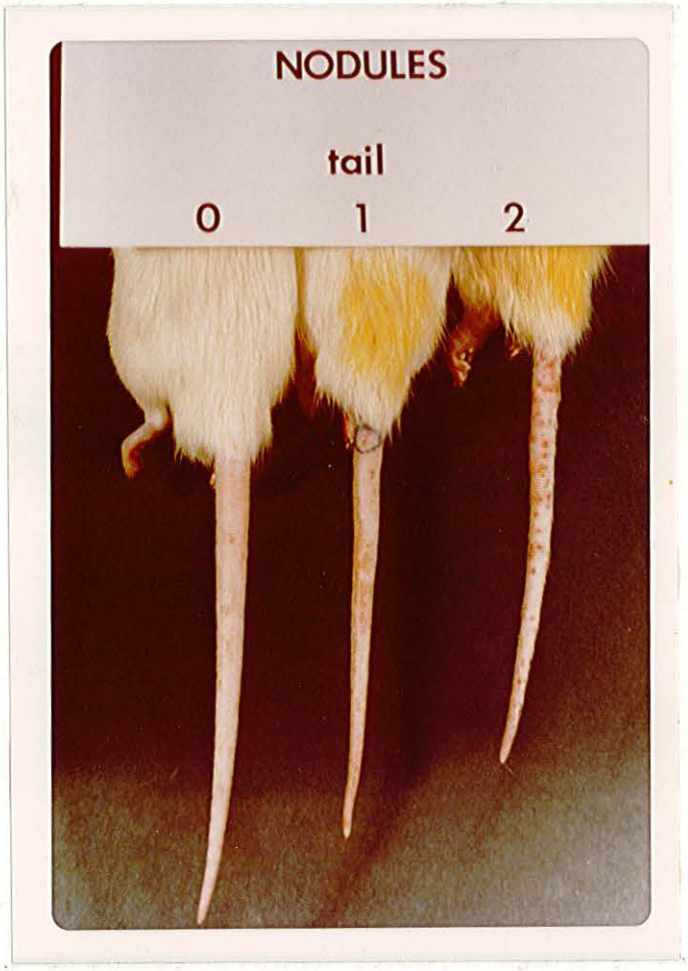


Figure 5.-- Inflammogram Scoring of Hindpaw Ankylosis (0 = 150° movement, 1 = >60-<150° movement, 2 = 30-60° movement, 3 = <30° movement).



a.-- Swelling: 0 = absent, 1 = mild-moderate and < 50% of tail area, 2 = severe and > 50% of tail area.



b.-- Nodules: 0 = absent, 1 = 1-2, 2 = > 2.

Figure 6.-- Inflammogram Scoring of Tail.

a.-- Erythema: 0 = absent, 1 = light-dull red and < 50% of ear area, 2 = bright red and > 50% of ear area.

b.-- Nodules: 0 = absent, 1 = 1-2, 2 = > 2.

Figure 7.-- Inflammogram Scoring of Ears.

Stepwise Calibration of Plethysmograph

1. Flip toggle switch A to "venous"
2. Flip toggle switch B to "off"
3. Flip toggle switch C to "normal"
4. Flip toggle switch D to "average"
5. Turn knob E to "2 mv/cm" (approximate setting)
6. Turn knob F to "x.1 mv/cm" (approximate setting)
7. Flip toggle switch G to "out"
8. Depress button H to "1 mm/sec"
9. Turn knob I to "operate" and allow at least 30 minutes for machine warm-up
10. Open clamp J and adjust mercury level in tube K with mercury level in reservoir L using syringe M
11. Close clamp J
12. Turn knob N and position pen 6 cm from top of paper
13. Flip toggle switch B to "-"
14. Turn knob O and position pen 6 cm from top of paper (pen should remain in position for step 12 at all times, and for step 14 only through step 14)
15. Immerse calibration rod[1] to the 1 ml mark
16. Turn knob O and position pen 5.5 cm from top of paper.
17. Immerse calibration rod to the 2 ml mark
18. Turn Knob P and position pen 5.0 cm from top of paper
19. Continue to immerse calibration rod in 1 ml increments, turning knob P to keep pen positioned at 0.5 cm intervals
20. Withdraw calibration rod
21. Keep repeating steps 15-19 until linearity is achieved and each 1 ml = 0.5 cm[2]

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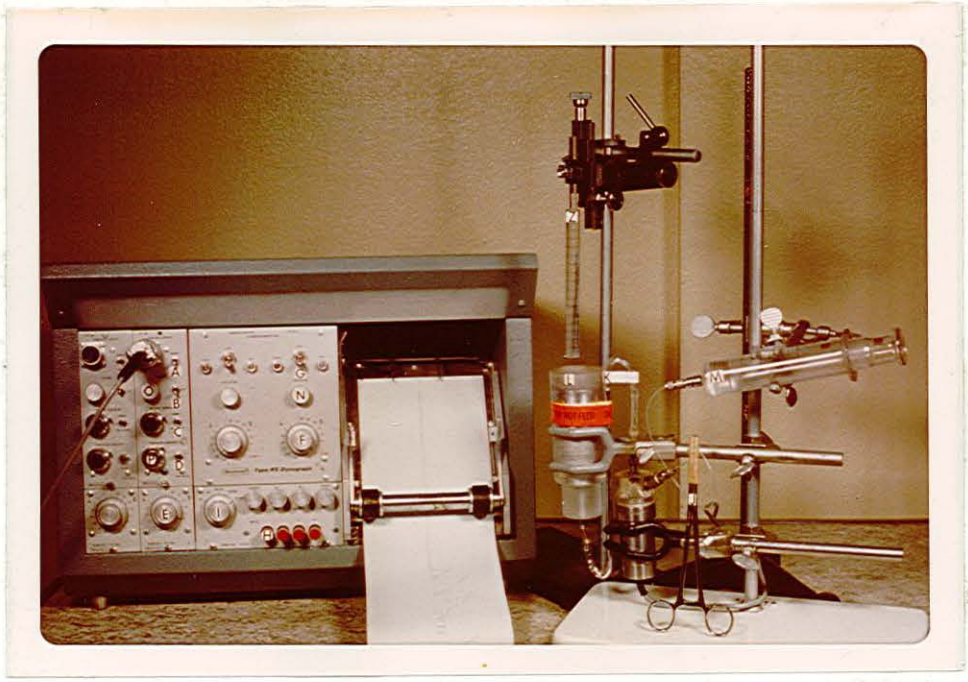
[1] The Perspex calibration rod of 1.27 cm diameter was scored using an electric lathe at 0.156 inch intervals. Volume = $\pi r^2 h$; therefore, when the radius = 0.635 cm, the height must = 0.790 cm for a 1 ml volume (0.156 inch = 0.5 ml volume).

[2] Machine must be checked for calibration and linearity frequently. When calibration is lost, repeat steps 12, 16, and 17-19. If all else fails, follow directions.



E. Preamplifier type 461B.

F. Dynograph amplifier type 462.

I. Control panel type A560.

L. Reservoir: depth = 9 cm, dia. = 4.3 cm.

O. Balance.

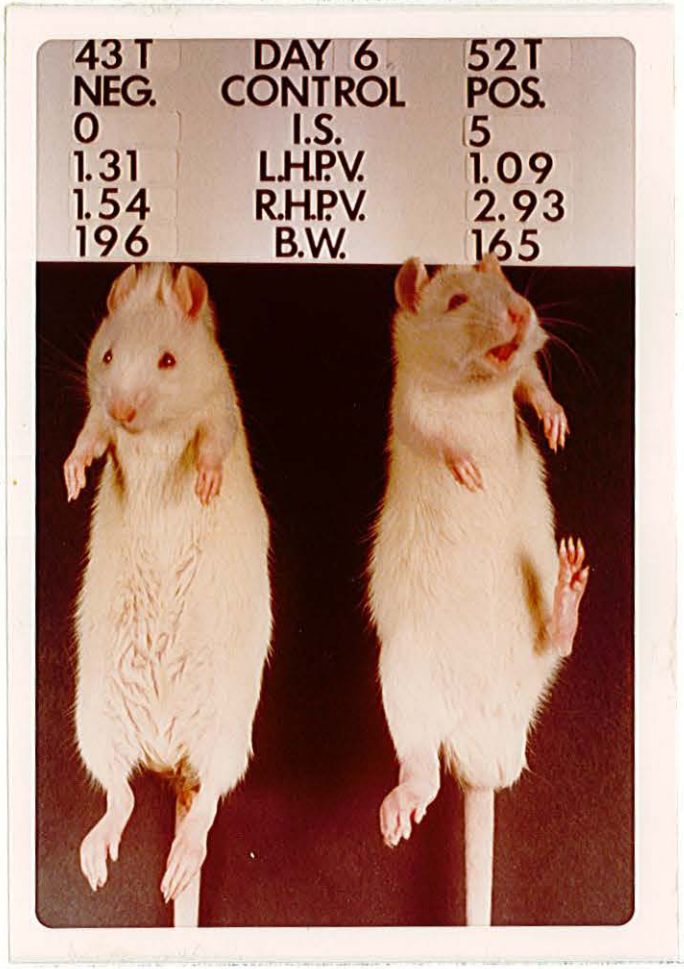
P. Transducer sensitivity.

Figure 8.-- Plethysmograph: Beckman Type RS Dynograph Connected to a Statham Strain Gauge Pressure Transducer (P23BB, 0-50 mm).

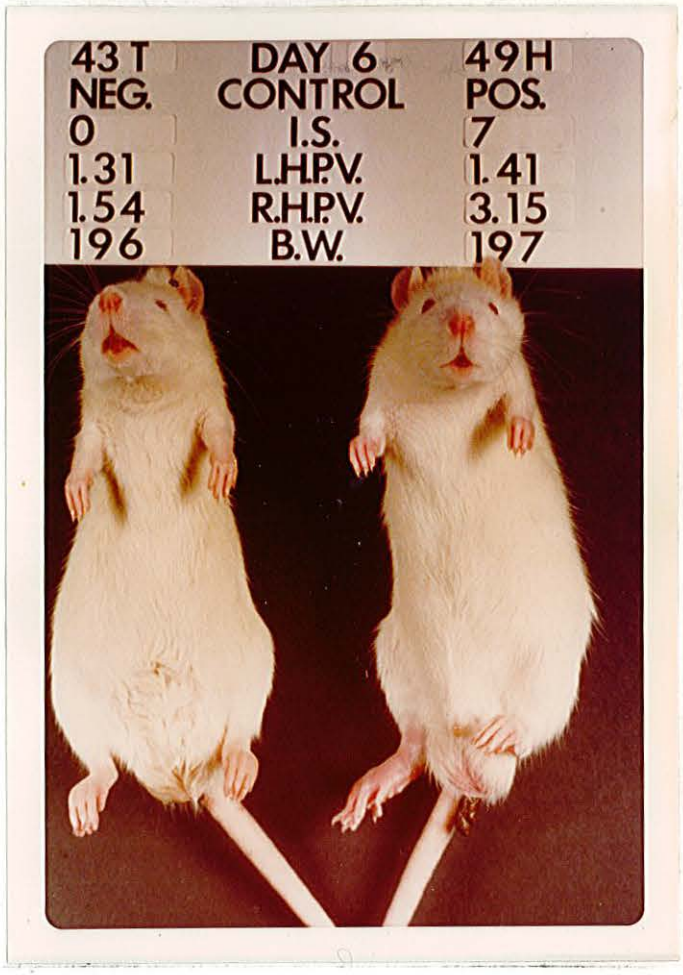
RESULTS

1. Developing Adjuvant Arthritis.-- The clinical course of adjuvant arthritis is illustrated in Figs. 9-13. Positive control animals 52T and 49H are compared with the same negative control animal (43T) on post-inoculation days 6,9,15,21 and 27. Inflammogram scores (IS), left (non-inoculated) hindpaw volumes (LHPV), right (inoculated) hindpaw volumes (RHPV) and body weights (BW) of each animal are noted. Animal 52T represents a strong reactor to the adjuvant inoculation, whereas animal 49H represents a weaker reactor. This difference can be noted in the progressively higher IS, LHPV, RHPV values and a greater decrease in the BW value for animal 52T during the 27 day period. An animal is depicted in Figs. 14-16 which received an inoculation of adjuvant and developed an acute reaction in the right (inoculated) hindpaw, yet failed to develop the delayed inflammatory symptoms. This refractory animal appears essentially equivalent to the negative control animal (43T) on day 27 (Fig. 16).

On day 0 there was no significant difference between

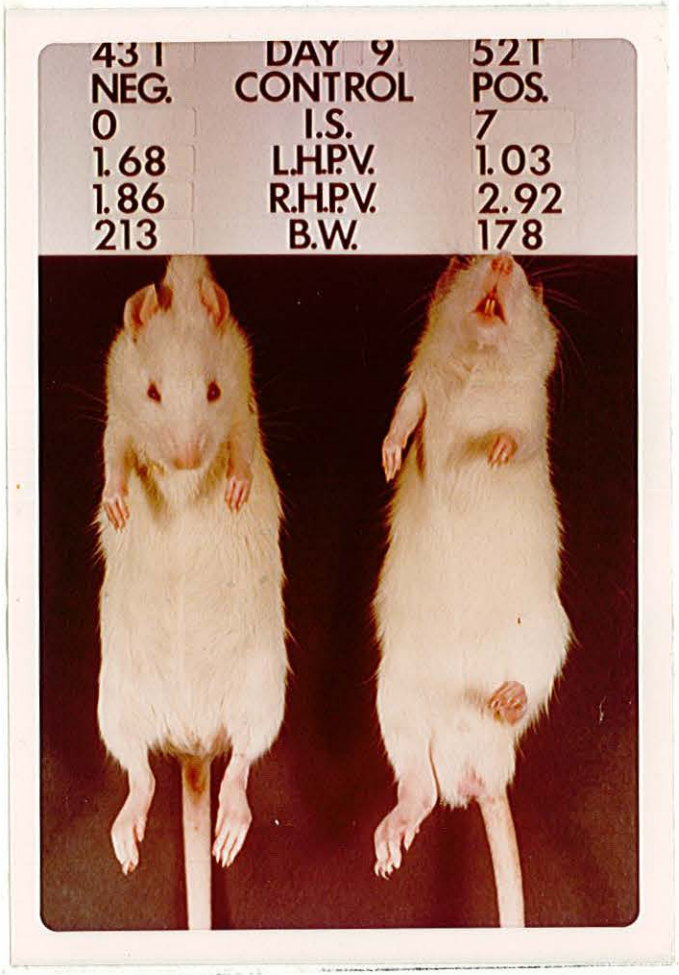


a.-- 43T vs. 52T.

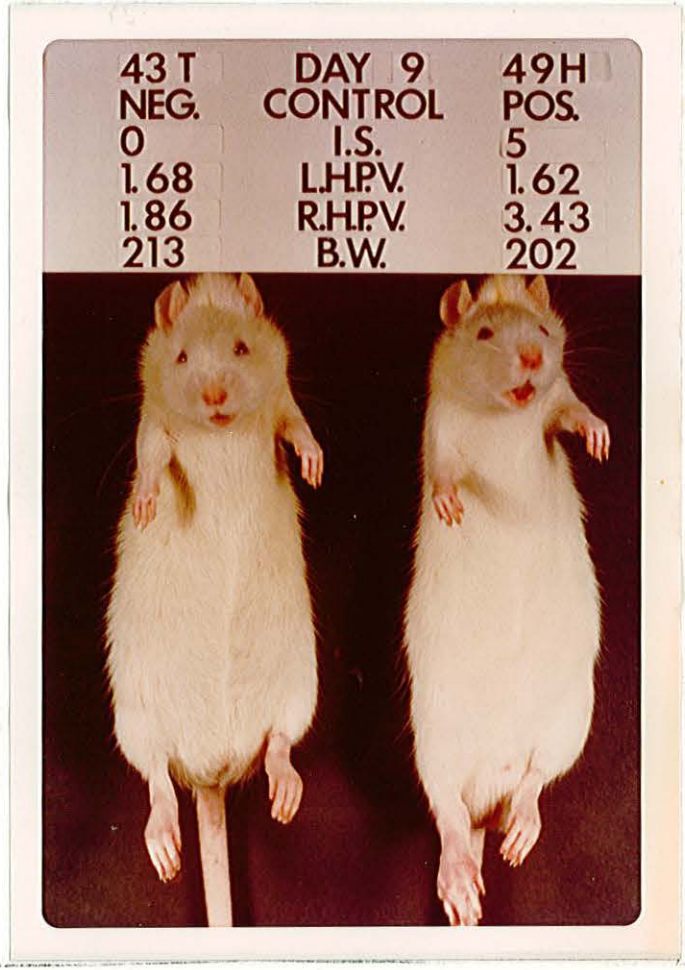


b.-- 43T vs. 49H.

Figure 9.-- Negative vs. Positive Control on Day 6.

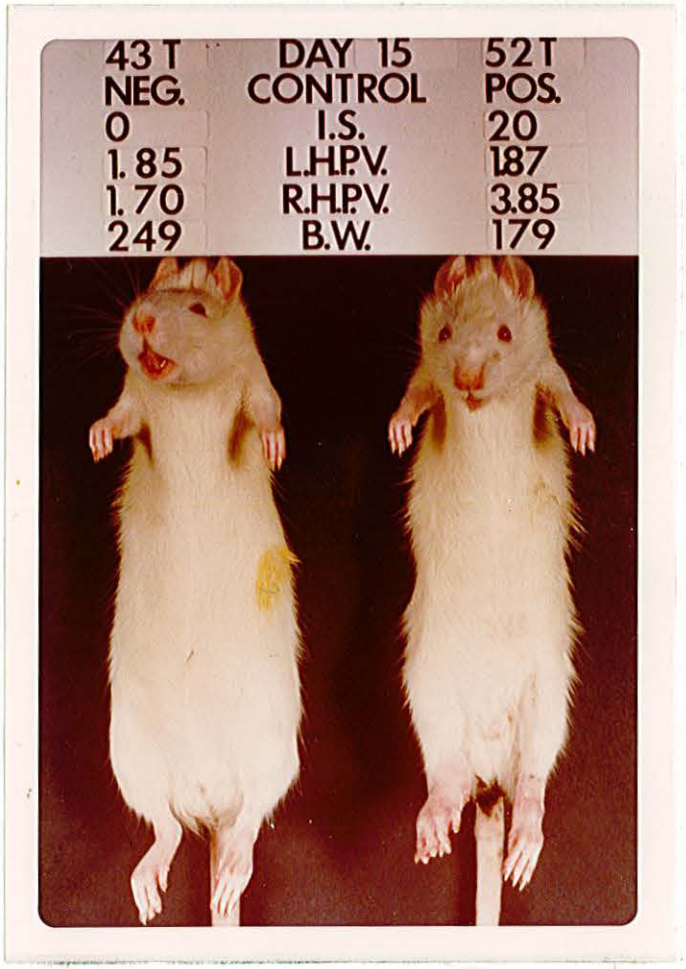


a.-- 43T vs. 52T.

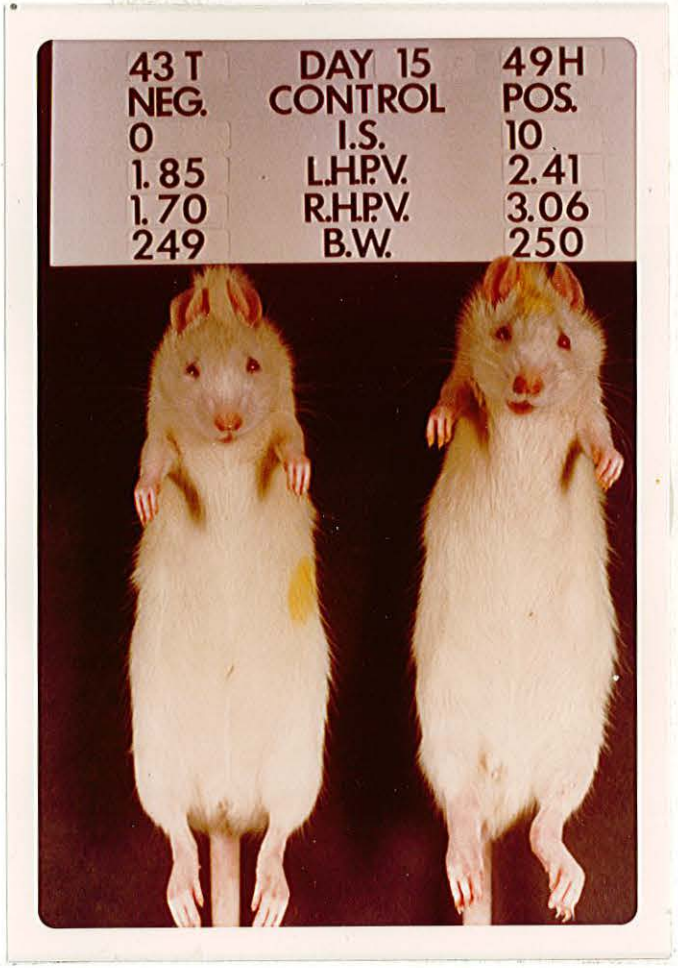


b.-- 43T vs. 49H.

Figure 10.-- Negative vs. Positive Control on Day 9.

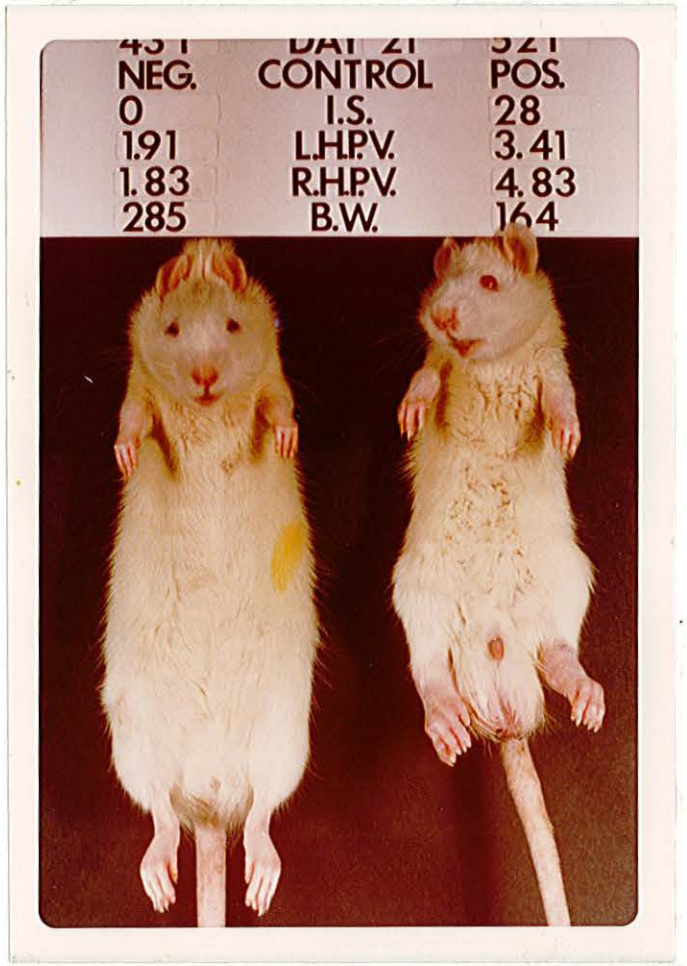


a.-- 43T vs. 52T.

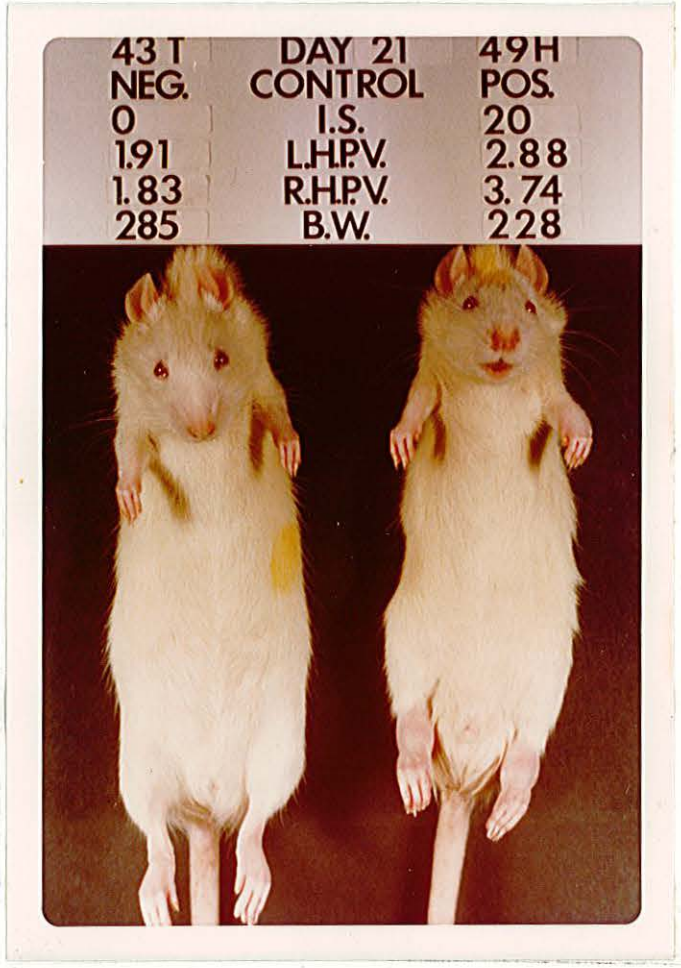


b.-- 43T vs. 49H.

Figure 11.-- Negative vs. Positive Control on Day 15.

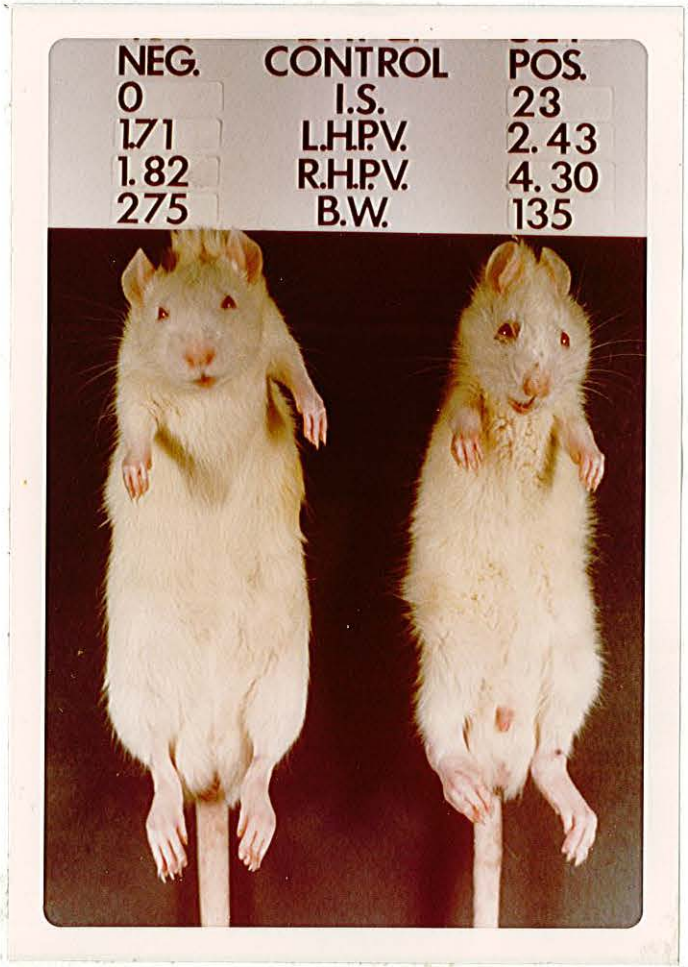


a.-- 43T vs. 52T.

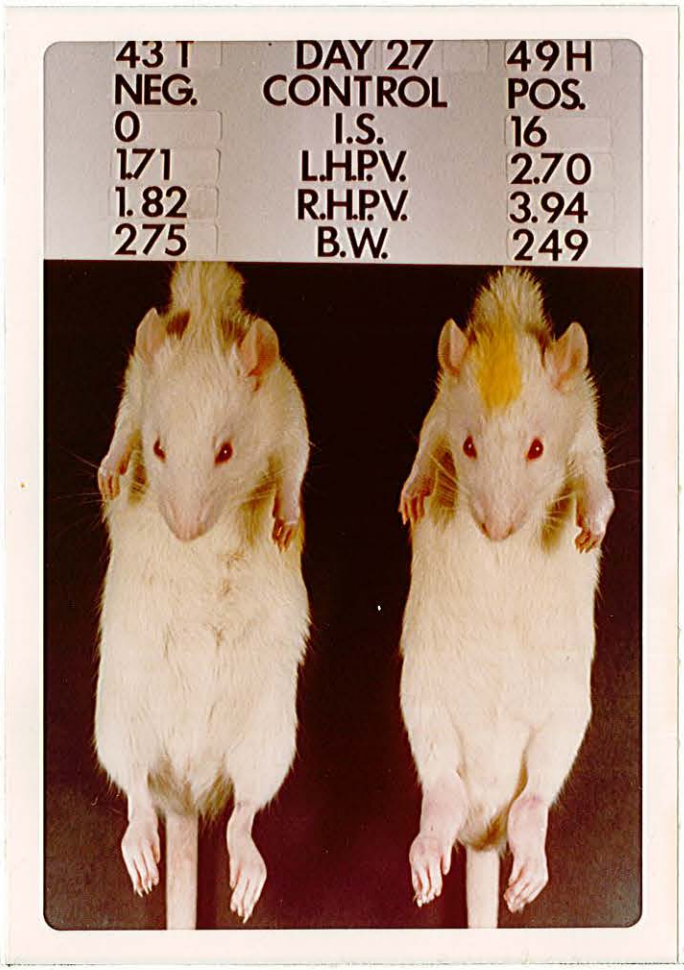


b.-- 43T vs. 49H.

Figure 12.-- Negative vs. Positive Control on Day 21.

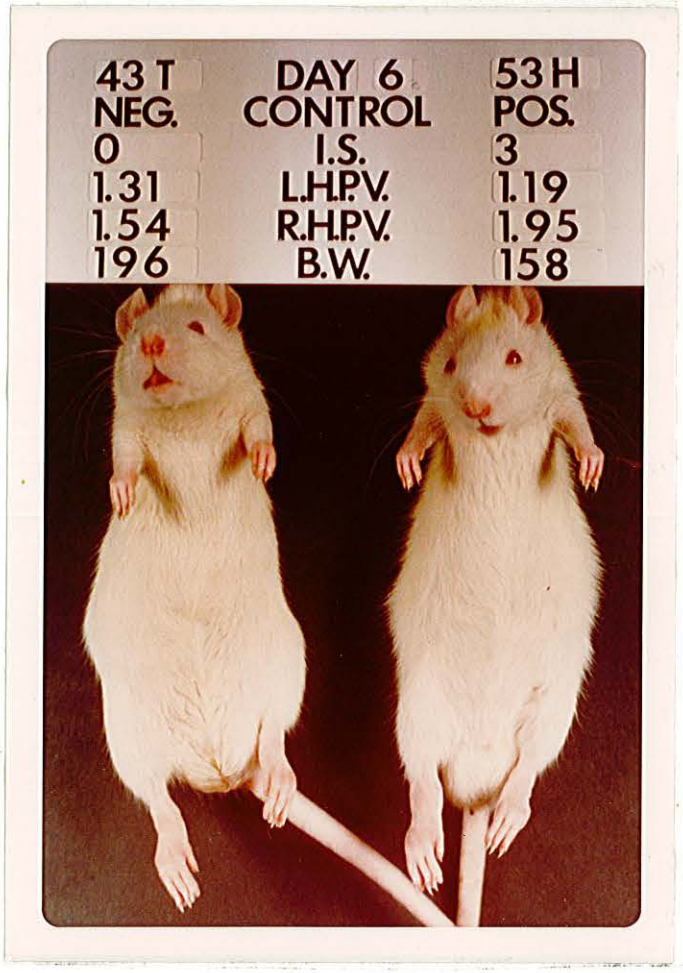


a.-- 43T vs. 52T.

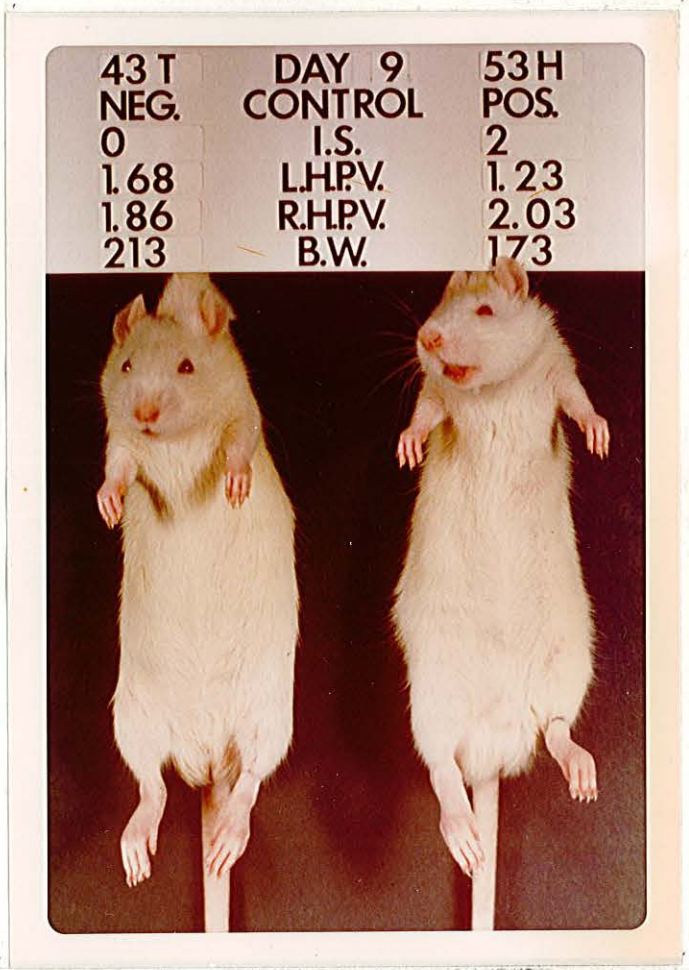


b.-- 43T vs. 49H.

Figure 13.-- Negative vs. Positive Control on Day 27.

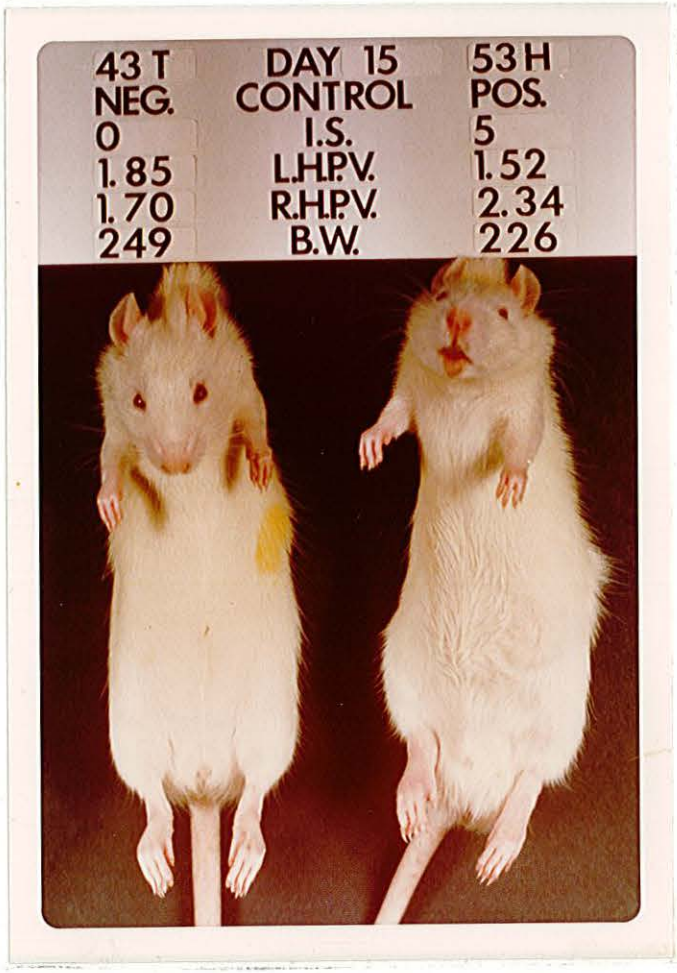


a.-- 43T vs. 53H on day 6.

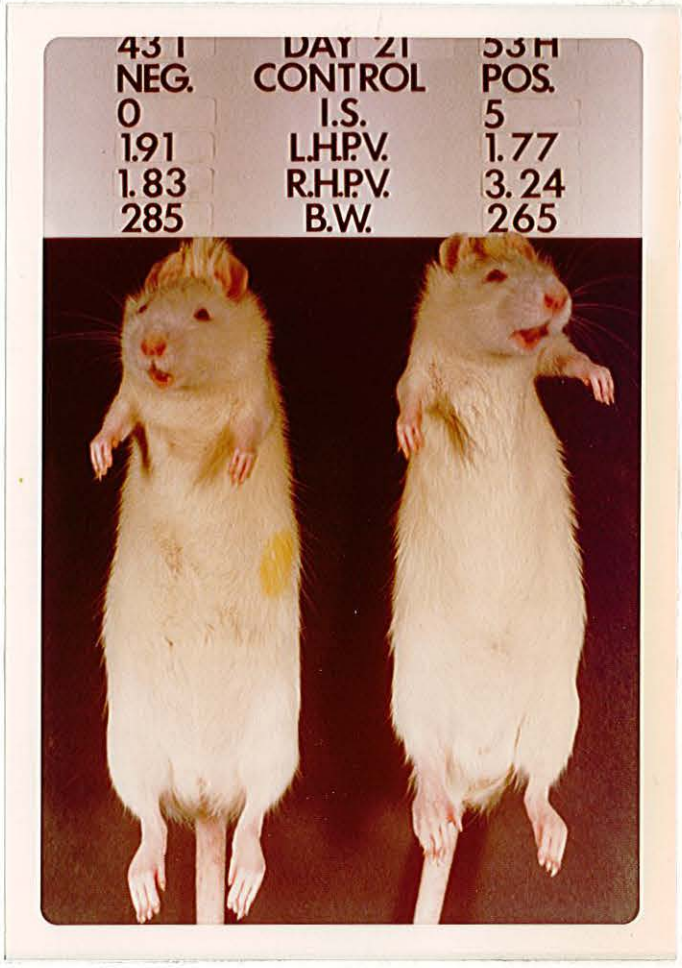


b.-- 43T vs. 53H on day 9.

Figure 14.-- Negative vs. Positive Control on Days 6 and 9.

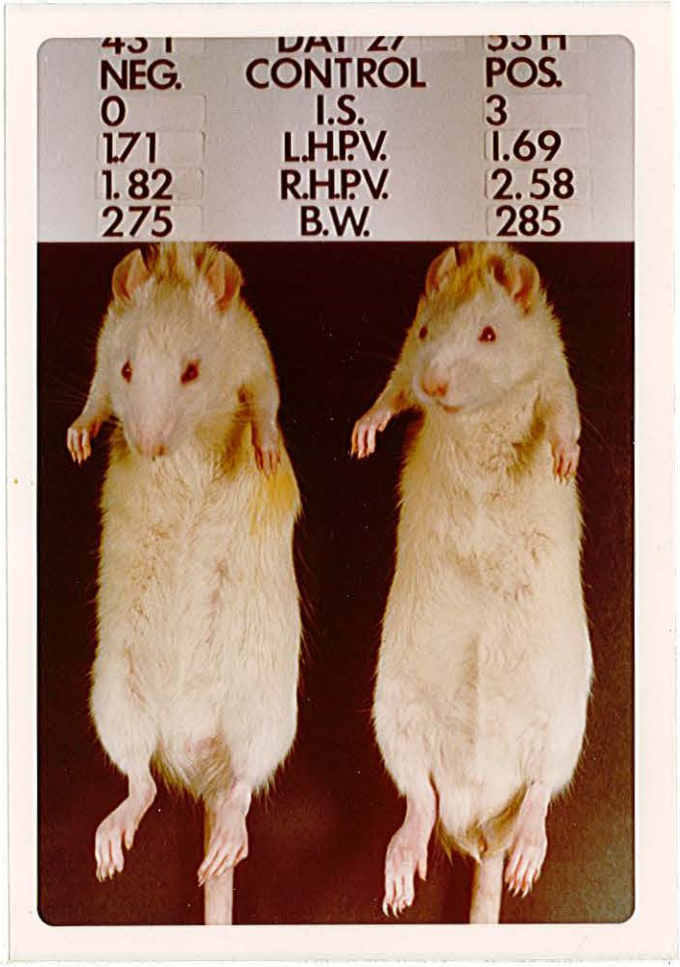


a.-- 43T vs. 53H on day 15.



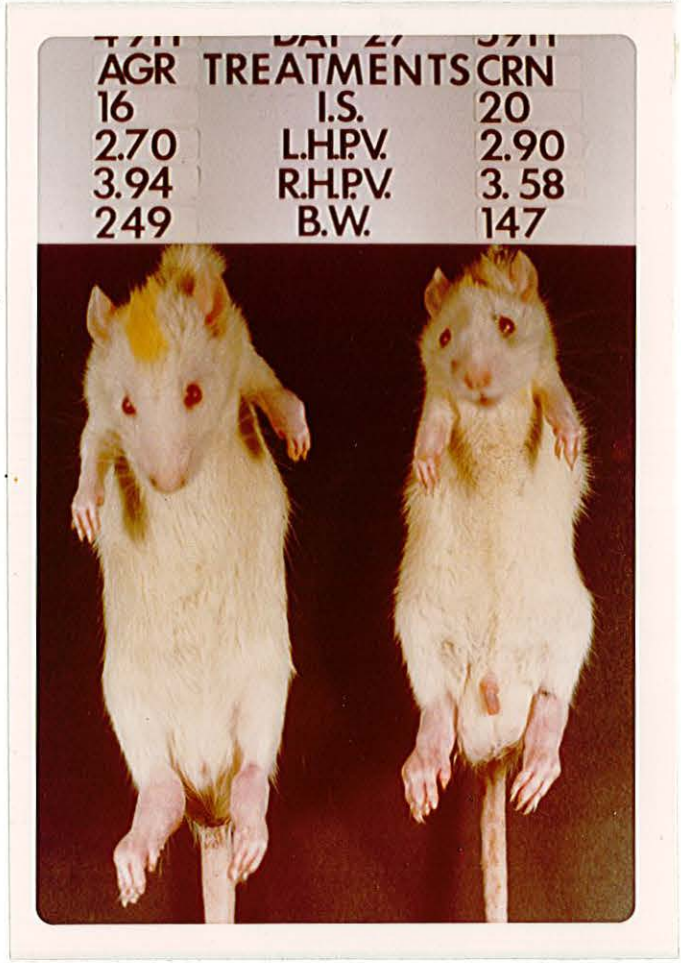
b.-- 43T vs. 53H on day 21.

Figure 15.-- Negative vs. Positive Control on Days 15 and 21.

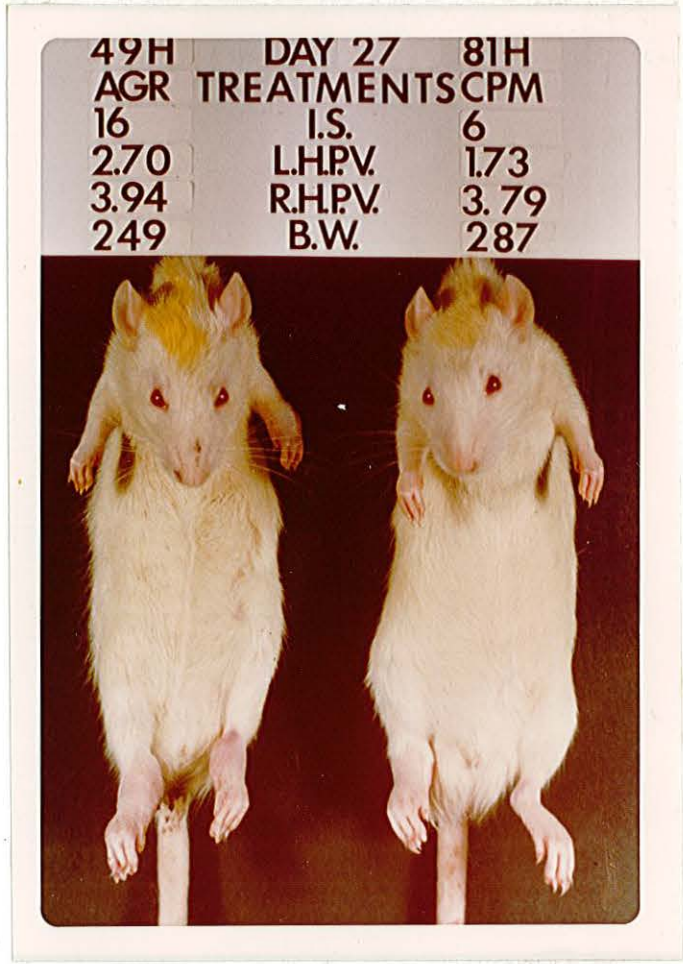


43T vs. 53H.

Figure 16.-- Negative vs. Positive Control on Day 27.

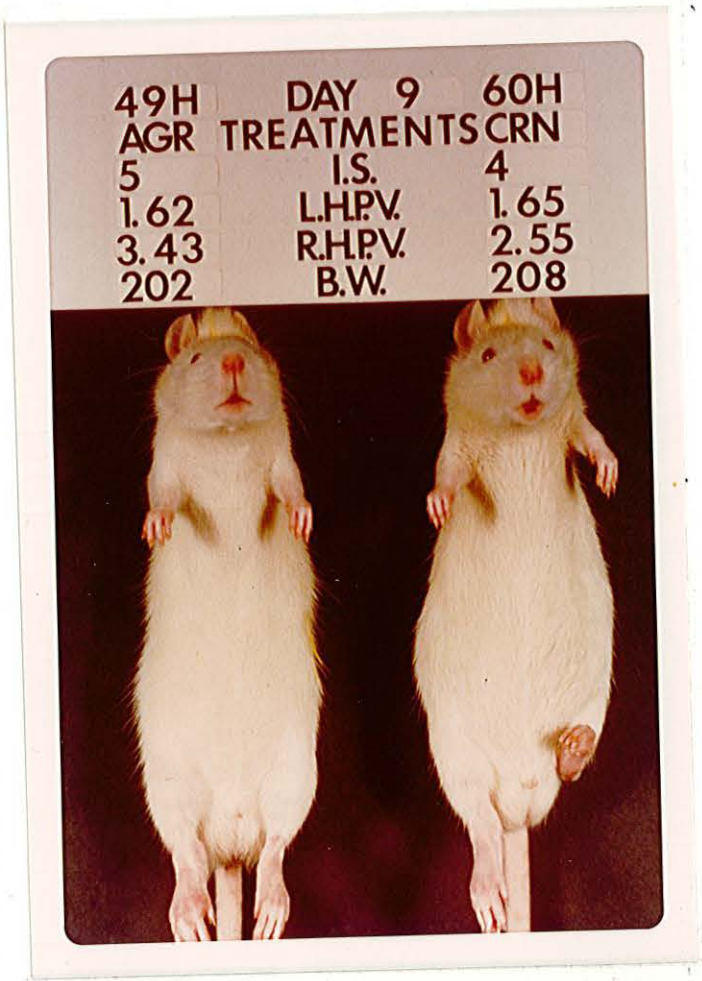


a.-- Agar vs. Cryogenine on Day 27.

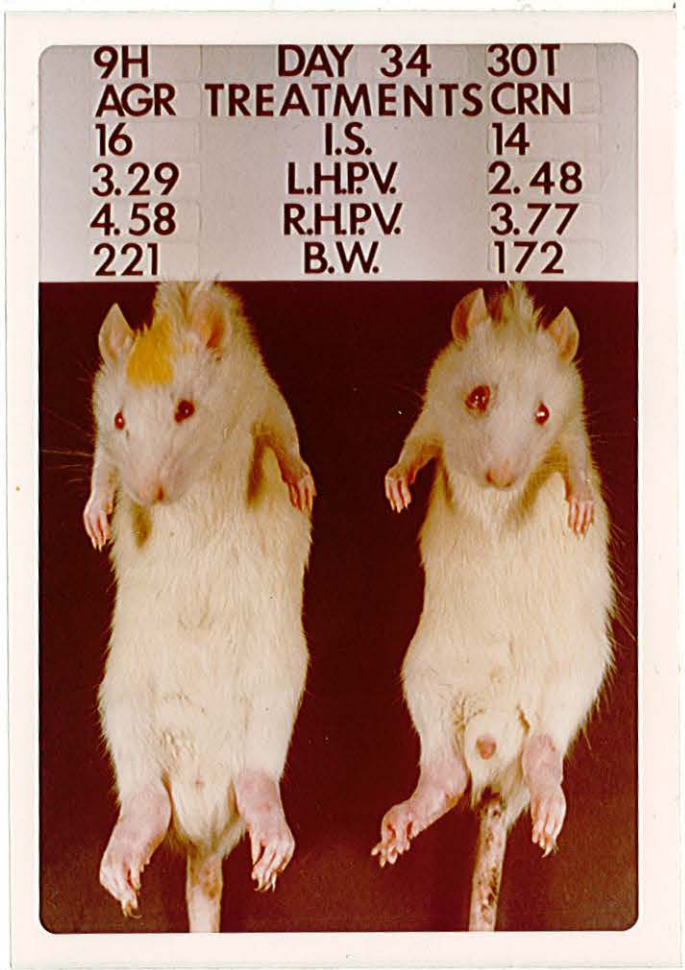


b.-- Agar vs. Cyclophosphamide on Day 27.

Figure 17.-- Post-Treatment Effects of Cryogenine and Cyclophosphamide on Developing AA.



a.-- Agar vs. Cryogenine on Day 9.



b.-- Agar vs. Cryogenine on Day 34.

Figure 18.-- Effects of Cryogenine Treatment on Developing and Established AA.

all treatment groups in terms of body weight (Table I)[1], inflammogram score (Table III), inoculated (right) hindpaw volume (Table VII), and non-inoculated (left) hindpaw volume (Table XI). Beginning on day 3, negative control animals (doubly housed) weighed significantly more ($P < .01$) than positive control animals. Negative controls (singly housed) weighed significantly more than positive controls beginning on day 9 ($P < .05$). A significant difference for both negative control groups continued (relative to the positive control group) throughout the balance of the experimental period. On day 30, the overall mean gain in body weight for the negative control animals (singly housed) was 170 gm versus 94 gm for the positive control group.

The mean inflammogram score for positive control animals during experimentation (Tables III-IV) followed a biphasic pattern as shown in Fig. 20[2]. The first phase (days 1-9) reached a peak on day 9. The second phase (days 12-30) reached a peak on day 21 and began to recede towards the 30th day.

The mean inoculated hindpaw volume for positive control animals during experimentation (Tables VII-VIII) followed a biphasic response roughly equivalent to that of

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[1] For convenience, Tables I-XIV are grouped together on pages 69-82.

[2] For convenience, Figs. 19-22 are grouped together on pages 83-90 and Figs. 23-26 on pages 100-107.

the mean inflammogram score as shown in Fig. 21. The inoculated hindpaw for the positive control group reached a peak in volume on day 9 (first phase). The volume then increased again to a maximum on day 24 (second phase) and began to recede towards the 30th day.

The non-inoculated hindpaw volume for positive control animals during experimentation (Tables XI-XII) followed a monophasic response as shown in Fig. 22. This response began on day 15, reached a maximum on day 21 and began to recede towards the 30th day.

Body weights for cryogenine, phenylbutazone and hydrocortisone-treated groups were never significantly different from positive control group weights (Tables I-II), excepting for the hydrocortisone group on day 9 (significantly higher, $P < .05$). This lack of significance was noted for the cyclophosphamide-treated group only through the 15th day. On day 18 the cyclophosphamide-treated group showed a significantly greater ($P < .05$) mean body weight when compared to the positive control group. This difference remained significant throughout the experimental period.

The first phase (days 1-9) of the inflammogram response was significantly depressed on day 9 by cryogenine and on days 3-9 by phenylbutazone (Table III). Hydrocortisone and cyclophosphamide did not significantly reduce the inflammogram score during the first phase (Table III).

Cyclophosphamide actually appeared to enhance inflammation on day 3 (Table III). The second phase (days 12-30) of the inflammogram response was not significantly depressed by cryogenine, phenylbutazone or hydrocortisone on any day, whereas cyclophosphamide caused a significant lowering on days 15-30 (Tables III-IV).

The first phase of the inoculated hindpaw swelling was significantly lowered by cryogenine on days 3,6,9 and 12 and on days 3,6 and 9 by phenylbutazone (Table VII). Hydrocortisone and cyclophosphamide did not significantly reduce the inoculated hindpaw swelling during the first phase (Table VII). The second phase (days 15-30) was not significantly reduced by cryogenine, phenylbutazone or hydrocortisone. Cyclophosphamide significantly reduced the second phase only on day 21 (Table VIII).

The monophasic response (days 15-30) of the non-inoculated hindpaw was not significantly decreased by either cryogenine, phenylbutazone or hydrocortisone (Tables XI-XII). Cyclophosphamide significantly reduced this phase on days 15-24 (Tables XI-XII).

Appendix D lists the data collected during the first 13 days of developing AA for "water comsumption" and "urine voided" of negative (NC) and positive (PC) controls and cryogenine (CGN) treated animals. The values listed under "water consumption" represent the mean difference in water bottle (containing water) weights for a particular group

recorded between 24 hr intervals. "Urine voided" represents the mean volume of urine collected at 24 hr intervals for a particular group.

Table I.-- Drug Effects on Body Weight in Developing Adjuvant Arthritis

Treatment Group	Mean Body Weight, gm ±S.E.					
	Day 0	Day 3	Day 6	Day 9	Day 12	Day 15
Negative Control (singly housed)	144 ± 5	163 ± 7	180 ± 7	207 ± 8[a]	226 ± 8[b]	248 ± 10[b]
Negative Control (doubly housed)	152 ± 3	166 ± 4[b]	189 ± 5[b]	213 ± 6[c]	235 ± 7[c]	256 ± 8[c]
Positive Control	142 ± 4	148 ± 4	163 ± 5	179 ± 5	188 ± 7	198 ± 9
Cryogenine	140 ± 5	140 ± 5	158 ± 4	175 ± 5	182 ± 5	191 ± 5
Phenylbutazone	148 ± 5	153 ± 5	174 ± 6	194 ± 7	210 ± 8	220 ± 8
Hydrocortisone	155 ± 3	159 ± 4	176 ± 4	194 ± 4[a]	198 ± 4	198 ± 6
Cyclophosphamide	155 ± 2	154 ± 4	171 ± 4	187 ± 5	203 ± 6	219 ± 6

Significantly different from Positive Control values; [a] $P < .05 \rightarrow .01$; [b] $P < .01 \rightarrow .001$; [c] $P < .001$.

Table II.-- Drug Effects on Body Weight in Developing Adjuvant Arthritis

Treatment Group	Mean Body Weight, gm ±S.E.				
	Day 18	Day 21	Day 24	Day 27	Day 30
Negative Control (singly housed)	267 ± 11[b]	285 ± 12[b]	287 ± 15[a]	302 ± 17[a]	314 ± 19[a]
Negative Control (doubly housed)	280 ± 7[c]	291 ± 10[c]	308 ± 11[c]	331 ± 10[c]	344 ± 10[c]
Positive Control	199 ± 11	204 ± 14	207 ± 17	214 ± 18	236 ± 19
Cryogenine	195 ± 8	200 ± 11	194 ± 12	204 ± 14	217 ± 18
Phenylbutazone	218 ± 9	225 ± 11	236 ± 10	247 ± 11	260 ± 12
Hydrocortisone	199 ± 8	201 ± 11	203 ± 13	219 ± 9	226 ± 11
Cyclophosphamide	229 ± 8[a]	243 ± 9[a]	256 ± 10[a]	272 ± 12[a]	295 ± 13a

Significantly different from Positive Control values: [a] $P < .05 > .01$; [b] $P < .01 - > .001$; [c] $P < .001$.

Table III.-- Drug Effects on Inflammogram Scoring in Developing Adjuvant Arthritis

Treatment Group	Mean Inflammogram Score ±S.E.					
	Day 0	Day 3	Day 6	Day 9	Day 12	Day 15
Negative Control (singly housed)	0	0	0	0	0	0
Negative Control (doubly housed)	0	0	0	0	0	0
Positive Control	0	5.2 ± 0.3	6.1 ± 0.6	6.3 ± 0.6	10.2 ± 2.0	14.8 ± 2.7
Cryogenine	0	4.6 ± 0.3	4.8 ± 0.4	4.5 ± 0.4[a]	8.3 ± 1.7	13.2 ± 2.7
Phenylbutazone	0	4.2 ± 0.2[a]	3.7 ± 0.4[b]	4.1 ± 0.5[a]	6.5 ± 1.1	15.3 ± 1.9
Hydrocortisone	0	5.8 ± 0.4	4.5 ± 0.6	4.7 ± 0.5	9.4 ± 1.4	18.6 ± 2.1
Cyclophosphamide	0	6.1 ± 0.3[a]	6.6 ± 0.4	6.7 ± 0.4	6.1 ± 0.4	6.4 ± 0.5[a]

Significantly different from Positive Control values: [a] $P < .05 - > .01$; [b] $P < .01 - > .001$.

Table IV.-- Drug Effects on Inflammogram Scoring in Developing Adjuvant Arthritis

Treatment Group	Mean Inflammogram Score ±S.E.				
	Day 18	Day 21	Day 24	Day 27	Day 30
Negative Control (singly housed)	0	0	0	0	0
Negative Control (doubly housed)	0	0	0	0	0
Positive Control	17.6 ± 3.0	18.0 ± 2.4	16.4 ± 3.0	15.3 ± 2.9	14.6 ± 3.3
Crycgenine	16.7 ± 2.7	18.0 ± 2.4	17.1 ± 2.8	14.9 ± 2.0	14.3 ± 2.2
Phenylbutazone	17.6 ± 2.4	15.6 ± 2.1	14.2 ± 1.9	13.0 ± 1.8	12.1 ± 2.1
Hydrocortisone	19.0 ± 2.0	19.8 ± 2.2	18.1 ± 2.6	18.0 ± 1.7	17.8 ± 1.5
Cyclophosphamide	9.0 ± 2.0[a]	10.4 ± 2.4[a]	4.8 ± 0.7[b]	5.0 ± 0.7[b]	6.4 ± 1.8[a]

Significantly different from Positive Control values: [a] $P < .05 - > .01$; [b] $P < .01 - > .001$.

Table V.-- Drug Effects on Inflammogram Scoring in Developing Adjuvant Arthritis

Treatment Group	Percent Change in Inflammogram Score[a]					
	Day 0	Day 3	Day 6	Day 9	Day 12	Day 15
Negative Control (singly housed)	---	---	---	---	---	---
Negative Control (doubly housed)	---	---	---	---	---	---
Positive Control	---	---	---	---	---	---
Cryogenine	---	-12	-21	-28	-19	-11
Phenylbutazone	---	-19	-39	-35	-36	+ 3
Hydrocortisone	---	+12	-26	-25	- 8	+26
Cyclophosphamide	---	+17	+ 8	+ 6	-40	-57

[a] Percent change calculated from Positive Control values.

Table VI.-- Drug Effects on Inflammogram Scoring in Developing Adjuvant Arthritis

Treatment Group	Percent Change in Inflammogram Score[a]				
	Day 18	Day 21	Day 24	Day 27	Day 30
Negative Control (singly housed)	---	---	---	---	---
Negative Control (doubly housed)	---	---	---	---	---
Positive Control	---	---	---	---	---
Cryogenine	- 5	0	+ 4	- 3	- 2
Phenylbutazone	0	-13	-13	-15	-17
Hydrocortisone	+ 8	+10	+10	+18	+22
Cyclophosphamide	-49	-42	-71	-67	-56

[a] Percent change calculated from Positive Control values.

Table VII.-- Drug Effects on Hindpaw Swelling in Developing Adjuvant Arthritis

Treatment Group	Mean Inoculated Hindpaw Volume, ml ± S.E.					
	Day 0	Day 3	Day 6	Day 9	Day 12	Day 15
Negative Control (singly housed)	1.08±0.16	1.46±0.06[c]	1.46±0.07[c]	1.95±0.09[b]	1.86±0.11[b]	1.66±0.06[b]
Negative Control (doubly housed)	1.12±0.04	1.48±0.06[c]	1.65±0.05[c]	1.71±0.05[c]	1.74±0.05[c]	1.80±0.06[c]
Positive Control	1.03±0.05	2.76±0.10	3.17±0.20	3.37±0.24	3.26±0.24	3.41±0.30
Cryogenine	1.05±0.06	2.33±0.12[a]	2.50±0.09[b]	2.55±0.08[b]	2.59±0.13[a]	2.84±0.12
Phenylbutazone	1.16±0.04	1.97±0.07[c]	2.32±0.10[b]	2.62±0.12[a]	2.71±0.12	3.02±0.13
Hydrocortisone	1.03±0.04	2.86±0.12	3.01±0.16	3.12±0.17	3.04±0.15	3.70±0.16
Cyclophosphamide	1.18±0.03	2.86±0.16	3.18±0.11	3.34±0.16	3.09±0.16	2.91±0.18

Significantly different from Positive Control values: [a] $P < .05 - > .01$; [b] $P < .01 - > .001$; [c] $P < .001$.

Table VIII.-- Drug Effects on Hindpaw Swelling in Developing Adjuvant Arthritis

Treatment Group	Mean Inoculated Hindpaw Volume, ml ±S.E.				
	Day 18	Day 21	Day 24	Day 27	Day 30
Negative Control (singly housed)	1.86±0.06[b]	1.74±0.09[b]	1.77±0.06[b]	1.85±0.07[b]	2.01±0.10[a]
Negative Control (doubly housed)	1.87±0.04[c]	1.81±0.06[c]	1.77±0.06[c]	2.02±0.06[c]	1.91±0.06[c]
Positive Control	3.69±0.32	4.12±0.38	4.36±0.42	4.20±0.45	4.24±0.58
Cryogenine	3.20±0.11	3.73±0.20	3.97±0.21	4.00±0.28	4.10±0.32
Phenylbutazone	3.88±0.18	4.20±0.21	4.15±0.24	4.18±0.25	3.98±0.31
Hydrocortisone	4.12±0.21	4.49±0.25	4.57±0.21	5.06±0.21	5.13±0.22
Cyclophosphamide	3.27±0.25	3.12±0.16[a]	3.40±0.33	3.34±0.25	3.02±0.21

Significantly different from Positive Control values: [a] $P < .05 - > .01$; [b] $P < .01 - > .001$; [c] $P < .001$.

Table IX.-- Drug Effects on Hindpaw Swelling in Developing Adjuvant Arthritis

Treatment Group	Percent Change of Inoculated Hindpaw Volume					
	Day 0	Day 3	Day 6	Day 9	Day 12	Day 15
Negative Control (singly housed)	---	---	---	---	---	---
Negative Control (doubly housed)	---	---	---	---	---	---
Positive Control	---	---	---	---	---	---
Cryogenine	---	-26	-32	-36	-31	-25
Phenylbutazone	---	-53	-46	-38	-30	-22
Hydrocortisone	---	+ 6	- 8	-11	-10	+12
Cyclophosphamide	---	- 3	- 7	- 8	-14	-27

Table X.-- Drug Effects on Hindpaw Swelling in Developing Adjuvant Arthritis

Treatment Group	Percent Change of Inoculated Hindpaw Volume				
	Day 18	Day 21	Day 24	Day 27	Day 30
Negative Control (singly housed)	---	---	---	---	---
Negative Control (doubly housed)	---	---	---	---	---
Positive Control	---	---	---	---	---
Cryogenine	-19	-13	-12	- 7	- 5
Phenylbutazone	- 2	- 2	-10	- 5	-12
Hydrocortisone	+16	+12	+ 6	+27	+28
Cyclophosphamide	-22	-37	-33	-32	-43

Table XI.-- Drug Effects on Hindpaw Swelling in Developing Adjuvant Arthritis

Treatment Group	Mean Non-Inoculated Hindpaw Volume, ml ±S.E.					
	Day 0	Day 3	Day 6	Day 9	Day 12	Day 15
Negative Control (singly housed)	1.12±0.04	1.23±0.15	1.24±0.07	1.65±0.09	1.71±0.08	1.60±0.08
Negative Control (doubly housed)	1.19±0.05	1.30±0.04	1.36±0.05	1.46±0.05	1.49±0.03	1.63±0.06[a]
Positive Control	1.07±0.04	1.27±0.07	1.30±0.07	1.41±0.06	1.78±0.14	2.18±0.21
Cryogenine	1.07±0.04	1.23±0.07	1.18±0.07	1.30±0.06	1.44±0.13	1.72±0.16
Phenylbutazone	1.21±0.04	1.35±0.07	1.45±0.06	1.43±0.06	1.60±0.06	1.94±0.10
Hydrocortisone	1.15±0.03	1.42±0.07	1.40±0.04	1.41±0.03	1.68±0.09	2.29±0.18
Cyclophosphamide	1.30±0.93	1.28±0.06	1.34±0.03	1.33±0.04	1.44±0.04[a]	1.47±0.04[b]

Significantly different from Positive Control values: [a] $P < .05 - > .01$; [b] $P < .01 - > .0001$.

Table XII.-- Drug Effects on Hindpaw Swelling in Developing Adjuvant Arthritis

Treatment Group	Mean Non-Inoculated Hindpaw Volume, ml ±S.E.				
	Day 18	Day 21	Day 24	Day 27	Day 30
Negative Control (singly housed)	1.68±0.98	1.72±0.08	1.65±0.06	1.74±0.05	1.87±0.07
Negative Control (doubly housed)	1.68±0.05[a]	1.67±0.05[b]	1.65±0.04[b]	1.75±0.05[b]	1.67±0.08[a]
Positive Control	2.20±0.21	2.46±0.23	2.41±0.25	2.33±0.22	2.30±0.30
Cryogenine	2.09±0.20	2.60±0.30	2.69±0.33	2.65±0.33	2.43±0.35
Phenylbutazone	2.24±0.16	2.41±0.20	2.40±0.24	2.59±0.30	2.53±0.35
Hydrocortisone	2.69±0.22	2.74±0.30	2.67±0.25	2.95±0.32	3.36±0.42
Cyclophosphamide	1.61±0.03[a]	1.62±0.05[b]	1.60±0.04[a]	1.81±0.15	1.86±0.19

Significantly different from Positive Control values: [a] $P < .05 - > .01$; [b] $P < .01 - > .001$.

Table XIII.-- Drug Effects on Hindpaw Swelling in Developing Adjuvant Arthritis

Treatment Group	Percent Change of Non-Inoculated Hindpaw Volume					
	Day 0	Day 3	Day 6	Day 9	Day 12	Day 15
Negative Control (singly housed)	---	---	---	---	---	---
Negative Control (doubly housed)	---	---	---	---	---	---
Positive Control	---	---	---	---	---	---
Cryogenine	---	-20	-52	-32	-48	-42
Phenylbutazone	---	-30	+ 4	-35	-45	-34
Hydrocortisone	---	+35	+ 9	-24	-25	+ 3
Cyclophosphamide	---	+110	-83	-91	-80	-85

Table XIV.-- Drug Effects on Hindpaw Swelling in Developing Adjuvant Arthritis

Treatment Group	Percent Change of Non-Inoculated Hindpaw Volume				
	Day 18	Day 21	Day 24	Day 27	Day 30
Negative Control (singly housed)	---	---	---	---	---
Negative Control (doubly housed)	---	---	---	---	---
Positive Control	---	---	---	---	---
Cryogenine	-10	+10	+21	+25	+10
Phenylbutazone	- 9	-14	-11	+10	+ 7
Hydrocortisone	+36	+14	+13	+43	+80
Cyclophosphamide	-73	-77	-78	-60	-54

Figure 19.-- Drug Effects on Body Weight During Developing AA.

Key -- Negative Control (doubly housed) 0.25% agar 10 ml/kg ○--○;
Positive Control 0.25% agar 10 ml/kg ●—●;
Cryogenine 100 mg/kg △--△; Phenylbutazone 100 mg/kg ▲—▲;
Hydrocortisone 10 mg/kg □--□; Cyclophosphamide 6 mg/kg ■—■.

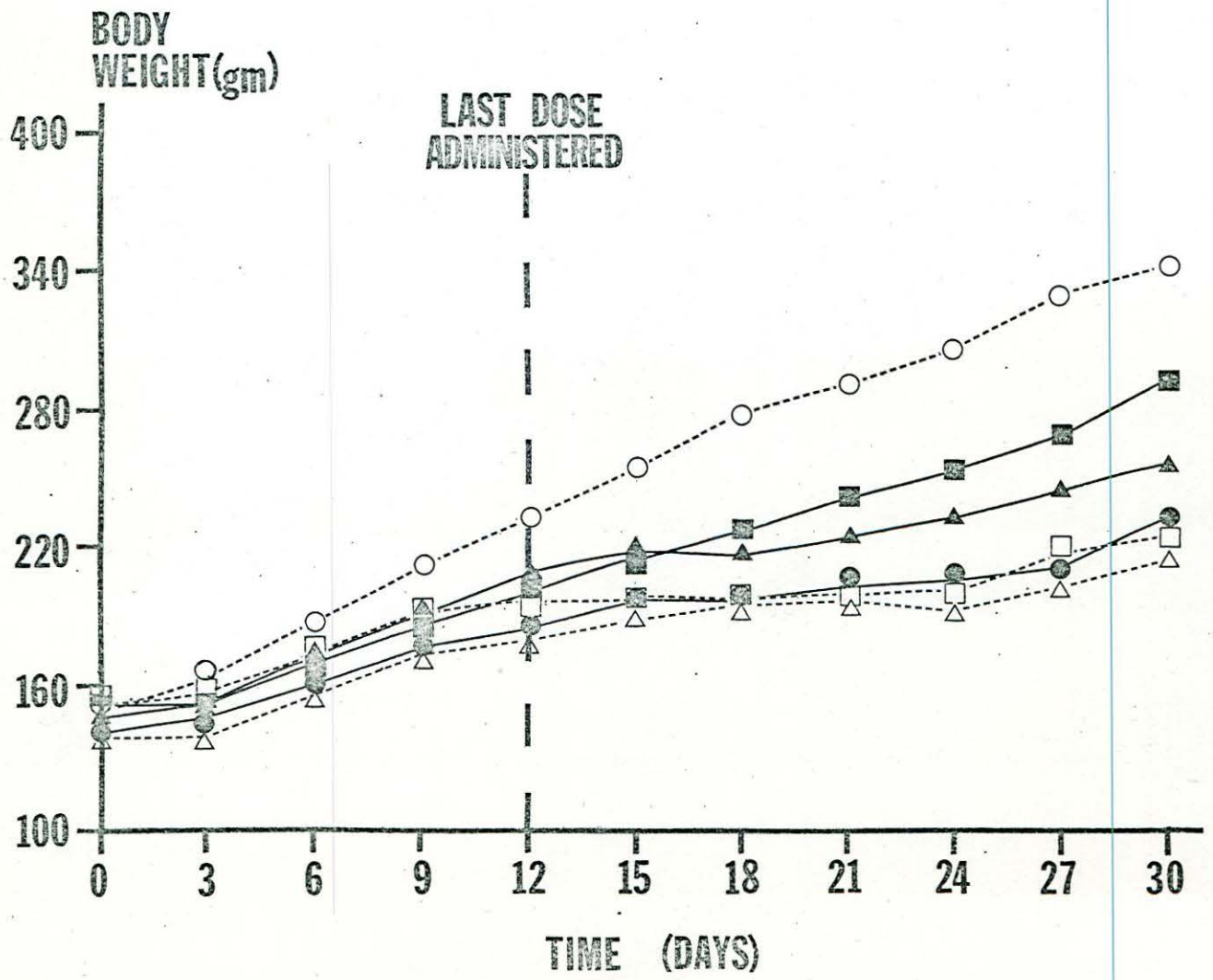

BODY
WEIGHT(gm)
LAST DOSE
ADMINISTERED
400
340
280
220
160
100
0
3
6
9
12
15
18
21
24
27
30
TIME (DAYS)

Figure 20.-- Drug Effects on Inflammogram Scoring During Developing AA.

Key -- Negative Control (doubly housed) 0.25% agar 10 ml/kg ○–○;
Positive Control 0.25% agar 10 ml/kg ●—●;
Cryogenine 100 mg/kg △–△; Phenylbutazone 100 mg/kg ▲—▲;
Hydrocortisone 10 mg/kg □–□; Cyclophosphamide 6 mg/kg ■—■.

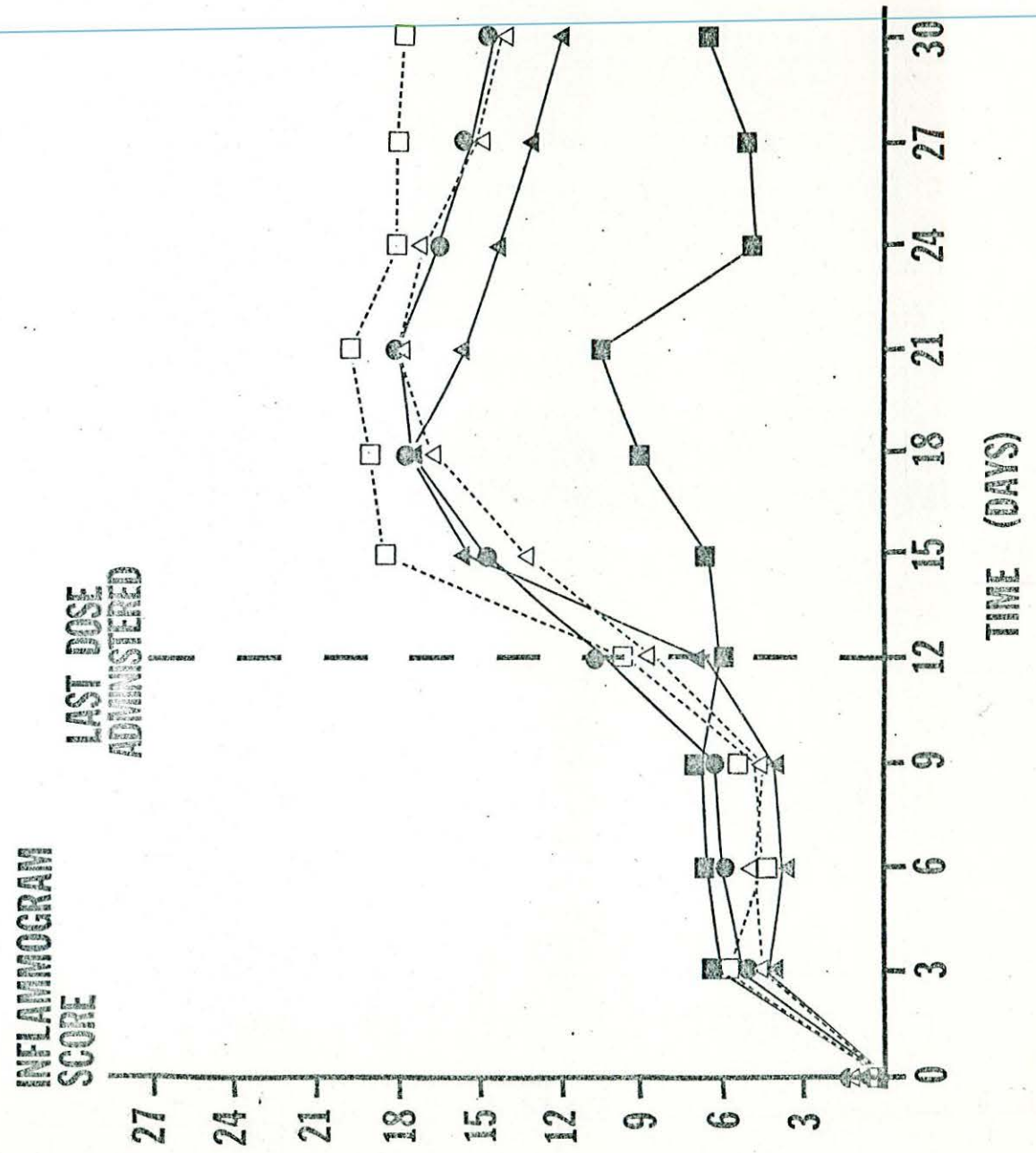

INFLAMMOGRAM
SCORE
LAST DOSE
ADMINISTERED
TIME (DAYS)
0
3
6
9
12
15
18
21
24
27
30

Figure 21.-- Drug Effects on Inoculated Hindpaw Volume During Developing AA.

Key -- Negative Control (doubly housed) 0.25% agar 10 ml/kg ○--○;
Positive Control 0.25% agar 10 ml/kg ●—●;
Cryogenine 100 mg/kg △--△; Phenylbutazone 100 mg/kg ▲—▲;
Hydrocortisone 10 mg/kg □--□; Cyclophosphamide 6 mg/kg ■—■.

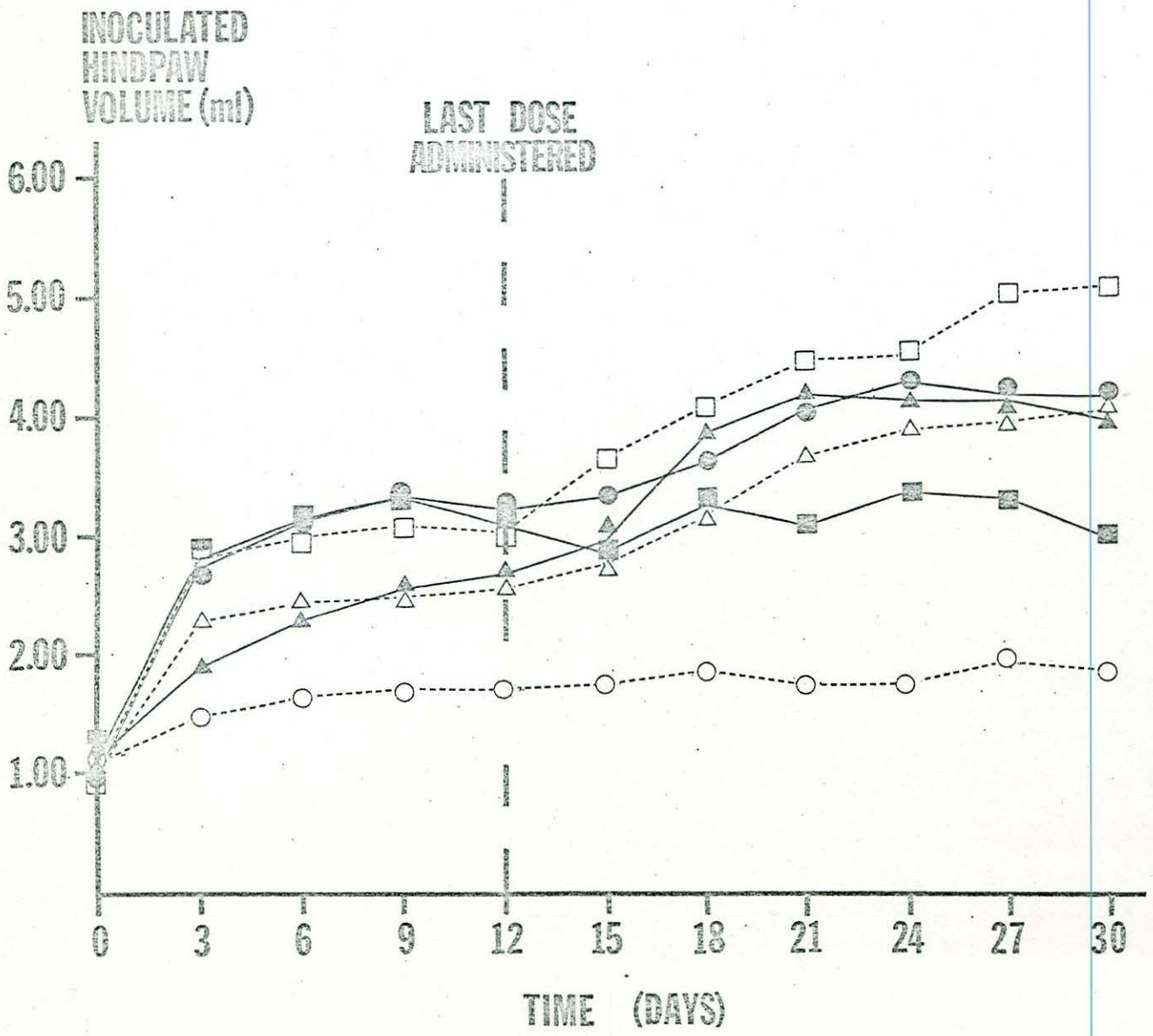
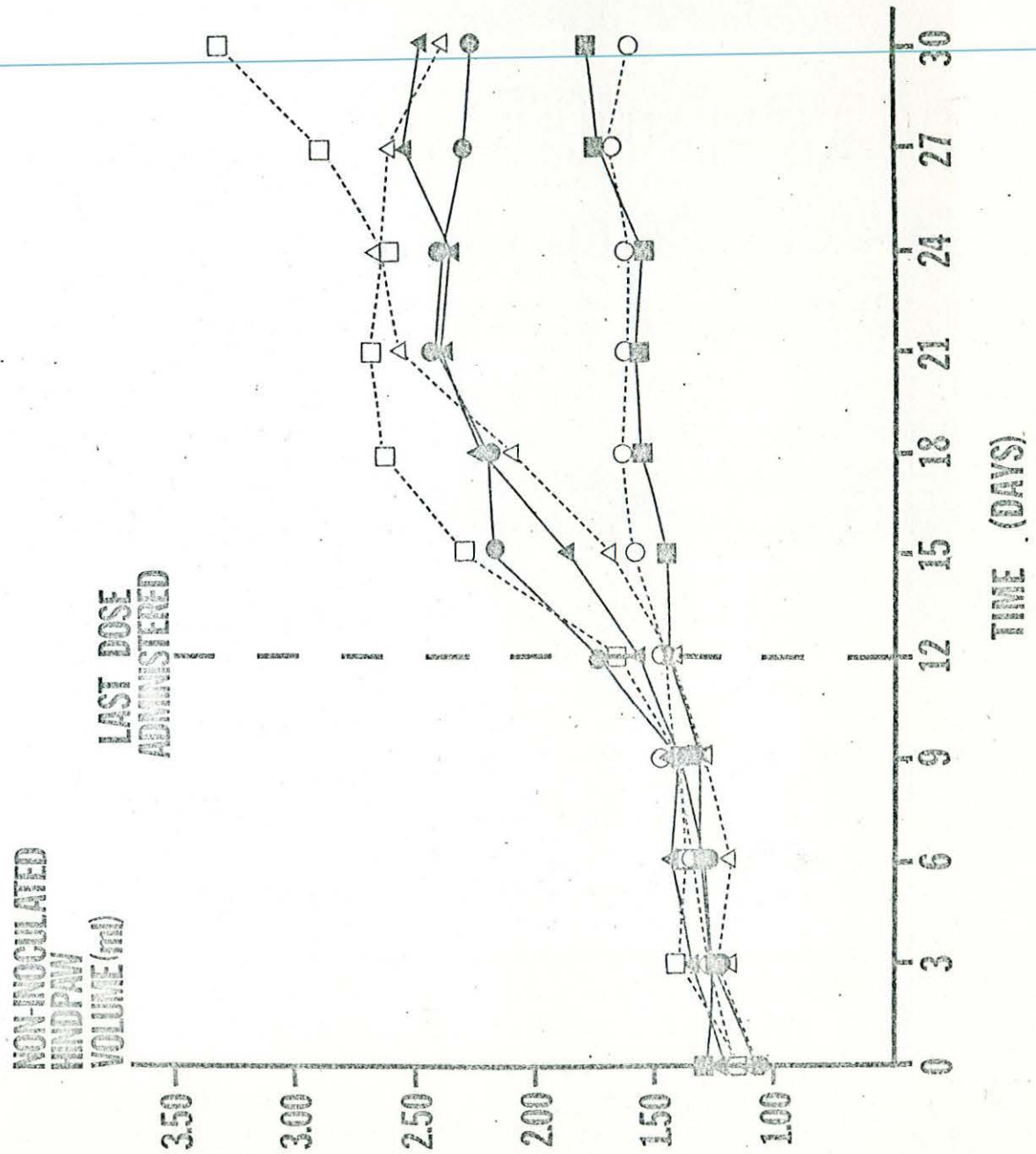

INOCULATED HINDPAW VOLUME (ml)
LAST DOSE ADMINISTERED
6.00
5.00
4.00
3.00
2.00
1.00
0
3
6
9
12
15
18
21
24
27
30
TIME (DAYS)

Figure 22.-- Drug Effects on Non-Inoculated Hindpaw Volume During Developing AA.

Key -- Negative Control (doubly housed) 0.25% agar 10 ml/kg ○--○;
Positive Control 0.25% agar 10 ml/kg ●—●;
Cryogenine 100 mg/kg △--△; Phenylbutazone 100 mg/kg ▲—▲;
Hydrocortisone 10 mg/kg □--□; Cyclophosphamide 6 mg/kg ■—■.


NON-INOCULATED HINDPAW VOLUME (ml)
3.50
3.00
2.50
2.00
1.50
1.00
LAST DOSE ADMINISTERED
0
3
6
9
12
15
18
21
24
27
30
TIME (DAYS)

2. Established Adjuvant Arthritis.-- Between days 16 and 31, the negative control animals weighed significantly more ($P < .001$) than the positive control animals (Table XV)[1] and had significantly lower inoculated ($P < .001$) and non-inoculated ($P < .01$) hindpaw volumes (Tables XVIII and XX). On day 16, (prior to actual drug treatment) all positive control and drug treatment groups were not significantly different in terms of the various experimental parameters, (Tables XV, XVI, XVIII and XX) with the exception of the cryogenine group which weighed significantly less ($P < .05$) than the positive control group.

During the experimental period, all drug treated groups remained equivalent in body weight to the positive control group. While the cryogenine-treated group weighed significantly less before and during drug administration, this difference became not significant on day 31 (Table XV).

The inflammogram score was significantly reduced ($P < .05$) on day 31 by phenylbutazone, but remained equivalent to the positive control group for all other drug-treatment groups (Table XVI).

The mean inoculated hindpaw volume was significantly reduced on days 28 and 31 by cryogenine and hydrocortisone ($P < .05$) and on days 22-31 by phenylbutazone ($P < .01$) (Table

---

[1] For convenience, Tables XV-XXI are grouped together on pages 93-99.

XVIII). Cyclophosphamide did not significantly reduce the inoculated hindpaw volume during the experimental period.

The mean non-inoculated hindpaw volume was significantly reduced only by phenylbutazone on days 22,25 and 28 ($P < .05 - > .01$). While cryogenine and hydrocortisone reduced the response, this activity was not significant (Table XX).

Table XV.-- Drug Effects on Body Weight in Established Adjuvant Arthritis

Treatment Group	Mean Body Weight, gm ±S.E.					
	Day 16	Day 19	Day 22	Day 25	Day 28	Day 31
Negative Control	261 ± 10[b]	281 ± 10[b]	298 ± 11[b]	310 ± 12[b]	323 ± 11[b]	329 ± 12[b]
Positive Control	203 ± 6	198 ± 6	194 ± 7	200 ± 8	208 ± 10	212 ± 12
Cryogenine	177 ± 7[a]	171 ± 8[a]	164 ± 9[a]	169 ± 10[a]	174 ± 13[a]	174 ± 16
Phenylbutazone	196 ± 10	187 ± 9	192 ± 12	208 ± 12	209 ± 14	231 ± 15
Hydrocortisone	192 ± 7	193 ± 7	203 ± 8	202 ± 7	220 ± 9	233 ± 11
Cyclophosphamide	186 ± 6	180 ± 7	178 ± 9	181 ± 11	184 ± 13	204 ± 14

Significantly different from Positive Control values: [a] $P < .05 - > .01$; [b] $P < .001$.

Table XVI.-- Drug Effects on Inflammogram Scoring in Established Adjuvant Arthritis

Treatment Group	Mean Inflammogram Score ±S.E.					
	Day 16	Day 19	Day 22	Day 25	Day 28	Day 31
Negative Control	0	0	0	0	0	0
Positive Control	18.3±2.6	20.3±2.0	19.4±1.5	20.1±1.5	19.9±1.6	20.1±1.7
Cryogenine	21.9±2.7	21.9±2.7	20.1±2.3	18.3±2.5	18.5±2.7	16.1±3.0
Phenylbutazone	20.7±3.0	19.6±2.5	18.4±2.0	16.6±2.0	14.4±2.2	13.6±1.8[a]
Hydrocortisone	19.2±2.5	21.4±2.3	19.9±2.4	19.0±2.3	18.1±2.4	16.4±1.7
Cyclophosphamide	18.1±2.3	19.4±2.0	19.3±1.9	16.5±2.6	18.0±2.9	16.8±3.0

Significantly different from Positive Control values: [a] $P < .05 - > .01$.

Table XVII.-- Drug Effects on Inflammogram Scoring in Established Adjuvant Arthritis

Treatment Group	Percent Change in Inflammogram Score[a]					
	Day 16	Day 19	Day 22	Day 25	Day 28	Day 31
Negative Control	---	---	---	---	---	---
Positive Control	---	---	---	---	---	---
Cryogenine	---	+ 8	+ 4	- 9	- 7	-20
Phenylbutazone	---	- 3	- 5	-17	-28	-32
Hydrocortisone	---	+ 5	+ 2	- 5	- 9	-18
Cyclophosphamide	---	- 4	0	-18	-10	-16

[a] Percent change calculated from Positive Control values.

Table XVIII.-- Drug Effects on Hindpaw Swelling in Established Adjuvant Arthritis

Treatment Group	Mean Inoculated Hindpaw Volume, ml ±S.E.					
	Day 16	Day 19	Day 22	Day 25	Day 28	Day 31
Negative Control	1.75±0.05[c]	1.75±0.03[c]	2.01±0.06[c]	1.91±0.05[c]	1.89±0.07[c]	1.86±0.05[c]
Positive Control	3.59±0.18	3.99±0.20	4.20±0.22	4.57±0.25	4.56±0.27	4.63±0.25
Cryogenine	3.80±0.23	4.02±0.26	3.63±0.24	3.78±0.32	3.71±0.26[a]	3.53±0.38[a]
Phenylbutazone	3.90±0.22	3.67±0.17	3.18±0.13[b]	3.12±0.18[c]	3.01±0.17[c]	3.20±0.21[c]
Hydrocortisone	3.87±0.22	4.00±0.21	3.96±0.24	3.91±0.25	3.74±0.22[a]	3.73±0.27[a]
Cyclophosphamide	3.54±0.24	4.03±0.25	4.03±0.32	4.10±0.39	3.98±0.35	3.72±0.38

Significantly different from Positive Control values: [a] $P < .05 - > .01$; [b] $P < .01 - > .001$; [c] $P < .001$.

Table XIX.-- Drug Effects on Hindpaw Swelling in Established Adjuvant Arthritis

Treatment Group	Percent Change of Inoculated Hindpaw Volume					
	Day 16	Day 19	Day 22	Day 25	Day 28	Day 31
Negative Control	---	---	---	---	---	---
Positive Control	---	---	---	---	---	---
Cryogenine	---	+ 3	-19	-24	-25	-33
Phenylbutazone	---	-13	-37	-46	-50	-45
Hydrocortisone	---	- 2	-10	-22	-28	-30
Cyclophosphamide	---	+ 2	- 6	-15	-18	-28

Table XX.-- Drug Effects on Hindpaw Swelling in Established Adjuvant Arthritis

Treatment Group	Mean Non-Inoculated Hindpaw Volume, ml ±S.E.					
	Day 16	Day 19	Day 22	Day 25	Day 28	Day 31
Negative Control	1.63±0.06[b]	1.64±0.06[c]	1.79±0.07[c]	1.75±0.09[b]	1.66±0.04[c]	1.69±0.06[b]
Positive Control	2.58±0.26	2.92±0.24	3.03±0.25	3.07±0.30	3.07±0.28	3.12±0.33
Cryogenine	2.85±0.29	2.79±0.24	2.60±0.19	2.60±0.23	2.47±0.19	2.56±0.26
Phenylbutazone	2.82±0.33	2.62±0.20	2.35±0.14[a]	2.30±0.11[a]	2.18±0.13[a]	2.41±0.15
Hydrocortisone	2.67±0.29	2.77±0.26	2.81±0.22	2.87±0.32	2.78±0.20	2.99±0.27
Cyclophosphamide	2.65±0.22	2.88±0.18	2.81±0.20	3.02±0.27	3.32±0.36	3.04±0.33

Significantly different from Positive Control values: [a] $P < .05 - > .01$; [b] $P < .01 - > .001$; [c] $P < .001$.

Table XXI.-- Drug Effects on Hindpaw Swelling in Established Adjuvant Arthritis

	Percent Change of Non-Inoculated Hindpaw Volume					
Treatment Group	Day 16	Day 19	Day 22	Day 25	Day 28	Day 31
Negative Control	---	---	---	---	---	---
Positive Control	---	---	---	---	---	---
Cryogenine	---	- 3	-21	-23	-30	-27
Phenylbutazone	---	-19	-40	-44	-52	-40
Hydrocortisone	---	-11	-14	-13	-18	- 9
Cyclophosphamide	---	- 1	-11	- 1	+16	- 3

Figure 23.-- Drug Effects on Body Weight During Established AA.

Key -- Negative Control (doubly housed) 0.25% agar 10 ml/kg ○--○;
Positive Control 0.25% agar 10 ml/kg ●—●;
Cryogenine 100 mg/kg △--△; Phenylbutazone 100 mg/kg ▲—▲;
Hydrocortisone 10 mg/kg □--□; Cyclophosphamide 6 mg/kg ■—■.

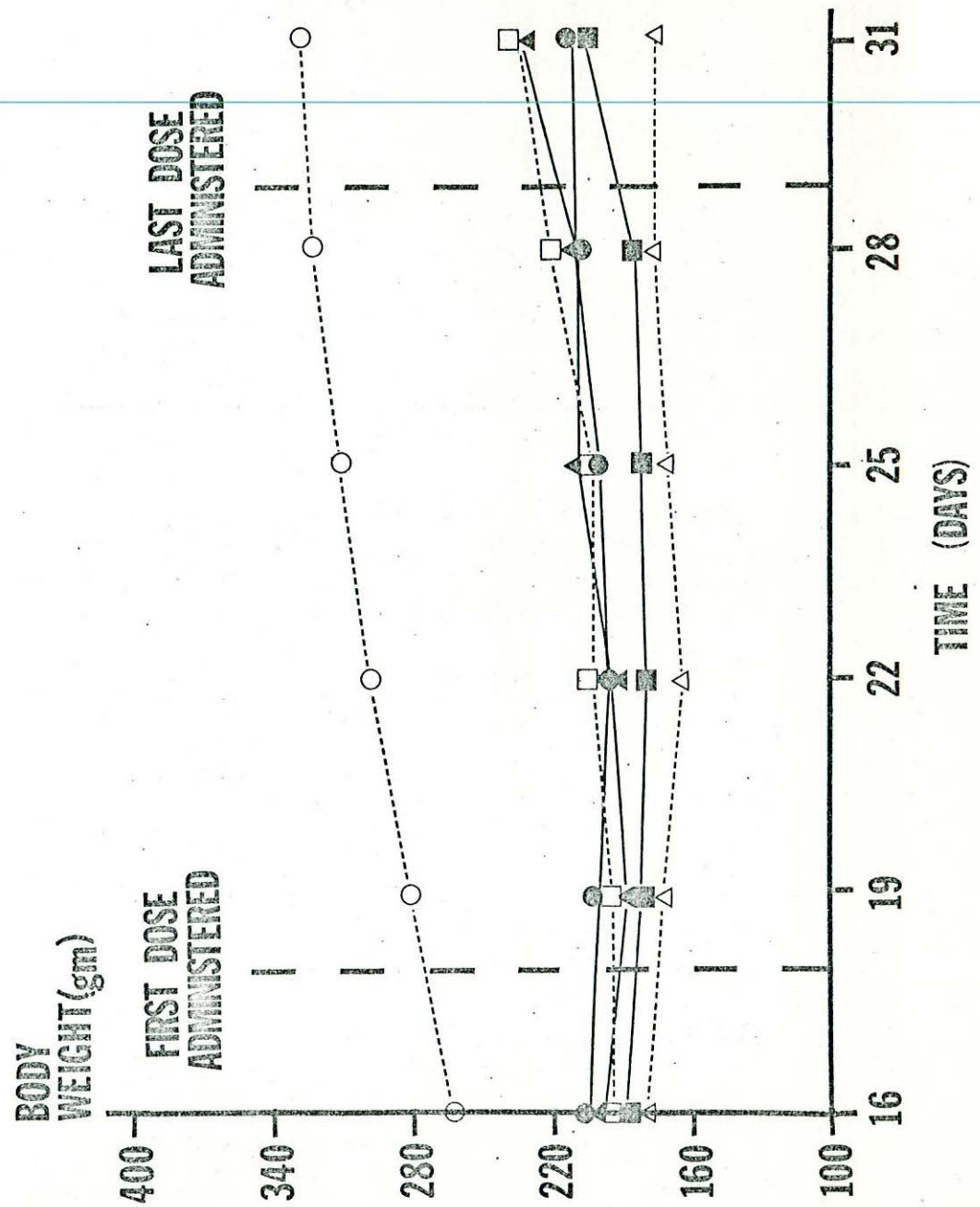

BODY WEIGHT (gm)
400
340
280
220
160
100
FIRST DOSE ADMINISTERED
LAST DOSE ADMINISTERED
16
19
22
25
28
31
TIME (DAYS)

Figure 24.-- Drug Effects on Inflammogram Score During Established AA.

Key -- Negative Control (doubly housed) 0.25% agar 10 ml/kg ○—○;
Positive Control 0.25% agar 10 ml/kg ●—●;
Cryogenine 100 mg/kg △—△; Phenylbutazone 100 mg/kg ▲—▲;
Hydrocortisone 10 mg/kg □—□; Cyclophosphamide 6 mg/kg ■—■.

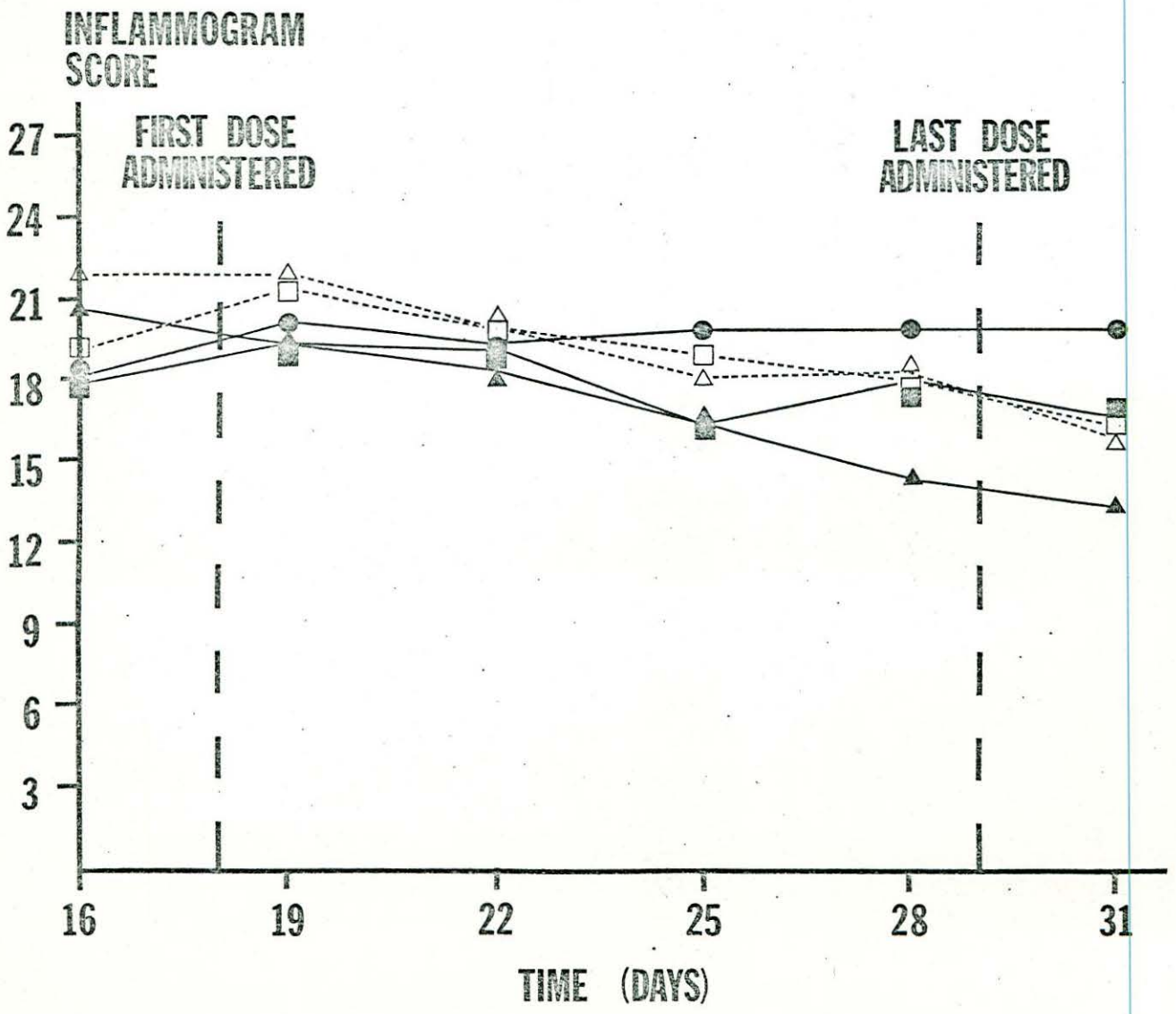

INFLAMMOGRAM SCORE
27
24
21
18
15
12
9
6
3
FIRST DOSE ADMINISTERED
LAST DOSE ADMINISTERED
16
19
22
25
28
31
TIME (DAYS)

Figure 25.-- Drug Effects on Inoculated Hindpaw Volume During Established AA.

Key -- Negative Control (doubly housed) 0.25% agar 10 ml/kg ○–○;
Positive Control 0.25% agar 10 ml/kg ●—●;
Cryogenine 100 mg/kg △–△; Phenylbutazone 100 mg/kg ▲—▲;
Hydrocortisone 10 mg/kg □–□; Cyclophosphamide 6 mg/kg ■—■.

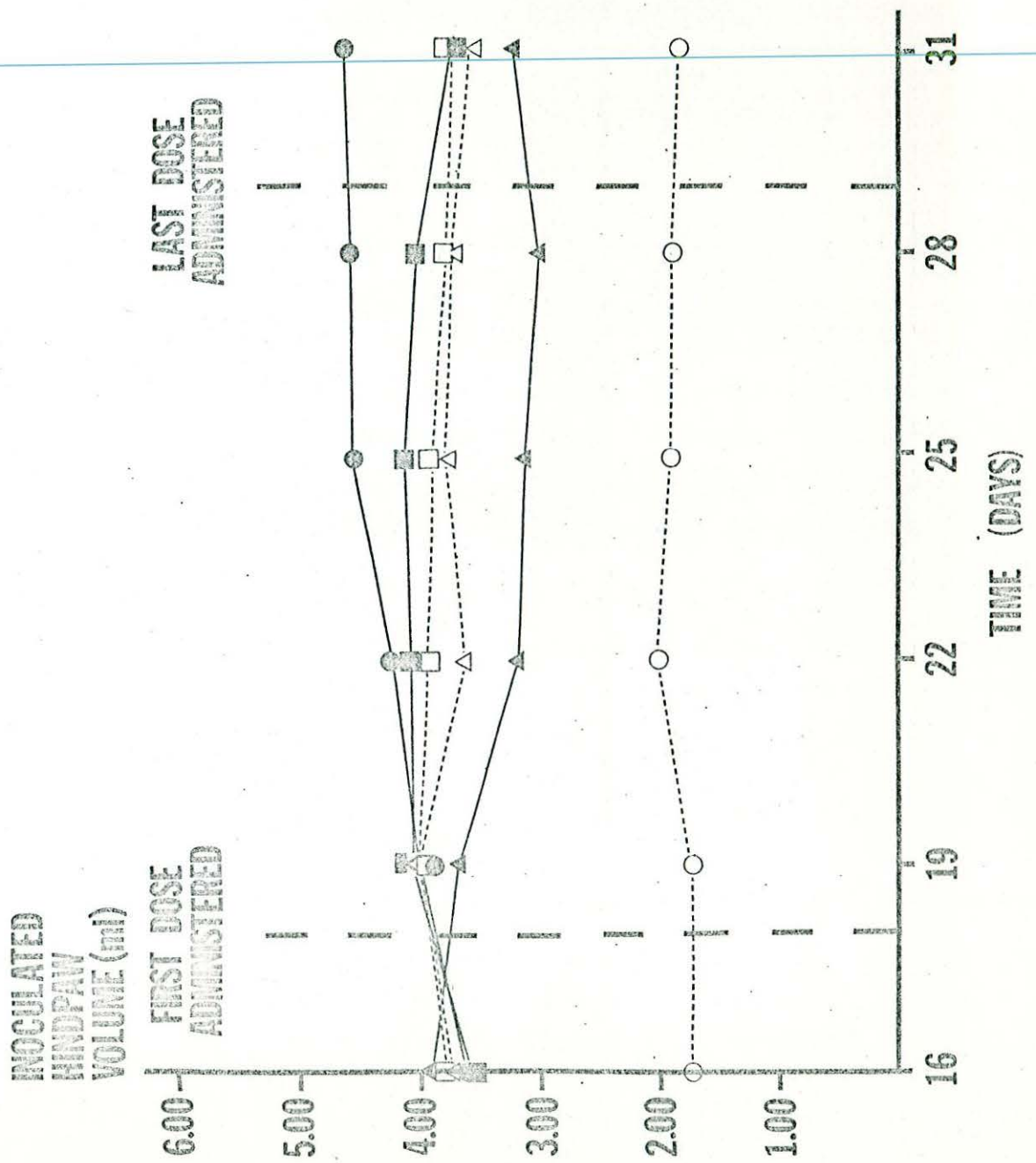
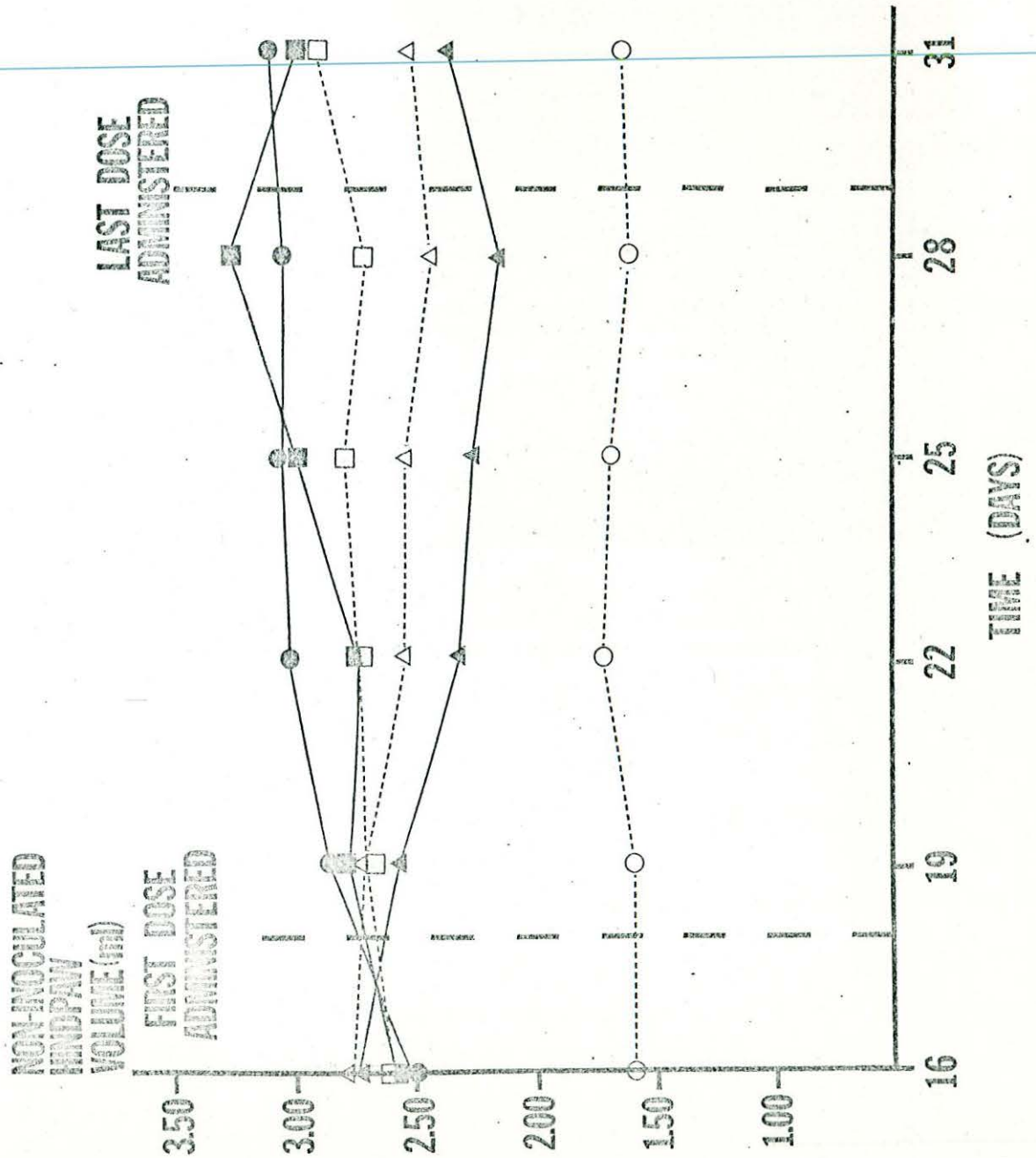

INOCULATED HINDPAW VOLUME (ml)
6.00
5.00
4.00
3.00
2.00
1.00
FIRST DOSE ADMINISTERED
LAST DOSE ADMINISTERED
16
19
22
25
28
31
TIME (DAYS)

Figure 26.-- Drug Effects on Non-Inoculated Hindpaw Volume During Established AA.

Key -- Negative Control (doubly housed) 0.25% agar 10 ml/kg ○--○;
Positive Control 0.25% agar 10 ml/kg ●—●;
Cryogenine 100 mg/kg △--△; Phenylbutazone 100 mg/kg ▲—▲;
Hydrocortisone 10 mg/kg □--□; Cyclophosphamide 6 mg/kg ■—■.


NON-MODULATED
MIDPAW/
VOLUME(ml)
FIRST DOSE
ADMINISTERED
LAST DOSE
ADMINISTERED
3.50
3.00
2.50
2.00
1.50
1.00
16
19
22
25
28
31
TIME (DAYS)

DISCUSSION

The present investigation is directed towards resolving the question of whether cryogenine possesses: (i) only AI activity, or (ii) mixed AI-IS activities. A drug administered in conjunction with an ongoing immune process and which significantly prevents the development of correlated inflammatory manifestations is defined in this study as an IS drug. A drug administered in conjunction with an ongoing inflammatory process and which brings about a significant reduction in the inflammatory manifestations correlated with that process is defined here as an AI drug.

Drugs administered during developing AA (days -1 to 12) are present during the formation of immunocompetent lymphocytes and obviously not present during the subsequent establishment (days 18-29) of the immune-mediated inflammation. This statement is further supported by the observations that: (i) AA can only be transferred via sensitized lymph node cells between the 8th-14th post-inoculation days (19), and (ii) the immune-mediated inflammation never appears before 11-14 days after inoculation (4). A true IS drug administered during developing AA should significantly prevent the development of the immune-mediated inflammation.

Cyclophosphamide represents the most widely used alkylating agent in both experimental and human therapy (90). Experimental autoimmune encephalomyelitis and thyroiditis in rats and systemic lupus erythematosus and rheumatoid arthritis in man represent immune-mediated, chronic inflammatory disorders amenable to treatment with cyclophosphamide (90,91). The secondary responses seen in the inflammogram score (Fig. 20 and the non-inoculated hindpaw volumes (Fig. 22) represent quantified expressions of immune-mediated inflammation. In the present study, cyclophosphamide administered during developing AA significantly prevented the development of immune-mediated inflammation. This is evidenced in Figs. 20 and 22. These results are in agreement with those reported by Perper et al. (55). A significant reduction in the inoculated hindpaw occurred only on day 21 with cyclophosphamide (Table VIII). It was noted by Ward and Jones (15) relatively high concentrations of adjuvant remain at the site of inoculation throughout the observation period. The relatively greater inoculated hindpaw volume (compared to the non-inoculated hindpaw volume, (Tables VII vs. XI and VIII vs. XII) undoubtedly reflects protraction of the earlier non-immune-mediated, acute inflammatory reaction. Therefore, an IS drug would not be expected to be completely effective since the inflammation is only partially attributable to an immune reaction. Positive control animals grew at a reduced rate relative to negative controls during

the course of AA (Fig. 19). Since a decreased weight gain is consistent with a severe chronic inflammatory disease, and since cyclophosphamide significantly prevented the AA disease, it was also predictable to find cyclophosphamide-treated animals developing a much larger overall mean gain in body weight (cyclophosphamide-treatment = +140 gm, agar-treatment = +94 gm).

Phenylbutazone and hydrocortisone represent clinically effective AI drugs useful in a variety of chronic inflammatory disorders. Paulus et al. (92) have indicated that while the glucocorticoids cause a lymphocytopenia and are capable of modifying delayed hypersensitivity responses, this action is believed to result from a non-specific, anti-inflammatory effect rather than direct suppression of the underlying immunological phenomenon. In the present study, phenylbutazone and hydrocortisone administered during developing AA provided no significant protection against the development of the immune-mediated inflammation (Figs. 20-22). Consistent with this lack of protection was a lack of significance between body weights of positive control animals and drug treated animals. These results are in essential agreement with those of Perper et al. (55).

As previously discussed, Koserky et al. (79) have shown that cryogenine provided a relatively long lasting anti-inflammatory effect after termination of treatment in AA. In this instance, the drug was administered during the

developing period (days -1-12) and through the 20th post-inoculation day. Two explanations are possible for this long lasting effect: (i) since the drug was administered through the sensitizing period (days 1-14) and during the period of established inflammation (days 15-20), this observation was due to an unusually long acting anti-inflammatory effect, or (ii) since the drug was administered during the sensitizing period this observation was due to mixed immune suppression and anti-inflammatory effectiveness. In the present study, cryogenine administered during developing AA provided no significant protection against the development of the immune-mediated inflammation (Figs. 20-22), nor reversal of decreased body weight gain. Thus, it can be deduced that cryogenine's long lasting effect in the study by Kosersky et al. (83) was due to a specific suppression of the chronic inflammation and not to immune suppression.

Drugs administered during established AA (days 18-29) are present during the course of a chronic inflammatory reaction. The histological features include a mononuclear inflammatory cell reaction coupled with an intensive connective tissue reaction (7). The inability to transfer AA after the 14th post-inoculation day (19) suggests that this period is not associated with an immune reaction. Therefore, a true AI drug administered during established AA should significantly reduce the inflammatory symptoms. In the

present study, cryogenine, phenylbutazone and hydrocortisone significantly reduced this established inflammation in the inoculated hindpaw as shown in Fig. 25. On days 28 and 31 cryogenine reduced the inoculated hindpaw volume by -25% and -33% (Table XIX). Also on days 28 and 31 hydrocortisone reduced the inoculated hindpaw volume by -28% and -30% (Table XIX). Phenylbutazone was effective on days 22 (-37%), 25 (-46%), 28(-50%) and 31 (-45%). Moreover, phenylbutazone produced a significant lowering of the inflammogram score on day 31 (-32%) (Tables XVI and XVII), and brought about a significant reduction in the non-inoculated hindpaw on days 22 (-40%), 25 (-44%) and 28 (-52%) (Tables XX and XXI). The overall effects for all three drugs can be seen in Figs. 24-26. Cyclophosphamide did not produce any significant reduction in the established inflammation which would appear to be consistent with its immunosuppressive effects during developing AA. These results are in essential agreement with those of Perper *et al.* (55).

Since the acute inflammation which follows the hindpaw inoculation (days 1-9) does not depend upon an immune reaction, AI drugs administered during this period should reduce the inflammatory symptoms. The first phases of the inflammogram score (Fig. 20) and the inoculated hindpaw volume (Fig. 21) represent quantified expressions of this non-immune inflammatory reaction. Cryogenine and phenylbutazone significantly reduced this acute inflammation as shown in

Figs. 20 and 21. Cryogenine was active against the inoculated hindpaw volume on days 3 (-26%), 6 (-32%), 9 (-36%) and 12 (-31%). Phenylbutazone was active on days 3 (-53%), 6 (-46%), and 9 (-38%). Both drugs also significantly reduced the inflammogram score: cryogenine on day 9 (-28%) and phenylbutazone on days 3 (-19%), 6 (-39%) and 9 (-35%). Hydrocortisone and cyclophosphamide were not significantly active during the acute phase. These results are consistent with those of Waltz et al. (56) and generally confirm the AI activity of cryogenine and phenylbutazone determined during established AA. Hydrocortisone appeared to have some anti-inflammatory effect but the degree was not statistically significant. A higher daily dosage of hydrocortisone probably would have yielded a positive anti-inflammatory reaction, since hydrocortisone has been shown by Kaplan et al. (71) to reduce this response.

In summary, cryogenine at an effective anti-inflammatory dose possessed no capacity for suppressing an immune reaction of the DHS type. However, cryogenine did suppress the acute inflammation and the chronic, established inflammation during developing and established AA. This confirms previous studies theorizing cryogenine's non-immune anti-inflammatory activity.

As seen in Appendix D, during developing AA, significantly less water was consumed by both cryogenine-treated animals (CGN) ($P < .05- > .01$) and the positive control group

(PC) ($\underline{P} < .01 - > .001$) relative to the negative controls (NC). There was no significant difference in urine voided between groups. Despite the facts that lythrine is a geometric isomer of cryogenine (Fig. 1) and has been reported to possess potent diuretic activity (84), cryogenine itself does not appear to possess diuretic potential. Further studies should be done to establish this in normal animals.

CONCLUSION

The interval between the 1st-12th post-inoculation day in rat AA represents the time needed for the induction and release of immunocompetent lymphocytes. The generalized articular (non-inoculated hindpaw) and non-articular (tail and ears) inflammatory symptoms occurring between the 12th and 30th day represent manifestations of a delayed hypersensitivity reaction mediated by these immunocompetent lymphocytes. In the present investigation, cyclophosphamide administered only during this induction period, significantly prevented the development of both articular and non-articular inflammatory manifestations (Fig. 17b). This protection was also indirectly evidenced by the significant body weight gain seen in cyclophosphamide-treated animals (relative to untreated, positive control animals) between the 18th and 30th day. Cryogenine as well as clinically effective AI drugs (phenylbutazone and hydrocortisone) administered during the induction period provided no protection against the delayed immune-mediated inflammation (Fig 17a).

Since the sustained chronic inflammation present in rat AA between the 18th and 29th post-inoculation days

occurs after the sensitizing period, true AI drugs administered during this interval act only to reduce the inflammatory symptomology. On the other hand true IS drugs administered in this time period would not be expected to be effective. Cryogenine, phenylbutazone and hydrocortisone were effective during this interval, while cyclophosphamide was without any significant effect (Fig. 18b).

The inoculated hindpaw between days 1-9 presents an acute inflammatory reaction mediated by the non-specific intradermal intrusion of the mycobacterium adjuvant. Again, since this reaction is not associated with a sensitizing period, then one should expect results analogous to those for AI and IS drugs administered only during the established period (18-29). Cryogenine and phenylbutazone provided significant reduction of the inoculated hindpaw swelling during the acute period, hydrocortisone was only marginally effective, and cyclophosphamide provided no anti-inflammatory activity (Fig. 18a).

Past investigators have documented cryogenine's anti-inflammatory activity in non-immune mediated experimental inflammatory models (e.g. carrageenin pedal edema assay). The present investigation forwards the thesis that cryogenine's anti-inflammatory activity in experimental rat AA is not attributable to immune suppression, but appears to result from suppression of one or more of the non-specific mediators and/or events of the inflammatory process.

Cryogenine does not appear to have any diuretic activity during the treatment of developing AA.

APPENDIX A

Definitions of Abbreviations Used in the Text

AA = adjuvant arthritis

AGR = agar

AI = anti-inflammatory

b.p. = boiling point

BSA = bovine serum albumin

BW = body weight

^{o}C = degree Centigrade

CDF = trade name for a highly inbred rat species

CGN = cryogenine

cm = centimeter

CNS = central nervous system

CPM = cyclophosphamide

DHS = delayed hypersensitivity

dia = diameter

ESR = erythrocyte sedimentation rate

^{o}F = degree Fahrenheit

gm = gram

h = height

H = head

HEA = hen egg albumin

hr = hour

IP = intraperitoneal

IR = infrared

IS = immunosuppressive

IS = inflammogram score

kg = kilogram

lbs = pounds

LD = lethal dose

LHPV = left hindpaw volume

mcg = microgram

mg = milligram

ml = milliliter

mm = millimeter

mv = millivolt

NC = negative control

N.F. = National Formulary

P = probability

PC = positive control

pH = negative logarithm of the hydrogen ion concentration

PMNs = polymorphonuclear leukocytes

PPLO = pleuropneumonia-like organisms

r = radius

RA = rheumatoid arthritis

R_F = relative fractional distance

RHPV = right hindpaw volume

R.P.M. = revolutions per minute

S.E. = standard error

sec = second

T = tail

TLC = thin layer chromatography

APPENDIX B

Isolation and Purification of Cryogenine[1]

1. Petroleum Ether Extraction.-- A Wiley mill[2] reduced the dried overground plant[3] to a semifine powder which was defatted by continuous extraction using petroleum ether, b.p. 30-60°C and a large volume soxhlet[4]. Eight to twelve defattings (400 gm of powdered material defatted/extraction) were accomplished using the same petroleum ether solvent. The defatted material was air dried overnight.

2. Methanol Extraction.-- The material was extracted with absolute methanol continuously for 48 hr in a large volume soxhlet. Six extractions were accomplished with the same absolute methanol. The thimble solution was checked with Wagner's I for completion of extraction. The methanol

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1 Petroleum ether and methanol extractions were previously done by Stanley T. Omaye and Roy G. Knickelbein. The present work began with the methanol extract and proceeded according to the general scheme used by Omaye (85) while incorporating several modifications.

2 Arthur H. Thomas Co., Philadelphia, Pennsylvania. Standard model #3, 2 mm mesh screen.

3 S.B. Penick & Co., 520 New York Ave., Lyndhurst, New York. Heimia salicifolia Lot BKC 752.

4 Kontes Glass Co., 2809 Tenth St., Berkeley, California. Extraction apparatus, Soxhlet K-58500.

extract was poured into large evaporating dishes, placed under an exhaust hood and allowed to sit for about one year.

3. Diethyl Ether Extraction.-- One part warm (40°C) singly distilled water and one part methanol extract were mixed together, acidified to pH 2[1] (10% hydrochloric acid) and stirred for about one hour. This acidified aqueous extract was diluted approximately 100% in singly distilled water (pH re-adjusted to 2) and filtered through Celite[2] with suction. The precipitate was washed with singly distilled water producing an approximate 50% increase in the filtrate volume. The filtrate (pH re-adjusted to 2) was defatted in a liquid-liquid ether extractor[3] for at least 48 hr. Upon completion, the clear ether layer was removed and the aqueous layer basified to pH 9 (8% ammonium hydroxide).

4. Chloroform Extraction.-- The alkaline aqueous suspension was extracted in a liquid-liquid chloroform extractor[4] for 48 hr. The chloroform was evaporated *in vacuo* at 40°C (constant temperature water bath) with a rotating flash-evaporator. The resultant light yellow residue represented

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1 Corning Glass Works, Medfield, Massachusetts. Corning Scientific Instruments pH meter, model 10.

2 J.T. Baker Chemical Co., New Jersey. Celite 503, Lot No. 2-3723.

3 Ace Glass Inc., Vineland, New Jersey. Ether extraction apparatus #6840, 2000 ml.

4 Kontes Glass Co., 2809 Tenth St., Berkeley, California. Chloroform extraction apparatus K-587000.

an approximate 4% yield from the methanol extract.

5. Column Chromatography.-- The dry chloroform extract was reduced to a fine powder in a glass mortar. The powder was dissolved in a minimum volume of boiling chloroform, and passed through a Buchner filter under suction. The filtrate was reduced in volume using the flash-evaporator and added in increments to a warm evaporating dish containing neutral alumina oxide[1] (pre-treated with chloroform). Residual chloroform was removed in an oven (60°C) leaving an admixture of chloroform extract to alumina of 1:2. The admixture was sprinkled into a glass column (length = 65 cm, diameter = 4.5 cm) containing neutral alumina oxide wet packed with benzene (an excess of benzene was allowed to accumulate at the top of the alumina so that the admixture would settle in even layers). The chloroform extract to alumina column packing represented a ratio of 1:30. Elution was accomplished with the following sequence of solvents: (i) benzene (1000 ml), (ii) 2% absolute ethanol in benzene (1000 ml), (iii) 4% absolute ethanol in benzene (2000 ml), (iv) chloroform (1000 ml) and (v) 1:1 chloroform to absolute methanol (1000 ml). Fractions collected were spotted with reference cryogenine[2]

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[1] Brinkmann Instruments, Inc., Cantiague Rd., Westbury, New York. EM reagents-aluminum oxide active neutral (activity 1). Art. 1077.

[2] Supplied by Dr. A.E. Schwarting, School of Pharmacy, University of Connecticut, Storrs, Connecticut.

on thin layer chromatography[1] (TLC) plates and developed using a 9:1, benzene to absolute ethanol solvent system. Fractions containing cryogenine were collected in 30 ml volumes, (in general, the alkaloid was first detected just after 2.85 liters of effluent had been collected).

6. Crystallization.-- Column fractions were flash-evaporated in vacuo at 40°C (constant temperature water bath) and re-dissolved in a minimum of boiling chloroform. A 100% excess of chloroform was added and the resultant solution passed through a Buchner filter under suction. The filtrate volume was reduced approximately 75% by boiling and the solution placed in the cold. Crystals were collected on a Buchner filter under suction and washed two to three times with cold chloroform. Re-crystallization involved the following unvarying procedure: (i) the crystals were dissolved in a minimum of hot chloroform using a water bath maintained between 52°C and 54°C, (ii) the solution was covered and placed in the cold, and (iii) the resulting crystals were collected as before on a Buchner filter, washed two to three times with cold chloroform and air dried on the filter under reduced suction. This procedure was repeated five to six times. Each yield was checked for purity using the previously described TLC system. Final re-crystallization

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[1] Brinkmann Instruments, Inc., Cantiague Rd., Westbury, New York. TLC-plates-aluminum oxide (Type E) F_{254}, pre-coated, 5 x 20 cm.

was accomplished with either 95% ethanol or absolute methanol. The procedure was the same as before, except that the water bath was increased in temperature to the boiling point of the solvent. Using methanol, the solution volume had to be reduced by about 50% (by boiling) and the vessel scratched and/or seeded to effect crystallization. The final batch of crystals were dried *in vacuo* at 110°C with phosphorus pentoxide[1]. The yield from the methanol extract was about 0.3% or about 4% from the chloroform extract.

7. *Analysis*.-- Cryogenine base melted at 248-250°C (reference cryogenine reported at 253°C).[2]

R_F values were determined using the previously described TLC system with the following modifications: (*i*) 24 mcg spotted on activated alumina oxide plates, (*ii*) the solvent system consisted of a 9:1 mixture of benzene and 95% ethanol, (*iii*) a pre-saturated developing tank (16 cm x 6.5 cm x 19 cm) filled to a depth of about 1.5 cm and (*iv*) the allowance of a 10 minute overrun for the solvent after a distance of 10 cm had been covered. Cryogenine ran as a single spot; R_F 0.70 (reference cryogenine, 0.71).

Amounts of 48 mcg and 96 mcg of cryogenine were

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[1] Fisher Scientific Co., Fair Lawn, New Jersey. Phosphoric anhydride (purified), F.W. 141.95, lot 702704.

[2] Arthur H. Thomas Co., Philadelphia, Pennsylvania. Thomas Hoover capillary melting point apparatus.

spotted on two-dimensional TLC[1] using absolute methanol and 95% ethanol as developing solvents. As before, the alkaloid migrated as a single spot with some tailing, being more noticeable with the larger sample.

Infrared absorption spectra[2] (IR) for both cryogenine and the reference standard are depicted on pages 127-8. Blomster et al. (67) have reported the presence of major absorption bands at 3500 cm^{-1} (OH), 1720 cm^{-1} (carbonyl) and two bands at 1605 cm^{-1} and 1510 cm^{-1} (indicative of aromatic ring absorption).

It was noticed on TLC that after final re-crytallization some samples of cryogenine contained a second, lighter spot running about mid-way between cryogenine and the origin. This spot was generally detectable only when sample amounts of 100 mcg or more were spotted. The presence of a small amount of impurity might be consistant with the slight reduction seen in both R_F and melting point values.

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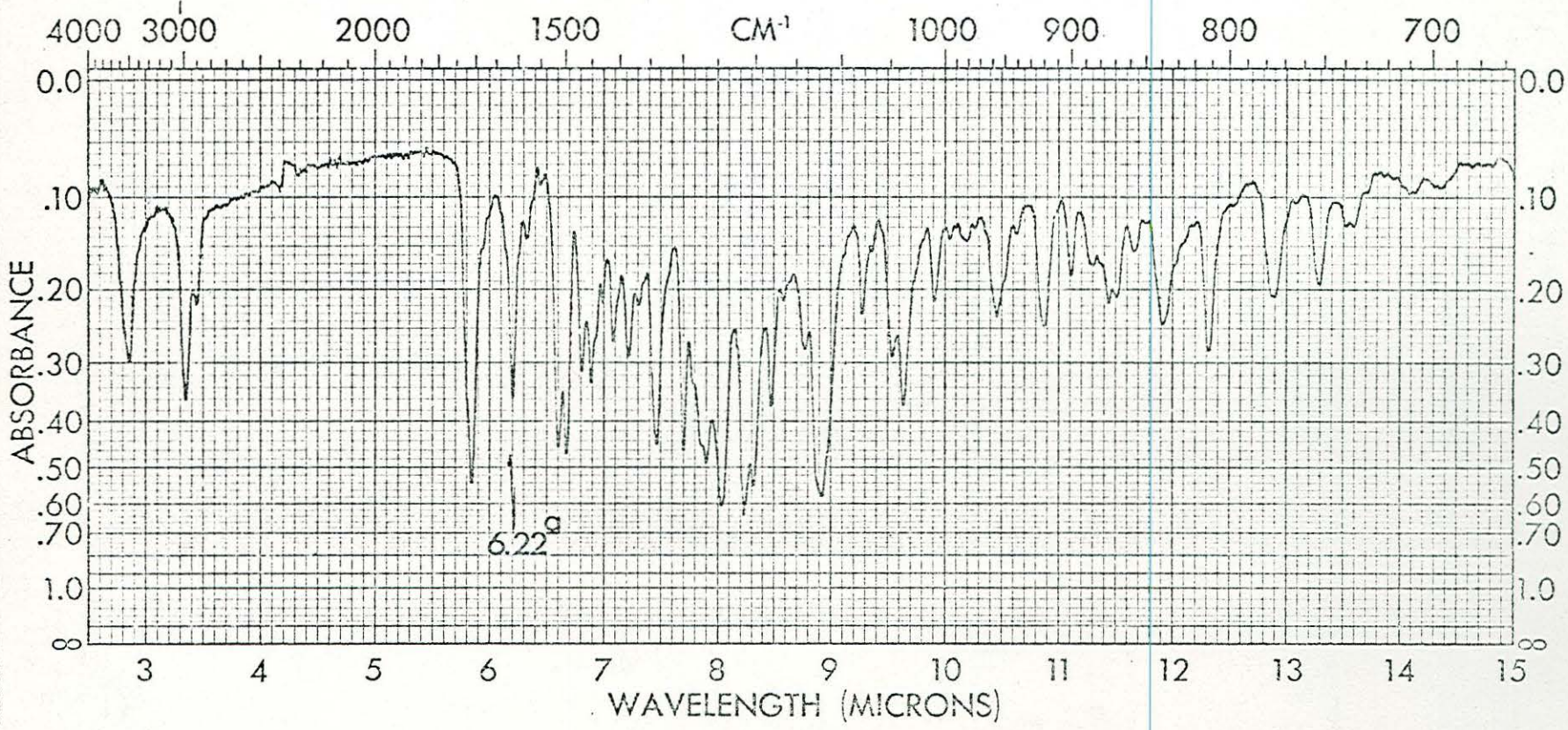
[1] Brinkmann Instruments Inc., Cantiague Rd., Westbury, New York. TLC plates silica gel, pre-coated, 20 x 20 cm.

[2] The Perkin-Elmer Corp., Norwalk, Connecticut. Part No. 137-1280. Scan Speed = F, slit = N, KBr pellet.

APPENDIX C

Infrared Spectra of Reference and Isolated Cryogenine



[a] Polystyrene calibration mark.

Infrared Spectrum of Cryogenine Isolated by the Present Investigator

APPENDIX D

Effects of Cryogenine on "Water Consumption" and "Urine Voided" During Developing Adjuvant Arthritis

Day	Mean ml Water Consumption			Mean ml Urine Voided ±S.E.		
	NC	PC[a]	CGN[b]	NC	PC	CGN
0	28	32	25	5.1±0.5	4.7±0.8	5.1±1.5
1	23	25	23	4.3±0.6	6.8±2.2	4.0±1.2
2	30	28	24	3.8±0.4	4.6±0.6	4.8±1.4
3	35	31	33	8.7±0.9	9.7±0.7	9.6±1.7
4	31	27	27	11.1±1.5	9.2±0.7	8.2±1.0
5	37	28	20	8.3±0.9	8.2±0.9	6.6±1.0
6	29	28	30	7.4±2.0	6.6±0.8	5.6±1.2
7	36	30	31	8.2±1.9	5.5±0.7	6.2±1.2
8	34	26	37	6.9±1.3	5.8±0.7	6.2±0.9
9	36	28	25	7.1±1.0	5.1±0.7	3.7±0.6[c]
10	30	23	27	9.6±0.9	8.6±0.7	7.2±0.9
11	38	32	31	12.8±1.9	13.6±1.1	10.5±3.4
12	32	30	25	10.0±0.6	11.1±0.8	6.4±1.2
13	36	30	27	8.6±1.5	11.6±1.8	8.3±2.9

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[a] Significantly different from Negative Control $P < .01- > .001$ (days 0-13).

[b] Significantly different from Negative Control $P < .05- > .01$ (days 0-13).

[c] Significantly different from Negative Control $P < .02- > .01$ (day 9).

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