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Hematologic effects of cryogenine and certain selected anti-inflammatory agents

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HEMATOLOGIC EFFECTS OF CRYOGENINE AND CERTAIN SELECTED ANTI-INFLAMMATORY AGENTS

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INTRODUCTION

Inflammation appears to be an attempt by the organism to re-establish homeostasis as a response to local reactive change in tissues following injury or irritation. This injury or irritation (whether caused by micro-organisms, toxins, antigens, etc.) first leads to an increased passage of fluid through the walls of the microvasculature, followed by stasis of circulation within the affected area. This, in turn, is followed by migration of leukocytes into the area and finally concluded by connective tissue proliferation leading to the deposition of granulation tissue (1,2,3).

There are many experimental models employed for the evaluation of drugs with possible anti-inflammatory activity. Since many of these systems employ whole animals, it seems rational that various hematological parameters could be applied. Our purpose was to test such parameters in two selected systems (i.e., adjuvant-induced arthritis and cotton pellet granuloma formation) during drug evaluation. The parameters under investigation included: total white blood cell count, differential leukocyte count, sedimentation rate and serum protein patterns.

A number of known anti-inflammatory agents and certain selected experimental drugs were subjected to this investigation. These compounds included: phenylbutazone,

mefenamic acid, indomethacin, paramethasone, hydrocortisone, 6-mercaptopurine, benzquinamide, and tetrabenazine (see Figure 1.).

Of three "unknown" compounds chosen for study, cryogenine was selected because of its unusual anti-inflammatory characteristics. While most anti-inflammatory agents are acidic compounds, cryogenine is one of eight alkaloids isolated from a plant growing in subtropical-tropical areas of South America and Mexico (4,5,6). The plant is Heimia salicifolia Link and Otto (7) from the family Lythraceae.

Since the preliminary pharmacologic studies by Robichaud et al. (8), a great deal of information has been gathered on the action of cryogenine and its analogs. Such actions include selected CNS depression in high doses, antispasmodic blocking of acetylcholine-induced contraction of smooth muscle, inhibition of pressor effects produced by exogenously-administered epinephrine, plus some antihistaminic and antibradykinin activity (9,10).

In 1966, Jiu noted that extractives of Heimia salicifolia possessed significant anti-inflammatory activity. The models he employed were yeast-induced foot edema in rats and cotton pellet granuloma in guinea pigs (11). In 1967, Kaplan and Malone published a paper (12) which demonstrated that cryogenine was effective in reducing inflammation in the following inflammatory models: paw edema induced by Mycobacterium adjuvant in rats, carrageenin- and serotonin-induced pedal edema in rats, and histamine-induced intradermal wheal responses in the rabbit. Cryogenine was also

Figure 1.— Chemical structures of compounds under investigation.

a. Tetrabenazine

b. RO 4-1284

c. Benzquinamide

d. RO 1-9564

e. Phenylbutazone

f. Cryogenine

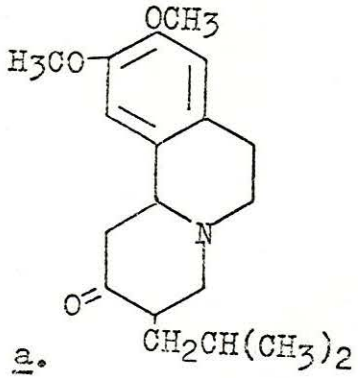
g. Indomethacin

h. Hydrocortisone

i. Mefenamic Acid

j. 6-Mercaptopurine

k. Paramethasone Acetate



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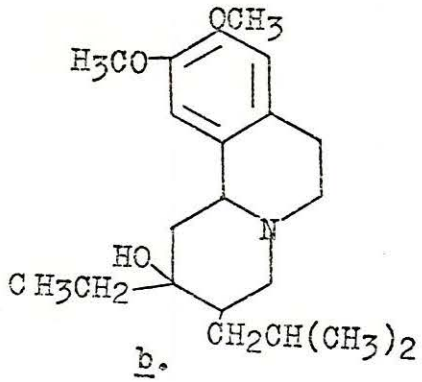

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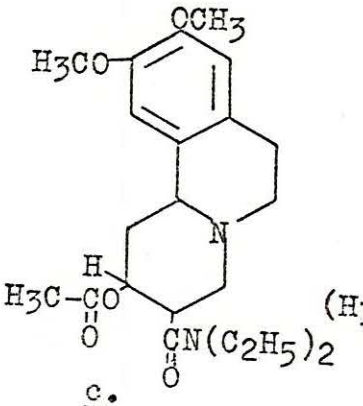

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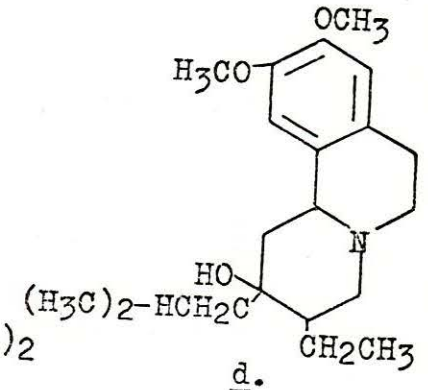

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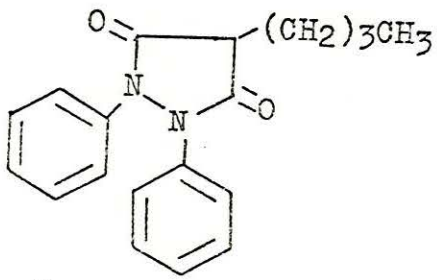

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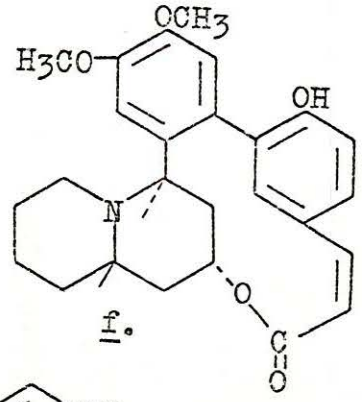

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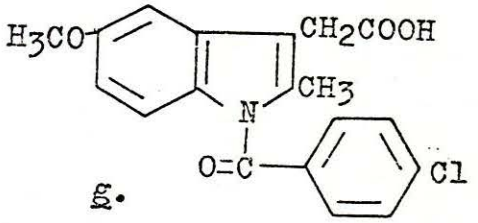

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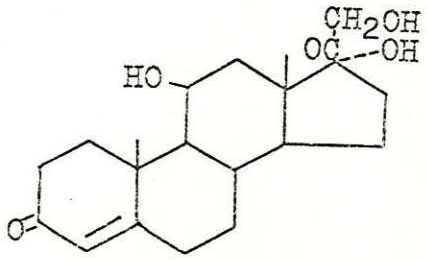

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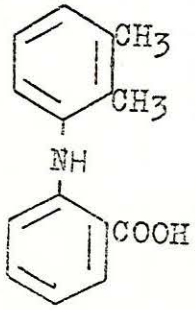

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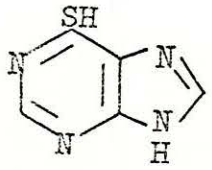

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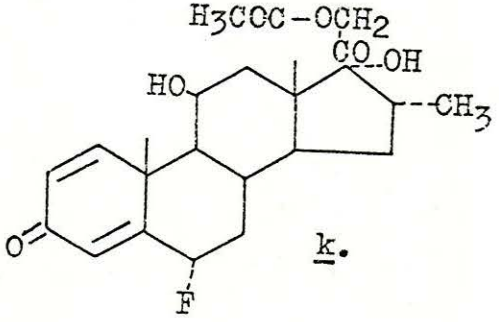

k.

shown to exhibit a low order of analgesic and antipyretic efficacy. Other studies in this laboratory documented that cryogenine lacked fibrinolytic activity, was ineffective in reversing stress-induced hyperglycemia, did not deplete central or peripheral tissue levels of norepinephrine and serotonin, and would not produce histopathologic lesions on chronic dosage (9,13,14).

The other two "unknown" compounds under investigation were: RO 4-1284 (2-ethyl-2,3,4,6,7,11b-hexahydro-2-hydroxy-3-isobutyl-9,10-dimethoxy-1H-benzo [h] quinolizine [also appearing in the literature as: (i) 2 hydroxy-2 ethyl-3-isobutyl-9,10 dimethoxyl-1,2,3,4,6,7-hexahydro-11b H-benzoquinolizine, (ii) derivative of 1,2,3,4,6,7-hexahydro-11bH-benzo [a] quinolizine, carbinol I.)] and RO 1-9564 (3-ethyl-2,3,4,6,7,11b-hexahydro-2-hydroxy-2-isobutyl-9,10-dimethoxy-1H-benzo[h] quinolizine [also appearing in the literature as: (i) 2 hydroxy-2 isobutyl-3 ethyl-9-10 dimethoxy-1,2,3,4,6,7-hexahydro-11b H-benzoquinolizine, (ii) derivative of 1,2,3, 4,6,7-hexahydro-11bH-benzo [a] quinolizine, carbinol III.)]. RO 4-1284, along with tetrabenazine, belongs to a distinct class of benzoquinolizine ataractic agents which deplete serotonin and norepinephrine from brain tissue and evoke typical reserpine-like responses (15). However, RO 1-9564 and benzquinamide belong to another distinct class of benzoquinolizine ataractic agents which do not deplete brain amines at therapeutic dosage levels and possess no reserpine-like syndrome (16,17). It is of interest to note that the

compounds of the first class, unlike the second, are able to stimulate adrenocortical activity (18). Cryogenine, like the second class of benzoquinolizine ataractic agents, seems to act by a more direct mechanism than by alteration of monoamine metabolism. There is also some indirect evidence that cryogenine may not act via the adenohyophyseal-adrenocortical axis (14).

Total white blood cell count, differential leukocyte counts and sedimentation rates were studied using the adjuvant-induced inflammatory model, since changes in these parameters are found in human rheumatoid arthritis. In this method, tubercule bacilli derived from human strains PN, DT and C were grown for 8 weeks, killed by steam, and dried in a vacuum oven (19). The dead mycobacteria were then suspended in an oil vehicle and injected subcutaneously into the subplantar surface of a rat's hind paw. The severity of the resulting polyarthritis was determined plethysmographically (20). This method is said to produce a syndrome more closely related to human rheumatoid arthritis than any other test (19).

Serum proteins were studied in rats with cotton pellet implantations, since changes in serum protein patterns have been observed in human inflammatory diseases. It is generally considered that the cotton pellet granuloma assay measures an anti-inflammatory agent's ability to interfere with the proliferative component of the inflammatory process. Steroidal and non-steroidal inflammatory inhibition has been demonstrated in this system by many investigators (21,22,23).

Tre histological evaluation of cotton pellet granuloma has been reported by Eichhorn and Sniffen (23). In general, the granuloma is composed of two layers: an outer zone comprised of fibroblastic material infiltrated with collagen fibrils and new capillaries plus a core composed of a large number of fibrin strands. Several studies have indicated that an apparent correlation exists between the appearance of collagen and disappearance of hexosamine and the appearance of collagen and an increase in the number of fibroblasts (24).

A brief review of the complexity of the "normal" inflammatory process may be useful at this point. The initial vascular phase begins with transitory vasoconstriction and local anemia. This is rapidly replaced by microvessel dilation. This subsequent hyperemia is transitory and eventually gives way to stasis of flow or stagnation. Leukocytes begin to orient themselves along the vessel wall in preparation for migration into the area of injury.

After the stagnation phase, the "walling off" stage of inflammation begins. This event occurs with the impaction of pores in the capillaries and lymphatic walls with precipitated soft fibrin. This event seems to be necessary to allow time for the phagocytic cells to assemble in the inflammed area (25). The net result can be summed up in one word--edema.

Once the "walling off" process is well under way, the third phase begins. This is the immigration of leukocytes --starting with neutrophils, followed by monocytes, lympho-

cytes, and plasma cells. Leukocytes seem to help resolve the inflammation by ridding the area of the inflammatory stimulus; however, on the other hand, they serve to promote the process by releasing inflammation-promoting substances (22,23,24,26).

The final or organization phase (26) is the repair phase of the inflammatory process. This begins with proliferation of fibroblasts and small vessels from connective tissue and endothelial cells. The result is a highly vascularized, reddish mass, or granulation tissue.

MATERIALS AND METHODS

A Revised Procedure For the Isolation and Purification of Cryogenine

Extraction.---The dried overground plant[1] was ground to a semifine powder with a Wiley Mill[2] (2 mm mesh screen). This material was then defatted by continuous extraction with petroleum ether, b.p. 30-60°C. With the large volume soxhlet[3] used in this study, each thimble made of clean bleached flour sacks held about 400 gm of powdered material per extraction. Usually 8 to 12 defattings could be done using the same petroleum ether solvent. After about eight defattings, the refluxing solvent became so saturated that bumping occurred. A few boiling stones (renewed after each extraction) prevented bumping.

After the material had been defatted, it was air dried overnight and extracted with absolute methanol continously for 48 hr (large volume soxhlet). To check for completion of the extraction procedure, Wagner's I (a nonspecific alkaloid stain) was used to check the thimble solution. When boiling stones were used, this extraction could

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[1] *Heimia salicifolia* Lot BKC 752; S.B. Penick & Co., 520 New York Ave., Lyndhurst, NY.

[2] Wiley Mill, Standard Model #3; Arthur H. Thomas Co., Philadelphia, PA.

[3] Extraction Apparatus, Soxhlet, K-58500; Kontes, 2809 Tenth St., Berkeley, CA.

be done at least six times before changing the absolute methanol solvent.

The methanol extract was then concentrated *in vacuo* with the aid of a rotating flash evaporator. Concentration was hastened by using a 40°C water bath. The intermittant addition of distilled water was useful to completely remove all the methanol; however, such additions must only be done after most of the methanol has been removed. When the methanol/water ratio was very high, frothing occurred under reduced pressure, resulting in the loss of product.

The remaining aqueous extract was then acidified to pH 2 with 10% hydrochloric acid using either pHydrion[4] paper or a pH meter. The acidified aqueous extract was filtered through Celite with suction and the precipitate washed with distilled water. The aqueous acidic filtrate was then further defatted in a continuous liquid-liquid ether extractor[5] (diethyl ether) for at least 24 hr or until the ether layer became clear. The aqueous acidic layer was separated from the ether layer and the pH adjusted to 9 with a 28% solution of ammonium hydroxide.

The alkaline aqueous solution was extracted continuously with a liquid-liquid chloroform extractor.[6] An indication of extraction completion could be noted by a loss of

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[4] pHydrion set #60783, Van Waters & Rogers, 3745 Bay Shore Blvd., Brisbane, CA.

[5] Ether Extraction Apparatus #6840, 2000 ml; Ace Glass Inc., Vineland, NJ.

[6] Chloroform Extraction Apparatus K-587000, 1000 ml; Kontes, 2809 Tenth St., Berkeley, CA.

yellow color in the chloroform layer just below the aqueous layer. To insure complete alkaloid removal, the extraction proceeded 48 hr beyond the above indication. The chloroform extract was then dried *in vacuo* at 40°C in a rotating flash evaporator over a water bath.

Purification.--The crude alkaloid mixture was then taken up in a minimal amount of methanol and applied to the top of pre-packed neutral alumina column[7] (about 30 gm of alumina per gm of alkaloid mixture with the starting weight of the total alkaloid mixture about 80 gm). Elution was conducted with the following liquids in the following sequence: benzene (1000 ml), 2% ethanol in benzene (1000 ml), 4% ethanol in benzene (2000 ml), chloroform (1000 ml), and 1:1 chloroform:methanol (1000 ml). Fractions of 20 ml each were collected in glass containers, paying particular attention to fractions #80 and beyond (4). All fractions were checked for cryogenine by thin layer chromatography (TLC) techniques. The TLC system consisted of aluminum oxide plates[8] and a developement solvent of 9:1 benzene:ethanol. Migration of spots were observed under short wave length illumination using pure cryogenine as a standard.

Chloroform proved to be the best initial solvent for crystallization and recrystallization. The sample was taken up in hot chloroform, air dried to 3/4 of its volume,

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[7] Aluminum Oxide, Active Neutral (Activity I) #1077, E. Merck Laboratories Inc., 500 Executive Blvd., Elmsford, NY.

[8] TLC plates, Aluminium Oxide (Type E) F_{254}, Pre-coated, E. Merck Laboratories Inc., 500 Executive Blvd., Elmsford, NY.

filtered and cooled. The resultant crystals were collected by filtration using a micro bell jar and the procedure repeated at least 5 to 6 times. Purification was checked by the above TLC procedure and melting point determinations. Final recrystallization was accomplished in 95% ethanol. The crystals were dried *in vacuo* at 100°C for 4 to 6 hr before physical studies were made.

Analysis of Cryogenine.—The dried base provided sharp melting points at 252-254°C. Mixed melting points of 1:9, 1:4 and 1:2 showed little variation from the above melting range. Infrared absorption spectrum (IR) analysis showed among others the presence of bands at 3500 cm^{-1} (OH), at 1720 cm^{-1} (carbonyl) and two bands at 1605 cm^{-1} and 1510 cm^{-1} (indicative of aromatic ring absorption)(4,27). All analysis were made using a reference standard of cryogenine for comparison.

Erythrocyte and Leukocyte Counts

The counting of erythrocytes and leukocytes by electronic means was more attractive time- and methodology-wise than methods using counting chambers and hemocytometers. The Coulter Counter was originally developed specifically for blood cell enumeration. This instrument is capable of determining both the number and size of particles suspended in some conductive media. The cells are counted by drawing a measured volume of the suspended cells through a small aperture having an immersed electrode on either side. As cells pass between the electrodes and displace electrolyte,

they introduce a change of resistance in the electrical circuit. As this occurs, a voltage pulse proportional to the particle size is produced which one can visualize on an oscilloscope. Such pulses can be electronically scaled and counted.

Red Blood Cell Counting.---Blood was taken from the heart of rats and placed in test tube pretreated with EDTA.[9] The blood was then diluted by taking 20 µl up in an automatic pipette[10] which produced a 1:500 dilution in isotonic saline.[11] This was followed by taking up 100 µl of the 1:500 dilution by the automatic pipette and diluting this 1:100 using isotonic saline again. The rest of the 1:500 solution was put aside for subsequent use (see methodology for white blood cell counting).

The Coulter Counter cannot distinguish between blood cells and particles of debris; therefore, all glassware and diluting fluids had to be free of dust particles and any other contaminating material. It is possible to limit the background count due to debris by use of the threshold dial. This permits selection of an aperture size consistent with the minimal size of the cells to be counted. The dilution of cells is necessary, since the instrument is only designed to count cell numbers up to 50,000 per 0.5 ml. It is also necessary to dilute the cells in order to reduce the coin-

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[9] Blood Collecting Tube, B-D Vacutainer, #3274QS, Becton, Dickinson & Co., Columbus, NB.

[10] Dilutor 2D, manufactured by Dade Reagents Inc., Delaware Parkway, Miami, FL.

[11] Isoton, manufactured by Coulter Diagnostic Inc., Hialeah, FL.

cidence of two or more cells entering the aperture simultaneously since the instrument counts such incidents as single cells. Reduction in count by normal occurrence of coincidence can be predicted and a correction table for such coincidence is supplied by the manufacturer.

A Coulter Counter, Model "F"[12] was used with the attenuator set at 0.707, and with an aperture setting of 16. Two to three runs were made per sample and an average taken. Corrections were made from the error tables for all counts over 10,000.

White Blood Cell Counting.--The remainder of the diluted blood sample was then used to determine the leukocyte count. Three drops of a saline solution containing saponin[13] was added to the sample in order to lyse the erythrocytes. The solution was lightly swirled to permit very thorough dispersion. This solution was not allowed to sit for more than 20 min., since lysing of the leukocytes will then occur. The same procedure used in counting erythrocytes was then used. Two to three runs were made for each determination and an average was calculated. Corrections were made for counts over 10,000 cells using the error tables provided by the manufacturer.

The Coulter Counter has been reported to reduce the time and increase the accuracy of counting erythrocytes and

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[12]Coulter Counter Model "F", manufactured by Coulter Electronics, Inc., Hialeah, FL.

[13]"Zap-Isoton" #B3145-5, manufactured by Coulter Electronics, Inc., Hialeah, FL.

leukocytes when compared to traditional methods (28,29). Tests comparing electronic and hemocytometer counts on sheep, swine and cow blood consistently showed less variation in sets of duplicate counts by the electronic method (30). However, erythrocyte counts obtained by the electronic counter are consistently lower than by hand count (31).

Differential Leukocyte Counts

Differentiation of blood cells is generally conducted by microscope using the high-dry magnification. However, dried blood smears were observed in this study without coverslips and covered with a thin film of immersion oil. This permitted clear and distinct images. The oil could be removed by wiping the slide with facial tissue or by immersing the slide in a container of xylene. The use of oil-immersion magnification was considered necessary in order to see the fine details that allow for accurate differentiation of cells.

Blood Smears.---Making good blood smears is vital for successful examination of leukocyte types. The blood film should be made as soon as the sample is drawn from the animal. Such smears may be prepared on either a slide or on a coverslip.

In the slide method used here, a clean microscope slide was placed on clean, level surface, and a small drop of thoroughly mixed[14] blood placed near one end of the slide. A round applicator stick was used to transfer blood from the

[14]The blood was kept mixing by the continuous, slow rocking motion of a conventional shaker (20 rpm.).

vial to the slide and as means of controlling drop size. Then, the blood was spread in an even film using another slide. The spreader slide was laid on the inner surface of the index finger and held there by the thumb. The slide was pushed toward the drop of blood until contact was made. After contact, the blood will spread along the edge of the spreader slide which is then moved forward in a smooth glide. The angle at which the spreader slide is held determines the thickness of the blood smear (thickness of the smear is directly proportional to the angle of the spreader slide). An angle of 30 degrees was used in this study. Rapid drying of the slide was accomplished by waving it in the air. Slow drying results in loss of water out of the erythrocytes, with the movement out into the plasma. This results in crenation (indentations in the surface of the cell) of the erythrocytes.

When slides are made correctly, the smears appear smooth, even in appearance, and free of clear areas. Jerking movements while spreading the film will produce wavy smears. Clear areas in the smear usually are due to grease or dirt on the surface of the slide. If clear streaks are noticed in the smear, this may be the result of a chipped edge on the spreader slide.

Whenever possible, the smears were stained immediately. Otherwise, they were stored in protected place and then stained within 24 hr.

Staining Methods.---The smears were stained for five

minutes in a stain prepared by dissolving 1.5 gm of Tetrachrome[15] in 1000 ml of absolute methyl alcohol. During preparation, the solution was heated to 50°C (with continuous stirring) for about 3 min. After heating, the solution was allowed to stand for at least 24 hr before using. It is important to filter this stain each time before using. Some investigators have claimed that prolonged storage before use increases the staining qualities of the reagent.

After staining had been completed, the slides were transferred to a phosphate buffer for five minutes.[16] The phosphate buffer solution was prepared by dissolving 1.0 gm of the salt in 1000 ml of distilled water. Great care was taken in transferring the slides from the stain to the buffer so that no water would be introduced into the stain. Such dilution reduces the effectiveness of the staining qualities.

After fixing in the phosphate buffer solution, the slide was subjected to two short rinses: the first in fresh phosphate buffer solution, which was followed by a rinse in distilled water. The slides were then allowed to air-dry before examination under the microscope.

Cell Counting.---As blood is spread on the slide, there is a tendency for the neutrophils to rush to the edges of the smear. Lymphocytes tend to remain mainly in the body

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[15] Tetrachrome Stain #637, manufactured by Allied Chemical, MacNeal Corp., New York, NY.

[16] Phosphate buffer #4020, pH 6.4, manufactured by (Harleco) Hartman-Leddon Co., Philadelphia, PA.

of the smear, while monocytes and eosinophils tend to be distributed throughout. Therefore, there are three general methods for making the differential count for blood smears on slides (32). The first means of counting is the "straight edge method" where one follows the edge of the film. This method will give consistently higher neutrophil and lower lymphocyte counts than the second method or "cross-sectional method." The second method consists of counting back and forth across the slide. The last or "battlement" method gives values that compare very closely to counts made by examination of the entire blood smear. The battlement method consists of counts made of three horizontal edge fields, plus two fields toward the interior, followed by two fields in a horizontal and then two fields in the vertical direction to reach the edge again.

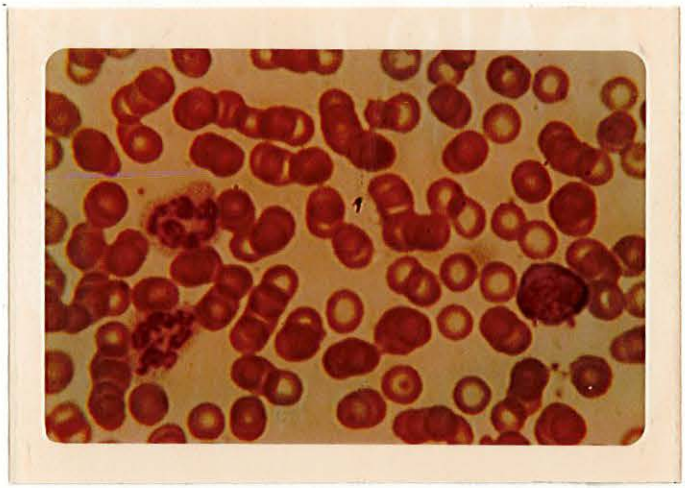
To insure reasonable accuracy, the number of cells differentiated should be in proportion to the total leukocyte count, e.g. where the total leukocyte count is 10,000 or less, one should differentiate 100 cells; and when total counts are between 10,000 and 20,000, the number of cells to be differentiated should be increased to 200.

The differential leukocyte count was expressed as the percentage of the number differentiated.

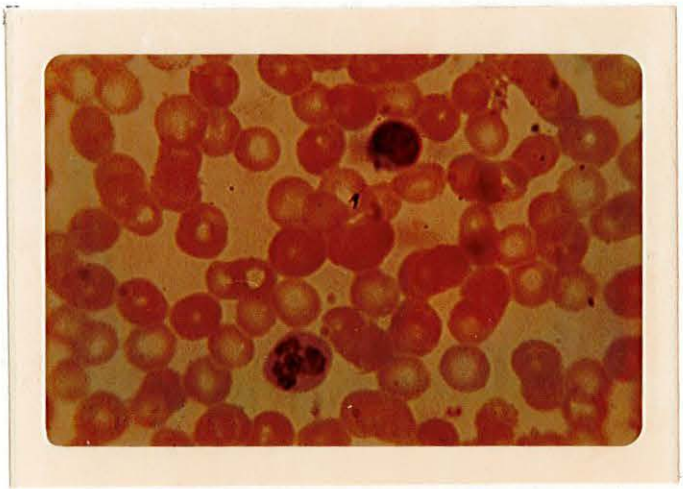
Types of Leukocytes.--There are basically five types of white blood cells: neutrophils, lymphocytes, monocytes, eosinophils and basophils (see Figures 2-8). Neutrophils have a polymorphous nucleus which is seen in younger cells as a twisted coil. The cytoplasm is essentially colorless

Figure 2.— White rat leukocytes: Two neutrophils (right) each with a polymorphous nucleus plus one lymphocyte (left).

Figure 3.— Human leukocytes: One neutrophil (bottom) with a polymorphous nucleus and one lymphocyte (top).



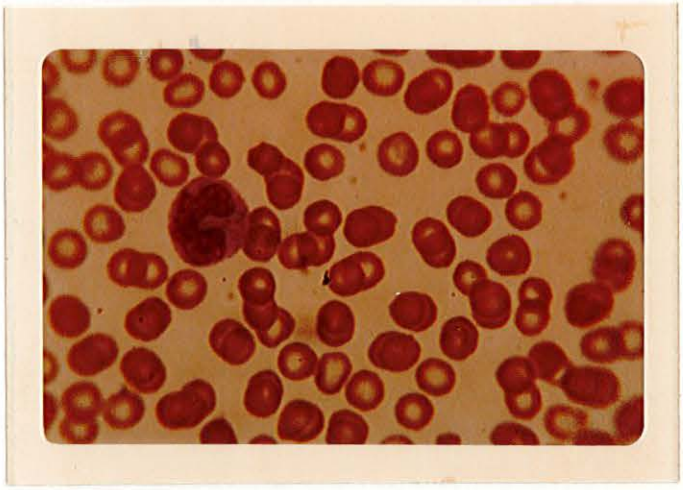
Oil-Immersion --- 1000x



Oil-Immersion --- 1000x

Figure 4.-- White rat leukocytes: Kidney bean-shaped monocyte.

Figure 5.-- Human leukocytes: Kidney bean-shaped monocyte.



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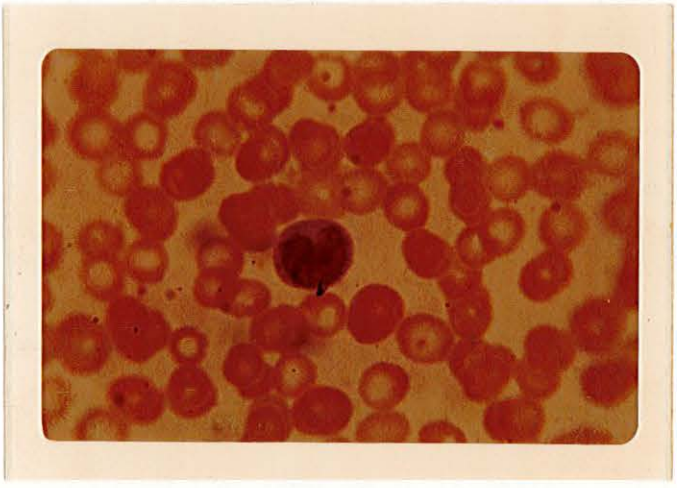
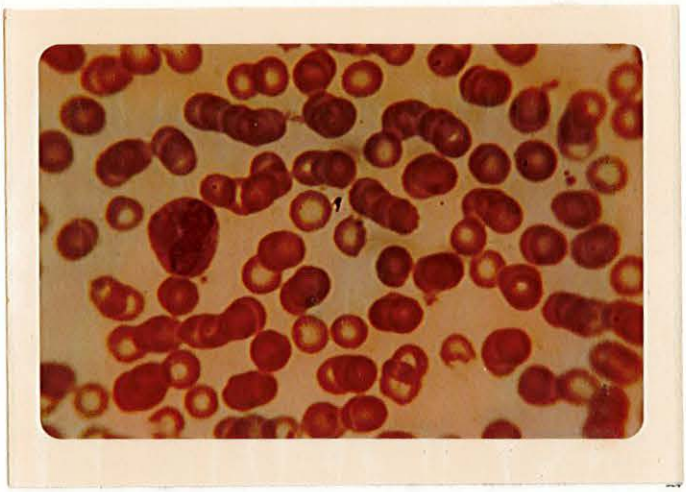


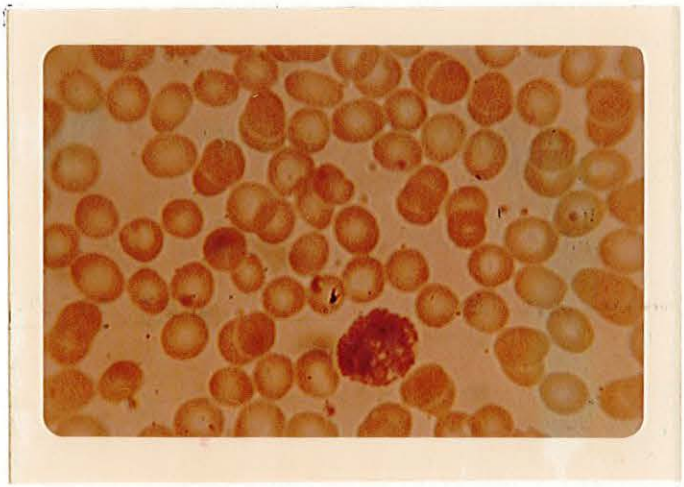
Oil-Immersion --- 1000x

Figure 6.— White rat leukocytes: Twisted U-shaped, granulated eosinophil.

Figure 7.— Human leukocytes: Granulated eosinophil.



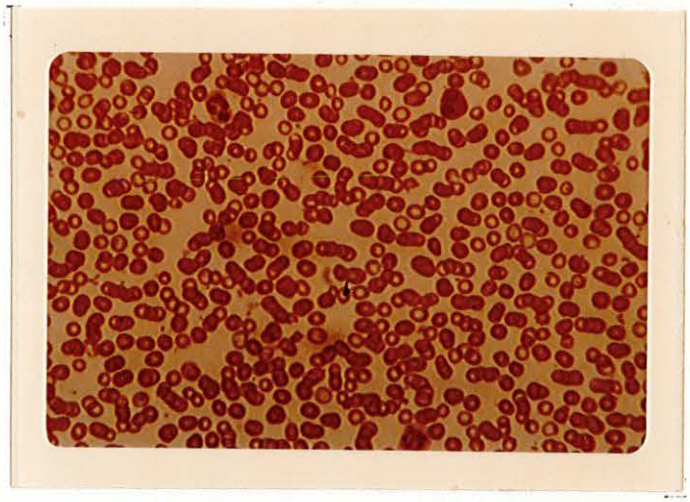
Oil-Immersion --- 1000x



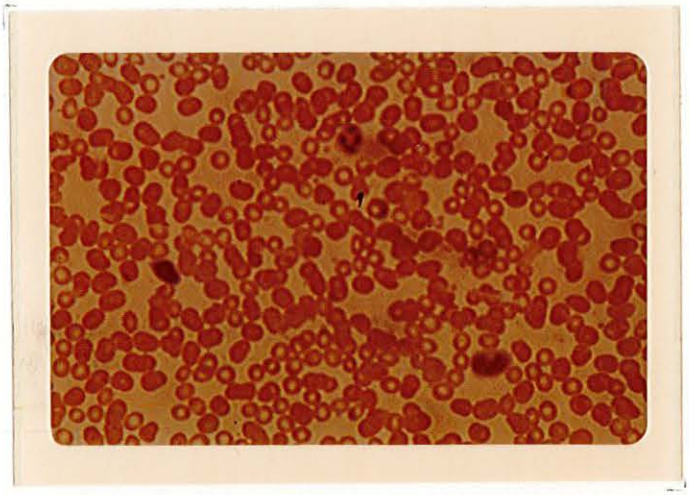
Oil-Immersion --- 1000x

Figure 8.— White rat leukocytes: Neutrophil (left) and eosinophil (right).

Figure 9.— Human leukocytes: Neutrophil (top), monocyte (right bottom) and lymphocyte (left bottom).



High-Dry --- 400x



High-Dry --- 400x

and with some granules. Lymphocytes vary in size from that equal to a red blood cell to that of a neutrophil. The cytoplasm stains light blue, and is often clustered with azurophilic granules when old. Monocytes are the largest of all leukocytes and have a nucleus that varies in shape, i.e. round to kidney bean. Their cytoplasm is basophilic and often vacuolated. An eosinophil is generally larger than a neutrophil and has its nucleus commonly in the form of a closed U. The nucleus stains a combination of light purple and pale blue. Its cytoplasm contains small, round, strongly acidophilic granules. The basophil has a nucleus that is almost invisible and a clear blue cytoplasm containing many basophilic metachromatic granules of uniform size (33).

Sedimentation Rate

This test was conducted routinely on all samples drawn. Blood was taken directly from the heart of rats and placed in small test tubes pretreated with EDTA. Tests were started within one hr after the blood had been taken; however, they could have been delayed for as long as six hours. The blood sample is placed in sedimentation tubes[17] kept in a perfectly vertical position and the height of the falling column of erythrocytes was checked at various time intervals.[18] Sedimentation is accelerated with any rise in temperature; therefore, the temperature of the laboratory was maintained

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[17] Tubes and sedimentation tube rack, "Sedirack", manufactured by Becton, Dickinson and Co., Rutherford, NJ.

[18] Data for this study was taken 2 hr after filling of the sedimentation rate tubes.

at 68°F. Sedimentation rate depends on the number of erythrocytes per unit volume of blood and the speed of settling is related inversely to the number of red cells. Therefore, the smaller the number the greater the speed of settling.

When many reticulocytes or many young forms of erythrocytes are present, a hazy reddish plasma without a clear cut line of separation between the settling red cell mass and the plasma can be noted (34). A clear plasma followed by a hazy reddish plasma which then gradually merges with the red cell mass is called a diphasic sedimentation reaction. Such a diphasic response develops when the blood contains a large number of red cells of abnormal shape.

Hematocrit

As an alternative to sedimentation rate, hematocrit can be determined on very limited amounts of blood. The term, hematocrit, means to separate blood. If one separates the blood into distinct compartments by centrifugation, three layers can be observed. The erythrocyte mass is found at the bottom and is called the packed cell volume (PCV). Just above the PCV is a white or gray layer, usually termed the buffy coat. This buffy coat is known to contain both leukocytes and thrombocytes. The third layer located above the buffy coat is the clear serum.

Microhematocrit would be useful in studies of this sort since the time required for the entire procedure is less than five minutes and the amount of blood required for each sample is minimal. Struma and Principato (35) have shown

a reproduceability of ±0.5% by microhematocrit, ±2.5% by the Wintrobe hematocrit method, ±5.9% by the Klett photoelectric colorimeter method and ±6.0% reproduceability by actual erythrocyte count. These data were obtained under optimal conditions with human blood; however, the investigators felt that under routine laboratory conditions the reproduceability might be even better.

A capillary tube (75 mm x 1 mm) was filled two-thirds to three-fourth of its capacity by capillary attraction. The outside was then wiped dry and one end sealed with clay. The tubes were then placed in a special centrifuge[19] and the automatic timer set for four minutes. After centrifugation at 11,000 r.p.m., the capillary tubes were taken out and read using an International Microcapillary Reader.

Total Protein

The Goldberg refractometer or TS meter was designed to provide a simple and accurate measurement of the total solids in urine, serum and plasma (36). The total solids (TS) meter[20] used in this study had a direct reading scale for refractive index. With this scale and a conversion chart, one could calculate protein concentration (gm/100 ml of plasma or serum). The refractive index is recorded as unity plus four digits to the right of the decimal. The TS

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[19] Hematocrit, High Speed, International Hematocrit Centrifuge, Model MB; Van Waters & Rogers, 3745 Bay Shore Blvd., Brisbane, CA.

[20] Manufactured by American Optical Company, #10401; Buffalo, NY.

meter is compensated for temperatures between 60-100°F, and measurements of total solids by this method has been reported to be accurate within ±0.1% (37). Total protein concentration can be made with a high degree of accuracy, as long as the serum or plasma is clear. While the total protein scale has been compensated for certain common nonprotein constituents of plasma and serum, lipemic plasma and cloudy plasma are not suitable for assay by this instrument. The refractive index is markedly increased by lipids, resulting in protein values that are erroneously high.

A single drop of serum or plasma was placed on the prism of the TS meter and viewed using natural or artificial illumination. A reading was made on the scale when the dividing line between bright and dark fields crossed the scale. The total time involved with filling, reading, and cleaning the instrument takes about three minutes for a single determination.

Serum Protein Electrophoresis

Electrophoresis is the movement of charged molecules in an electric field. There are three essential ingredients: an electric field, a charged particle, and a medium in which the movement may occur. There are basically two types of systems: free or "moving boundary" electrophoresis (where the serum proteins move in a liquid without the benefit of a solid matrix support) and "zone" electrophoresis (which uses a solid support medium, i.e. paper, starch, agar gel, acrylamide gel, or cellulose acetate). Zone electrophoresis

(cellulose acetate) was used for this study.

There are many types of cellulose acetate plates and many factors which account for the variety (e.g. the length of the cellulose chain, the degree of acetylation of the cellulose, pore size, gelation, chain coiling, and residual contaminents). To expect the same results with two different types of cellulose acetates under common test condition is like expecting to see two people with the same finger prints (38). Other influences include, electro-osmosis, evaporation, temperature, voltage, current and ionic strength, all of which contribute to protein movement (39)

Separation of Serum Protein Fractions.---Titan III cellulose acetate plates[21] were found to be very suitable for our studies. The Titan III has greater gelation which results in a matrix more easily traversed by a charged molecule. High gelation also affords better resolution and results in tighter bands. The Titan III cellulose acetate plate is composed of two layers. The top is cellulose acetate while the bottom is an inert polyester plastic sheet of mylar. The mylar is a supportive layer to make the cellulose acetate film easier to handle. Otherwise the cellulose acetate would be extremely flimsy and fragile when wet. A second advantage of the mylar layer is that it restricts evaporation during electrophoresis to just one surface.

Wetting the Strip.---It is important that the

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[21] Titan III Cellulose Acetate plate #3023, Zip Zone (2 3/8" x 3") box of 25; Helena laboratories, P.O. Box 752 Beaumont, TX.

wetting should produce a uniform medium. Titan III must be slowly dipped into the buffer, otherwise blistering of the medium will result. Such air ridges distort protein movement through the plate. To make sure each strip was in complete equilibrium, they were allowed to soak for 20 min before using. The buffer used for this study was a commercially available product (pH 8.6 Barbital, Ionic Strength 0.075).[22]

Blotting the Strip.---The Titan III cellulose acetate has a high affinity for buffer trapped in its pores and must be blotted firmly enough to remove all excess surface buffer. If this blotting is not firm enough, the serum sample will not be readily accepted and may run. After blotting, the sample should be quickly applied, otherwise evaporation will cause withdrawal of buffer from the gel. If white blotches occur, one must be sure to allow the strip ample time to rewet while resting in the chamber prior to electrophoresis. If this is not done, air pockets will occur which cause a difference in resistance in these areas resulting in very irregular separation.

Sample Application.---When applying the sample, it is important to keep the application as narrow and uniform as possible. The Zip Zone Applicator[23] can apply 8 samples at a time, which are uniform, narrow and sharp. The applicator should be primed before using in order to leave

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[22] Electra B2 Buffer #5207; Helena Laboratories, P.O. Box 752, Beaumont, TX.

[23] Zip Zone Applicator (#4080), Sample Plate (#4081), Aligning Base (#4082); Helena Laboratories, P.O. Box 752, Beaumont, TX.

a residual layer of protein on the applicator; this makes the second loading much more uniform.

Dead center application neutralizes the buffer movement forces from each side. Since diffusion begins immediately upon application, electrophoresis should begin as soon as possible.

Electrophoresis.---Once the strip has been placed in the chamber,[24] the voltage power supply[25] was set at 180 volts. If one exceeds 7 milliamps per 2 3/8" x 3" Titan III strip, ice must be used to prevent excess heat build up. In this study, the strip was allowed to run for 25 min and then stained.

Staining.---The Titan III strip was floated cellulose acetate side down in the stain (Ponceau S, 0.5% in 7.5% TCA[26]). This technique provides even and continuous staining. The background immediately assumes the color of the stain while the protein remains white and unstained---this is known as negative staining. When the staining is complete, the protein fractions become the same color as the background. Negative staining allows one to "preview" separation results.

Destaining and Clearing.---Destaining was accomplished by 3 successive washes of 5% acetic acid for 2 min

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[24]Zip Zone Chamber #1283; Helena Laboratories, P.O. Box 752, Beaumont, TX.

[25]Ponceau S #5525; Helena Laboratories, P.O. Box 752, Beaumont, TX.

[26]Regulated Power Supply, Model ip-32; Heathkit, Benion Harbor, MI.

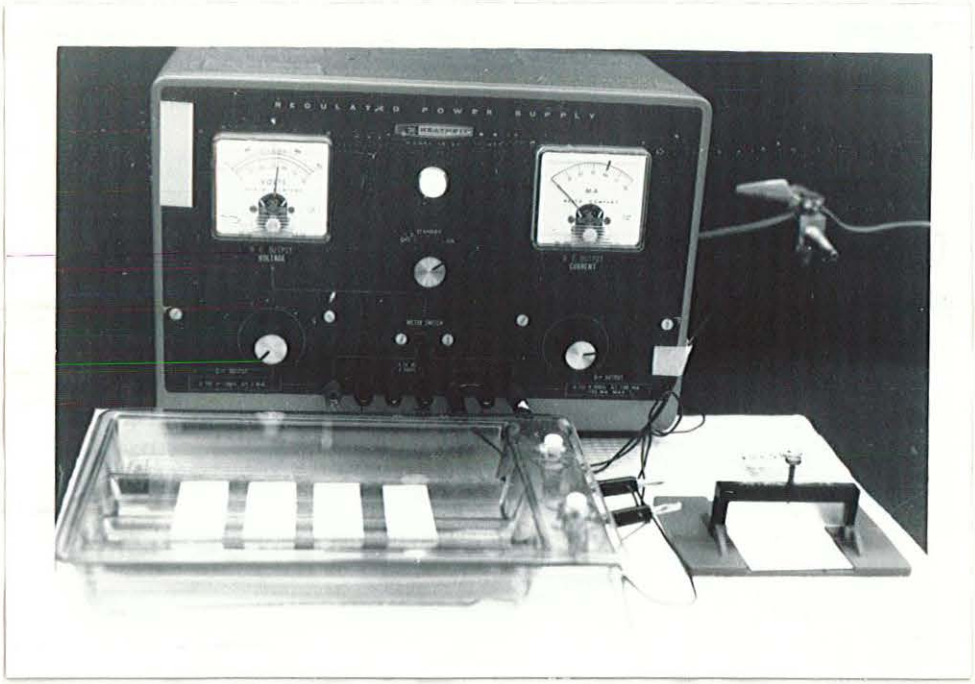

Figure 10.-- Electrophoresis power supply, cell and applicator set up.

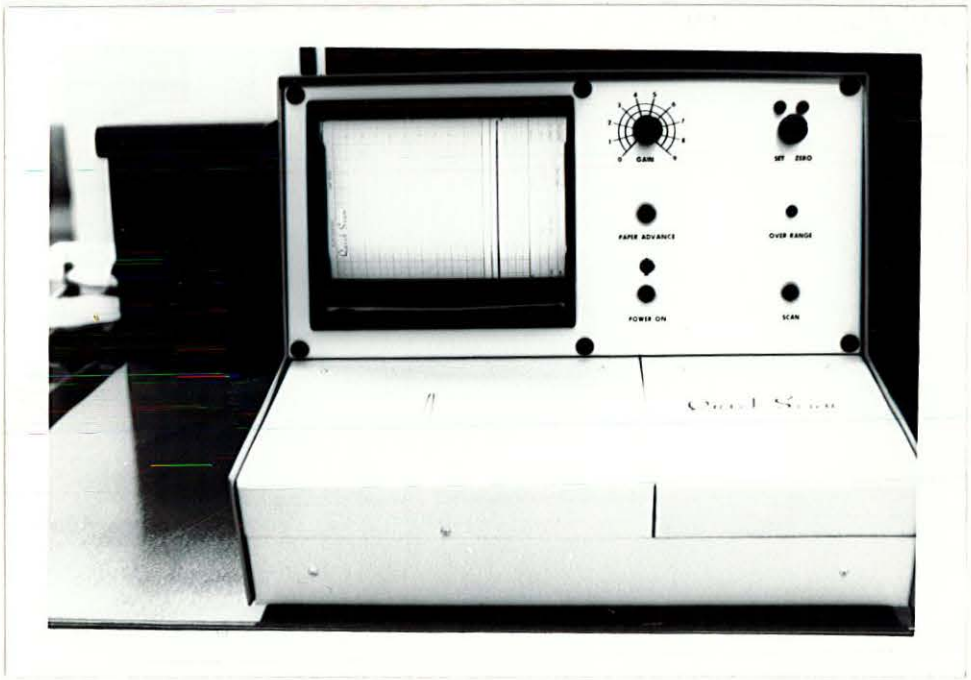

Figure 11.-- The "Quick Scan" electrophoresis densitometer.

each or until the background was white. The strips were then placed in absolute methanol for 2 min to dehydrate. Clearing was accomplished by putting the strips in 1:3 glacial acetic acid:methanol for 10 min. After the clearing, the strips were put aside to dry.

Quantitation.---The most popular method of quantitation is to stain the protein with a visible dye, then measure the intensity of the dye and correlate this intensity to the protein concentration. Ponceau S is the most popular stain for serum protein studies on cellulose acetate strips. It is inexpensive, convenient to use, and has many advantages. A vivid color is produced with very small amounts of protein (as low as 0.05 µg). Ponceau S can also be used simultaneously with a fixative agent (i.e., trichloroacetic acid). In this study, the Quick Scan Densitometer by Helena Laboratories[27] was employed for all serum quantitation. This particular instrument is relatively low in cost and offers some advantages in regard to speed of operation, versatility and precision (40).

Once the strips have been scanned, it is a simple procedure to calculate the concentration of each protein fraction. Chart paper has two tracings: the top trace representing the optical density pattern of the serum protein fractions, and the lower trace representing the integrator output and recording the area under the curve. Usually,

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[27]"Quick Scan" Electrophoresis Densitometer, Model 2-20, #1020; Helena Laboratories, P.O. Box 752 Beaumont, TX.

the lowest point between two peaks is picked as the boundary between two fractions. At these valleys, lines are drawn parallel to the vertical lines of the chart paper which are extended to the bottom of the integrator graph. Lines should also be drawn at the beginning and end of each scan. To determine the peak area, one must remember that the output of the electronic integrator is such that the number of spaces traversed by the integrator pen is proportional to the area under the curve traced by the recorder pen. Once the area under the curve has been determined, one calculates the percentage of each fraction and from total protein studies each fraction can be converted to a gram-percent value:

$$\text{\% Serum Protein Fraction} \times \text{Total Serum Protein (gm/100 ml)} = \text{Protein Fraction gm/100 ml}$$

To insure accuracy of determinations, periodical checks were made by using standard chemistry control serums.[28]

Adjuvant Arthritis

Oral administration of all drugs started 1 day before adjuvant injection and continued daily for 21 days. Dosage vehicle was 0.25% agar (5 ml/kg). Young adult, Sprague-Dawley female rats were lightly anesthetized with sodium pentobarbital (30 mg/kg, ip) and 0.05 ml of adjuvant[29] injected into the subplantar surface of the left hind paw. The rats were maintained with free access to Purina laboratory rat chow and tap water. Progress of inflammation in the injected

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[28] Q-PAK Chemistry Control Serum, Special; Hyland Div., Travenol Laboratories, Inc., Costa Mesa, CA.

[29] Difco #0640-33, 5 mg/ml; Difco Laboratories, Detroit, MI.

and contralateral paw was measured plethsmographically (41).

Cotton Pellet Granuloma

This method was similar to that of Meier et al. (42), and identical to the procedure currently used in the laboratory of Syntex Research (Palo Alto, CA) to screen for anti-inflammatory activity. Non-sterile, chopped raw-cotton pellets (10.0 mg$\pm$0.5 mg) were hand rolled. Ether-anesthetized, male Sprague-Dawley rats were shaved on their backs and a single transverse skin incision was made in the dorso-caudal region, where two pellets were placed in the dorso-lateral superficial fascia about 2 cm caudal to the shoulders. The incision was closed with metal wound clips. The rats were maintained with free access to Purina Laboratory Chow and tap water. Where adrenalectomized rats were needed, the animals were adrenalectomized and pellets were implanted with two incisions instead of the one above. Animals were maintain on 1% salt water and dosed orally with drugs suspended in 0.25% agar at 10 ml/kg. To prevent infection, 1% potassium penicillin G and 1% streptomycin were injected subcutaneously (0.1 ml/rat). Six days later, the rats were sacrificed by decapitation, blood was collected and the cotton pellets excised. Both wet and dry weights of the granuloma were measured. Dry granuloma weight was determined after heating the excised mass in a drying oven at 80°C for 18 hr (43).

Drugs Used in This Investigation

The following drugs were used in this study: hydro-

cortisone base (Sigma Chemical Co. #H4001), indomethacin (Merck Sharp & Dohme #L-590226-00A104), phenylbutazone (Geigy Corp. #1000), tetrabenazine (Hoffmann-La Roche #C/421), benzquinamide (Chas. Pfizer & Co., Inc. #p-2647), paramethasone (Lilly #Y03201), 6-mercaptopurine (Atlas Chemical Co.), RO 4-1284 or 2-ethyl-2,3,4,6,7,11b-hexahydro-2-hydroxy-3-isobutyl-9,10-dimethoxy-1H-benzo[h] quinolizine (Hoffmann-La Roche), RO 1-9564 or 3-ethyl-2,3,4,6,7,11b-hexahydro-2-hydroxy-2-isobutyl-9,10-dimethoxy-1H-benzo[h] quinolizine (Hoffmann-La Roche), and mefenamic acid (Parke-Davis & Co. Lot #405200).

Statistics

All statistical methods in this study were based on standard analysis of variance methods.

RESULTS

Adjuvant Arthritis

Sedimentation rates were determined at +6, 15, 21 and 30 days after adjuvant administration. Drugs were administered orally daily for 21 days beginning 1 day prior to adjuvant injection. All drugs were given at theoretically equieffective anti-inflammatory dosages (see Table I) based on preliminary screening. In animals injected with Mycobacterium adjuvant, cryogenine, phenylbutazone, hydrocortisone and paramethasone were found to be effective in suppressing increases in sedimentation rate (Figure 12). The increased sedimentation rates of positive control animals (rats receiving adjuvant but no drug treatment) within 6 days after adjuvant injection can be associated with the primary inflammatory response in the injected foot (53). Increased elevation in sedimentation rates were also noted on day 15 (associated with the acute phase of the disease) and on day 21 (when full manifestation of secondary lesions occurred in the contralateral foot and other tissues). Such elevated rates continued for the remainder of the observation period to day 30.

The dual-phasic inflammatory response also was documented in Table I and Table II which show an increase in total white cell count (peaking on day 21) associated with

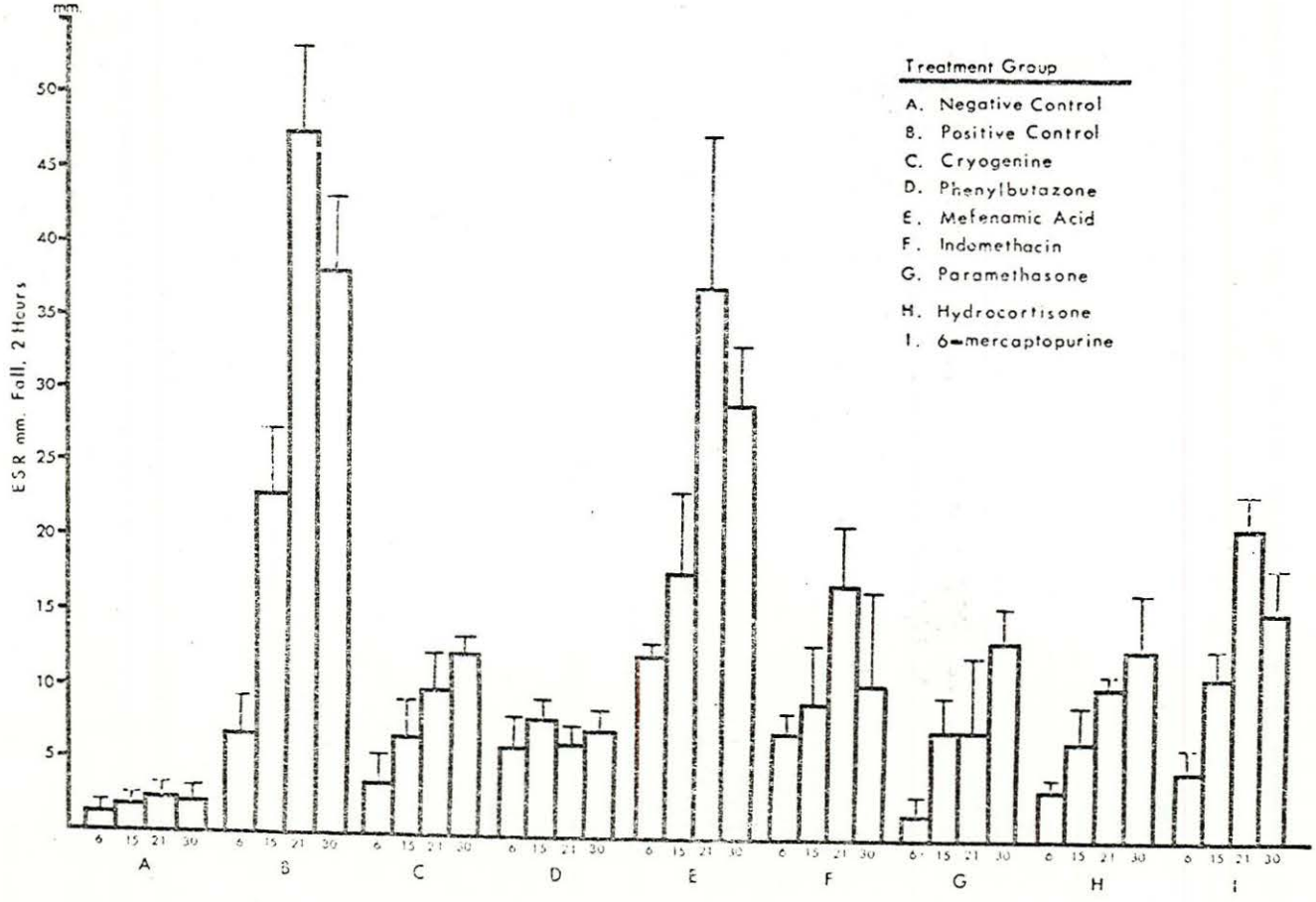
TABLE I: Effects of Selected Drug Treatments on Inflammation and Total White Cell Counts During Adjuvant-Induced Polyarthritis in Rats

Treatment Group	Daily Dosage, mg/kg	Swelling Inhibition[a] Contralateral Foot,%			Mean Leukocytes(thousands/mm^3)±S.E.			
		Day 15	Day 21	Day 30	Day 6	Day 15	Day 21	Day 30
Negative Control[b]	---	---	---	---	19.3±0.8	15.4±0.9	17.3±1.1	15.3±0.9
Positive Control[b]	---	---	---	---	24.7±2.1	25.2±3.0	29.2±1.1	24.5±3.2
Cryogenine	100.0	93.5	86.4	55.6	16.6±3.4	18.3±1.9	22.0±3.3	20.6±1.7
Phenylbutazone	100.0	74.2	83.1	67.9	21.4±0.9	22.2±3.0	24.4±2.0	23.9±2.1
Mefenamic Acid	25.0	112.9	89.8	37.0	21.4±1.8	25.3±0.9	29.3±3.1	26.3±2.0
Indomethacin	1.0	71.0	66.1	45.7	23.3±3.9	23.0±3.3	26.4±1.1	26.1±2.3
Paramethasone	0.5	125.8	105.1	95.1	21.8±2.2	10.1±2.3	13.4±0.3	18.3±1.8
Hydrocortisone	10.0	90.3	86.4	59.3	12.5±1.1	12.3±0.3	16.5±1.9	19.5±1.4
6-Mercaptopurine	2.0	103.0	91.5	75.3	14.2±1.9	24.2±2.1	26.6±2.2	25.0±2.7

[a]Foot swelling inhibition data were made available for this study, courtesy of Dr. D.S. Kosersky (44).

[b]Negative controls were animals receiving no drug treatment and no adjuvant, while positive controls are animals receiving adjuvant but no drug treatment.

Figure 12.-- Effects of selected drug treatments on erythrocyte sedimentation rates in control and adjuvant-injected rats.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Treatment Group
A. Negative Control
B. Positive Control
C. Cryogenine
D. Phenylbutazone
E. Mefenamic Acid
F. Indomethacin
G. Paramethasone
H. Hydrocortisone
I. 6-mercaptopurine

TABLE II: Effects of Drug Treatments on White Cell Counts During Adjuvant-Induced Polyarthritis in Rats

Treatment Group	Differential White Blood Cell Counts,%							
	Day 6		Day 15		Day 21		Day 30	
	Lymph.	Neutr.	Lymph.	Neutr.	Lymph.	Neutr.	Lymph.	Neutr.
Negative Control[a]	83	12	78	17	81	15	81	17
Positive Control[a]	51	43	56	43	53	37	63	32
Cryogenine	68	28	67	27	66	29	71	25
Phenylbutazone	65	30	63	35	68	28	74	21
Mefenamic Acid	57	39	52	42	61	31	59	37
Indomethacin	51	47	57	37	65	29	63	33
Paramethasone	46	48	49	47	49	44	61	33
Hydrocortisone	42	53	51	45	57	39	73	22
6-Mercaptopurine	58	37	69	21	69	27	69	25

[a]Negative controls were animals receiving no drug treatment and no adjuvant, while positive controls were animals receiving adjuvant but no drug treatment.

lymphopenia and neutrophilia in the positive control group animals. The first cell count increase on day 6 can be associated with primary swelling in the injected foot. This was followed by a second increase on day 21 which could be associated with secondary lesion development.

While all of the agents in Table I were roughly equieffective at the doses tested (with the exception of highly effective paramethasone) in preventing inflammation in the contralateral foot by day 30, only hydrocortisone and paramethasone appeared to prevent leukocytosis (as seen in day 21). Paramethasone actually decreased leukocyte count below negative control levels. Cryogenine clearly seemed to have prevented the drastic elevation of leukocyte count seen in the positive control animals, while mefenamic acid, indomethacin, and 6-mercaptopurine clearly did not affect adjuvant-induced leukocytosis. The effect of phenylbutazone at this dosage level was equivocal. There is some indication that the effects of 6-mercaptopurine (day 6) and paramethasone (day 15 and day 21) may be time dependent.

In Table II, the leukocytosis exhibited above (in positive controls) seemed to be the result of an increase in neutrophils. This increase in polymorphonuclear leukocytes seemed to be moderately reduced with cryogenine, phenylbutazone, mefenamic acid, indomethacin, and 6-mercaptopurine. However, there did not seem to be any reversal of the increase in polymorphonuclear leukocytes seen with the negative controls by the compounds, hydrocortisone and paramethasone.

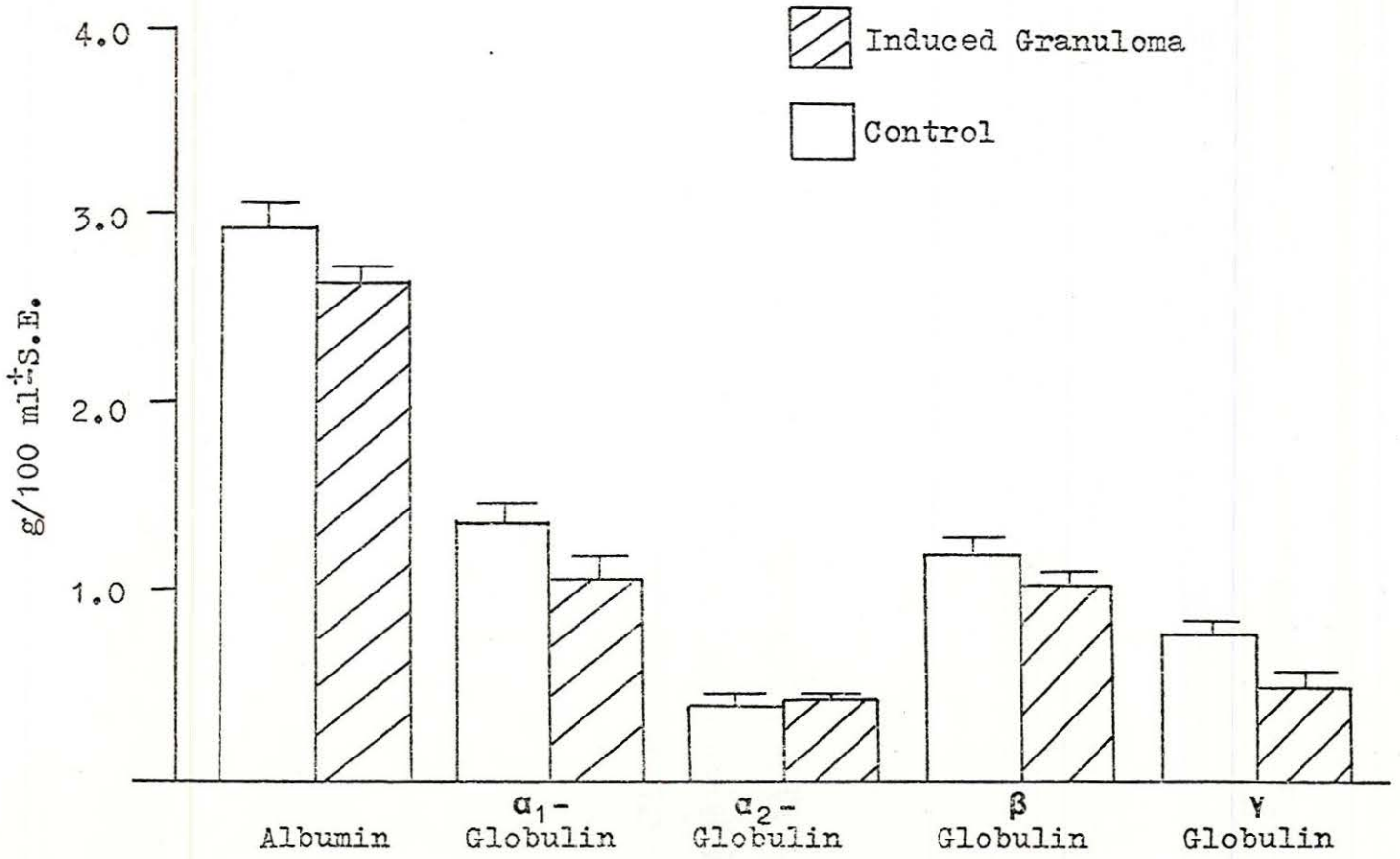
Cotton Pellet Granuloma

Since a number of serum protein studies have already been documented in the literature regarding adjuvant-induced inflammation, this study undertook the investigation of the serum protein changes produced by cotton pellet implantation (45,46,47,48,53). Positive controls (those implanted with cotton pellets but receiving no drug) showed significant decreases in all serum protein fractions except the alpha$_2$-fraction (see Figure 13).

Tables III to VII (pages 49-66) and Figures 14 to 18 (pages 51-68) document the effects of phenylbutazone, indomethacin, hydrocortisone and cryogenine on serum protein fractions of adrenalectomized and non-adrenalectomized rats with induced cotton pellet granuloma. Table VIII (page 69) shows the effects of these drugs in preventing granuloma formation.

In non-adrenalectomized rats, phenylbutazone (Table III to VIII and Figure 14 to 18) prevented the lowering of albumin with all three dosages and possibly on the gamma-fraction (median dosage); however, there were no major changes in gamma-globulin levels with both the low and top dosages. Adrenalectomized rats showed reversal occurring in the alpha$_2$-globulin and albumin fractions, with overshooting of the alpha$_1$-, beta and gamma-fractions. The dramatic rise in globulin proteins (excepting the alpha$_2$-fraction) in adrenalectomized rats was associated mainly with the low dosage of phenylbutazone and was followed by a decrease in globulin proteins with higher doses of the drug. Non-adrenalectomized (Table III) ani-

Figure 13.--Electrophoretic patterns of serums from rats with induced cotton pellet granuloma.



mals showed 11.4%, 23.3% and 22.9% granuloma inhibition (corresponding to 10 mg, 30 mg and 90 mg/kg of phenylbutazone), while adrenalectomized animals showed 11.7%, 21.9% and 33.1% inhibition, respectively. Granuloma inhibition in both instances appeared to be somewhat dose-responsive (an increase in dosage producing an increased anti-inflammatory effect).

With indomethacin-treated (Table III to VII and Figure 14 to 18), non-adrenalectomized rats, serum proteins seemed to be elevated towards normal levels in the albumin, beta (where dose-responsive decreases seemed to occurred), and the gamma-fractions. There appeared to be no reversal apparent in indomethacin-treated, adrenalectomized animals, except possibly with the albumin fraction at the highest dosage level. There was a further depression in the gamma-fraction with the median dose of 0.6 mg/kg in adrenalectomized animals, but this interesting and unexpected effect was not seen again with the high dose of indomethacin. In the granuloma studies (Table VIII), 1.6%, 12.5% and 25.3% of inhibition was documented in non-adrenalectomized animals, while adrenalectomized animals showed 3.4%, 11.6% and 28.5% inhibition (corresponding to 0.2, 0.6 and 1.8 mg/kg of indomethacin, respectively). Granuloma inhibition was somewhat less than that seen above with phenylbutazone.

Hydrocortisone (Table III to VII and Figure 14 to 18) seemed to be both very effective in the reversal of serum protein fractions to negative control levels and in the inhibi-

tion of granuloma production. In non-adrenalectomized animals, all serum fractions were returned to normal values with significant overshoots in all fractions. Step-wise increases (apparently dose-responsive) were apparent with the alpha$_1$- and gamma-globulins. Adrenalectomized animals showed effective returns to normal values; and with the high dose of hydrocortisone, significant overshoots were noted for all fractions except the beta-globulin. All elevations seemed to be somewhat dose-related. Corresponding inhibition of granuloma formation (Table VIII) was 20.4% and 40.4% for non-adrenalectomized and 23.5% and 28.3% in adrenalectomized rats with 2.5 mg and 7.5 mg/kg of drug given, respectively.

Somewhat like indomethacin, cryogenine showed a nearly complete return to normal of the gamma-fraction in non-adrenalectomized animals. In regard to albumin, the highest dose of cryogenine did not show a significant difference from the value seen with the negative controls; however, the albumin levels of all cryogenine-treated animals were not significantly different from positive controls. The data does seem to indicate that with doses greater than 189 mg/kg of cryogenine, significant differences from positive control might be seen in the albumin fraction. Only the alpha$_1$-serum protein fraction seemed to return towards normal (overshooting at the highest dose) in adrenalectomized rats. It is interesting to note that albumin levels seemed to decrease with increasing levels of cryogenine, with a very significant drop noted in animals receiving the highest dose. Granuloma

TABLE III: Effects of Selected Drug Treatments on Serum Albumin of Rats With Induced Cotton Pellet Granuloma (gm/100 ml ± S.E.)

Treatment Group	N	Non-Adrenalectomized	N	Adrenalectomized	P
Negative Control	15	2.98±0.07	8	2.41±0.03	<0.001
Positive Control	24	2.64±0.04^{d*}	7	2.31±0.08^{a*}	<0.01
Phenylbutazone (10mg/kg/day)	8	2.95±0.07^{a*d**}	7	2.53±0.04^{b*b**}	<0.001
Phenylbutazone (30mg/kg/day)	8	2.98±0.08^{a*d**}	8	3.04±0.14^{d*d**}	>0.05
Phenylbutazone (90mg/kg/day)	8	2.84±0.05^{a*a**}	8	3.24±0.13^{d*d**}	<0.05
Indomethacin (0.2mg/kg/day)	8	2.69±0.04^{c*a**}	8	2.38±0.09^{a*a**}	<0.01
Indomethacin (0.6mg/kg/day)	7	2.80±0.04^{b*c**}	8	2.39±0.08^{a*a**}	<0.001
Indomethacin (1.8mg/kg/day)	8	2.77±0.03^{b*b**}	7	2.58±0.04^{c*b**}	<0.01
Hydrocortisone (2.5mg/kg/day)	7	3.24±0.08^{b*d**}	8	2.74±0.08^{c*c**}	<0.001
Hydrocortisone (7.5mg/kg/day)	8	3.24±0.08^{b*d**}	7	3.51±0.11^{d*d**}	>0.05
Cryogenine (21mg/kg/day)	8	2.60±0.13^{b*a**}	8	2.29±0.06^{a*a**}	<0.05
Cryogenine (63mg/kg/day)	8	2.70±0.03^{c*a**}	8	2.24±0.10^{a*a**}	<0.001
Cryogenine (189mg/kg/day)	8	2.78±0.07^{a*a**}	8	1.00±0.16^{d*d**}	<0.001

TABLE III (continued)

---

[a]Not significantly different ($P \geqslant 0.05$).

[b]Significantly different ($P = 0.01\text{-}0.05$).

[c]Highly significantly different ($P = 0.001\text{-}0.01$).

[d]Very highly significantly different ($P = < 0.001$).

*Indicates statistical comparison with the negative control---animals without cotton pellet implantation and dosed with 0.25% agar (10 ml/kg).

**Indicates statistical comparison with the positive control--animals with cotton pellet implantation and dosed with 0.25% agar (10 ml/kg).

Figure 14.--Effects of selected drug treatment on serum albumin of rats with induced cotton pellet granuloma.

Treatment Group	Drug Dosage mg/kg Low(L)	Medium(M)	High(H)
Control (Con)	---	---	---
Phenylbutazone (PBZ)	30.0	60.0	90.0
Indomethacin (IND)	0.2	0.6	1.8
Hydrocortisone (HYC)	2.5	---	7.5
Cryogenine (CRY)	21.0	63.0	189.0

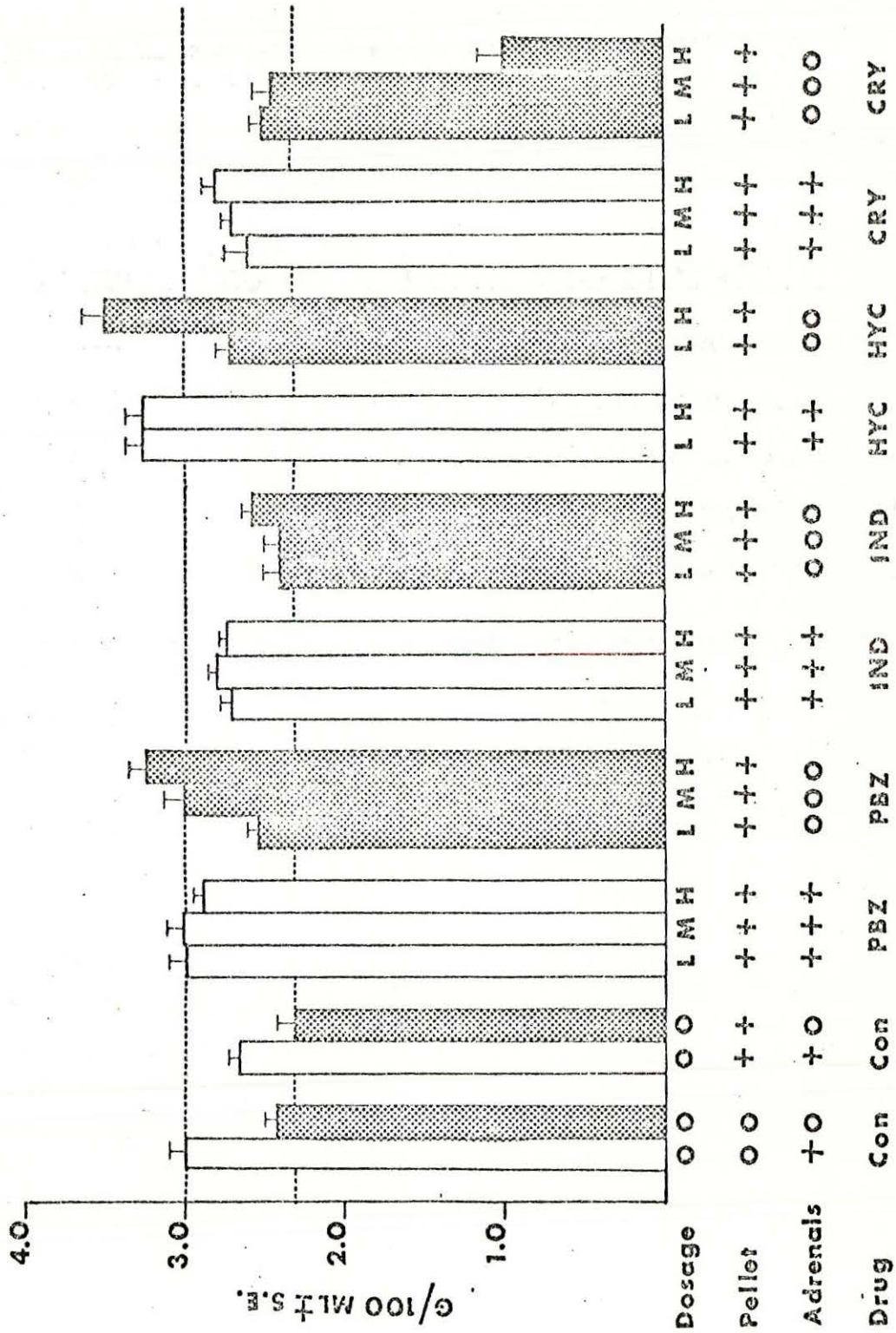

g/100 ML ± S.E.
4.0
3.0
2.0
1.0
Dosage
Pellet
Adrenals
Drug
Con
Con
PBZ
PBZ
IND
IND
HYC
HYC
CRY
CRY
L M H
L M H
L M H
L M H
L M H
L M H
L M H
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TABLE IV: Effects of Selected Drug Treatments on Serum Alpha$_1$-Globulin Proteins of Rats With Induced Cotton Pellet Granuloma (gm/100 ml ± S.E.)

Treatment Group	N	Non-Adrenalectomized	N	Adrenalectomized	P
Negative Control	15	1.26 ± 0.04	8	1.10 ± 0.05	<0.05
Positive Control	24	$0.97 \pm 0.03^{d*}$	7	$1.09 \pm 0.02^{a*}$	<0.01
Phenylbutazone (10mg/kg/day)	8	$0.98 \pm 0.03^{d*a**}$	7	$1.84 \pm 0.04^{d*d**}$	<0.001
Phenylbutazone (30mg/kg/day)	8	$1.07 \pm 0.04^{c*a**}$	8	$1.56 \pm 0.07^{d*d**}$	<0.001
Phenylbutazone (90mg/kg/day)	8	$1.05 \pm 0.04^{c*a**}$	8	$1.67 \pm 0.06^{d*d**}$	<0.001
Indomethacin (0.2mg/kg/day)	8	$0.99 \pm 0.05^{d*a**}$	8	$1.02 \pm 0.05^{a*a**}$	>0.05
Indomethacin (0.6mg/kg/day)	7	$1.02 \pm 0.03^{d*a**}$	8	$1.08 \pm 0.03^{a*a**}$	>0.05
Indomethacin (1.8mg/kg/day)	8	$1.05 \pm 0.03^{d*a**}$	7	$1.15 \pm 0.04^{a*a**}$	>0.05
Hydrocortisone (2.5mg/kg/day)	7	$1.43 \pm 0.03^{c*d**}$	8	$1.31 \pm 0.07^{d*b**}$	>0.05
Hydrocortisone (7.5mg/kg/day)	8	$1.59 \pm 0.11^{b*d**}$	7	$1.47 \pm 0.06^{d*d**}$	>0.05
Cryogenine (21mg/kg/day)	8	$1.00 \pm 0.04^{d*a**}$	8	$1.19 \pm 0.05^{a*a**}$	>0.05
Cryogenine (63mg/kg/day)	8	$0.99 \pm 0.02^{d*a**}$	8	$1.21 \pm 0.10^{a*a**}$	<0.05
Cryogenine (189mg/kg/day)	8	$1.06 \pm 0.04^{c*a**}$	8	$1.38 \pm 0.05^{c*d**}$	<0.001

TABLE IV (continued)

---

[a]Not significantly different ($P \geqslant 0.05$).

[b]Significantly different ($P = 0.01-0.05$).

[c]Highly significantly different ($P = 0.001-0.01$).

[d]Very highly significantly different ($P = < 0.001$).

*Indicates statistical comparison with the negative control--animals without cotton pellet implantation and dosed with 0.25% agar (10 ml/kg).

**Indicates statistical comparison with the positive control--animals with cotton pellet implantation and dosed with 0.25% agar (10 ml/kg).

Figure 15.---Effects of selected drug treatment on serum alpha$_1$-globulin proteins of rats with induced cotton pellet granuloma.

Treatment Group	Drug Dosage mg/kg Low(L)	Medium(M)	High(H)
Control (Con)	---	---	---
Phenylbutazone (PBZ)	30.0	60.0	90.0
Indomethacin (IND)	0.2	0.6	1.8
Hydrocortisone (HYC)	2.5	---	7.5
Cryogenine (CRY)	21.0	63.0	189.0

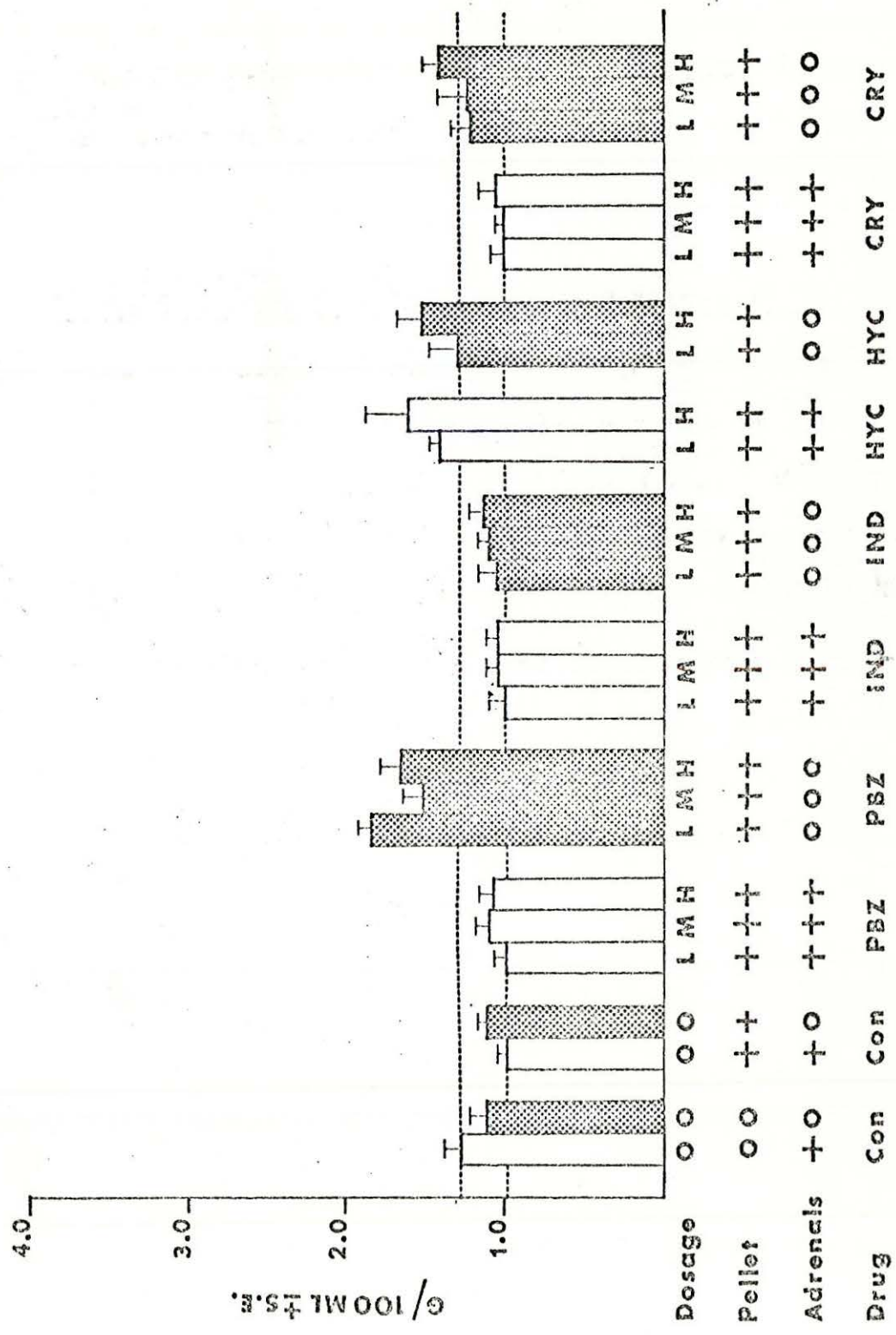
g/100 ML ±S.E.
4.0
3.0
2.0
1.0
Dosage
Pellet
Adrenals
Drug
Con
PBZ
IND
HYC
CRY
L M H

TABLE V: Effects of Selected Drug Treatments on Serum Alpha$_2$-Globulin Proteins of Rats With Induced Cotton Pellet Granuloma (gm/100 ml $\pm$ S.E.)

Treatment Group	N	Non-Adrenalectomized	N	Adrenalectomized	P
Negative Control	15	0.44 ± 0.03	8	0.45 ± 0.02	>0.05
Positive Control	24	$0.41 \pm 0.01^{a*}$	7	$0.34 \pm 0.02^{d*}$	<0.01
Phenylbutazone (10mg/kg/day)	8	$0.43 \pm 0.01^{a*a**}$	7	$0.47 \pm 0.02^{a*d*}$	>0.05
Phenylbutazone (30mg/kg/day)	8	$0.36 \pm 0.02^{b*b**}$	8	$0.46 \pm 0.02^{a*c*}$	<0.001
Phenylbutazone (90mg/kg/day)	8	$0.40 \pm 0.12^{a*a**}$	8	$0.54 \pm 0.02^{c*d*}$	>0.05
Indomethacin (0.2mg/kg/day)	8	$0.37 \pm 0.02^{a*a**}$	8	$0.30 \pm 0.02^{d*a**}$	<0.05
Indomethacin (0.6mg/kg/day)	7	$0.39 \pm 0.03^{a*a**}$	8	$0.31 \pm 0.02^{d*a**}$	<0.05
Indomethacin (1.8mg/kg/day)	8	$0.36 \pm 0.03^{a*a**}$	7	$0.30 \pm 0.01^{d*a**}$	>0.05
Hydrocortisone (2.5mg/kg/day)	7	$0.51 \pm 0.03^{a*c**}$	8	$0.45 \pm 0.02^{a*c**}$	>0.05
Hydrocortisone (7.5mg/kg/day)	8	$0.54 \pm 0.03^{b*d**}$	7	$0.59 \pm 0.03^{c*d**}$	>0.05
Cryogenine (21mg/kg/day)	8	$0.35 \pm 0.01^{c*d**}$	8	$0.37 \pm 0.02^{b*a**}$	>0.05
Cryogenine (63mg/kg/day)	8	$0.40 \pm 0.02^{a*a**}$	8	$0.39 \pm 0.02^{a*a**}$	>0.05
Cryogenine (189mg/kg/day)	8	$0.38 \pm 0.01^{a*b**}$	8	$0.35 \pm 0.02^{c*a**}$	>0.05

TABLE V (continued)

---

[a]Not significantly different ($P \geqslant 0.05$).

[b]Significantly different ($P = 0.01-0.05$).

[c]Highly significantly different ($P = 0.001-0.01$).

[d]Very highly significantly different ($P = \leq 0.001$).

*Indicates statistical comparison with the negative control--animals without cotton pellet implantation and dosed with 0.25% agar (10 ml/kg).

**Indicates statistical comparison with the positive control--animals with cotton pellet implantation and dosed with 0.25% agar (10 ml/kg).

Figure 16.--Effects of selected drug treatment on serum alpha$_2$-globulin proteins of rats with induced cotton pellet granuloma.

Treatment Group	Drug Dosage mg/kg Low(L)	Medium(M)	High(H)
Control (Con)	---	---	---
Phenylbutazone (PBZ)	30.0	60.0	90.0
Indomethacin (IND)	0.2	0.6	1.8
Hydrocortisone (HYC)	2.5	---	7.5
Cryogenine (CRY)	21.0	63.0	189.0

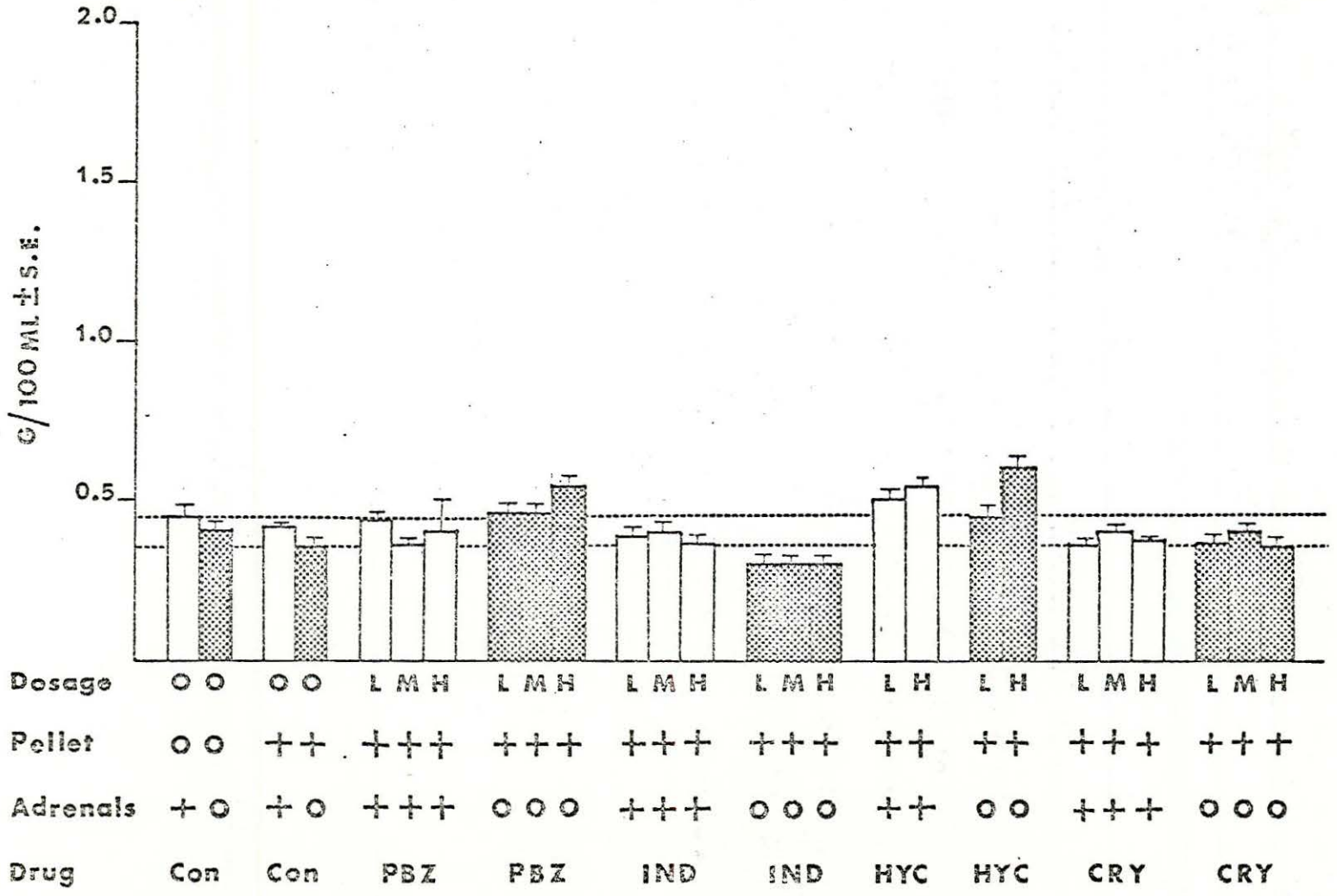
2.0
1.5
1.0
0.5
C/100 ML ± S.E.
Dosage O O O O L M H L M H L M H L M H L H L H L M H L M H
Pellet O O + + + + + + + + + + + + + + + + + +
Adrenals + O + O + + + O O O + + + O O O + + O + + + O O O
Drug Con Con PBZ PBZ IND IND HYC HYC CRY CRY

TABLE VI: Effects of Selected Drug Treatments on Serum Beta-Globulin Proteins of Rats With Induced Cotton Pellet Granuloma (gm/100 ml ± S.E.)

Treatment Group	N	Non-Adrenalectomized	N	Adrenalectomized	P
Negative Control	15	1.20±0.03	8	1.12±0.03	>0.05
Positive Control	24	1.09±0.02^{c*}	7	1.01±0.04^{b*}	>0.05
Phenylbutazone (10mg/kg/day)	8	1.05±0.03^{c*a**}	7	1.55±0.08^{d*d**}	<0.001
Phenylbutazone (30mg/kg/day)	8	1.07±0.04^{b*a**}	8	1.33±0.05^{c*d**}	<0.01
Phenylbutazone (90mg/kg/day)	8	1.09±0.04^{b*a**}	8	1.24±0.06^{a*c**}	>0.05
Indomethacin (0.2mg/kg/day)	8	1.19±0.04^{a*b**}	8	0.95±0.06^{b*a**}	<0.01
Indomethacin (0.6mg/kg/day)	7	1.16±0.04^{a*a**}	8	0.94±0.02^{d*a**}	<0.001
Indomethacin (1.8mg/kg/day)	8	1.04±0.03^{c*a**}	7	0.97±0.03^{c*a**}	>0.05
Hydrocortisone (2.5mg/kg/day)	7	1.36±0.04^{c*d**}	8	1.09±0.02^{a*a**}	<0.001
Hydrocortisone (7.5mg/kg/day)	8	1.34±0.04^{b*d**}	7	1.25±0.04^{b*c**}	>0.05
Cryogenine (21mg/kg/day)	8	1.07±0.03^{c*a**}	8	1.10±0.02^{a*a**}	>0.05
Cryogenine (63mg/kg/day)	8	1.00±0.03^{d*b**}	8	1.05±0.04^{a*a**}	>0.05
Cryogenine (189mg/kg/day)	8	0.89±0.01^{d*d**}	8	1.10±0.03^{a*a**}	<0.001

TABLE VI (continued)

---

[a]Not significantly different ($P \geqslant 0.05$).

[b]Significantly different ($P = 0.01\text{-}0.05$).

[c]Highly significantly different ($P = 0.001\text{-}0.01$).

[d]Very highly significantly different ($P = < 0.001$).

*Indicates statistical comparison with the negative control--animals without cotton pellet implantation and dosed with 0.25% agar (10 ml/kg).

**Indicates statistical comparison with the positive control--animals with cotton pellet implantation and dosed with 0.25% agar (10 ml/kg).

Figure 17.--Effects of selected drug treatment on serum beta-globulin proteins of rats with induced cotton pellet granuloma.

Treatment Group	Drug Dosage mg/kg Low(L)	Medium(M)	High(H)
Control (Con)	---	---	---
Phenylbutazone (PBZ)	30.0	60.0	90.0
Indomethacin (IND)	0.2	0.6	1.8
Hydrocortisone (HYC)	2.5	---	7.5
Cryogenine (CRY)	21.0	63.0	189.0

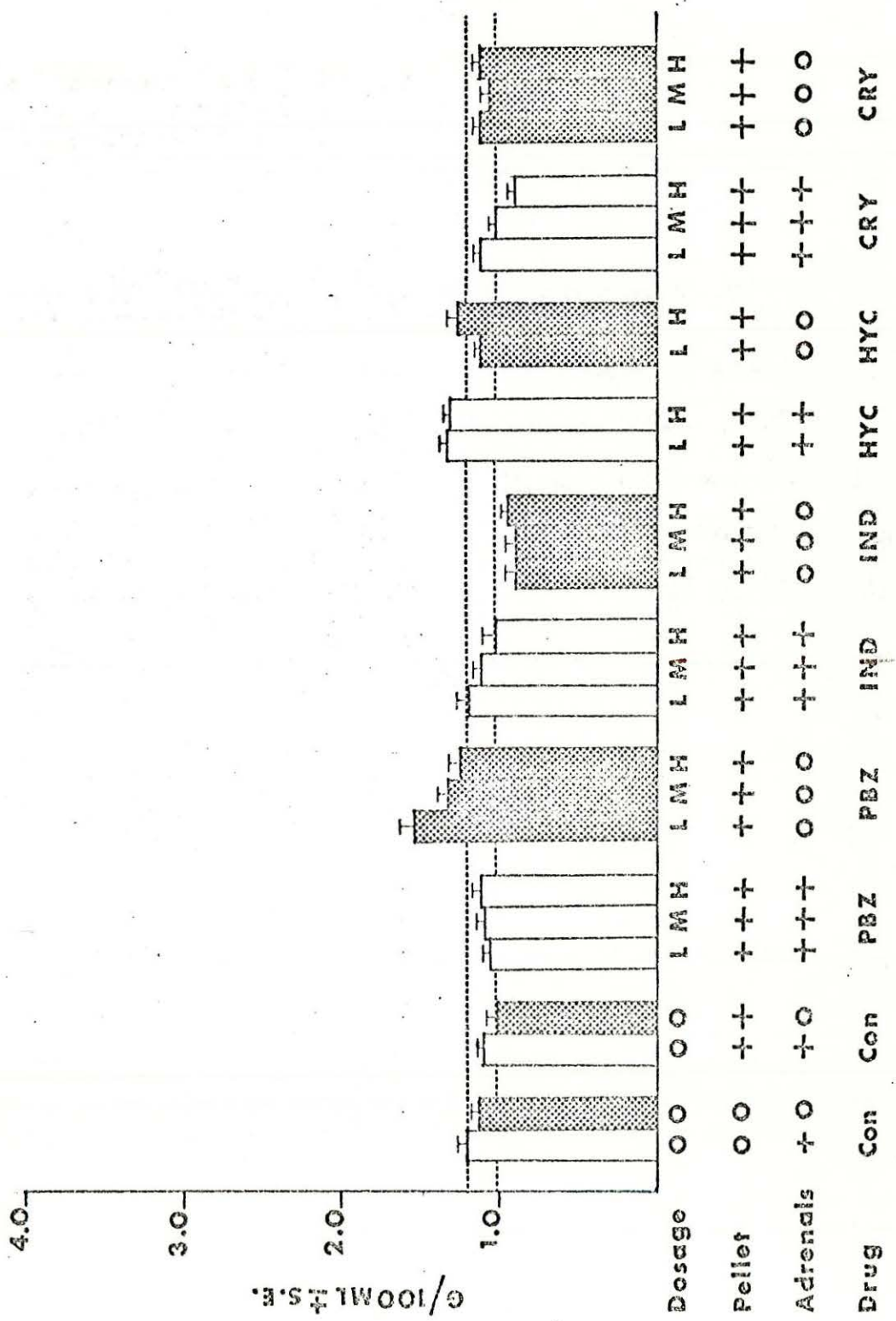
4.0
3.0
2.0
1.0
g/100ML ± S.E.
Dosage 0 0 0 0 L M H L M H L M H L M H L M H L H L H L M H L M H
Pellet 0 0 +
Adrenals + 0 + 0 + + + + + + + + 0 0 0 0 + + + + 0 0 + + + + 0 0 0
Drug Con Con PBZ PBZ IND IND HYC HYC CRY CRY

TABLE VII: Effects of Selected Drug Treatments on Serum Gamma-Globulin Proteins of Rats With Induced Cotton Pellet Granuloma (gm/100 ml±S.E.)

Treatment Group	N	Non-Adrenalectomized	N	Adrenalectomized	P
Negative Control	15	0.70 ± 0.04	8	0.45 ± 0.03	<0.001
Positive Control	24	$0.46 \pm 0.02^{d*}$	7	$0.62 \pm 0.04^{c*}$	<0.01
Phenylbutazone (10mg/kg/day)	8	$0.51 \pm 0.04^{c*a**}$	7	$0.99 \pm 0.06^{d*d**}$	<0.001
Phenylbutazone (30mg/kg/day)	8	$0.66 \pm 0.04^{a*d**}$	8	$0.74 \pm 0.08^{c*a**}$	>0.05
Phenylbutazone (90mg/kg/day)	8	$0.50 \pm 0.06^{b*a**}$	8	$0.79 \pm 0.10^{c*a**}$	<0.05
Indomethacin (0.2mg/kg/day)	8	$0.63 \pm 0.04^{a*d**}$	8	$0.64 \pm 0.10^{a*a**}$	>0.05
Indomethacin (0.6mg/kg/day)	7	$0.64 \pm 0.07^{a*b**}$	8	$0.45 \pm 0.05^{a*b**}$	<0.05
Indomethacin (1.8mg/kg/day)	8	$0.62 \pm 0.05^{a*c**}$	7	$0.54 \pm 0.05^{a*a**}$	>0.05
Hydrocortisone (2.5mg/kg/day)	7	$0.82 \pm 0.06^{a*d**}$	8	$0.71 \pm 0.05^{d*a**}$	>0.05
Hydrocortisone (7.5mg/kg/day)	8	$0.88 \pm 0.10^{a*d**}$	7	$0.95 \pm 0.12^{b*b**}$	<0.05
Cryogenine (21mg/kg/day)	8	$0.68 \pm 0.03^{a*d**}$	8	$0.58 \pm 0.06^{a*a**}$	>0.05
Cryogenine (63mg/kg/day)	8	$0.64 \pm 0.07^{a*b**}$	8	$0.52 \pm 0.05^{a*a**}$	>0.05
Cryogenine (189mg/kg/day)	8	$0.65 \pm 0.08^{a*b**}$	8	$0.58 \pm 0.05^{b*a**}$	>0.05

TABLE VII (continued)

---

[a]Not significantly different ($P \gg 0.05$).

[b]Significantly different ($P = 0.01-0.05$).

[c]Highly significantly different ($P = 0.001-0.01$).

[d]Very highly significantly different ($P = < 0.001$).

*Indicates statistical comparison with the negative control---animals without cotton pellet implantation and dosed with 0.25% agar (10 ml/kg).

**Indicates statistical comparison with the positive control---animals with cotton pellet implantation and dosed with 0.25% agar (10 ml/kg).

Figure 18.---Effects of selected drug treatment on serum gamma-globulin proteins of rats with induced cotton pellet granuloma.

Treatment Group	Drug Dosage mg/kg Low(L)	Medium(M)	High(H)
Control (Con)	---	---	---
Phenylbutazone (PBZ)	30.0	60.0	90.0
Indomethacin (IND)	0.2	0.6	1.8
Hydrocortisone (HYC)	2.5	---	7.5
Cryogenine (CRY)	21.0	63.0	189.0

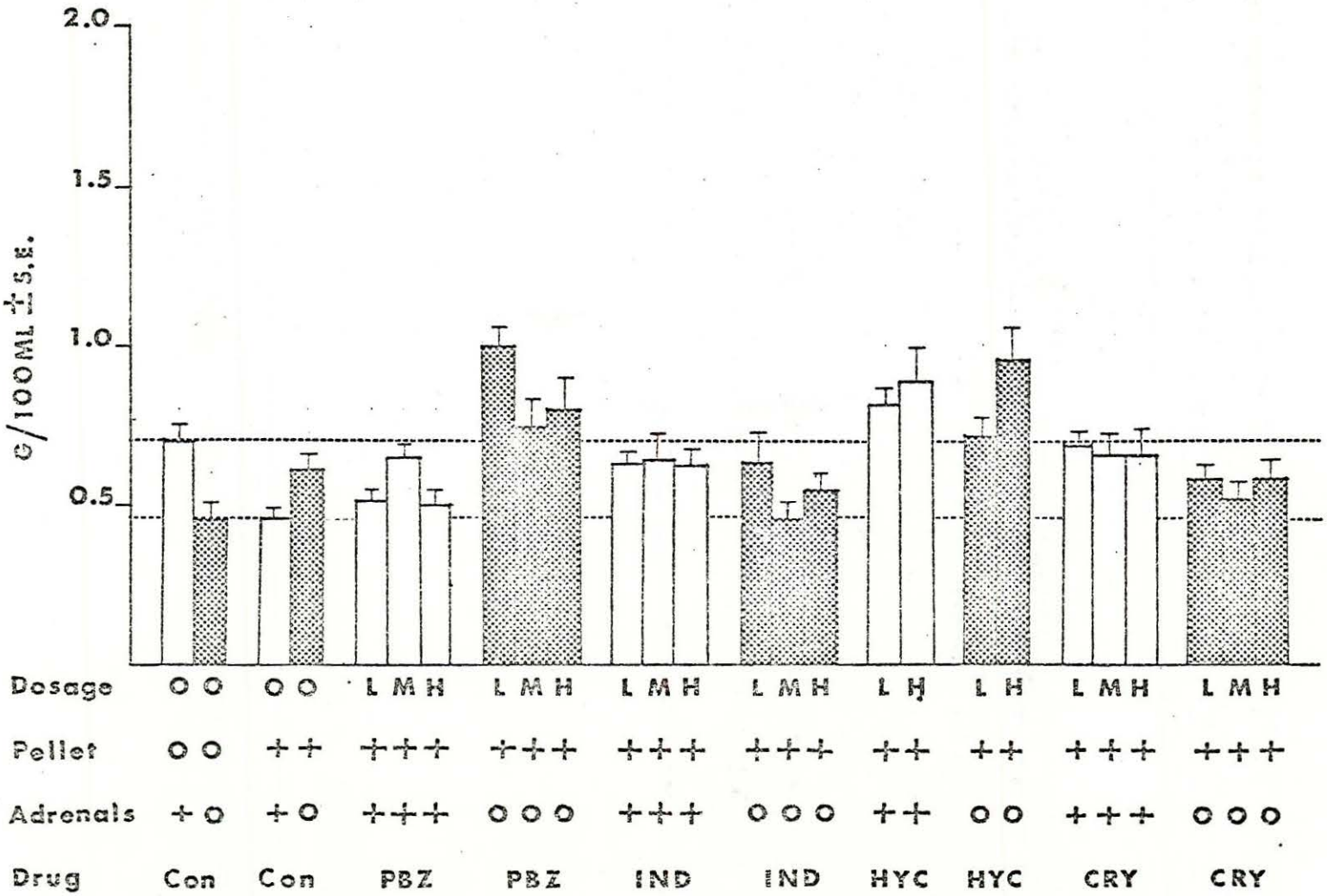
2.0
1.5
1.0
0.5
c/100ML ± S.E.
Dosage O O O O L M H L M H L M H L M H L H L H L M H L M H
Pellet O O + + + + + + + + + + + + + + + + + +
Adrenals + O + O + + + O O O + + + O O O + + O O + + + O O O
Drug Con Con PBZ PBZ IND IND HYC HYC CRY CRY

TABLE VIII: Effects of Selected Drug Treatments on Rats With Induced Cotton Pellet Granuloma (Dry Granuloma Wt. mg/100 g Body Wt. ± S.E.)[a]

Treatment Group	Non-Adrenalectomized	Adrenalectomized
Positive Control	41.9±2.83	48.0±2.78
Phenylbutazone (10mg/kg/day)	37.1±2.14	42.4±3.75
Phenylbutazone (30mg/kg/day)	31.3±3.50	37.5±1.99
Phenylbutazone (90mg/kg/day)	32.7±2.40	32.1±2.08
Positive Control	25.6±2.13	43.8±3.36
Indomethacin (0.2mg/kg/day)	25.2±2.22	42.3±3.76
Indomethacin (0.6mg/kg/day)	22.4±1.02	38.7±3.07
Indomethacin (1.8mg/kg/day)	19.1±1.61	31.3±1.17
Positive Control	34.2±2.96	48.0±2.78
Hydrocortisone (2.5mg/kg/day)	27.2±3.27	36.7±2.18
Hydrocortisone (7.5mg/kg/day)	20.4±1.33	24.4±2.63
Positive Control	30.2±2.45	43.8±3.36
Cryogenine (21mg/kg/day)	25.4±1.85	45.2±3.58
Cryogenine (63mg/kg/day)	26.7±2.06	40.0±2.08
Cryogenine (189mg/kg/day)	21.5±1.70	40.9±5.85

[a]Granuloma data were made available for this study courtesy of L. DeCato (43).

formation (Table VIII) inhibition seemed to correlate with the above data in that non-adrenalectomized animals showed 15.9%, 11.6% and 28.8% inhibition, while adrenalectomized rats showed 3.2%, 8.7% and 6.8% of granuloma inhibition (for 21 mg, 63 mg and 189 mg/kg of cryogenine, respectively). Such results could indicate that part of the anti-inflammatory action of cryogenine may depend on the presence of the adrenals.

The results of tetrabenazine and RO 4-1284 are shown in Tables IX and X, respectively. Tetrabenazine showed significant recovery in albumin (overshooting with the high dose) and gamma-fractions while RO 4-1284 showed recovery in albumin, beta- and gamma-fractions. In both cases the serum protein fraction recovery also appeared to be significantly different from negative control values. Inhibition of granuloma formation with tetrabenazine (going from lowest to highest dose) were 17.1%, 14.4% and 28.5% while RO 4-1284 showed 20.0%, 16.1% and 35.7% inhibition, respectively. There seems to be some similarity between the effects of tetrabenazine and the experimental compound, cryogenine, on serum protein fraction changes.

Benzquinamide and RO 1-9564 showed some similarity in serum protein changes and inhibition of granuloma tissue formation (Tables XI and XII). With benzquinamide, there was a return (no significant difference between negative control and drug-treated animals) to negative control values for the following serum protein fractions: beta- and gamma-globu-

Treatment Scheme for Table IX.

Treatment Group	Dosage[d] (mg/kg/day)	Population
Negative Control[b]	----	15
Positive Control	----	24
Tetrabenazine	7	8
Tetrabenazine	21	8
Tetrabenazine	63	8

[d]In 0.25% agar, at 10 ml/kg.

TABLE IX: Effects of Selected Drug Treatments on Serum Proteins of Rats With Induced Cotton Pellet Granuloma (gm/100 ml±S.E.)

Treatment Group	Albumin	α_1-globulin	α_2-globulin	β-globulin	γ-globulin	Dry Granuloma Wt. mg/100 gm±S.E.[a]
Negative Control[b] (No Drug)	2.98±0.07	1.26±0.04	0.44±0.03	1.20±0.03	0.70±0.04	---
Positive Control (No Drug)	2.64±0.04[c]	0.97±0.03[c]	0.41±0.01	1.09±0.02[c]	0.46±0.02[c]	26.3±1.32
Tetrabenazine (7mg/kg/day)	2.70±0.05[c]	1.00±0.04[c]	0.41±0.02	1.06±0.03[c]	0.62±0.08	21.8±1.10
Tetrabenazine (21mg/kg/day)	2.78±0.06[c]	0.95±0.02[c]	0.43±0.02	1.10±0.03[c]	0.77±0.05[d]	22.5±1.66
Tetrabenazine (63mg/kg/day)	3.17±0.10[d]	1.00±0.04[c]	0.41±0.03	1.10±0.03[c]	0.78±0.05[d]	18.8±1.23

[a]Granuloma data were made available for this study courtesy of L. DeCato (43).

[b]No cotton pellet-induced granuloma.

[c]Significantly different from negative control values ($P < 0.05$).

[d]Significantly different from positive control values ($P < 0.05$).

Treatment Scheme for Table X.

Treatment Group	Dosage[d] (mg/kg/day)	Population
Negative Control[b]	----	15
Positive Control	----	24
RO 4-1284	5	7
RO 4-1284	15	8
RO 4-1284	45	5

[d]In 0.25% agar, at 10 ml/kg.

TABLE X: Effects of Selected Drug Treatments on Serum Proteins of Rats With Induced Cotton Pellet Granuloma (gm/100 ml±S.E.)

Treatment Group	Albumin	α_1-globulin	α_2-globulin	β-globulin	γ-globulin	Dry Granuloma Wt. mg/100 gm±S.E.[a]
Negative Control[b] (No Drug)	2.98±0.07	1.26±0.04	0.44±0.03	1.20±0.03	0.70±0.04	---
Positive Control (No Drug)	2.64±0.04[c]	0.97±0.03 [c]	0.41±0.01	1.09±0.02[c]	0.46±0.02[c]	28.5±1.37
RO 4-1284 (5mg/kg/day)	3.09±0.11[d]	0.97±0.05[c]	0.36±0.02[c,d]	1.13±0.06	0.55±0.08[c]	22.8±0.91
RO 4-1284 (15mg/kg/day)	3.20±0.09[d]	0.90±0.03[c]	0.36±0.02[c,d]	1.25±0.03[d]	0.59±0.06[d]	23.9±1.43
Ro 4-1284 (45mg/kg/day)	3.48±0.10[c,d]	0.85±0.04[c,d]	0.36±0.02[c,d]	1.16±0.04	0.73±0.08[d]	18.6±1.81

[a] Granuloma data were made available for this study courtesy of L. DeCato (43).

[b] No cotton pellet-induced granuloma.

[c] Significantly different from negative control values ($P < 0.05$).

[d] Significantly different from positive control values ($P < 0.05$).

Treatment Scheme for Table XI.

Treatment Group	Dosage[d] (mg/kg/day)	Population
Negative Control[b]	---	15
Positive Control	---	24
Benzquinamide	20	8
Benzquinamide	60	8
Benzquinamide	180	8

[d]In 0.25% agar, at 10 ml/kg.

TABLE XI: Effects of Selected Drug Treatments on Serum Proteins of Rats With Induced Cotton Pellet Granuloma (gm/100 ml±S.E.)

Treatment Group	Albumin	α_1-globulin	α_2-globulin	β-globulin	γ-globulin	Dry Granuloma Wt. mg/100 gm±S.E.[a]
Negative Control[b] (No Drug)	2.98±0.07	1.26±0.04	0.44±0.03	1.20±0.03	0.70±0.04	---
Positive Control (No Drug)	2.64±0.04[c]	0.97±0.03[c]	0.41±0.01	1.09±0.02[c]	0.46±0.02[c]	32.0±2.32
Benzquinamide (20mg/kg/day)	2.80±0.03[cd]	0.98±0.04[c]	0.40±0.03	1.12±0.04	0.64±0.09	27.4±1.91
Benzquinamide (60mg/kg/day)	2.57±0.07[c]	0.98±0.04[c]	0.42±0.02	1.22±0.04[d]	0.71±0.09[d]	27.8±1.98
Benzquinamide (180mg/kg/day)	2.74±0.07[c]	1.01±0.06[c]	0.37±0.02	1.24±0.03[d]	0.63±0.06[d]	24.6±1.75

[a] Granuloma data were made available for this study courtesy L. DeCato (43).

[b] No cotton pellet-induced granuloma.

[c] Significantly different from negative control values ($P < 0.05$).

[d] Significantly different from positive control values ($P < 0.05$).

Treatment Scheme for Table XII.

Treatment Group	Dosage[d] (mg/kg/day)	Population
Negative Control[b]	---	15
Positive Control	---	24
RO 1-9564	10	8
RO 1-9564	30	8
RO 1-9564	90	7

[d] In 0.25% agar, at 10 ml/kg.

TABLE XII: Effects of Selected Drug Treatments on Serum Proteins of Rats With Induced Cotton Pellet Granuloma (gm/100 ml±S.E.)

Treatment Group	Albumin	α_1-globulin	α_2-globulin	β-globulin	γ-globulin	Dry Granuloma Wt. mg/100 gm±S.E.[a]
Negative Control[b] (No Drug)	2.98±0.07	1.26±0.04	0.44±0.03	1.20±0.03	0.70±0.04	---
Positive Control (No Drug)	2.64±0.04[c]	0.97±0.03[c]	0.41±0.01	1.09±0.02[c]	0.46±0.02[c]	28.5±1.37
RO 1-9564 (10mg/kg/day)	3.02±0.06[d]	0.90±0.05[c]	0.34±0.02[cd]	1.10±0.04	0.73±0.09[d]	25.0±1.52
RO 1-9564 (30mg/kg/day)	3.01±0.07[d]	0.94±0.03[c]	0.34±0.02[cd]	1.02±0.06[c]	0.66±0.11	21.6±1.60
RO 1-9564 (90mg/kg/day)	3.01±0.09[d]	0.89±0.03[c]	0.33±0.02[cd]	1.12±0.04	1.01±0.04[cd]	19.0±1.19

[a]Granuloma data were made available for this study courtesy L. DeCato (43).

[b]No cotton pellet-induced granuloma.

[c]Significantly different from negative control values ($P < 0.05$).

[d]Significantly different from positive control values ($P < 0.05$).

lins. Only beta and gamma-globulins, however, showed significant differences from the positive control. Alpha$_2$-globulin levels were not significantly depressed in positive control (having cotton pellet implantations) and there appeared to be no effect of benzquinamide on this globulin. The granuloma inhibition data showed (going from low dose to high dose) 14.3%, 13.1% and 23.1% inhibition in benzquinamide-treated animals while compound RO 1-9564 showed 20.4%, 17.1% and 29.8% inhibition, respectively.

In Table XIII, results are summarized regarding the effects of various types of stress. There appeared to be some lowering of albumin and alpha$_1$-globulin in sham-operated animals when compared with the negative controls (no treatment, no cotton pellet implantation and with intact adrenals). These slight changes may indicate some edema formation due to the surgery itself. Starvation (off food 2 days) produced significant increases in albumin, alpha$_1$-, and gamma-globulins from positive control levels; however, only the albumin was significantly higher than the negative control. Dehydration (off water 2 days) showed a significant increase of all protein fractions from positive control values and a significant increase in all fractions except the albumin when compared to the negative controls. This observation in the dehydrated animals may be just an indication of reduced blood volume which would explain the increased proteins.

Treatment Scheme for Table XIII.

Treatment Group	Dosage[f] (mg/kg/day)	Population
Negative Control[b]	----	15
Positive Control	----	24
Negative Control[c]	----	8
Sham Operation	----	8
Off Food 2 Days	----	8
Off Water 2 Days	----	6

[f]In 0.25% agar, at 10 ml/kg.

TABLE XIII: Effects of Selected Drug Treatments on Serum Proteins of Rats With Induced Cotton Pellet Granuloma (gm/100 ml±S.E.)

Treatment Group	Albumin	α_1-globulin	α_2-globulin	β-globulin	γ-globulin	Dry Granuloma Wt. mg/100 gm±S.E.[a]
Negative Control[b] (No Drug)	2.98±0.07	1.26±0.04	0.44±0.03	1.20±0.03	0.70±0.04	---
Positive Control (No Drug).	2.64±0.04[d]	0.97±0.03[d]	0.41±0.01	1.09±0.02[d]	0.46±0.02[d]	30.2±2.43
Negative Control[c] (No Drug)	2.42-0.03[d]	1.10-0.05[d]	0.45±0.02	1.12±0.03	0.52±0.03[d]	---
Sham Operation[b] (No Drug)	2.73±0.07[d]	1.05±0.04[d]	0.40±0.03	1.17±0.07	0.68±0.07	---
Off Food 2 Days (No Drug)	3.26±0.09[de]	1.29±0.06[e]	0.42±0.02	1.13±0.07	0.76±0.12[e]	26.4±1.79
Off Water 2 Days (No Drug)	3.01±0.06[e]	1.50±0.07[de]	0.55±0.02[de]	1.49±0.04[de]	1.04±0.11[de]	27.7±1.35

[a]Granuloma data were made available for this study courtesy of L. DeCato (43).

[b]No cotton pellet-induced granuloma.

[c]Adrenalectomized but no cotton pellet-induced granuloma.

[d]Significantly different from first negative control values ($P < 0.05$)

[e]Significantly different from positive control values (P 0.05).

DISCUSSION

Leukocytes are formed in the lymph nodes (lymphocytes and monocytes) and in the bone marrow (granulocytes). One can think of them as the protective mobile units of the body which can be transported to any area of inflammation. The granulocytes are granular in appearance and contain polymorphous nuclei. There are three types of granulocytes: polymorphonuclear neutrophils, polymorphonuclear eosinophils and polymorphonuclear basophils. Monocytes along with granulocytes can ingest (phagocytosis) invading organisms that threaten the body. There is one other type of white blood cell known as megakaryocyte which produces platelets. Platelets are important in the body defense by activating the blood clotting mechanism (49).

Chemotaxis is defined as the process whereby some chemical released at a local site causes the movement of leukocytes to or away from (positive and negative chemotaxis, respectively) that site. Chemical substances responsible for chemotaxis generally are bacterial toxins or degenerated tissue polysaccharides produced by inflammation.

Once at the site of disturbance, leukocytes accomplish their job by phagocytosis. Macrophages, which are derived from monocytes, are the power houses of phagocytic cells. Such cells are able to engulf whole red blood cells,

malarial parasites, and many other objects simultaneously. The neutrophils are only capable of ingesting particles no larger than a bacteria. In chronic infections, the macrophages are very important in removing necrotic tissue. In such cases, lymphocytes enter the area, increase in size over a period of hours and become macrophages.

Proteolytic enzymes known as lysosomes are responsible for the digestion of particles once inside the neutrophil or macrophage. Phagocytic cells are also known to contain lipases and bacteriocidal agents to break down and kill bacteria. A single neutrophil can usually phagocytize 5 to 12 bacteria, and a macrophage engulf 100 bacteria before the breakdown products of the foreign particles accumulate and kill the cells.

During inflammation, a globulin substance known as the "leukocytosis promoting factor" is liberated (50). This can cause neutrophilia by stimulating the bone marrow to release granulocytes, especially neutrophils. The histocytes (tissue monocytes) change to macrophages and migrate to the site by ameboid movement. Such histocytes are known as the body's first line of defense. In 6 to 12 hr, monocytes from the blood reinforce the first line of defense by changing to macrophages and move chemotactically towards the damaged site. By the 10--12th hour, lymphocytes develop into macrophages and carry on the fight. In chronic infection, the affected area becomes acidic which is unsuitable for the existence of neutrophils but very suitable for macrophages. This is why

neutrophils may only be useful in the initial stages of inflammation while macrophages are advantageous later.

There is little known about the role of eosinophils. They exhibit chemotaxis, but are very weak phagocytes. Since they enter the blood when foreign protein is injected into the system, it has been suggested that their function is to detoxify proteins (50). They may also release profibrinolysin which forms fibrinolysin, the substance responsible for dissolution of clots. There is a profound increase in eosinophils during allergic reactions, and the eosinophils seem to collect at sites of antigen-antibody reactions in the tissues (51).

Basophils on the other hand are known to be very similar to mast cells. Like mast cells, basophils release heparin which functions to prevent blood coagulation. The total number of basophils in the blood stream ordinarily is very small. However, the population of basophils does increase during the healing phase of inflammation. There is a tendency for red blood cells to clump during inflammation, and it is thought that the increase in basophils is a natural means of combating agglutination (51).

The step-wise increase in leukocytes from day 6 to day 21 reported by Newbould and Piliero *et al.* (52,53) was also noted in the present study (Table I). Leukocytosis persisted through out the 30 day observation period. It is evident that cryogenine was effective in maintaining near normal levels during drug treatment. From the differential white

blood cell counts, it was apparent that the increases in total leukocyte count in controls and drug-treated rats were primarily due to increases in neutrophils. This maybe due to lymphocytes being depleted from the blood into the site of inflammation--a characteristic of the latter phases of inflammation. Cryogenine was effective in modifying the polymorphonuclear leukocyte increase towards normal. The rises in total leukocyte count in adjuvant-injected rats with time parallel the increases seen in erythrocyte sedimentation rate. This relationship may be due to increased electrostatic changes on the erythrocytes induced by increases in plasma proteins. Similar changes have been reported by Browning and co-workers (54) in patients with rheumatoid arthritis.

With normal rat serum, electrophoretic separation results in five distinct components. These fractions are referred to as: the albumin fraction (with the greatest mobility--traveling furthest from the origin towards the anode), the $\underline{\text{alpha}}_1$-, $\underline{\text{alpha}}_2$-, beta- and gamma-globulins. The globulin fractions are named in order of decreasing mobility, the gamma-globulins being the slowest. Not only does the albumin have the greatest mobility, but it also stains the most intensely. The $\underline{\text{alpha}}_1$-globulin forms a narrow band just adjacent to the albumin. The $\underline{\text{alpha}}_2$-globulin is just distinguishable following the $\underline{\text{alpha}}_1$-globulin. The beta-globulins forms characteristically discrete and compact bands. The gamma-globulin forms a broad band which, due to

endosmotic flow (39), usually lines just to the cathodal side of the site of application.

Since electrophoresis of plasma yields an extra band (fibrinogen) between the beta- and gamma-globulins, only serum was used in this study. Any hemolysed serums were discarded since hemoglobin introduces a band in the beta-region.

Normal globulin patterns (negative controls) in this study were in agreement with prior studies (47,48). Positive controls were somewhat in agreement with granuloma exudate values of Glenn et al. (48). D-α-tocopherol-induced granuloma exudates have been shown to have reduced serum globulins in all fractions except the $alpha_2$-fraction where slight increases were noted. Biochemical studies in the same paper (48) noted that these reduced levels of serum globulins might be due to reduced levels of glycoproteins and mucoproteins.

Sunderman (55) has shown that patients with scleroderma also exhibit a lowering of all serum globulin fractions. Scleroderma is characterized by fibrosis or fibrous tissue formation. A relationship may exist with the fact that granuloma formation occurs with an increase proliferation of fibroblasts.

Hypoalbuminemia was observed (in positive controls) in this investigation, which is in accordance with findings in serum studies performed using other forms of inflammation. Low levels of the albumin serum fraction is very characteristic of edema formation. Models of inflammation and certain

clinical situations that they tend to mimic (e.g. adjuvant arthritis and rheumatoid arthritis) show characteristic increases in $alpha_1$-, $alpha_2$-, beta- and gamma-globulin fractions. Such increases are directly proportional to the intensity of the disease state. Piliero et al. (53) has noted that rises in beta- and gamma-globulin fractions in adjuvant rats may be related to the production of antibodies. Also, they further speculated that the increases in $alpha_2$-globulins may reflect a transport function associated with antibodies. As one would expect if the above is true, all of these fractions are restored following successful drug treatment (53).

Another trait generally reported for experimental inflammatory models is a decrease in calculated albumin/globulin ratios. In our study of cotton pellet granuloma, decreases in albumin and all globulin fractions except the $alpha_2$-globulin were noted (Figure 13). Upon calculation, the albumin/globulin (Figure 19) ratios appeared to increase in rats with induced granuloma. Both observations would seem to be possible if one takes into account that: (a) cotton pellet granuloma-induced inflammation is not an immunological disease state like adjuvant arthritis, and (b) granuloma formation is a proliferative or repairative phenomenon. One should also note that any granuloma tissue proliferation occurring in adjuvant arthritis, might be masked by the destructive elements of the "disease" (i.e. necrosis induced by lysosomal enzyme release).

Figure 19.--Effects of selected drug treatment on albumin/globulin ratios (A/G) of rats with induced cotton pellet granuloma.[a]

Treatment Group	Code
Control	CON
Phenylbutazone	PBZ
Indomethacin	IND
Hydrocortisone	HYC
Cryogenine	CRY
Tetrabenazine	TEB
RO4-1284	RO4
Benzquinamide	BEN
RO1-9564	RO1
Sham Operation	Sham Op

[a]See Table III to XIII for low (L), medium (M) and high (H) dosages of each compound.

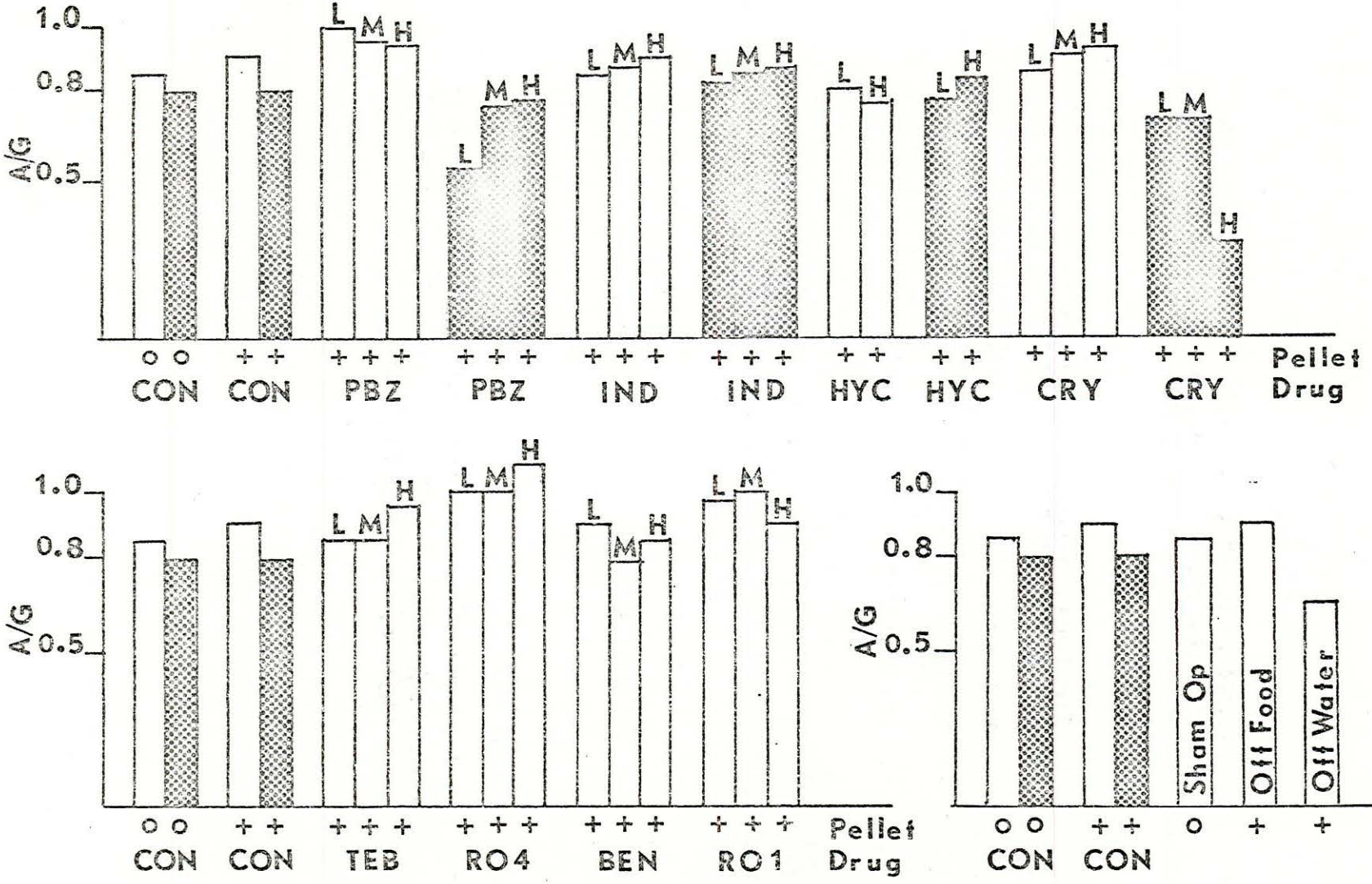
1.0
0.8
0.5
A/G
L M H
L M H
L M H
L M H
L M H
L H
L H
L M H
L M
H
o o
+ +
+ + +
+ + +
+ + +
+ + +
+ +
+ +
+ + +
+ + +
Pellet
CON CON PBZ PBZ IND IND HYC HYC CRY CRY
Drug
1.0
0.8
0.5
A/G
L M H
L M H
L M H
L M H
L M H
L M H
Pellet
CON CON TEB RO4 BEN RO1
Drug
1.0
0.8
0.5
A/G
Sham Op
Off Food
Off Water
o o
+ +
o
+
+
CON CON

Figure 19 also document the effects of various drug treatments on albumin/globulin (A/G) ratios. Phenylbutazone-treated, non-adrenalectomized rats seemed to exhibit an initial A/G increase, with gradual decreases with higher doses. On the other hand, phenylbutazone-treated, adrenalectomized animals showed an initial decrease of A/G ratio followed by dose responsive increases. Both adrenalectomized and non-adrenalectomized, indomethacin-treated animals showed qualitatively similar, modest, dose-related increases in A/G ratios. Hydrocortisone showed a dose-related decrease in A/G ratios in non-adrenalectomized rats and an apparent dose-related increase in A/G ratios in adrenalectomized rats. This unusual dose-response relationship exhibited by hydrocortisone was qualitatively exactly opposite to the effects produced by cryogenine. Cryogenine-treated, non-adrenalectomized rats showed a pattern similar to indomethacin, tetrabenazine and RO 4-1284, in that A/G ratios exhibited gradual dose-responsive increases. However, in adrenalectomized, cryogenine-treated animals the A/G ratios exhibited decreases and with an extreme drop noted with the highest dose. Benzquinamide and RO 1-9564 were somewhat unusual in that their A/G ratios did not seem to follow a dose-response depression or elevation. One also notes from Figure 19 that food deprivation for two days produced an increase in A/G ratio while dehydration (off water 2 days) produced an decrease in A/G ratio. Sham operated animals showed no apparent changes from negative (non-adrenalectomized) control values.

The relationship between A/G ratio and drug treatment probably depends on which protein fraction the drug binds to (i.e. albumin or globulin proteins).

Cortisol and its analogs are able to suppress many manifestations of the inflammatory process. They have been shown to inhibit edema, exudation of plasma proteins, leukocyte emigration, capillary permeability and the proliferative stage of inflammation. Possible mechanisms of glucocorticoids include lysosomal stabilization, inhibition of kinins, alteration of connective-tissue metabolism, alteration of the immune response and inhibition of leukocyte response (56). However, no universally accepted unifying theory for glucocorticoid action has yet been found.

Phenylbutazone, an acidic compound, has marked anti-inflammatory action. Like cortisol, phenylbutazone's mechnism of action is unknown. It has been shown to inhibit various endogenous mediators of inflammation, interfere with several enzymes or cofactors responsible for cellular metabolism, uncouple oxidative phosphorylation, and inhibit mucopolysaccharide synthesis (57,58). Some believe the last action might be due to its interactions with reactive lysyl, amine and thiol groups.

Indomethacin is also an acidic compound and is reputed to have an anti-inflammatory potency 4.1 times that of hydrocortisone in the cotton pellet granuloma inhibition system (59). As with the above agents, indomethacin's anti-inflammatory mechanim of action is still unknown. Indometha-

cin is able to uncouple oxidative phosphorylation, suppress the vascular hyperpermeability properties of some endogenous mediators of inflammation, inhibit leukocyte migration, and alter serum proteins (21).

Both tetrabenazine and benzquinamide have no significant therapeutic use in treating inflammation at this time. Tetrabenazine is a synthetic analogue of reserpine and also the prototype model from which benzquinamide (Quantril) was constructed. Tetrabenazine has reserpine-like actions on the central nervous system and will deplete peripheral tissues of noradrenaline and serotonin. Tetrabenazine is less potent than reserpine and its actions appear more rapidly and persist for a shorter time. Serious side affects restrict it from being substitued for reserpine. Benzquinamide, beside possessing reserpine's tranquilizing effects, has antihistaminic, anticholinergic and antiserotonin actions. It does not, however, alter the levels of brain amines (60).

In this study, non-adrenalectomized rats showed a definite return of gamma-globulin levels and possible return of albumin levels to negative control values with all compounds except phenylbutazone and benzquinamide (i.e. no significant difference between drug-treated and negative control, yet with a significant difference between drug-treated and positive control). With cryogenine and tetrabenazine, reversal of albumin depletion was seen only at the highest dose levels. Hydrocortisone was the only compound in non-adrenalectomized animals which was successful in returning all serum globulin

fractions back to normal levels; there was actually overshooting beyond the "normal" values of the negative controls. Indomethacin, benzquinamide, RO 4-1284 and RO 1-9564 showed some tendency to reverse the beta-globulin fraction toward normal. There seemed to be some indications that the experimental compound, cryogenine, acts in a manner similar to tetrabenazine and possibly to RO 4-1284 i.e. similar changes in albumin and beta-fraction and a similar shift of A/G ratios. DeCato (43) has speculated that cryogenine may owe part of of its anti-inflammatory action to adrenocortical stimulation. The present study seems to provide further support for DeCato's hypothesis since in regard to albumin and gamma-globulin fractions, adrenalectomized animals given cryogenine showed less return to normal (negative control) levels of serum protein fractions than non-adrenalectomized rats. In adrenalectomized animals, hydrocortisone showed complete recovery or slight overshoots of globulins over normal levels--- a totally expected effect under the circumstances. Phenylbutazone seemed most effective in returning albumin levels back toward negative control values. In general cryogenine was most sucessful in reversing the gamma-globulin fraction back to negative control levels.

CONCLUSION

Sedimentation rates, total white cell counts, differential leukocyte counts and serum proteins studies all provided valuable supporting data for the validity of the inflammation/anti-inflammation system under investigation.

In the Mycobacterium adjuvant arthritis study, cryogenine, phenylbutazone, hydrocortisone and paramethasone were effective in suppressing sedimentation rate increases. Indomethacin and 6-mercaptopurine were somewhat less effective. The positive control rats (receiving no drug) showed an increase in total cell count which was associated with lymphopenia and neutrophilia. Cryogenine, phenylbutazone, hydrocortisone, and paramethasone were effective in reversing inflammatory tissue changes, while mefenamic acid, indomethacin and 6-mercaptopurine were less so at the dosage levels tested here.

In cotton pellet granuloma testing, positive controls (receiving no drug) showed significant decreases in all serum protein fractions excepting the $alpha_2$-globulin fraction. Hydrocortisone proved to be the most effective agent in both adrenalectomized and non-adrenalectomized animals in reversing serum proteins back to negative control values. Phenylbutazone seemed to be the most effective agent in reversing albumin levels back to normal values in both adrenalectomized

and non-adrenalectomized rats. Indomethacin exhibited significant mitigating effects in regard to albumin and gamma-globulin fractions (cryogenine-like).

Like tetrabenazine and the experimental compound RO 1-9564, benzquinamide produced a significant reversal of low gamma-globulin and of low albumin fractions. RO 4-1284 also showed definite reversal of albumin and significant reversal in gamma-globulin. Benzquinamide, RO 4-1284 and possibly RO 1-9564 may show significant returns to normal values in the beta-globulin fraction but such an effect was not exhibited with tetrabenazine.

Cryogenine produced reversal of granuloma-lowered albumin and gamma-globulin serum fractions; however, like tetrabenazine, cryogenine did not show beta-globulin fraction reversal. Cryogenine was less effective as an anti-inflammatory agent in adrenalectomized animals in that albumin, beta- and gamma-globulin levels did not return to negative control values. Cryogenine and tetrabenazine also exhibited similar changes in albumin/globulin (A/G) ratios. Therefore, this study seems to support the hypothesis of DeCato (43) that cryogenine may owe part of its anti-inflammatory activity to adrenocortical stimulation.

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