

1973

Pharmacological Evaluation of an Extract from Eisenia Bicyclis

David Michael Whitaker
University of Rhode Island

Follow this and additional works at: <https://digitalcommons.uri.edu/theses>

Terms of Use

All rights reserved under copyright.

Recommended Citation

Whitaker, David Michael, "Pharmacological Evaluation of an Extract from Eisenia Bicyclis" (1973). *Open Access Master's Theses*. Paper 229.

<https://digitalcommons.uri.edu/theses/229>

This Thesis is brought to you by the University of Rhode Island. It has been accepted for inclusion in Open Access Master's Theses by an authorized administrator of DigitalCommons@URI. For more information, please contact digitalcommons-group@uri.edu. For permission to reuse copyrighted content, contact the author directly.

PHARMACOLOGICAL EVALUATION OF AN EXTRACT
FROM EISENIA BICYCLIS

BY

DAVID MICHAEL WHITAKER

A THESIS SUBMITTED IN PARTIAL FULFILLMENT
OF THE REQUIREMENTS FOR THE DEGREE OF
MASTER OF SCIENCE
IN
PHARMACOLOGY AND TOXICOLOGY

UNIVERSITY OF RHODE ISLAND

1973

EVALUATION OF AN EISENIAN EXTRACT

MASTER OF SCIENCE THESIS
OF
DAVID MICHAEL WHITAKER

Approved:

Thesis Committee:

Major Professor

Gay S. Carlson

Guzon Shingiz

~~_____~~

Charles L. Smith

A. A. A. A.

Dean of the Graduate School

UNIVERSITY OF RHODE ISLAND

1973

ABSTRACT

An extract from Eisenia bicyclis containing principally a polyphenolic polymer was examined for pharmacological activity. The investigation centered around the established activity of a model polyphenolic compound, phloroglucinol. Studies on inflammation and alteration of hepatic microsomal enzyme activity were also performed.

Heat denaturation of a 0.15% bovine serum albumin solution was inhibited 96% by the addition of the material at a final concentration of 0.32 mg/ml. Since stabilization of such solutions is often indicative of anti-inflammatory activity, direct measurements of this activity were made. The inhibition of formation of carrageenin-induced rat paw edema was utilized as a test system. As determined by paw volume changes, administration of a dose of 75 mg/kg (ip) one hour prior to carrageenin injection inhibited edema production by 88%. The potent anti-inflammatory agent phenylbutazone, at a dose of 90 mg/kg (ip), provided 100% protection. The protection given by the Eisenian extract apparently was not due to pituitary-adrenal stimulation, since the protection is 4-fold better than that given by a dose of 50 mg/kg (ip) of cortisone. The effect cannot be attributed to changes in body temperature, which remains unchanged. The effect can be partially but not completely attributed to the irritant

properties of the material since buffering a dose of 100 mg/kg (ip) provided 42% protection. The effect is partially due to stabilization of the lysosomal membrane since in vitro 309 ug of the extract per milliliter of incubation media inhibited by 64% the release of the marker enzyme β -glucuronidase from lysosomes. The compound administered at a dose of 75 mg/kg (ip) daily for 17 days offered no protection against rat paw edema induced by Freund's complete adjuvant.

Capillary fragility was determined by mouse lung hemoglobin content following sudden decompression. No decrease in fragility was observed 1.5 hours after pretreatment with the extract at a dose of 300 mg/kg (ip).

The material administered to mice (300 mg/kg, ip) 30 minutes prior to administration of lethal doses of curare exhibited protection when the curare was also given by the intraperitoneal route. No protection was exhibited when the curare was administered subcutaneously. This suggested a chemical rather than a pharmacological antagonism. This hypothesis was supported when no antagonism was found to the action of curare on the cat gastrocnemius muscle in vivo by jugular infusion of 180 mg of the extract, and when a dose of 300 mg/kg (ip) one hour prior to sacrifice was found to have no effect on the activity of rat brain cholinesterase. The extract offered no antagonism to the action of either strychnine or tetrodotoxin.

No change in rat liver microsomal enzyme activity, as

determined by the extent of metabolism of O-ethyl O-(4-nitrophenyl) phenylphosphonothioate (EPN), was found after the administration of 300 mg/kg (ip) of the material daily for 3 days. Also, a single injection of 300 mg/kg (ip) 1 hour prior to sacrifice for measurement of activity was without effect.

Estrogenic activity was assayed by changes in uterine weight in the immature female rat. The administration of 300 mg/kg of the extract intraperitoneally daily for 3 days did not alter uterine weight.

Rat liver homogenates were assayed for tryptophan pyrrolase activity by measuring the formation of the end-product kynurenine. The administration of 300 mg/kg (ip) of the extract 1.5 hours prior to sacrifice caused 33% inhibition of the enzyme activity. The presence of the extract at a concentration of 486 µg/ml resulted in an in vitro inhibition of 82%.

ACKNOWLEDGEMENTS

The author wishes to express his gratitude to his wife Rosemary for her support and understanding.

Dr. Gary P. Carlson provided invaluable guidance throughout this study. Mr. Steven Zelenski and Dr. Leonard Worthen were kind enough to supply the Eisenian extract.

This work was supported by the Sea Grant College Program at the University of Rhode Island.

TABLE OF CONTENTS

ABSTRACT.....	1
ACKNOWLEDGEMENTS.....	1v
TABLE OF CONTENTS.....	1
LIST OF TABLES.....	3
LIST OF FIGURES.....	5
I. INTRODUCTION.....	6
II. REVIEW OF THE LITERATURE.....	8
III. EXPERIMENTAL.....	12
A. Animals.....	12
B. Materials.....	12
C. Analytical Procedures.....	13
1. Turbidimetric Determination of Heat Denaturation of Bovine Serum Albumin...	13
2. Plethysmographic Rat Paw Volume Determination.....	13
3. Carrageenin-Induced Rat Paw Edema.....	14
4. Determination of Thermogenic Activity..	14
5. Harvesting of Lysosomes and Determin- ation of the Activities of β - glucuronidase and Acid Phosphatase.....	15
6. Freund's Adjuvant-Induced Rat Paw Edema.....	17
7. Induction of Mouse Lung Hemorrhage and Lung Hemoglobin Determination.....	17
8. <u>In vivo</u> Antagonism of Curare, Strychnine, or Tetrodotoxin in Mice....	18

9.	Determination of Acetylcholinesterase Activity.....	18
10.	Cat Sciatic Nerve-Gastrocnemius Muscle Preparation.....	19
11.	Determination of EPN Detoxification.....	20
12.	Determination of L-Tryptophan Pyrrolase Activity.....	21
13.	Determination of Estrogenic Activity....	22
D.	Statistical Methods.....	22
IV.	RESULTS.....	23
V.	DISCUSSION.....	46
VI.	SUMMARY AND CONCLUSIONS.....	55
VII.	REFERENCES.....	58

LIST OF TABLES

Table	Page
1. Effect of c40SA on heat denaturation of bovine serum albumin.....	24
2. Effect of phloroglucinol on heat denaturation of bovine serum albumin.....	25
3. Effect of drugs on carrageenin-induced rat paw edema.....	26
4. Effect of neutralized or buffered ec40SA on carrageenin-induced rat paw edema.....	29
5. Effect of drugs on lysosomal membrane stability <u>in vitro</u>	31
6. Effect of f40SA and refined 40SA on lysosomal membrane stability <u>in vitro</u>	32
7. Antagonism of Freund's adjuvant-induced rat paw edema.....	33
8. Effect of drugs on mouse lung hemorrhage induced by sudden decompression.....	34
9. Protection against curare poisoning in mice by e40SA.....	36
10. Protection against curare poisoning in mice by ec40SA.....	37
11. Effect of ec40SA <u>in vivo</u> and <u>in vitro</u> on rat brain cholinesterase activity.....	38
12. Effect of ec40SA on strychnine poisoning in mice.....	40
13. Effect of f40SA on tetrodotoxin poisoning in mice.....	41
14. Effect of drugs on EPN detoxification.....	42
15. Effect of drugs <u>in vivo</u> and <u>in vitro</u> on rat liver tryptophan pyrrolase activity.....	43

Table

Page

16. Effect of drugs on rat uterine weight..... 45

LIST OF FIGURES

Figure	Page
1. Effect of e40SA on rat body temperature.....	28

I INTRODUCTION

Many naturally derived compounds are used extensively in medicine. Some outstanding examples are digitalis, reserpine, atropine, and colchicine. Despite the awareness of the origins of such drugs from terrestrial plants, until recently there has been relatively little effort made to examine marine species for pharmacological agents. Extensive research is needed in this area, considering that of the 500,000 species which inhabit the water, less than one percent have been examined for biological activity.

The drugs which have been isolated from marine plants exhibit a wide variety of pharmacological activity. The following are some representative examples (Der Marderosian, 1969). The fungus Cephalosporium acremonium is the source of cephalothin, an antibiotic which is active against various penicillin-resistant microbes. The algae Lyngbya majuscula has been implicated in outbreaks of dermatitis among swimmers, and toxicity in fish and mice. However, extracts of this algae have also shown antimicrobial, antiviral, fungicidal, and other types of growth-inhibitory properties in preliminary pharmacological studies. The algae Digenea simplex has yielded the anthelmintic kainic acid. Chondrus crispus is the source of the linear polysaccharide carrageenin,

which exhibits antipeptic, anticoagulant, and antithrombic properties. More thorough screening programs would undoubtedly uncover more agents from just these four species.

In this study, an extract from the algae Eisenia bicyclis (Kjellman) Setchell, native to the shores of Korea, containing principally a polyphenolic polymer, has been examined for pharmacological activity. The investigation centered around the established activity of a model polyphenolic compound, phloroglucinol, or 1,3,5-trihydroxybenzene. Studies on inflammation and alteration of hepatic microsomal enzyme activity were also performed.

II REVIEW OF THE LITERATURE

The pharmacological activity of phloroglucinol (1,3,5-trihydroxybenzene) has been investigated by several groups. Okanishi and Shimaoka (1952) determined by gross observation that phloroglucinol pretreatment could decrease mouse lung capillary fragility, utilizing a decompression hemorrhaging technique developed by Majovski et al. (1944). To induce lung hemorrhage, mice were placed in a chamber which was decompressed to 40 mm Hg. The animals were removed from the chamber after two minutes and the lungs were excised and judged for extent of hemorrhage according to an arbitrary rating system. A capillary stabilizing effect uncovered by this fragility screen might find application in hypobaric environments or in the treatment of hypertension.

Frieden et al. (1961) reported that phloroglucinol non-competitively blocks the conversion of L-tryptophan to kynurenine by the liver enzyme tryptophan pyrrolase. Eighty-one percent inhibition was achieved at a concentration of 3.0 mM. Such an inhibition in vivo could result in nicotinic acid deficiency.

Mogey and Young (1949) investigated the in vitro effect of phloroglucinol on the curarized rat diaphragm. In this experiment, the AD66/33 of phenol, i.e., that amount of phenol which reduces by half the activity of an amount of

d-tubocurarine which by itself would have effected 66.7 percent paralysis, was established as a basis for the comparison of the antagonism offered by other phenols. The AD66/33 for phenol was found to be 1.52 mg. The relative activity, on a weight for weight basis, for phloroglucinol was found to be one-tenth that of phenol. Thus the authors concluded that phloroglucinol acted as a very weak curare antagonist. No inhibition of rat brain acetylcholinesterase or pseudo (horse serum) cholinesterase was exhibited, nor was the pH of the medium affected enough to alter the action of the curare. Thus the mechanism of the antagonism was unexplained.

It has been demonstrated that a variety of phenolic substances such as genistein and coumestrol possess estrogenic activity (Biggers and Curnow, 1954; Bickoff et al., 1958). The administration of such agents could result in infertility due to suppressed hypophyseal gonadotropin resulting in a disturbed estrous cycle.

Conney (1967) discussed extensively the pharmacological implications of the alteration of the activity of the drug-metabolizing liver microsomal enzyme system. Specifically, the administration of certain drugs, such as phenobarbital and 3-methylcholanthrene, results in enhanced metabolism of other agents due to induction of the enzyme system. Because of this important source of drug interaction, it appears important to test all drugs and chemical agents for the ability to induce or inhibit drug metabolizing activity. According to Neal and DuBois (1965), metabolism of the

pesticide 0-ethyl 0-(4-nitrophenyl) phenylphosphonothioate (EPN) by rat liver microsomal enzymes provides an indication of the activity of that system, thus providing a method to investigate the effect of drugs on this metabolic pathway.

Measurement of the anti-inflammatory activity of drugs is a complex problem and many methods and models have been proposed and utilized. Grant et al. (1970) demonstrated that anti-inflammatory drugs such as aspirin and indomethacin are often capable of inhibiting the heat denaturation of bovine serum albumin solutions. Winter et al. (1962) concluded that the polysaccharide carrageenin was highly suited as a phlogistin in the rat paw edema model of inflammation. Pearson (1956) showed that Freund's complete adjuvant, a suspension of heat-killed Mycobacterium butyricum in paraffin, when injected into the paw of the rat, could elicit a polyarthrititis syndrome which could serve to further evaluate anti-inflammatory agents. Van Arman et al. (1965) described a highly sensitive mercury displacement plethysmograph which could be employed to quantitate rat paw volumes in conjunction with paw edema models of inflammation. The device is essentially a mercury-filled syringe connected to a blood pressure transducer which is in turn wired to a polygraph amplifier. The introduction of objects into the mercury results in a linear response on the amplifier recorder.

Many mechanisms have been proposed for the action of anti-inflammatory agents. Labilization of the lysosomal

membrane and subsequent tissue dissolution due to the release of degradative lysosomal enzymes has been implicated as a mechanism underlying several types of inflammation, ranging from heat and chemical burns to arthritis (Houck and Forscher, 1968). Ignarro (1971), utilizing the release of the marker enzymes β -glucuronidase and acid phosphatase from the lysosome, has demonstrated that anti-inflammatory drugs are often capable of stabilizing the membrane of that organelle. Such stabilization may therefore partially explain the mechanism of action of many anti-inflammatory agents.

Garattini et al. (1965) indicate that many chemical agents, such as hypertonic saline, acetic acid, and formaldehyde, when injected by a general route, possess anti-inflammatory activity by virtue of their irritant properties, which allow for a minimization of local edema formation. The screening of new anti-inflammatory agents must therefore account for possible irritant effects.

III EXPERIMENTAL

A. Animals

Sprague-Dawley derived random bred albino rats were obtained from Charles River Breeding Laboratories, Wilmington, Massachusetts. Male rats employed weighed 200-250 g while female rats weighing 55-65 g were used. Male mice, 35-42 g, were obtained from the same breeder. The animals were housed in quarters maintained at 21-23°C with room lights on alternating 12 hour light-dark cycles. Commercial laboratory chow and water were allowed ad libitum. Animals were used no earlier than 3 days following receipt from the supplier.

B. Materials

Analytical grade chemicals or equivalent were used throughout the study. Cofactors (NADP and glucose-6-phosphate) and substrates (L-tryptophan, p-nitrophenyl phosphate, phenolphthalein glucuronide, and acetylcholine chloride) were obtained from Calbiochem or Sigma Chemicals. Phenylbutazone and EPN were gifts of Geigy Pharmaceuticals and DuPont respectively. Drugs were obtained from their respective manufacturers. Water employed as a solvent was double-distilled.

The molecular weight of the Eisenian extract has been

determined to be about 10,000. The extract will be referred to as 40SA, preceded by letters which indicate the extraction batch.

Recent evidence suggests that an integral part of the polyphenolic polymer is polysaccharide (Zelenski, 1973).

C. Analytical Procedures

All incubations except for determination of cholinesterase activity were carried out in a Dubnoff metabolic shaker under an atmosphere of air. All absorbance determinations were made utilizing a Beckman DB-G spectrophotometer. Unless otherwise indicated, all 40SA fractions were dissolved in water or saline.

1. Turbidimetric Determination of Heat Denaturation of Bovine Serum Albumin

The extent of heat-induced denaturation of bovine serum albumin (BSA) was quantitated by a method suggested by Grant et al. (1970). Equal amounts of 0.3% BSA and buffer or drug solution were heated at 70°C for 4 minutes, cooled in ice water for 3 minutes, maintained at room temperature for 15 minutes, and the resultant degree of turbidity was determined by the absorbance at 660 nm. The buffer employed was 0.05 M Tris acetate, pH 6.0, and was employed as the solvent for c40SA. Phloroglucinol was dissolved in buffer containing 2.5% dimethylformamide.

2. Plethysmographic Rat Paw Volume Determination

Rat paw volume was determined by a plethysmographic technique described by Van Arman et al. (1965).

A 30 cc capacity syringe was filled with mercury and connected by means of cannula tubing to a Statham blood pressure transducer which in turn was wired to a Grass polygraph amplifier. The introduction of objects into the mercury resulted in a linear recorder output response from the amplifier. The apparatus was calibrated by the introduction of objects of known volume. Rat paws were prepared for volume measurement by marking just above the topmost callus pad. Paws were immersed to this mark in the mercury during measurement.

3. Carrageenin-Induced Rat Paw Edema

Carrageenin was utilized as a phlogistin to induce rat paw edema as suggested by Winter et al. (1962).

Male rats were administered (ip) either saline, 90 mg/kg phenylbutazone, 50 mg/kg cortisone, or 75 mg/kg c40SA. One hour later, 0.1 ml of 1.0% carrageenin in saline was injected into the sub-plantar tissue of the hind paw, using a 26 gauge needle. The paw volume was determined immediately plethysmographically, and again three hours later, and edema formation was calculated in mm³.

4. Determination of Thermogenic Activity

Thermogenic activity was determined by monitoring the rectal temperature of rats using a Yellow Springs Instrument Co. thermistor thermometer, model 8420. Saline or 75 mg/kg or 150 mg/kg e40SA was administered (ip) to male rats and rectal temperature was determined (2.5 cm probe

insertion) immediately and every hour thereafter for four hours. Animals were housed three per cage; ambient temperature was 24°C.

5. Harvesting of Lysosomes and Determination of the Activities of β -Glucuronidase and Acid Phosphatase

Lysosomal fractions were prepared according to the method suggested by Ignarro (1971). The determination of the activities of the lysosomal enzymes β -glucuronidase and acid phosphatase was also performed by the method reported by that author.

The liver from a decapitated and exsanguinated male rat was excised and a 10% homogenate prepared in cold 0.25M sucrose-0.02 M Tris acetate buffer, pH 7.4. The homogenate (40 ml) was then centrifuged at 600 g for 5 min at 4°C in a Sorvall RC2-B centrifuge (# 34 rotor). The resulting supernatant was decanted and diluted with an equal volume of the same buffer, and centrifuged at 3500 g for 15 min at 4°C. The intact pellet was rinsed gently twice in 0.45 M sucrose-0.04% glycogen-0.02 M Tris acetate buffer, pH 7.4, and resuspended in 4.0 ml of the same buffer. The suspensions were maintained at 4°C.

Labilization or stabilization of lysosomes was ascertained by determining the release of lysosomal marker enzymes. The lysosomal suspension was warmed to 25°C for 5 min and 0.2 ml aliquots were added to 20 ml glass beakers containing 2.0 ml of 0.18 M sucrose-0.04 M Tris acetate buffer, pH 7.4, with either 0.1 ml drug solution or water,

and the beakers were incubated at 37°C for 0 or 15 min in a Dubnoff metabolic shaker. The incubation was terminated by high speed centrifugation (27,000 g for 15 min at 4°C) after transfer of the samples to polyethylene tubes. When appropriate the direct effect of the drugs on the marker enzymes was determined. In this case the drugs were added to the system after the incubation period. To determine the total enzyme activity of the system, 2.0 ml of 0.2% (v/v) Triton X-100 in 0.04 M Tris acetate buffer, pH 7.4, replaced the 2.0 ml of buffer in the incubation.

Acid phosphatase and β -glucuronidase were employed as lysosomal marker enzymes. The formation of p-nitrophenol from p-nitrophenyl phosphate was measured to determine acid phosphatase activity. The formation of phenolphthalein from phenolphthalein glucuronide was estimated to assay β -glucuronidase activity. One milliliter of high speed supernatant and 1.0 ml of distilled water were added to 1.0 ml of 0.3 M citrate buffer, pH 4.8. Incubation (at 37°C for 20 min) was initiated by the addition of 0.04 ml of freshly prepared substrate (either p-nitrophenyl phosphate, 79 mg/ml in distilled water, or phenolphthalein glucuronide, 63 mg/ml in distilled water). Blanks received water in place of substrate. Acid phosphatase determinations were terminated by the addition of 0.2 ml of 4 N NaOH, and absorbances were then determined at 405 nm. β -glucuronidase assays were terminated by the addition of 0.4 ml of 2.2 M

glycine-NaOH buffer, pH 12, and absorbances were then measured at 540 nm. The absorbances obtained were compared with those of standard solutions of phenolphthalein and p-nitrophenol.

6. Freund's Adjuvant-Induced Rat Paw Edema

Polyarthritis was induced in rats by treatment with complete Freund's adjuvant according to the method of Pollock and Brown (1971).

Male rats were treated daily for 17 days with either saline (ip), 75 mg/kg f40SA adjusted to pH 7.0 with NaOH (ip), or 2 mg/kg triamcinolone (sc). On the second treatment day, 0.1 ml Freund's complete adjuvant, containing 10 mg heat-killed Mycobacterium butyricum per milliliter of 15% mannide monooleate in paraffin oil, was injected into the sub-plantar tissue of the hind paw, using a 26 gauge needle. The paw volume was determined immediately plethysmographically and again on day 18, and edema formation calculated.

7. Induction of Mouse Lung Hemorrhage and Lung Hemoglobin Determination

Lung hemorrhage was induced by sudden decompression, a method developed by Majovski et al. (1944). A 30 liter vacuum sink was evacuated to 40 mm Hg. Mice in a 1.5 liter test chamber at atmospheric pressure were subjected to the lower pressure by the opening of a stopcock. Within 20 seconds the pressure in the two chambers equilibrated at 70 mm Hg. The mice died within 60-90 seconds and were removed from the chamber after two minutes. The lungs were

rapidly excised, weighed, homogenized in 25 volumes of cyanomethemoglobin reagent and centrifuged to sediment tissue. The supernatant was examined for hemoglobin content by determining absorbance at 540 nm. The remaining homogenate was dried to constant weight at 85°C. Results were reported as mg hemoglobin per mg dry lung. The resulting values were compared to those of control animals maintained at atmospheric pressure. Animals were administered (ip) saline, 300 mg/kg e40SA, 300 mg/kg phloroglucinol, or 100 mg/kg phenylbutazone 1.5 hours prior to sacrifice.

8. In vivo Antagonism of Curare, Strychnine, or Tetrodotoxin in Mice

Lethal doses of curare, strychnine, or tetrodotoxin were administered to mice to investigate antagonism to these agents. The Eisenian extract at a dose of 300 mg/kg or saline was administered intraperitoneally to mice 30 minutes prior to the administration (ip or sc) of either curare (0.28-0.60 mg/kg), strychnine (2.0 mg/kg), or tetrodotoxin (12 µg/kg). These doses are close to the respective LD₅₀'s. The number of animals injected and the number of survivors were recorded.

9. Determination of Acetylcholinesterase Activity

The activity of rat brain acetylcholinesterase was estimated by the manometric method suggested by DuBois and Mangun (1947) employing the Warburg apparatus.

Male rats were administered (ip) either saline or 300 mg/kg ec40SA one hour prior to sacrifice for brain excision.

Ten percent brain homogenates were prepared in calcium-free Ringer-bicarbonate buffer. One-half milliliter of homogenate and 2.2 ml buffer were transferred to the main compartment of the Warburg flask. In vitro experiments included the addition of ec40SA at a concentration of 5 mg/ml to the main compartment. The flasks were flushed for 5 minutes with 95:5 N₂:CO₂ while they equilibrated to the temperature of the water bath (38°C). The reaction was initiated by tipping into the main compartment the contents of the side-arm (0.3 ml of 0.1 M acetylcholine chloride). After another 5 minute equilibration, the manometer readings of the amount of CO₂ produced by the enzymatic hydrolysis of the acetylcholine were recorded at 5 minute intervals for 30 minutes. The CO₂ production was corrected for both tissue and acetylcholine blanks and for thermo-barometer changes.

10. Cat Sciatic Nerve-Gastrocnemius Muscle Preparation

The in vivo cat sciatic nerve-gastrocnemius muscle preparation was utilized to quantitate curare antagonism.

A 3.2 kg cat was anesthetized with 1.5 g/kg urethane (ip). The left jugular vein was cannulated and used for infusion of drugs. The right carotid artery was cannulated and connected to a Statham blood pressure transducer which was in turn wired to a Grass polygraph amplifier to monitor blood pressure. Forty units/kg of heparin were infused to prevent clotting. An automatic respirator was connected to a tracheal cannula. The left gastrocnemius muscle was

connected to a Grass force transducer which was in turn wired to a Grass polygraph amplifier to record muscle activity. The left sciatic nerve received a 1.0 V stimulus lasting 0.1 msec at a rate of 12/minute. This stimulus effected an initial contractile force of 980 dynes. d-Tubocurarine chloride was infused until a 70% reduction in contractile force was obtained. The following drugs were infused, in order, over periods of 3, 6, or 9 minutes respectively: 0.18 mg physostigmine, 30 mg phloroglucinol, or 180 mg e40SA. Additional curare was infused between drugs as needed to reinstate the reference curarization level.

11. Determination of O-Ethyl O-(4-nitrophenyl) phenylphosphonothioate Detoxification.

The detoxification of O-ethyl O-(4-nitrophenyl) phenylphosphonothioate (EPN) was estimated by measuring the formation of the resultant p-nitrophenol according to the method of Neal and DuBois (1965).

To an incubation mixture containing 1.3 μ moles NADP, 3.3 μ moles glucose-6-phosphate, 0.2 ml EPN-buffer and sufficient water to make a total volume of 0.8 ml was added 0.1 ml 20% rat liver homogenate in 1.15% KCl containing 0.25% nicotinamide. The EPN-buffer was prepared by dissolving 7.0 mg EPN in 1.0 ml 1:4 ethanol: propylene glycol and adding 0.5 ml of this solution to 5.0 ml of 0.1 M phosphate buffer, pH 7.8.

Incubation was carried out at 37°C for 60 min. The

reaction was terminated by adding 2.5 ml cold acetone to the reaction flask. The flask contents were transferred to centrifuge tubes containing 0.2 ml glycine-NaOH buffer (0.5 M, pH 9.5). The tubes were centrifuged and the absorbance of the supernatant was determined at 410 nm versus a blank to which tissue had been added at the termination of the incubation. The amount of *p*-nitrophenol formed was determined by comparison with the absorbance of known quantities of the chemical.

12. Determination of L-Tryptophan Pyrrolase Activity

L-tryptophan pyrrolase activity of rat liver homogenates was estimated through assay of the end product kynurenine, according to the method of Knox and Mehler (1950).

Incubations were carried out at 37°C for 60 minutes in media containing 7.5 mmoles L-tryptophan, 2.0 ml of 0.1 M phosphate buffer, pH 7.0, and 0.8 ml 25% liver homogenate in 0.15 M KCl in a total volume of 4.0 ml. For in vitro investigation, 1.94 mg of phloroglucinol or f40SA was added. To examine the in vivo effects of drugs on the enzyme, animals were injected (ip) 1.5 hours prior to sacrifice with saline, 300 mg/kg phloroglucinol, or 300 mg/kg e40SA; or with 35 mg/kg hydrocortisone four hours prior to sacrifice for liver excision.

The reaction was terminated by the addition of 2.0 ml of 15% metaphosphoric acid. The flask contents were filtered and a 3.0 ml aliquot of the filtrate was

neutralized with 1.0 ml of 1 N NaOH. The absorbance was then determined at 365 nm versus a blank to which the homogenate had been added after the metaphosphoric acid. The amount of kynurenine formed was calculated from the absorbance of solutions of kynurenine of known concentration.

13. Determination of Estrogenic Activity

To determine estrogenic activity, immature female rats were injected daily for three days with either saline (ip), 300 mg/kg f40SA (ip), or 25 µg/animal estradiol-17-β(sc). The estradiol was prepared by diluting 1.0 ml of a 25 mg/100 ml 95% ethanol stock solution with 4.0 ml saline. On the fourth day, the uterus was excised, weighed, and the uterine-to-body weight ratio calculated.

D. Statistical Methods

The 2-tailed Student's "t" test for independent means was used to examine experimental means for statistical significance. The level of significance (P) was determined by comparison of the computed "t" value with values from standard tables. All calculated "t" values were tested at both the 0.05 and 0.01 probability levels for rejection of the null hypothesis.

IV RESULTS

Inflammation

The extract was initially evaluated by means of the albumin denaturation test, which is used to screen new drugs for anti-inflammatory activity. The results of this screening procedure are shown in Tables 1 and 2. Table 1 shows that the heat denaturation of a 0.15% bovine serum albumin solution was inhibited 96% by the addition of the extract at a final concentration of 0.32 mg/ml, and that there was a dose dependent relationship at lower concentrations. Table 2 shows that the model compound, phloroglucinol, enhanced by at least 24% the denaturation process.

Since the extract exhibited activity in the in vitro anti-inflammatory test system, determination of its effect in an intact animal model of inflammation would be valuable. Table 3 summarizes the results of an anti-inflammatory study employing as a model carrageenin-induced rat paw edema. The extract, administered at a dose of 75 mg/kg (ip) one hour prior to carrageenin injection, inhibited edema formation by 88%. The administration of the potent anti-inflammatory agent phenylbutazone (90 mg/kg) inhibited edema production by 100% and also allowed for a partial absorption of the injected carrageenin. Administration of

Table 1. Effect of C40SA on heat denaturation of bovine serum albumin.

<u>c40SA CONCENTRATION mg/ml</u>	<u>ABSORBANCE^a</u>	<u>% CONTROL</u>
0.0	.620	100
0.005	.550	89
0.01	.560	90
0.02	.340	55
0.04	.345	56
0.08	.049	8
0.16	.036	6
0.32	.022	4
0.64	.090	15

^aDetermined at 660 nm.

Table 2. Effect of phloroglucinol on heat denaturation of bovine serum albumin.

PHLOROGLUCINOL CONCENTRATION mg/ml	ABSORBANCE ^a	% CONTROL
0.0	0.76	100
0.50	1.00	132
0.25	1.00	132
0.125	1.00	132
0.062	1.00	132
0.031	1.00	132
0.015	1.10	145
0.0075	0.95	125
0.0037	0.96	126
0.0018	0.94	124
0.0009	1.10	145

^aDetermined at 660 nm.

Table 3. Effect of drugs on carrageenin-induced rat paw edema.

<u>TREATMENT</u> ^a	<u>MEAN EDEMA ± SE, mm³ (4)</u> ^b	<u>-% CONTROL</u>
Saline	1363 ± 206	100
c40SA (75 mg/kg, ip)	163 ± 69 ^c	12
Phenylbutazone (90 mg/kg, ip)	-100 ± 71 ^c	-7
Cortisone (50 mg/kg, ip)	738 ± 97 ^d	54

^aDrugs were administered 1 hour prior to carrageenin injection.

^bNumber of animals.

^cP < .01

^dP < .05

the steroid cortisone (50 mg/kg) inhibited edema formation by 46%. The effects of the above drugs were significant at the $P < .05$ level.

Several experiments were undertaken to determine the mechanism of action of the extract as an anti-inflammatory agent in the carrageenin paw edema system. The first of these, a study of the effect of the extract on rat body temperature, is summarized in Figure 1. The extract, at a dose of either 75 or 150 mg/kg, had a negligible effect on body temperature over a period of four hours.

Since some compounds have been shown to possess anti-inflammatory activity due to irritant properties, an experiment was conducted to determine whether the extract was an irritant by virtue of its acidity. The carrageenin paw edema model was again employed, and the extract, at a dose of 100 mg/kg, was either buffered at or neutralized to pH 7.4. Table 4 shows the results of this study. The neutralized extract did not offer statistically significant protection (76% of control) while the buffered material inhibited edema formation by 42% ($P < .05$).

Stabilization of the lysosomal membrane has also been invoked as a mechanism of action of various anti-inflammatory agents. The degree of stabilization or labilization can be inferred from the extent of release of the lysosomal marker enzymes acid phosphatase and β -glucuronidase. The extract was so examined for its effect on the lysosomal membrane. The results of this study are shown in Tables 5 and 6.

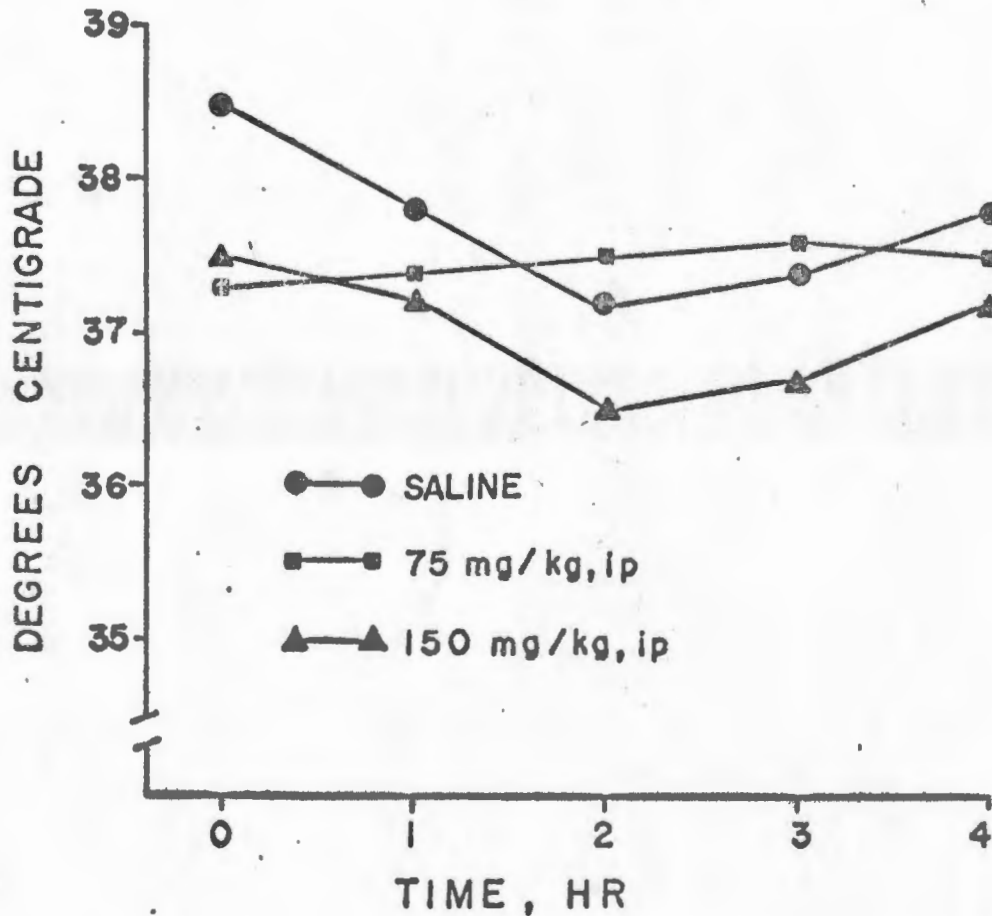


Figure 1. Effect of e4OSA on rat body temperature. Animals were administered either saline or e4OSA (75 or 150 mg/kg, ip) and rectal temperature was monitored over a period of 4 hours. Each point represents the mean temperature of 3 animals.

Table 4. Effect of neutralized or buffered ec40SA on carrageenin-induced rat paw edema.

<u>TREATMENT</u> ^a	<u>MEAN EDEMA ± SE, mm³, (6)</u> ^b	<u>% CONTROL</u>
Saline	1174 ± 99	100
Neutralized ^c (100 mg/kg, ip)	898 ± 181	76
Buffered ^d (100 mg/kg, ip)	678 ± 176 ^e	58

^aDrugs were administered 1 hour prior to carrageenin injection.

^bNumber of animals.

^cNeutralized to pH 7.4 with NaOH.

^dBuffered at pH 7.4 with 0.2 M KH_2PO_4 - Na_2HPO_4 .

^e $P < .05$

The enzyme activities reported are corrected for direct drug inhibition, if any. Table 5 shows that acid phosphatase activity is insensitive as an indicator of lysosomal membrane condition, since the powerful stabilizing agent phenylbutazone inhibited enzyme release by only 7%. However, phenylbutazone (0.309 mg/ml) and the extract at a concentration of either 3.087 or 0.309 mg/ml offered significant ($P < .01$) inhibition of release of β -glucuronidase (30, 36, and 36 per cent of control respectively). Phloroglucinol and 0.031 mg/ml f40SA were ineffective.

Table 6 shows that the extract retains its lysosomal membrane stabilizing ability even when further chemically purified. The refined material offered statistically significant ($P < .01$) inhibition of release of β -glucuronidase (43% of control).

The extract was further tested for anti-inflammatory activity by means of the Freund's adjuvant-induced rat paw edema model of arthritis. Table 7 summarizes the results of this study. The potent steroid triamcinolone inhibited edema formation by 77% ($P < .05$), in contrast to the extract, which provided negligible protection against this type of inflammation.

Capillary fragility

The effect of the extract on capillary fragility as determined by lung hemoglobin content following sudden decompression was examined. Table 8 is a summary of this study. None of the drugs tested, which included e40SA,

Table 5. Effect of drugs on lysosomal membrane stability in vitro.

<u>DRUG</u>	ENZYME ACTIVITY ^a ± SE (4) ^b (% CONTROL)	
	<u>ACID PHOSPHATASE^c</u>	<u>β-GLUCURONIDASE^d</u>
None	4.46 ± 0.16 (100)	0.66 ± 0.02 (100)
Phloroglucinol (0.309 mg/ml)	4.48 ± 0.41 (100)	0.68 ± 0.02 (103)
Phenylbutazone (0.309 mg/ml)	4.13 ± 0.08 (93)	0.20 ± 0.01 (30) ^f
f40SA (3.087 mg/ml)	3.66 ± 0.22 (82) ^e	0.24 ± 0.03 (36) ^f
f40SA (0.309 mg/ml)	3.64 ± 0.16 (82) ^e	0.24 ± 0.01 (36) ^f
f40SA (0.031 mg/ml)	3.83 ± 0.22 (86)	0.64 ± 0.02 (97)

^aExpressed as μmoles product/ml lysosomal fraction/20 min.

^bNumber of animals.

^cDetermined at 405 nm.

^dDetermined at 540 nm.

^eP < .05.

^fP < .01.

Table 6. Effect of f40SA and refined 40SA on lysosomal membrane stability in vitro.

<u>DRUG</u>	<u>β-GLUCURONIDASE ACTIVITY^a \pm SE (4)^b (%CONTROL)</u>
None	0.53 \pm 0.04 (100)
f40SA (0.309 mg/ml)	0.20 \pm 0.03 (38) ^c
Refined 40SA (0.309 mg/ml)	0.23 \pm 0.02 (43) ^c

^aExpressed as μ moles phenolphthalein/ ml lysosomal fraction/ 20 min., determined at 540 nm.

^bNumber of animals.

^cP < .01.

Table 7. Antagonism of Freund's adjuvant-induced rat paw edema

<u>TREATMENT</u> ^a	<u>MEAN EDEMA ± SE, mm³ (6)</u> ^b	<u>% CONTROL</u>
Saline	3920 ± 983	100
Triamcinolone (2 mg/kg, sc)	911 ± 153 ^c	23
r40SA ^d (75 mg/kg, ip)	3079 ± 286	79

^aDrugs were administered daily for 17 days; adjuvant injected on second treatment day.

^bNumber of animals.

^cP < .05

^dAdjusted to pH 7.0 with NaOH.

Table 8. Effect of drugs on the mouse lung hemorrhage induced by sudden decompression.

<u>TREATMENT</u> ^a	<u>MEAN mg HEMOGLOBIN^b/mg DRY LUNG ± SE(4)^c</u>	
	<u>TABLE</u>	<u>CHAMBER</u>
Saline	0.136 ± 0.005	0.154 ± 0.007
e40SA (300 mg/kg, ip)	0.145 ± 0.009	0.164 ± 0.006
Phloroglucinol (300 mg/kg, ip)	0.129 ± 0.005	0.152 ± 0.008
Phenylbutazone (100 mg/kg, ip)	0.165 ± 0.025	0.170 ± 0.006

^aDrugs were administered 1.5 hours prior to decompression.

^bDetermined at 540 nm.

^cNumber of animals.

phloroglucinol, and phenylbutazone, reduced capillary fragility in this test system.

Curare, strychnine, and tetrodotoxin poisoning

The extract was tested for its ability to protect against poisoning in mice induced by curare, strychnine, or tetrodotoxin. Table 9 shows that if both curare and the extract were administered intraperitoneally, there existed protection against curare lethality. As shown in Table 10, if the curare was administered subcutaneously and the extract intraperitoneally, no protection was exhibited by the extract.

Further experimentation was performed to investigate the mechanism of action of the apparent curare antagonism of the extract. Table 11 shows that the material had a negligible in vivo effect on rat brain cholinesterase activity, in contrast to an in vitro inhibition of 53% ($P < .01$). The extract was also tested for its in vivo effect on the curarized cat gastrocnemius muscle. Jugular infusion of 180 mg of e40SA over a period of nine minutes exhibited no effect on the curarized muscle, in contrast to the anticholinesterase agent physostigmine, which completely restored contractile force after infusion of 0.18 mg over a period of three minutes.

To determine whether the extract might protect against poisoning due to other agents, its effects on strychnine and tetrodotoxin lethality were examined. In both cases,

Table 9. Protection against curare poisoning in mice by e40SA.

<u>CURARE DOSE</u> (mg/kg, ip)	<u>NUMBER OF SURVIVORS</u>	
	<u>SALINE</u>	<u>e40SA (300 mg/kg, ip)^a</u>
0.30	5/5 ^b	5/5
0.37	3/5	5/5
0.45	2/5	4/5
0.52	1/5	5/5
0.60	0/5	5/5

^aAdministered 30 minutes prior to curare injection.

^bNumber of survivors/number injected.

Table 10. Protection against curare poisoning in mice by ec40SA.

<u>CURARE DOSE</u> (mg/kg, sc)	NUMBER OF SURVIVORS	
	<u>SALINE</u>	<u>ec40SA (300 mg/kg, ip)^a</u>
0.28	4/5 ^b	5/5
0.36	3/5	4/5
0.44	2/5	2/5
0.52	1/5	0/5
0.60	0/5	0/5

^aAdministered 30 minutes prior to curare injection.

^bNumber of survivors/number injected.

Table 11. Effect of ec40SA in vivo and in vitro on rat brain cholinesterase activity.

<u>TREATMENT</u>	<u>MEAN ENZYME ACTIVITY^a ± SE (3)^b</u>	<u>% CONTROL</u>
Saline	2.96 ± 0.20	100
ec40SA (300 mg/kg, ip) ^c	3.17 ± 0.16	107
ec40SA (5 mg/ml)	1.38 ± 0.16 ^d	47

^aExpressed as μ moles ACh/50 mg brain/10 min.

^bNumber of animals.

^cAdministered one hour prior to sacrifice.

^d $P < .01$

the poison and the extract were administered by the intra-peritoneal route. Tables 12 and 13 show that the extract does not protect against the lethality of either strychnine or tetrodotoxin, respectively.

Liver microsomal enzymes

The possible effect of the extract on the activity of the drug-metabolizing hepatic microsomal enzyme system was determined by measuring the effect of the extract on the metabolism of the pesticide EPN. Table 14 summarizes the results of the experiment. The classical inducing agent phenobarbital significantly increased microsomal enzyme activity by more than 4-fold ($P < .01$). The extract was ineffective in inducing this system after three days of treatment or in inhibiting the activity when given 60 minutes before sacrificing the animals.

Tryptophan pyrrolase

Table 15 summarizes the effects of various drugs on rat liver tryptophan pyrrolase activity. In vivo administration of hydrocortisone and f40SA resulted in increased enzyme activity (322% control, $P < .01$) and inhibition (67% control, $P < .01$), respectively. In vivo administration of phloroglucinol had no effect on the enzyme activity, while the in vitro effect of phloroglucinol was that of inhibition (48% control, $P < .01$). The presence of the Eisenian extract resulted in an in vitro inhibition of 82% ($P < .01$).

Table 12. Effect of ec40SA on strychnine poisoning in mice.

<u>STRYCHNINE ROUTE</u> (2 mg/kg)	<u>NUMBER OF SURVIVORS</u>	
	<u>SALINE</u>	<u>ec40SA (300 mg/kg, ip)^a</u>
ip	0/4 ^b	0/4
sc	0/4	0/4

^aAdministered 30 minutes prior to strychnine injection.

^bNumber of survivors/number injected.

Table 13. Effect of f40SA on tetrodotoxin poisoning in mice.

<u>TREATMENT</u> ^a	<u>NUMBER OF SURVIVORS</u>
Saline	1/4 ^b
f40SA (300 mg/kg, ip)	1/6

^aAnimals were administered 12 µg/kg (ip) tetrodotoxin 30 min after protecting agent.

^bNumber of survivors/number injected.

Table 14. Effect of drugs on EPN^a detoxification.

<u>TREATMENT</u>	<u>MEAN ENZYME ACTIVITY^b ± SE</u>	<u>% CONTROL</u>
Saline	1.8 ± 0.1 (6) ^c	100
Phenobarbital (3 days, 50 mg/kg, ip)	7.8 ± 0.5 (3) ^d	433
e40SA (3 days, 300 mg/kg, ip)	1.4 ± 0.3 (3)	77
e40SA (1 hr., 300 mg/kg, ip)	2.0 ± 0.1 (3)	111

^aO-ethyl 0-4-nitrophenyl phenylphosphonothioate

^bActivity expressed as µg PNP/50 mg liver/hr.

^cNumber of animals.

^d_p < .01

Table 15. Effect of drugs in vivo and in vitro on rat liver tryptophan pyrrolase activity.

<u>TREATMENT</u>	<u>MEAN ENZYME ACTIVITY^a ± SE (4)^b</u>	<u>% CONTROL</u>
Saline ^c	20.6 ± 0.5	100
Hydrocortisone ^d (35 mg/kg, ip)	66.4 ± 1.9 ^e	322
Phloroglucinol ^c (300 mg/kg, ip)	19.8 ± 0.3	96
f40SA (300 mg/kg, ip) ^c	13.7 ± 0.2 ^e	67
Phloroglucinol (486 µg/ml)	9.9 ± 0.3 ^e	48
f40SA (486 µg/ml)	3.7 ± 0.2 ^e	18

^aExpressed as µmoles kynurenine/g liver/hr.

^bNumber of animals.

^cAdministered 1.5 hours prior to sacrifice.

^dAdministered 4 hours prior to sacrifice.

^eP < .01

Rat uterus

The extract was tested for possible estrogenic activity by examining its ability to alter the uterine to body weight ratio in immature female rats. Table 16 shows the results of the study. Estradiol-17- β increased the ratio more than 2-fold ($P < .01$), while the extract had no effect on the ratio.

Table 16. Effect of drugs on rat uterine weight.

<u>TREATMENT</u> ^a	<u>Uterine Wt. / Body Wt.</u> x 10 ³ ± S.E. (5) ^b	<u>% CONTROL</u>
Saline (ip)	0.69 ± 0.06	100
Estradiol (0.5 µg/rat, sc)	1.64 ± 0.06 ^c	238
ec40SA (300 mg/kg, ip)	0.61 ± 0.04	88

^aDrugs were administered daily for 3 days.

^bNumber of animals.

^cP < .01

V DISCUSSION

Inflammation

The extract was initially evaluated for pharmacological activity utilizing the albumin heat denaturation screening test for anti-inflammatory drugs. A large number of anti-inflammatory drugs have been shown to inhibit the heat denaturation of albumin solutions (Grant et al., 1970). Heat denaturation is able to produce albumin aggregates which are immunologically distinct from the parent protein (Maurer, 1959). Such aggregates are known to occur spontaneously (Foster et al., 1965); these spontaneous aggregates behave as antigens in the etiology of at least one immuno-inflammatory disease, serum sickness nephritis (Lirenman et al., 1967).

The extract inhibited albumin denaturation by 96 %, as shown in Table 1, in contrast to the denaturation enhancement afforded by the model compound, phloroglucinol (Table 2). While the mechanism of such a stabilization is presently unknown, it is possible that hydrogen bonding occurs between the extract and the protein, thus increasing the stability of the molecular structure.

The in vitro anti-inflammatory screen was followed by in vivo anti-inflammatory determinations utilizing the carrageenin-induced rat paw edema model of inflammation,

which is least influenced by non-specific factors (Garattini et al., 1965). The mechanism by which carrageenin, a high molecular weight polysaccharide, induces paw edema is as controversial as the mechanisms of action of the agents which inhibit this edema formation. The following have been invoked as mediators of carrageenin-induced edema: kinins and 5-hydroxytryptamine (5-HT) (Crunkhorn and Meacock, 1971), histamine (DiRosa et al., 1971), and activation of the complement system (DiRosa, 1972). It is possible that the apparent complement system activation reflects a mild antigen-antibody response to carrageenin, since high molecular weight polysaccharides as well as proteins are capable of eliciting such a response (Porter, 1967). Thus, 5-HT and histamine release may be secondary to complement activation.

Administration of the extract one hour prior to carrageenin injection inhibited the formation of edema by 88 %. Accordingly, various experiments were undertaken to determine the mechanism of action of this protection. Since stimulation of the adrenal cortex, either directly or indirectly via the hypothalamus or pituitary, can be related to anti-inflammatory activity, the action of the extract was compared with that of a large dose of cortisone (50 mg/kg, ip), which inhibited edema production by 46 %. Since cortisone afforded only one-half the inhibition offered by the extract, it is unlikely that the extract could stimulate the production or release of sufficient corticosteroid to reduce inflammation by the extent exhibited.

The thermogenic activity of new anti-inflammatory compounds must be determined since the peripheral vasodilation which would accompany a rise in body temperature might either promote edema, due to increased capillary permeability, or reduce edema, by more rapid removal of phlogistin. The extract was tested for its effect on body temperature, and it can be concluded from Figure 1 that the extract had a negligible effect on body temperature over a period of four hours. Thus the anti-inflammatory activity of the extract is not based on thermogenic effects.

Garattini et al., (1965) have shown that various compounds, when injected by the intraperitoneal route, can inhibit edema formation in various models of inflammation due to their properties as irritants. A partial basis for this phenomenon is decreased availability of circulating fluid due to the draining of water into the peritoneal cavity. Accordingly, an experiment was undertaken to determine if the extract was an irritant by virtue of its acidity. Employing the carrageenin model, extract was tested which was maintained at pH 7.4 either by phosphate buffering or sodium hydroxide neutralization. The results of the experiment are shown in Table 4, and it is evident that although the anti-inflammatory potency of the extract has been decreased by neutralization, the buffered material retains statistically significant anti-inflammatory activity which is one-half that of non-adjusted, acidic extract solution. Thus the mechanism of action of the extract can

be partially, but not fully, explained on the basis of its irritancy.

Labilization of the lysosomal membrane is known to occur at sites of injury and has also been implicated as a major factor in the development of inflammation, since lysosomal enzymes possess the capacity to degrade completely the components of the connective tissue, such as collagen, protein-mucopolysaccharide complexes, glycoprotein, and elastin (Houck and Forscher, 1968; Ignarro, 1971). Many anti-inflammatory agents have been shown to stabilize the lysosomal membrane through their inhibition of the release of the marker enzymes β -glucuronidase and acid phosphatase from lysosomal preparations. The extract was examined in this manner. It can be concluded from the data in Table 5 that the extract is as effective as phenylbutazone as a lysosomal membrane stabilizing agent.

Since the crude extract might contain an impurity which would account for the lysosomal membrane stabilization, a refined sample of the extract was also tested for its ability to inhibit the release of β -glucuronidase from lysosomal preparations. The results, summarized in Table 6, show that the refined material is as effective as the crude in its ability to stabilize the lysosomal membrane.

The extract was further tested for anti-inflammatory activity by means of the adjuvant arthritis model, which is a delayed hypersensitivity reaction. Table 7 shows the results of the study, and it can be concluded that the

extract exerted no anti-inflammatory effect against this type of inflammation. The mechanism by which effective agents, such as triamcinolone, combat this model of arthritis is obscure, but may be in part due to lysosomal stabilization (Pollock and Brown, 1971).

An hypothesis may be formulated to account for the contrasting effects of the extract on the two in vivo models of inflammation. "Primary" membrane stabilization (stabilization of membranes of whole cells, e.g., basophils, platelets, or mast cells) and "secondary" membrane stabilization (stabilization of the membranes of cell organelles, e.g. lysosomes) apparently are involved in the etiology of both carrageenin and Freund's edema. The extract has been shown to stabilize at least one type of membrane, that of the lysosome. However, Freund's edema may be more difficult to suppress than carrageenin edema because of overwhelming participation of the immunity mechanism in the case of the former.

The extract, by virtue of its polysaccharide content, might also act as a counter-irritant.

Capillary fragility

The extract was examined for its ability to decrease capillary fragility. It should be noted that the method of Majovski (1944) was employed except in the comparison of different treatments, for which a quantitative spectrophotometric assay for hemoglobin was used in place of the arbitrary qualitative assay originally described.

The method employed is non-specific in that the underlying mechanism of a stabilizing agent cannot be differentiated from the known possibilities, which include effects on the 1) endothelial cell membrane, 2) endothelial cell junction, and 3) sources of possible chemical intermediates (such as basophils, and platelets, and plasma kinins).

None of the drugs tested enhanced capillary stability (Table 8) in contrast to the effect of phloroglucinol reported by Okanishi and Shimaoka (1952). Close examination of the results shows that the method is not sufficiently sensitive to quantitate the possible stabilizing effects of drugs, inasmuch as the chamber control animals exhibited only a 13% increase in lung hemoglobin compared to the non-chamber animals. This increase is not statistically significant.

Curare, strychnine, and tetrodotoxin poisoning

The extract was tested for its effectiveness as an antagonist against curare, strychnine, and tetrodotoxin, because of the report of Mogey and Young (1949) that phloroglucinol acted as a mild curare antagonist in vitro. These three agents act on the nervous system dissimilarly: 1) curare, the classical neuromuscular blocker, competitively blocks postjunctional acetylcholine receptor sites, 2) strychnine, a CNS stimulant, acts by blocking post-synaptic inhibition, and 3) tetrodotoxin selectively blocks axonal conduction.

Since the extract was ineffective in preventing curare

poisoning when administered via a different route than that of the curare, in contrast to the protection exhibited when the routes of administration were identical (Tables 9-10), a true pharmacological antagonism would seem an unlikely mechanism of action of the extract. This was supported by demonstrating that 1) the extract was an ineffective in vivo cholinesterase inhibitor (Table 11) and 2) the extract exerted no antagonism to the action of curare on the in vivo cat gastrocnemius muscle-sciatic nerve preparation.

An alternative mechanism of action might be based on the occurrence of a chemical interaction of the negatively charged extract molecule with the positively charged quaternary nitrogen of the curare molecule. Further experimentation in this context failed to support such a mechanism: while no protection was offered against the lethality of strychnine, which contains an uncharged tertiary nitrogen (Table 12), neither was there protection against poisoning by tetrodotoxin which contains a positively charged guanidinium group (Table 13). An untested hypothesis for the curare protection might be an inhibition of absorption of the curare from the peritoneal cavity.

Liver microsomal enzymes

In order to more fully characterize the action of a new drug, its interaction with other drugs must be examined. One potential site of drug interaction is the liver microsomal electron transport system, an enzyme

system which is responsible for the biotransformation of a wide variety of drugs and other chemical agents. Most important relative to drug interactions are the stimulation (induction) and inhibition of the system.

The extract was tested for possible microsomal induction or inhibition (Table 14) by measuring the effect on the metabolism of the insecticide EPN. The extract was observed to have no effect on microsomal enzymes after administration daily for three days or one hour following a single dose.

Tryptophan pyrrolase

Tryptophan pyrrolase, located in the soluble fraction of the liver cell, catalyzes the cleavage of the indole ring from the nutritionally essential amino acid tryptophan. The resultant product, formyl kynurenine, is readily hydrolyzed to kynurenine. Tryptophan pyrrolase is important not only for tryptophan degradation but also for the conversion of tryptophan to niacin. The enzyme is inducible; known inducing agents include adrenal corticosteroids and tryptophan itself.

Investigation of the effect of the extract on the activity of tryptophan pyrrolase would provide a more complete characterization of the effects of the extract on liver metabolism, in conjunction with the effects exerted on the microsomal enzyme system, especially since Frieden et al. (1961) reported that phloroglucinol inhibited tryptophan pyrrolase in vitro. Such an investigation was carried out and the results, shown in Table 15, indicate that the

extract, both in vivo and in vitro, caused enzyme inhibition. Such inhibition could result in nicotinic acid deficiency or in increased synthesis of serotonin, which might result in stimulation of cerebral activity.

Rat uterus

A variety of naturally occurring phenolic materials have been shown to exhibit estrogenic activity (Biggers and Curnow, 1954); the widespread metabolic consequences of such activity seemed sufficient justification for determining whether the extract acted in a similar manner.

The uterus, target organ for estrogen, is most frequently employed to bioassay for estrogenic activity. The response of this organ to estrogenic stimulation is biphasic in nature: initially, hyperemia and imbibition of water occur, followed later by hypertrophy and hyperplasia; thus an increase in the uterine to body weight ratio can be demonstrated.

The extract was found to cause neither estrogenic stimulation nor retrogression of this organ (Table 16).

VI SUMMARY AND CONCLUSIONS

1. An extract from Eisenia bicyclis containing principally a polyphenolic polymer inhibited the heat denaturation of a 0.15% bovine serum albumin by 96% when added to the incubation medium at a concentration of 0.32 mg/ml. This was suggestive of possible anti-inflammatory activity.
2. As determined by paw volume changes, administration of the extract at a dose of 75 mg/kg one hour prior to carrageenin injection inhibited edema production by 88%.
3. The ability of the extract to suppress carrageenin edema was apparently not due to pituitary-adrenal stimulation, since a large dose of cortisone (50 mg/kg) provided only one-half the protection afforded by the extract. Nor was the anti-inflammatory effect due to changes in body temperature, which remained unchanged over a period of four hours after administration of a dose of either 75 or 150 mg/kg.
4. The anti-inflammatory effect of the extract was partially due to its irritant properties, since buffering a dose of 100 mg/kg provided only one-half the protection afforded by a non-buffered dose. The extract may also act against inflammation by stabilizing the lysosomal

membrane, since in vitro 309 μ g of the extract per milliliter of incubation medium inhibited by 64% the release of the marker enzyme β -glucuronidase from rat liver lysosomes.

5. The extract administered at a dose of 75 mg/kg daily for 17 days offered no protection against rat paw edema induced by Freund's complete adjuvant.
6. No decrease in capillary fragility of the mouse lung, as determined by hemoglobin content following sudden decompression, was observed 1.5 hours after pretreatment with the extract at a dose of 300 mg/kg.
7. The material administered to mice at a dose of 300 mg/kg 30 minutes prior to administration of lethal doses of curare exhibited protection against curare poisoning only if the routes of administration were identical.
8. The mechanism of action of the apparent curare antagonism of the extract appeared to be chemical rather than pharmacological, since the material had no effect on either the curarized cat gastrocnemius muscle in vivo or the activity of rat brain cholinesterase in vivo. An exact chemical mechanism cannot be formulated because the material was ineffective in preventing poisoning by either another positively charged toxin, tetrodotoxin, or an uncharged poison, strychnine.
9. The extract was observed to have no effect on liver microsomal enzyme activity after administration of 300 mg/kg daily for three days or one hour following a

single dose of 300 mg/kg.

10. The administration of the extract at a dose of 300 mg/kg 1.5 hours prior to sacrifice resulted in 33% inhibition of the activity of tryptophan pyrrolase. The presence of the extract at a concentration of 486 µg/ml resulted in an in vitro inhibition of 82%.
11. The extract, administered at a dose of 300 mg/kg daily for three days, did not alter the uterine to body weight ratio in immature female rats.

VII REFERENCES

- Bickoff, E. M., et al.: Isolation of a new estrogen from ladino clover. J. Agric. Fd. Chem. 6: 536-539, 1958.
- Biggers, J. D. and D. H. Curnow: Oestrogenic activity of subterranean clover. Biochem. J. 58: 278-282, 1954.
- Conney, A. H.: Pharmacological implications of microsomal enzyme induction. Pharmacol. Rev. 19: 317-366, 1967.
- Crunkhorn, P. and S. C. R. Meacock: Mediators of the inflammation induced in the rat paw by carrageenin. Brit. J. Pharmacol. 42: 392-402, 1971.
- Der Marderosian, A.: Marine pharmaceuticals. J. Pharm. Sci. 58: 1-33, 1969.
- DiRosa, M.: Biological properties of carrageenan. J. Pharm. Pharmacol. 24: 89-102, 1972.
- DiRosa, M., et al.: Studies of the mediators of the acute inflammatory response induced in rats in different sites by Carrageenan and Turpentine. J. Path. 104: 15-29, 1971.
- DuBois, K. P. and G. H. Mangun: Effect of hexaethyl tetraphosphate on cholinesterase in vitro and in vivo. Proc. Soc. Exptl. Biol. Med. 64: 137-139, 1947.
- Foster, J. F., et al.: Microheterogeneity of plasma albumins. J. Biol. Chem. 240: 2495-2502, 1965.
- Frieden, E., et al.: Inhibition of tryptophan pyrrolase by serotonin, epinephrine, and tryptophan analogs. Arch. Biochem. Biophys. 92: 176-182, 1961.
- Garattini, S., et al.: Sensitivity of local edemas to systemic pharmacological effects. In Non-steroidal anti-inflammatory drugs, Excerpta Medica Foundation, Amsterdam, 155-161, 1965.
- Grant, N. H., et al.: Stabilization of serum albumin by anti-inflammatory drugs. Biochem. Pharmacol. 19: 715-722, 1970.

Houck, J. C. and B. K. Forscher, editors, Chemical Biology of Inflammation. Pergammon, Oxford, 1968.

Ignarro, L. J.: Effects of anti-inflammatory drugs on the stability of rat liver lysosomes in vitro. Biochem. Pharmacol. 20: 2847-2860, 1971.

Knox, W. E. and A. H. Mehler: The conversion of tryptophan to Kynurenine in liver. J. Biol. Chem. 187: 419-430, 1950.

Lirenman, D. S., et al.: Role of aggregated bovine serum albumin in pathogenesis of serum sickness nephritis in rabbits. Proc. Soc. Exp. Biol. Med. 125: 231-234, 1967.

Majovski, G. J., et al.: Vascular fragility and permeability as influenced by various agents. J. Pharm. Exptl. Ther. 80: 1-7, 1944.

Maurer, P. H.: Modified bovine serum albumin. VI. Immunochemical and physicochemical properties of BSA denatured by various agents. Archs. Biochem. Biophys. 79: 13-26, 1959.

Mogey, G. A. and P. A. Young: The antagonism of curarizing activity by phenolic substances. Brit. J. Pharmacol. 4: 359-365, 1949.

Neal, R. A. and K. P. DuBois: Studies on the mechanism of detoxification of cholinergic phosphorothioates. J. Pharmacol. Exptl. Ther. 148: 185-192, 1965.

Okanishi, T. and A. Shimaoka: Pharmacological studies of flavone derivatives I, capillary stabilizing effect. Ann. Repts. Shionogi Res. Labs. No 2: 162-166, 1952.

Pearson, C. M.: Development of arthritis, peri-arthritis, and periostitis in rats given adjuvants. Proc. Soc. Exptl. Biol. Med. 91: 95-101, 1956.

Pollock, S. H. and J. H. Brown: Studies on the acute inflammatory response. III. Glucocorticoids and Vitamin E in vivo attenuate thermal labilization of isolated hepatic lysosomes. J. Pharmacol. Exptl. Ther. 178: 609-615, 1971.

Porter, R. R.: The structure of antibodies. Sci. Am. 217: 81-90, 1967.

Van Arman, C., et al.: Some details of the inflammations caused by yeast and carrageenin. J. Pharmacol. Exp. Ther. 150: 328-334, 1965.

Winter, C. A., et al.: Carrageenin-induced edema in hind paw of the rat as an assay for anti-inflammatory drugs. Proc. Soc. Exptl. Biol. Med. 111: 544-547, 1962.

Zelenski, S. G.: Ph.D. Thesis, University of Rhode Island, 1973.