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Pharmacological Evaluation of an Asterosaponin Extract from Asterias Forbesi (Desor)

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PHARMACOLOGICAL EVALUATION OF AN ASTEROSAPONIN EXTRACT FROM ASTERIAS FORBESI (DESOR)

BY

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ASTEROSAPONIN

MASTER OF SCIENCE THESIS

OF

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ABSTRACT

An asterosaponin extracted from the Atlantic starfish Asterias forbesi (Desor) has been evaluated for pharmacological activity. The investigation has revealed that while the asterosaponin does not exhibit all the activities that would be expected, such as hemotoxicity to blood cells, cholinergic blocking activity, initiation of avoidance response reaction or production of autotomy, it does exhibit some unique properties not demonstrated by other asterosaponins. These activities include analgesia, anti-inflammation, and transient hypotension produced by a direct effect on the vascular bed.

The bovine serum albumin assay for anti-inflammatory activity showed that a concentration of 500 µg/ml of asterosaponin caused an 85% inhibition of protein denaturation. The rat paw edema assay demonstrated that pretreatment with asterosaponin at 75 mg/kg ip significantly reduced the edema caused by the carrageenin injection. In both cases the response was dose dependent.

The analgesic activity of the asterosaponin was analyzed using the Koster acetic acid writhing test. Significant analgesia was demonstrated with a dose of asterosaponin as low as 15 mg/kg while 25 mg/kg was capable of reducing the writhes by 99%.

The transient hypotensive activity of the asterosaponin was demonstrated in rats, cats, and dogs. Utilizing the cat it was demonstrated that very low doses of the asterosaponin (0.5 mg/kg) could significantly transiently lower the blood pressure, but had no effect on the nictitating membrane or on somatic muscle contraction. The rat was less sensitive to the intravenously administered asterosaponin then the cat, eliciting a hypotensive effect only when concentrations of 4.0 mg/kg or higher were administered. The sensitivity of the dog to asterosaponin was intermediate. The dog exhibited a drop in blood pressure when a dose of 2.0 mg/kg of asterosaponin was administered, and similar to the cat and rat showed a dose response relationship to the asterosaponin.

The mechanism by which the asterosaponin caused the transient drop in blood pressure was investigated by an analysis of vagal influence, the possibility of histamine release, direct action on the vasculature and the possible complex involvement of some type of adrenergic blockade or stimulation using an experimental set up monitoring blood pressure, heart rate and ECG. The data showed that the asterosaponin continued to exhibit its hypotensive action even when the vagus nerves were severed, the animal was pretreated with antihistamine, the alpha receptors were blocked, or when the beta receptors were blocked.

The hind leg perfusion experiment performed on the rat proved that the asterosaponin was acting directly on the vasculature. The experiment showed that the asterosaponin was capable of causing vasodilation directly when all neural influences were removed by cervical dislocation and severing, and was even capable of acting in the presence of beta blockade created by the administration of propranolol to levels which blocked isoproterenol effects.

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The author is indebted and thanks Dr. Gary P. Carlson for his guidance throughout this study. The author would also like to thank Dr. Yuzuru Shimizu who supplied the original asterosaponin and guided the author during the extraction of the asterosaponin.

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TABLE OF CONTENTS

LIST OF TABLES

Table Page

LIST OF FIGURES

I. INTRODUCTION

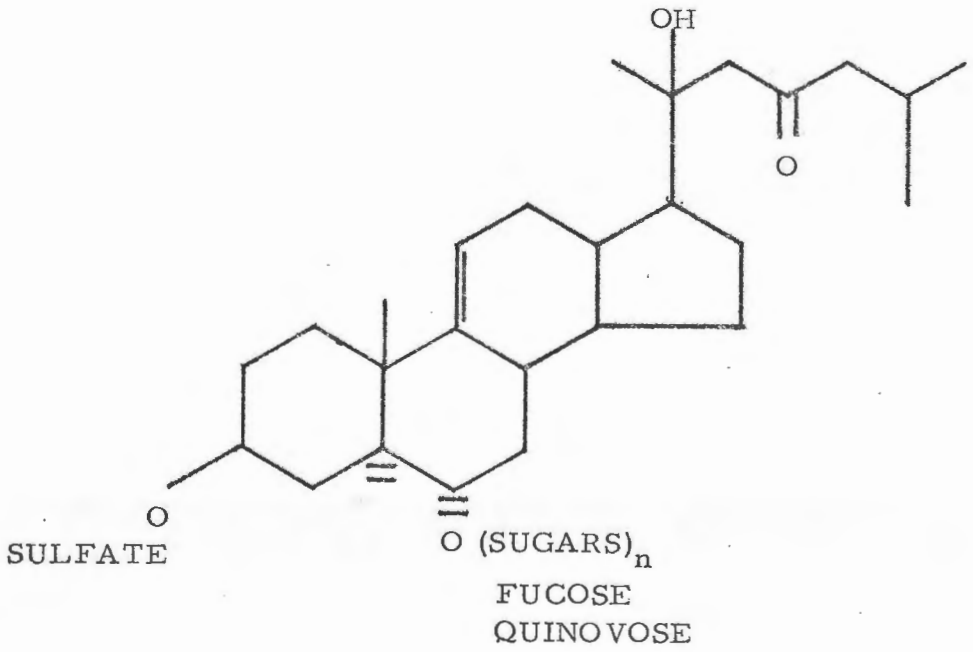
The pharmacological evaluation of compounds extracted from plant or animal sources is basic to the science of pharmacology. The employment of animals and plants from the marine environment as a source for biologically active compounds has created the field of marine pharmacology.

The isolation of toxic saponin compounds from starfish in the early 1960s (Hashimoto and Yasumoto, 1960) renewed the scientific argument as to whether starfish employ some type of toxin in order to facilitate prey procurement, specifically the opening of the bivalve shell. Further research into the pharmacological properties of these asterosaponin compounds (Rio et al., 1963; Yasumoto et al., 1964; Rio et al., 1965; Yasumoto et al., 1966; Friess et al., 1968; Friess, 1970; Shimizu, 1971, 1972) indicated that the asterosaponins were not a toxin used by the starfish to aid in the opening of bivalves but that these and related compounds did possess many pharmacological properties. These properties included activity as antiviral agents (Shimizu, 1971), anticoagulant agents (Baslow, 1969), cytotoxins (Ruggieri, 1965), reducers of sperm motility (Ruggieri, 1965), cholinergic blocking agents (Friess, 1970), hemotoxic agents

(Owellen et al., 1973), producers of an avoidance response reaction by mollusks (Mackie et al., 1968) and antifungal agents (Wolters, 1968).

Dr. Yuzuru Shimizu isolated an antiviral asterosaponin extract from the Atlantic starfish Asterias forbesi in 1971. This asterosaponin (Figure 1) is an aglycone with a sulfate group and sugars attached. That structure is characteristic of saponin compounds derived from starfish and is very similar to compounds extracted from Holothurins (Friess et al., 1965; Friess and Durant, 1965). The investigation of the pharmacological properties of this purified and identified asterosaponin from Asterias forbesi was the basis for this thesis. The investigation was aimed at evaluating the asterosaponin for pharmacological activities that had been exhibited by other asterosaponin or related compounds. The investigators were also interested in evaluating the extract for properties not previously associated with asterosaponins and to determine the mechanism by which these activities were produced in the hope that the compound might exhibit activities that would be of use as either a research tool or a possible drug.

FIGURE 1. STRUCTURE OF ASTEROSAPONIN FROM ASTERIAS FORBESI



II. LITERATURE REVIEW

Nigrelli (1952) isolated the first saponin of animal origin when he isolated the compound he later called holothurin from a sea cucumber. The Holothurian from which the saponin was isolated is a member of the phylum Echinodermata as are the Asteroidea or starfish. Holothurin was shown to exhibit toxic effects on the killifish and pearl fish, and the compound restricted the growth of sarcoma 180 in Swiss Webster mice. Nigrelli et al. (1955) reported on the chemical nature of this compound from the Holothurian and showed that this toxic compound had a steroid nucleus and a monosaccharide moiety. Sullivan et al. (1955) demonstrated that the compound had anti-tumor activity on the Krebs-2 Ascites Tumors in Swiss mice. The investigation of the pharmacologic properties of this toxin was continued with the work of Friess et al. (1959) who demonstrated that the toxin had irreversible blocking effects on the rat phrenic nerve-diaphram preparation.

Work concerning holothurin continued into the 1960s when Friess and Durant (1965) and Friess et al. (1965) demonstrated that the anticholinergic activity of the toxin demonstrated through the rat phrenic nerve experiments could be partially blocked by physostigmine and neostigmine.

Saponins from other animals were discovered in the early 1960s when Matsuno and Yamanouchi (1961) isolated a saponin from a different Holothurian then the one Nigrelli had used. This was done while the cholinergic involvement of toxic substances from echinoderms became even more well documented through the work of Mendes et al. (1963).

The isolation of toxic principles from the Echinodermata rekindled the long fought argument over the possible presence of some type of toxic substance in starfish which is used by the starfish to facilitate food procurement, specifically a toxin that would reduce the effort necessary for the starfish to open the bivalve shell.

The toxic substance investigated by this research was isolated from the Atlantic starfish Asterias forbesi and was tested on one of the starfish's common food sources, the quahog Mercenaria mercenaria. Thus the literature dealing with possible toxins possessed by starfish has focused on this particular starfish, and when possible in relation to the clam Mercenaria mercenaria.

The first hint of evidence for or against a toxin being used by the starfish Asterias forbesi comes from the work of Galtsoff and Loosanoff (1939). While investigating the problem of Asterias forbesi plundering the oyster and clam beds of the eastern shore they observed that, on occasion, specimens of Asterias would be

unable to open large mollusks and would leave the mollusk and go on. The conclusion could be made that if a toxin were present in the starfish, it would be unlikely for this response to be necessary. Further evidence against the presence of a toxic substance used by Asterias forbesi in food procurement has been presented by the work of Burnett (1955, 1960). Burnett showed that for Asterias forbesi there have been no evolutionary pressures to develop such a toxin because the starfish is fully capable of exerting a force greater than 5,500 grams, a full 1,000 grams greater then the force needed to open a quahog. Burnett demonstrated that the starfish does not pull on the shell of the quahog continuously and that the shell halves will frequently snap shut on the everted stomach, with no noticable adverse effects to the starfish. Furthermore, Burnett (1960) showed, using Asterias forbesi and Mercenaria mercenaria that the stomach of the starfish could pass through very small openings in the clam and that the starfish contained stomach enzymes capable of digesting the living clam. Burnett concluded (1960) that "Thus, there is strong evidence that Asterias secretes no poison before it begins to digest a clam."[1]

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[1]A. Burnett, "The mechanism employed by the starfish Asterias forbesi to gain access to the interior of the bivalve, Venus mercenaria," Ecology 41 (1960): 584.

Lavoie (1956) presented additional evidence that starfish employ no toxic compound in their acquisition of food by using extracts of Asterias forbesi on the mussel Mytilus edulis. Various extracts from the stomach and pyloric caeca were tested by a number of techniques. The extracts were tested by injection into the mantle cavity of the mussel, by injection into the adductor muscles, by addition to the sea water containing the mussel and the extract was even perfused on the exposed heart of the Mytilus. All of the experiments with all the extracts produced the same results; there was no difference in the effect seen with the starfish extract from the response seen with sea water used as a control. Lavoie also demonstrated that the starfish could enter the clam through a gap as small as 0.1 mm. However, the conclusion that was made by Lavoie was that, "The negative results of the experiments involving sea star extracts are not proof that asteroids do not secrete a toxin during predation, but they do indicate that no such substance can be separated from the sea star organs by the extraction methods used."[2] The conclusion indicates the dilemma these investigators face. It is impossible to prove that a toxin does not exist, and only possible to prove that one does exist when it is demonstrated unequivocally. This has not been done.

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[2]M. Lavoie, "How sea stars open bivalves," Biol. Bull. 111 (1956): 122.

Christensen (1957) also supported the mechanical theory of starfish access to mollusks, that is, that force was the "primary" method employed by seastars of the Asterias type to gain access to the tissue of the bivalve prey. The hesitance on the part of the researcher to completely dispel the idea of a toxin being present is evidenced here by the choice of the word "primary".

Nichols (1964) continues to include the idea that a toxic substance is being used by starfish in prey predation, and Aldrich presents observational data (Aldrich, 1954) in his Ph. D. dissertation that extracts from Asterias forbesi do indeed produce relaxation of the adductor muscles of Mytilus edulis and Modiolus demissus plicata. Aldrich's data however is based on observations of the response of Modiolus demissus to extracts of Asterias tissue where a gaping response was interpreted as being the result of a toxic substance present in extracts from pyloric diverticulum. Unfortunately no reason was given by Aldrich as to why the response of Modiolus was inconsistant. With both the filtered and dialized extracts there appears to have been specimens which were not affected by the extract. Observations of an Asterias feeding on a Mytilus edulis is the second source for Aldrich's belief that the starfish contains a toxin. The fact that the starfish stomach is everted at the begining of the attack supposedly suggests the role of a

chemical mediator prior to the mechanical phase of penetration.

Hashimoto and Yasumoto (1960) in studying an extract from the starfish Asterina pectinifera which had been shown to be toxic to oyster hearts, could hinder the ecdysis of fly maggots, and was toxic to fish and warm blooded animals, noticed that the water in the aquarium which held the starfish often turned foamy when starfish died. Hypothesizing that the extract that they had been studying might be responsible for this foaming, and knowing that one of the properties of saponins is their surfactant nature, they initiated a search for a saponin compound in the starfish. Indeed, when isolation procedures were completed the compound was classified as a saponin.

Continuing their work Yasumoto and Hashimoto, together with Watanabe (Yasumoto et al., 1964) showed that with better extraction procedures using the starfish Asterias amurensis a highly purified toxic saponin fraction could be obtained. This new asterosaponin proved to be hemolytic to rabbit blood and toxic to killifish, earthworms and fly maggots. When 80 mg of the starfish saponin was injected into the mantle cavity of three individual oysters through a hole in the edge of their shells, these shell fish, which were kept in an aquarium, showed no signs of relaxation of the adductor muscles for the six hour observation time.

These results of Yasumoto et al. (1964) suggest that the asterosaponin is not responsible for facilitating the starfish's ability to

open the clam or oyster, but that it may be present for another purpose as yet obscure. This corresponds with the opinion of Feder and Christensen (1966) who believe that there is no basis for the toxin theory. However they do believe toxic substances are present in starfish, but that they are not utilized for prey procurement.

Yasumoto et al. (1966) investigated whether starfish in general possess saponins since saponins had been believed to be restricted to the plant kingdom prior to the work of Nigrelli. Analyzing five different starfish species, they found that all the starfish analyzed did contain saponins. The saponins were found in all parts of the starfish body and were at highest concentrations in the summer months.

Rio et al. (1963) in Nigrelli's laboratory determined that the giant sunburst starfish Pycnopodia helianthoides from the Pacific north west also possessed a saponin, and that this asterosaponin differed from the Holothurian saponin. The compound was cytotoxic and hemolytic and contained a steroidal aglycone characteristic of the saponins.

A toxic extract from Asterias forbesi was demonstrated by Chaet in 1962. He showed that the scalded coeleomic fluid of Asterias contained a toxin which when injected into the peritoneal cavity of the starfish Asterias forbesi caused autotomy (the rejection of the starfish's own arms) and death. Toxic fractions from other species

of Asterias were examined by Fange (1963) who showed that an extract from Asterias rubens produced the irreversible contraction of the isolated Buccinum radula muscle. This fraction was inactivated by boiling.

Rio et al. (1965) evaluated the biological activities of extracts from five starfish species. Although one of the asterosaponins evaluated was extracted from Asterias forbesi, it is not believed that the compound is the same as the compound isolated by Shimizu. No structure for the compound was determined, but the extraction procedures are not the same. The Asterias forbesi toxin was shown to be toxic to Fundulus heteroclitus down to a level of 2 µg/ml. However, no marked eosinophilia or hemolytic activity was reported for the compound. The compound was shown to immobolize sperm and cytolyze unfertilized sea urchins eggs.

Friess, Durant and Chanley (1968) used the rat phrenic nerve-diaphram preparation on two saponins from the starfish Asterias amurensis to evaluate the cholinergic properties of the asterosaponins as they had done for the holothurin in 1959. Their work established that these two saponins demonstrated irreversible blockade of contractural responses elicited both directly and indirectly.

In 1970 Friess reviewed the literature on the steroidal saponin esters from Echinoderms (Friess, 1970). The compounds investigated were from sea cucumbers and starfish and all contained the steroidal

nucleus, polysaccharide moiety and esterified sulfuric acid residue, while some of the sugars present on the moiety included D-quinovose and D-fucose. Refering to the work of Hashimoto on Asterias amurensis Friess pointed out that both asterosaponins A and B extracted from the starfish contained the same aglycones and one sulfate group. The sulfate group was suggested to be the part of the compound responsible for the toxicity and anionic character. Compound A contained only the terminally reduced sugars fucose and quinovose but both asterosaponin A and B were "quite" powerful in destruction of excitability of a cholinergic neuromuscular preparation.

Shimizu in 1971 isolated an anti-viral substance from the starfish Asterias forbesi (Shimizu, 1971). This compound later (Shimizu, 1972) was established to be of the classical steroidal aglycone, polysaccharide moiety and sulfate derivative form, and is the compound that was used in this investigation.

Mention has already been made to the possibility of an avoidance response reaction being caused by the asterosaponin. The work of Fange (1963) Feder and Lasker (1964) Feder and Arvidsson (1967) and Mackie et al. (1968) clearly shows that extracts from starfish could initiate an escape response in mollusks. The observations of Pratt and Campbell (1956) suggested that the mollusk Mercenaria mercenaria may be able to sense the presence of Asterias forbesi

and initiate an avoidance or escape response. These beliefs were based on the observation that quahogs appear to burrow deeper when Asterias are in the vicinity of the bivalves.

Asterias forbesi often feeds on the clam Mercenaria mercenaria. There is evidence that asterosaponins can be cardioactive (Hashimoto and Yasumoto, 1960) and this is another side to the debate as to whether the starfish opens the bivalve with shear force or through the use of a toxin. In order to facilitate an evaluation of the bivalve toxicity, and the cardioactive and cholinergic properties of the asterosaponin background information on the anatomy and physiology of Mercenaria mercenaria, its heart, and the chemicals it is sensitive to was necessary. This information has been given by Prosser (1940), Greenberg and Jegal (1963), Greenberg (1965), Florey (1967) and Agarwal and Greenberg (1969).

Advancing the research covered by this thesis into activities of asterosaponins not already cited as being properties of asterosaponins it was decided to investigate the possibility of anti-inflammatory activity, analgesic activity, and the effects of the asterosaponin on the mammalian cardiovascular system. Reviewing the literature on anti-inflammatory assay procedures it was determined to use the procedures of Grant et al. (1970) for the in vitro assay and that of Winter et al. (1963) and Winter and Flataker (1965) for

the *in vivo* method. The procedure of Koster *et al.* (1959) was reviewed and chosen to be used to evaluate the analgesic activity of the asterosaponin.

III. EXPERIMENTAL

A. Animals

Male albino rats weighing between 150 to 500 grams and male mice, 30 to 50 grams (Charles River Breeding Laboratories, Wilmington, Massachuestts) were used. 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 three days following receipt from the supplier.

Cats and dogs from the University of Rhode Island College of Pharmacy's kennel were of mixed breed.

Clams, Mercenaria mercenaria, were obtained from Wickford Shell Fish Inc. (Wickford, Rhode Island). The clams were stored in aerated artificial sea water aquaria (Aquarium Systems, Inc., Cleveland, Ohio). The temperature of the sea water was maintained at 15-20°C with room lights on alternating 12 hour light-dark cycles.

The starfish (Asterias forbesi) that were used for the extraction procedure and for experimentation were collected from Narragansett Bay in the shallow areas (usually at low tide) off the rocks close to Hazzard Ave. in Narragansett, Rhode Island.

The snail Littorina littorea was collected on the rocky shore

of Narragansett Bay at low tide near the "Towers" of Narragansett, Rhode Island.

B. Materials

Analytical grade chemicals or equivalent were used throughout the study. Carrageenin was obtained from Marine Colloids, (Rockland, Maine).

Two samples of asterosaponin were used in this investigation. The original extract, produced by Dr. Yuzuru Shimizu of the Department of Pharmacognosy of the University of Rhode Island was only occasionaly used in the investigation as indicated. The major portion of the research was carried out on material that was extracted and purified by Les Goldsmith during the summer of 1973 under the guidance of Dr. Yuzuru Shimizu.

C. Analytical Procedures

The asterosaponin was dissolved in 0.9% saline for intravenous and intraperitoneal administration, artificial sea water for marine preparations, distilled water for in vitro preparations and Ringer-Locke solution for hind leg perfusion preparations. The asterosaponin was often used in doses of 1.5×10^{-power} g/ml because the estimated molecular weight of the compound was 1,500 (Shimizu, 1972) thus allowing for easy conversion to molar concentration.

1. Cholinergic Assay and the Effects of Asterosaponin on the Mercenaria mercenaria Heart

Large bivalves Mercenaria mercenaria were kept in aerated artificial sea water 15-20°C until used. The dissection was carried out under sea water. A bivalve specimen was selected and the edge of the shell taped until a small crack appeared. The clam was then opened by cutting the adductor muscles, and care was taken not to injure the portion of the clam close to the hinge, as this was the vicinity of the heart. After removal of extraneous tissue, the dorsal half of the clam was transfered into the dissection dish with the dorsal side (containing the heart) up and a superficial sagital cut was made along the dorsal body wall. The heart was then isolated and prepared for removal by cutting the intestine which passed through the heart on both sides. Ligatures were tied around both ends of the ventricle, so that eventually one could be attached to a transducer and the other to a glass hook. The heart was severed distal to both ligatures and immediately transfered to a 10 ml muscle bath with a surrounding jacket through which cooled (18°C) water was pumped to regulate the temperature of the heart and solutions. A constant but gentle stream of air bubbles continually oxygenated the heart preparation. The heart was held in the bath with one ligature connected to a glass hook at the bottom of the bath while the other ligature was connected to the A-715 transducer attached to a Physiograph

(E. M. Instrument Co., Houston, Texas).

Standard dilutions were made of acetylcholine in sea water to cover a range of 1.0×10^{-4} g/ml to 1.0×10^{-12} g/ml. One ml was added to 9 ml of sea water to reduce the concentration by 1 power of 10. The effect of the acetylcholine, or test drug was recorded for 30 seconds and then the heart was flushed two times with sea water and allowed to stabilize for one minute or longer. Starting with the 1.0×10^{-12} g/ml concentration of acetylcholine, the concentration was increased until the heart beat stopped. Intermediate concentrations between the level of acetylcholine that completely stopped the contractions and the next more dilute concentration were occasionally applied to exceptionally sensitive hearts to attain a finer comparison of activity.

The acetylcholine effect was then compared to the effect of different concentrations of the asterosaponin, following the same procedure. Samples of acetylcholine, giving known activity were administered intermittently to the test preparation during asterosaponin testing to monitor the deterioration of the sensitivity of the preparation. The anticholinesterase neostigmine and the anticholinergic atropine were used to gain more information from the preparation.

2. Effect of Asterosaponin on the Whole Clam Mercenaria mercenaria

Five large bivalves Mercenaria mercenaria had small holes

drilled in the ventral lateral section of their shell so that a small syringe needle could be inserted into the mantle cavity of the clam. Four of the clams were injected with 1 ml of 1.5×10^{-4} g/ml asterosaponin while the fifth received a 1 ml injection of sea water. The clams were all placed in a container of aerated sea water for 24 hours and observed for any type of reaction to the experimental procedure.

3. Avoidance Response Reaction

Experiments designed after the experiments of Mackie et al. (1968), were performed to determine if there were any escape response reactions of the local salt water species of gastropod, Littorina littorea to the asterosaponin.

The snails were placed in individual beakers of sea water that were positioned over pieces of graph paper. When normal exploratory activity of the snail had resumed, the asterosaponin was gently injected into the sea water close to the head of the snail with a syringe. The response of the snail was then recorded, using the graph paper squares to determine the movement of the snail. Injections of sea water via syringe into the snail's sea water environment at the proximity of his head were used as a control. A different snail was used for each dose of asterosaponin tested.

4. Blood Cell Lysing

The following procedure was used to determine if the Asterias

forbesi asterosaponin exhibited blood cell lysing properties similar to other asterosaponins. Rat blood obtained from tail veins was treated with concentrations of asterosaponin ranging from 0.3 to 10.0 mg/ml, or with the standard Merck saponin used for blood count determinations. The blood samples were placed in a Coulter Counter and the blood count determined.

5. Autotomy Caused by Asterosaponin

Chaet in 1962 had demonstrated the ability of a compound from the scalded coelomic fluid of Asterias forbesi to bring forth an autonomous loss of arms by that starfish, when the compound was injected into its peritoneal cavity. The procedure used in this research to investigate this property being possessed by the asterosaponin from Asterias forbesi was similar.

Intermediate sized starfish 8-12 cm in diameter were injected intraperitoneally with 1 ml of 1.5×10^{-4} g/ml asterosaponin and then placed in the aquarium for observation. Control starfish received intraperitoneal injections of 1 ml of seawater. The starfish were observed for four hours and abnormal reactions were recorded. The starfish were then observed periodically for the next twenty hours.

6. Analgesia

The method of Koster (1959) was the method used to evaluate the analgesic properties of the asterosaponin. Groups of 5 mice

were pretreated by ip injection with either; 1) saline (0.1 ml/g) 2) morphine (10 mg/kg) or 3) asterosaponin. The asterosaponin was dissolved in saline and was administered at 6 dosage levels ranging from 10 mg/kg to 50 mg/kg. Fifteen minutes after the pretreatment injections the mice received ip injections of 0.01 ml/g of 0.6% fresh acetic acid. Three minutes after the acetic acid injections the writhes exhibited by the group of mice were counted and totaled for twelve minutes. In this procedure reduction in writhes is equated with analgesia.

7. In Vitro Anti-inflammatory Assay

To evaluate possible anti-inflammatory activity by the asterosaponin the in vitro assay of Grant et al. (1970) was employed. The assay employs the use of bovine serum albumin. Many compounds that prevent inflammation also are capable of reducing the heat denaturation of albumin protein.

The test system contained 2 ml of 1% bovine serum albumin and 2 ml of 0.05 M Tris acetate buffer, pH 6.0 or test solution in a test tube. Control and test systems were heated in a constant temperature bath using the Haake FK2 constant temperature circulator at 69.5°C for exactly four minutes. The test tubes were then placed in ice and their turbidities were read at 660 nm on a Beckman model DB-G spectrophotometer. If the original turbidity was above scale, the sample was dilluted 9:1 with distilled water. The value of the

percent of control turbidity was calculated and an analysis of variance calculated to determine if ther was a significant drug effect.

8. In Vivo Anti-inflammatory Assay

Since positive results obtained from in vitro anti-inflammatory assays are inconclusive in determining the presence of anti-inflammatory activity for a compound, the in vivo anti-inflammatory procedure of Winter et al. (1963) was also employed. Rats weighing approximately 150 g received either no pretreatment, asterosaponin 75 mg/kg ip or phenylbutazone 90 mg/kg ip, one hour prior to edema production. Edema was produced by the injection of 0.05 ml of 1% carrageenin into the sub-planter tissue of a hind paw of a rat. The volume of the paw was then immediately determined by the use of a previously calibrated volume displacement apparatus which employed the use of a 50 ml syringe filled with mercury connected through a plastic catheter to a Statham P 23 transducer linked to a Grass polygraph (Van Arman et al., 1965).

9. Toxicity to Mice -- LD_{50}

The toxicity of the asterosaponin was evaluated in mice. Groups of 5 male mice were injected intraperitoneally with 50, 100, 200, or 400 mg/kg of the asterosaponin.

The response of the mice to the intraperitoneal asterosaponin injection was observed for two hours and then intermittant observations were made over the next three days. The number of deaths

were recorded and an LD_{50} estimated from the results.

10. Cat Blood Pressure and Nervous System Evaluation

The effect of the asterosaponin on blood pressure, respiration, the autonomic nervous system, and skeletal muscle response to neuronal stimulation were determined using a cat.

A cat was anesthetized with pentobarbital. The cat's respiration was monitored by the use of a sensitive thermocouple inserted into the plastic cannula used in the tracheotomy. The thermocouple was then connected to the Grass polygraph via a Grass model 7P1B low level D. C. pre-amplifier and a model 7DAE Grass polygraph D. C. driver amplifier. Blood pressure was monitored using the cannulation of the femoral artery connected to the same Grass polygraph and amplifier system by a model P 23 Dc 10 volt Statham transducer. The effect of stimulating the nictitating membrane was also recorded on the polygraph. The nictitating membrane response was tested via supramaximal stimulation (7 volts, 5 per second for 2. 0 milliseconds) of the cut preganglionic cervical sympathetic nerve. The response of a striated muscle (tibialis anterior) to supramaximal stimulation (10 volts, 1 per second for 2. 0 milliseconds) of the peripheral end of the cut peroneal nerve was also monitored. The tibialis anterior and the nictitating membrane were connected to a Grass force displacement transducer model FT 03C connected to the polygraph to record contractions of the muscle

or membrane.

The effects of the asterosaponin on the monitored parameters were evaluated after intravenous administration of the compound. Whenever the asterosaponin or other drug was administered the cannula was immediately flushed with saline.

11. Blood Pressure and ECG Responses of the Rat to Asterosaponin

The response of the cat to asterosaponin administration initiated interest into the cardiovascular effects of the asterosaponin. These cardiovascular effects of intravenous asterosaponin administration were further investigated primarily in the rat.

A rat was anesthetized with urethane (6 ml/kg of 20% solution, ip). A tracheal cannula (a 1.5" piece of PE tubing, size 280 or smaller) was then inserted and tied securely. The femoral vein, as it passed into the iliac vein was exposed and cannulated with PE 10 tubing and connected to a 1 ml syringe that was filled with saline. This cannula was used for intravenous drug administration. Each of the common carotids were exposed and separated from the surrounding tissues. Then the two nerves, the vagus and the cervical sympathetic which lie along the carotids, were carefully and gently separated from the section of the vessel to be used for blood pressure catherterization using PE 90 tubing. Prior to its insertion the cannula was attached to the saline filled transducer system in the usual manner and the tip filled with heparin solution. A blood pressure

bottle filled with 250 ml of saline and 2.5 ml of heparin (1000 units/ml) had been previously attached to this transducer. This system was used to record the blood pressure changes following drug administration.

To record the rat's ECG three needle electrodes were placed subcutaneously. The ground electrode was connected to the leg of the rat that was not used for the intravenous catheterization. The two remaining electrodes were placed on the chest of the rat or on his outstreached forepaws. The electrodes were connected to the Grass polygraph wide band A. C. pre-amplifier and integrator model 7 P3A which was connected to the Grass polygraph D. C. Driver amplifier model 7 DAE. The paper speed was kept at 2.5 mm/sec. except for recording at critical times at 100 mm/second.

12. Dog Blood Pressure, Heart Rate, and ECG in Response to Asterosaponin

The experimental set up to monitor the effects of the asterosaponin on the blood pressure, heart rate, and ECG of the dog was similar to that of the rat. The dog was anesthetized with pentobarbital 30 mg/kg and a tracheotomy performed. The femoral vein was cannulated on one leg of the dog for the administration of drug intravenously. The femoral artery on the other leg was cannulated and attached to a pressure transducer for the recording of the arterial blood pressure through a Grass polygraph. The ECG recording

responses to the vagus.

In a second part of the experiment, asterosaponin was administered after the vagus nerves had been severed, and the results recorded to compare them to the normal effects of the asterosaponin on a rat with intact vagus nerves.

14. Evaluation of the Histamine Releasing Potential of Asterosaponin

The possibility that the hypotensive effect seen with the asterosaponin administration could be caused by endogenous histamine release was evaluated. Rats were prepared for ECG monitoring and blood pressure determination. The antihistamine diphenhydramine was administered intravenously to the rat until sufficient amounts were present to prevent the hypotensive activity of intravenously administered histamine (0.5 mg/kg). When a protective level of antihistamine was attained asterosaponin (15 mg/kg) was administered intravenously to determine if the hypotensive action of the asterosaponin could be blocked by pretreatment with the antihistamine.

15. Evaluation of the Alpha Adrenergic Blocking Capabilities of Asterosaponin

An analysis of the influence of the asterosaponin on the alpha receptors of the adrenergic nervous system was possible from the cat nictitating membrane experiment. To gain more direct evidence for the possibility of this type of activity, a specific experiment was designed, incorporating the rat blood pressure preparation.

Rats were prepared for the usual ECG and blood pressure determinations. Phenoxybenzamine was then administered to the rat to the point where no further drop in blood pressure was seen. Then asterosaponin (15 mg/kg) was administered intravenously to determine if the asterosaponin could produce further hypotension even though the alpha receptors were blocked. The normal procedure of flushing each dose of administered drug with saline before the next drug was administered was maintained and ample time was allowed for each drug to have an effect.

16. Evaluation of Beta Stimulating Properties of Asterosaponin

The stimulation of the vascular beta receptors by adrenergic agonists can significantly alter peripheral resistance. The stimulation of these beta receptors leads to a drop in blood pressure through vasodilation. The determination of the possibility of asterosaponin acting as a peripheral vascular beta stimulant was carried out using the rat blood pressure technique. The possibility of beta influence was also investigated with the rat hind leg perfusion experiment (see below).

A rat was prepared for blood pressure and heart rate monitoring following the standard procedure for the blood pressure and ECG determination. The beta blocking agent propranolol was administered intravenously to the rat at very high doses (0.9 mg/kg), so that no stimulation of the beta receptors was possible. After this blocking

dose had been administered, asterosaponin was injected and the blood pressure measured to determine if the asterosaponin (15 mg/kg) could produce hypotension even though the beta receptors were blocked.

17. Evaluation of Asterosaponin Working Directly on the Vasculature -- The Rat Hind Leg Perfusion Procedure

The results of the previous experiments aimed at determining the site of asterosaponin action in connection with the production of transient hypotension made it necessary to further evaluate the possibility that the asterosaponin was acting directly on the vasculature. The perfused hind limb preparation makes this type of evaluation possible.

A rat was anesthetized and attached to a rat board that was mounted on approximately a 50° angle so that the scrotal sac of the rat was positioned at the lower end of the board. The abdominal cavity was opened and the skin pulled away from the musculature surrounding the scrotal sac and along one leg. The vena cava and decending aorta were carefully isolated below the level of the kidney and anterior to where they bifurcate into the lower appendages. The vena cava was tied just below the level of the kidney and the aorta was cannulated. The cannula to the aorta was attached to a three way stopcock permitting aerated body temperature Ringer-Locke solution to be perfused at a constant pressure into the artery.

The stopcock could also be turned to allow for the administration of drugs via a syringe. The veins of the leg that had the skin pulled away were severed so that the perfusate could travel through the arterioles and vascular bed and emerge through these severed veins. A small hole was placed at the bottom of the scrotal sac and an eye dropper inserted therein so that the perfusate from the leg would pass through the eyedropper, dropping through a drop counter connected to the polygraph. Since the pressure was constant, changes in the diameter of the vessels in the leg were reflected in the number of drops during a prescribed period of time.

Through the three way stopcock epinephrine (4.0 μg) and isoproterenol (0.1 mg) were administered to establish the reliability of the preparation. Standard amounts of Ringer-Locke solution were also administered to evaluate the effect of the injection process itself on the preparation. Asterosaponin was administered to record its effects on vascular resistance. Then the combination of adrenergic blocking and stimulating agents was used as a pretreatment before the asterosaponin to verify the hypothesized mechanism of action of the asterosaponin.

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 the 0.05 level for rejection of the null hypothesis. In some instances an analysis of variance, F test for experimental means was used to examine experimental means for statistical significance. The level of significance (P) was determined by comparison of the computed F value with values from standard tables. All calculated F values were tested at the 0.05 level for rejection of the null hypothesis.

IV. RESULTS

A. Mercenaria mercenaria Heart Evaluation for Cholinergic Activity by Asterosaponin

The clam heart bioassay established no activity by the asterosaponin for the dose range of 1.5×10^{-18} g/ml (1.0×10^{-18} M) to 1.5×10^{-7} g/ml (1.0×10^{-7} M) (Table 1). The injection procedure, the freshness of the toxin, freshness of the preparation, and the order of the administration of the drug had no effect on the results. This was demonstrated in several ways. The injection of artificial sea water into the bathing medium producing no adverse effects showing the injection procedure itself did not affect the contractions of the isolated heart. The order in which the different concentrations of the drug were administered likewise did not alter the results attained. In some instances the lowest concentrations of asterosaponin were administered first, working progressively toward higher concentrations. On other occasions the experiment was initiated using the middle concentration and at times using the highest concentration of drug. The possibility that the process of acetylcholine administration prior to asterosaponin prevented the effects of the asterosaponin was not seen. Table 1 shows that the reliability of the preparation was checked on every occasion for sensitivity to

TABLE 1. THE EFFECT OF ASTEROSAPONIN & ACETYLCHOLINE ON THE CONTRACTIONS OF THE ISOLATED HEART OF MERCENARIA MERCENARIA

	Acetylcholine concentrations 10^{power} g/ml									Toxin concentrations @ 1.5×10^{power} g/ml											
Power	-13	-12	-11	-10	-9	-8	-7	-6	-5	-18	-17	-16	-15	-14	-13	-12	-11	-10	-9	-8	-7	-6
1		X	X	Y	Z	Z				Y[a]		X	X	Y[a]	X	Y[a]	X					
2		X	X	X	Y	Z				X	X	O	Y	X	X						X	X
3	Y	X							Z					X			X		Y	X	Y[b]	
4		X	Y	Y	Z							X				X		X			X[c]	
5	X	X	X	Y	Z							X									X	
6				X	Y	Z								X						X	X	
7[d]		X	Y	Y	Z	Z																
8[e]				X	Y	Z							X					X			X	X
9			Z	Z												X				X	X	
10[f]	Z	Z												X					X			X

O = paired contraction effect. The amplitude of the first contraction is smaller then the amplitude of the second, then the pattern repeats

X = no effect; Y = slight drop in amplitude same frequency; Z = severe drop in amplitude & frequency

EXPLANATION OF TABLE 1

[a] This recording was recorded as a slight drop. However this was probably due to the instability of the preparation since after sea water replacement the contractions were larger then with the toxin for the first 3.5 minutes but then the amplitude of the beat was comparable to that exhibited with the toxin.

[b] The dose of 1.5×10^{-7} g/ml was repeated later in the experiment, after trials with both toxin and acetylcholine, with the same effect.

[c] The 1.5×10^{-7} g/ml dose of toxin by itself had no effect, and with neostigmine (0.25 mg) pretreatment it continued to show no effect.

[d] Atropine was used to assure that the preparation was responding correctly. When atropine was present at 1.0×10^{-6} M the heart stopped contracting.

[e] Atropine present at 1.0×10^{-7} M protected against the effect of 1.0×10^{-8} g/ml (5.5×10^{-8} M) acetylcholine.

[f] Toxin given before acetylcholine

acetylcholine, and on numerous occasions checked with atropine as an anticholinergic drug. The data in Table 1 show that the hearts were all sensitive to acetylcholine at the concentration of 1.0×10^{-9} g/ml (5.5×10^{-9}M) and many of the hearts were sensitive to concentrations as low as 1.0×10^{-13}g/ml (5.5×10^{-13}M). Table 1 contains data that also show that when atropine was present at 1.0×10^{-7}M it protected the preparation against the effect of 1.0×10^{-8}g/ml (5.5×10^{-8}M) acetylcholine. At doses of the asterosaponin ranging from 1.5×10^{-18}g/ml to 1.5×10^{-7}g/ml there was no effect produced on the heart. The administration of neostigmine (0.25 mg) to the test preparation did not alter the lack of effect on the preparation produced by 1.5×10^{-7}g/ml of asterosaponin.

B. Effect of Asterosaponin on the Whole Clam Mercenaria mercenaria

Neither the asterosaponin injection nor the sea water injection into the intact clam caused any abnormal reaction by the clam. Clams that were injected with 1.0 ml of 1.5×10^{-4}g/ml of asterosaponin did not appear to differ in any way from the clam receiving 1.0 ml of sea water.

C. Blood Cell Lysing Properties of Asterosaponin

Since many asterosaponins reviewed in the literature and saponins in general exhibit hemolytic activity it was determined that it would be necessary to evaluate this asterosaponin for hemolytic

activity. Table 2 shows that the asterosaponin possessed no cytotoxic activity towards rat leukocytes or erythrocytes at doses ranging up to 10 mg/ml.

D. Autotomy and Asterosaponin

Since another investigator (Chaet 1962) had shown that an extract from Asterias forbesi was capable of inducing autotomy in Asterias forbesi, the asterosaponin was evaluated for autotomy activity to determine if it was the active constituent. The data (Table 3) demonstrate that the asterosaponin from Asterias forbesi was incapable of causing autotomy in the starfish Asterias forbesi.

E. Escape or Avoidance Response to Asterosaponin

The ability of the asterosaponin to initiate an avoidance response in Littorina littorea was evaluated (Table 4). The results demonstrate that when 1.5×10^{-5} g of asterosaponin was injected into the water near the snails head no abnormal behavior was generated and no avoidance response observed.

F. Anti-inflammatory Activity

Because of the known anti-inflammatory activity of a wide variety of steroids the steroidal asterosaponin was tested for anti-inflammatory activity with two different tests, the in vitro bovine serum albumin denaturation assay and the in vivo carrageenin induced rat hind paw edema assay.

TABLE 2. EVALUATION OF BLOOD CELL LYSING PROPERTIES OF ASTEROSAPONIN

Treatment	Concentration[a]	Cytotoxic Activity RBC	WBC
Asterosaponin	10	no	no
Asterosaponin	5	no	no
Asterosaponin	2.5	no	no
Asterosaponin	1.2	no	no
Asterosaponin	0.6	no	no
Asterosaponin	0.3	no	no
Merck Saponin		yes	yes

[a] mg/ml of whole rat blood

TABLE 3. AUTOTOMY BY ASTERIAS FORBESI IN RESPONSE TO ASTEROSAPONIN INJECTION

Treatment[a]	N[b]	Concentration	Autotomy[c]
Asterosaponin	6	1.5×10^{-4} g/ml	no
Sea Water	2	100%	no

[a] 1.0 ml injection into the peritoneal cavity

[b] Number of starfish

[c] Starfish were observed for 24 hours

TABLE 4. AVOIDANCE RESPONSE INITIATION

PROPERTIES OF ASTEROSAPONIN IN LITTORINA LITTOREA

Treatment[a]	N[b]	Concentration	Escape Response
Asterosaponin	10	1.5×10^{-5} g/ml	no
Sea Water	3	100%	no

[a] 1.0 ml injection in immediate vicinity of the snails

[b] Number of animals

The bovine serum albumin assay for anti-inflammatory activity showed (Table 5) that a concentration of 500 µg/ml of asterosaponin caused an 85% inhibition of protein denaturation. Phenylbutazone, the well known anti-inflammatory agent, restricted the denaturation by over 80% at a concentration of 120 µg/ml. When the data was plotted (Figure 2) both the asterosaponin and phenylbutazone produced this restriction of denaturation in a log dose response manner. Since the asterosaponin results were positive for this test which is used to screen new drugs for anti-inflammatory activity, the in vivo carrageenin induced rat paw edema assay was chosen to determine if the asterosaponin was capable of restricting inflammation in the intact animal.

The rat paw edema assay (Table 6) showed that pretreatment with asterosaponin at 75 mg/kg ip reduced the edema caused by the carrageenin injection by 53 percent. Pretreatment with phenylbutazone (90 mg/kg) had a similar effect, decreasing inflammation by 52 percent.

G. Toxicity to Mice

The evaluation of the LD_{50} of any toxic compound is paramount to any type of drug testing procedure. The data of the LD_{50} determination (Table 7) for the asterosaponin demonstrate that the asterosaponin has a LD_{50} value in mice between 100 and 200 mg/kg.

TABLE 5. ANTI-INFLAMMATORY ACTIVITY OF ASTEROSAPONIN AND PHENYLBUTAZONE THROUGH THE BOVINE SERUM ALBUMIN ASSAY SYSTEM

Treatment[a]	N[b]	Percent of Control Absorbance[c]
Asterosaponin[d]		
30	2	70.34 ± 3.07
60	2	63.63 ± 0.00
120	4	55.06 ± 2.84
250	2	28.46 ± 4.83
500	2	14.31 ± 3.41
1000	2	11.92 ± 0.79
Phenylbutazone[d]		
30	2	53.58 ± 4.22
60	2	43.32 ± 2.23
120	4	17.49 ± 2.56
250	2	0.90 ± 0.90
500	2	0.90 ± 0.90
1000	2	0.90 ± 0.90

[a] µg/ml

[b] Number of replicates

[c] 660 nm

[d] Significant drug effect $P<0.05$ (Analysis of Variance)

FIGURE 2. INHIBITION OF BOVINE SERUM ALBUMIN DENATURATION BY ASTEROSAPONIN AND PHENYLBUTAZONE

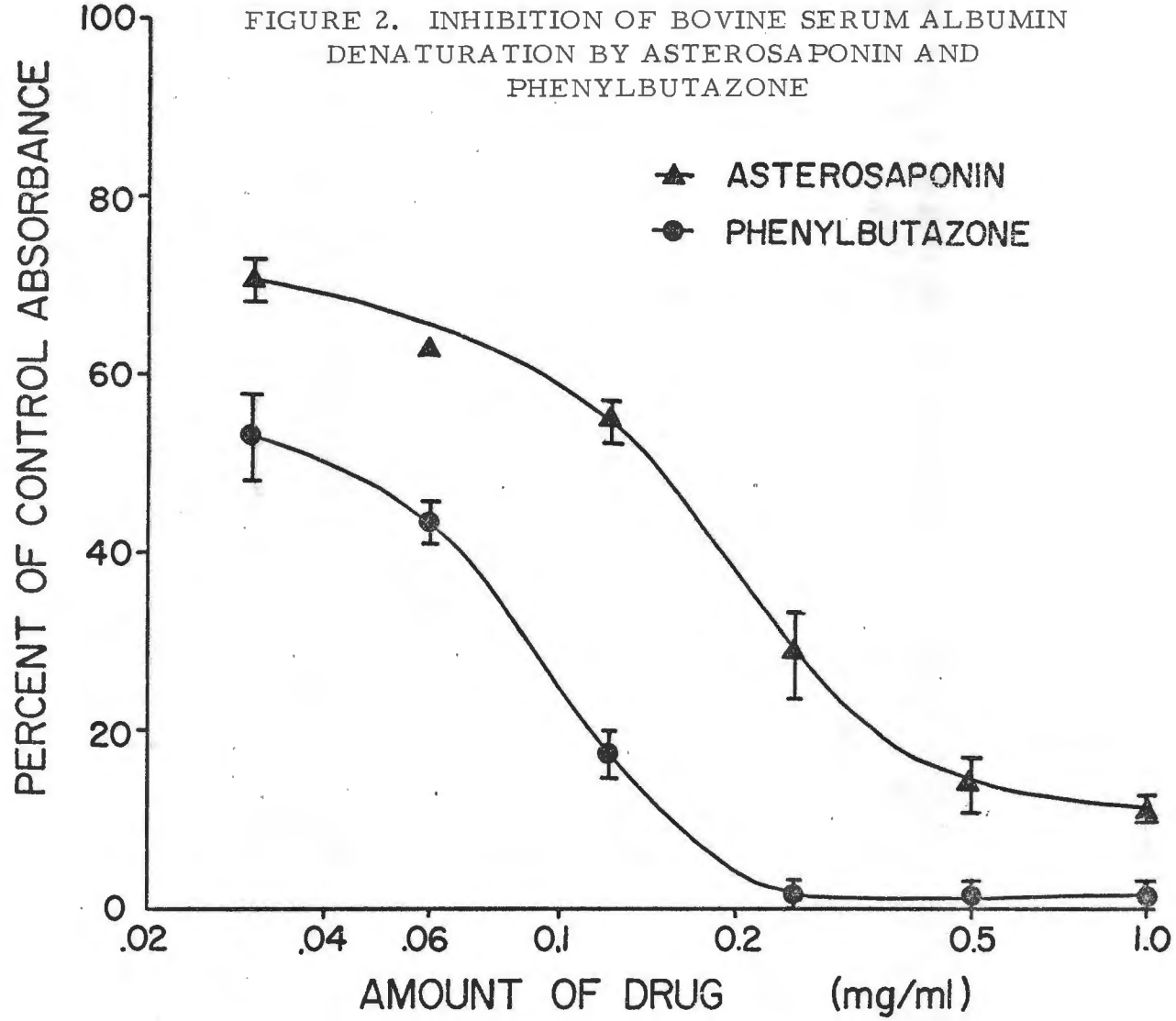


TABLE 6. RAT PAW EDEMA

Treatment[a]	N[b]	Volume[c] 0 hr	3 hr	(3 hr)-(0 hr)
Control	4	1401.1 ± 103.8	2201.2 ± 143.9	800.1 ± 61.5
Asterosaponin (75 mg/kg)	4	1468.6 ± 28.5	1846.3 ± 70.2	377.7 ± 81.5[d]
Phenylbutazone (90 mg/kg)	4	1476.0 ± 72.1	1860.4 ± 88.3	384.3 ± 47.8[d]

[a] Injection (ip) of test compound one hour prior to injection of 0.05 ml of 1% carrageenin into the sub planter tissue of the hind paw

[b] Number of rats

[c] Volume of paw in mm^3

[d] Significantly different from control, $P<0.05$

TABLE 7. LD_{50} IN MICE FOR ASTEROSAPONIN

Treatment	Dose[a]	N[b]
Asterosaponin	400	5/5
Asterosaponin	200	5/5
Asterosaponin	100	5/0
Asterosaponin	50	5/0

[a] mg/kg, ip

[b] Number of mice injected/number dead within 48 hours

H. Analgesic Activity

In a further evaluation of pharmacological activity, the analgesic activity of the asterosaponin was evaluated, employing the Koster acetic acid assay (Koster, 1959). The asterosaponin was effective in reducing the writhes exhibited by mice due to acetic acid injection (Table 8) and therefore extremely effective as an analgesic agent. While the 10 and 12 mg/kg doses did not reflect analgesic activity, the 15 mg/kg dose did reduce the writhes exhibited by the group by 71 percent. Higher doses possessed even greater analgesic activity as 25 mg/kg reduced the writhes by 99 percent and 50 mg/kg reduced writhes by 100 percent. These results of asterosaponin induced analgesia were compared to the results obtained with morphine which at 10 mg/kg reduced writhes by 100 percent.

I. Cat Blood Pressure and Nervous System

The effects of asterosaponin injected intravenously at different dosages upon a number of physiological processes were monitored in the cat. The effects of the toxin on blood pressure, respiration, preganglionic sympathetic nerve stimulation, and stimulation of a somatic motor nerve to a striated muscle were all recorded.

Figure 3 shows the polygraph recordings that were taken of a cat prior to any drug administration. The chart shows an initial blood pressure of 100 mm Hg, 11.5 breaths per minute, and the

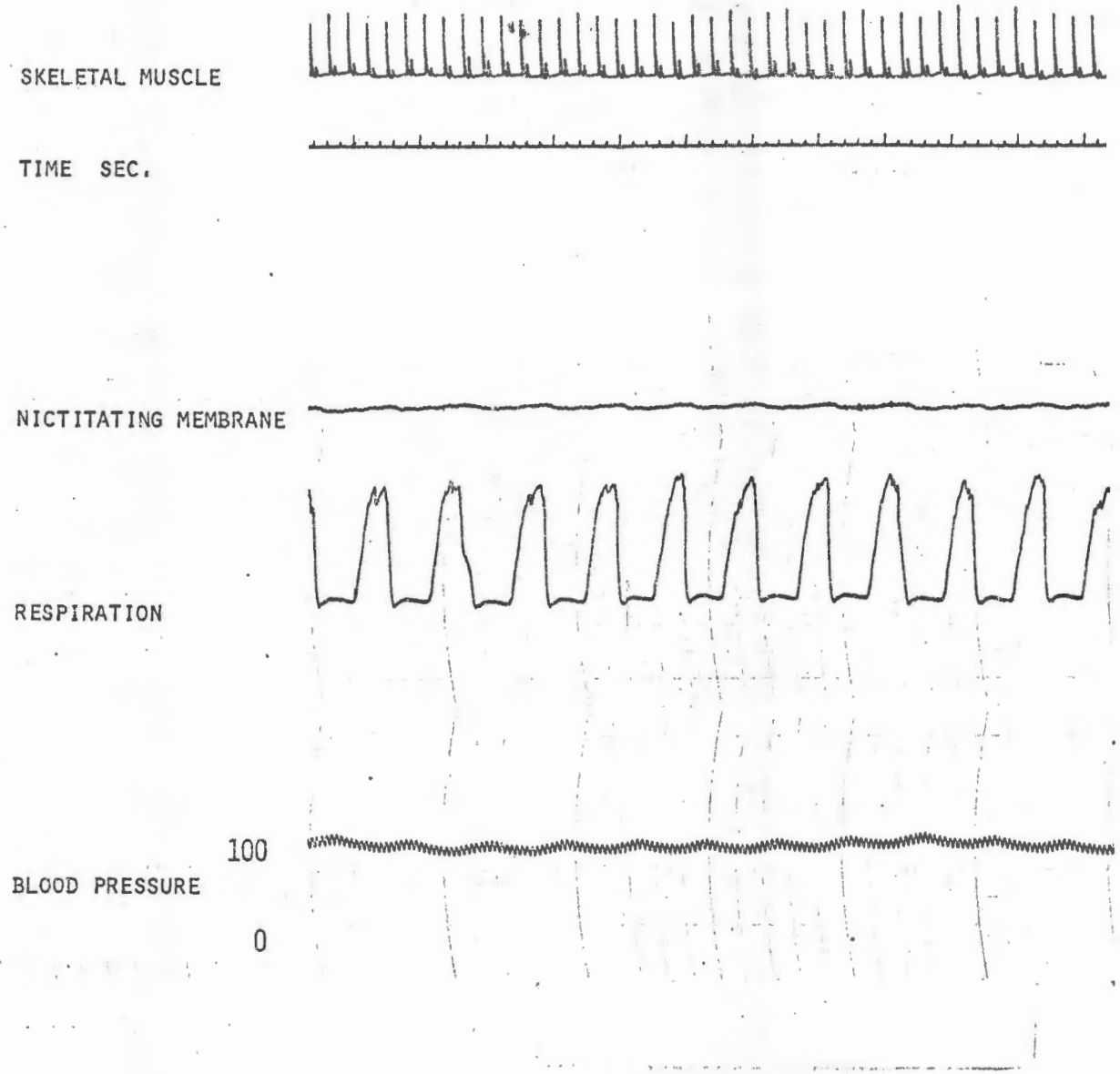
TABLE 8. MOUSE ANALGESIA AS EVALUATED FROM WRITHES FOLLOWING ACETIC ACID INJECTIONS

Pretreatment[a]	Dose	Trials[b]	Writhes[c]	Percent of Control
Saline 0.9%	0.01 ml/g	4	146	--
Morphine	10 mg/kg	2	0	0
Asterosaponin	10 mg/kg	1	143	98
Asterosaponin	12 mg/kg	1	139	95
Asterosaponin	15 mg/kg	1	43	29
Asterosaponin	20 mg/kg	1	11	7
Asterosaponin	25 mg/kg	1	2	1
Asterosaponin	50 mg/kg	1	0	0

[a] ip injections 15 minutes prior to treatment with 0.01 mg/g of 0.6% acetic acid

[b] Number of times that dose of compound was tested

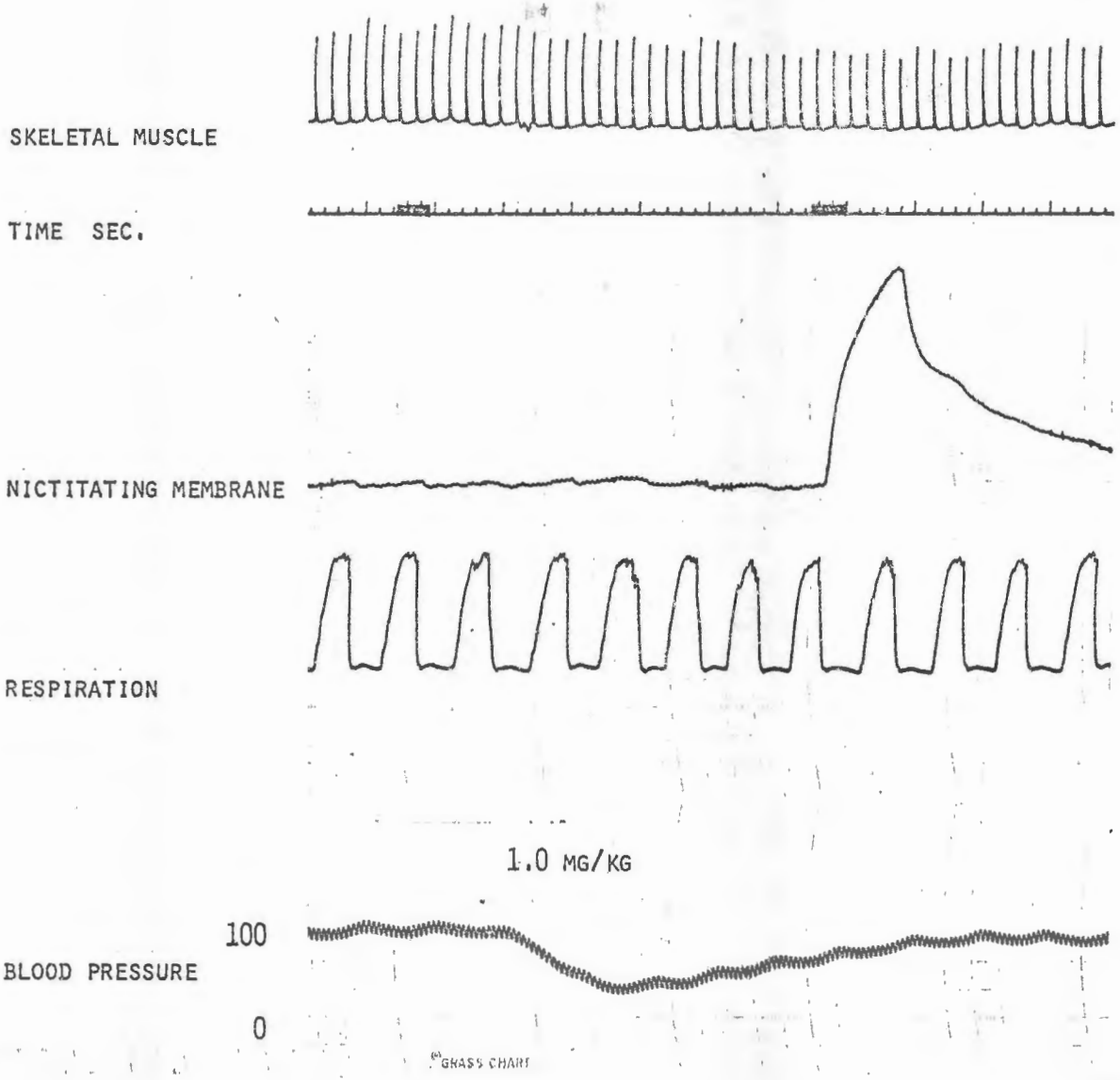
[c] Mean number of writhes exhibited by the group of 5 mice during the 12 minute period starting 3 minutes after acetic acid injection

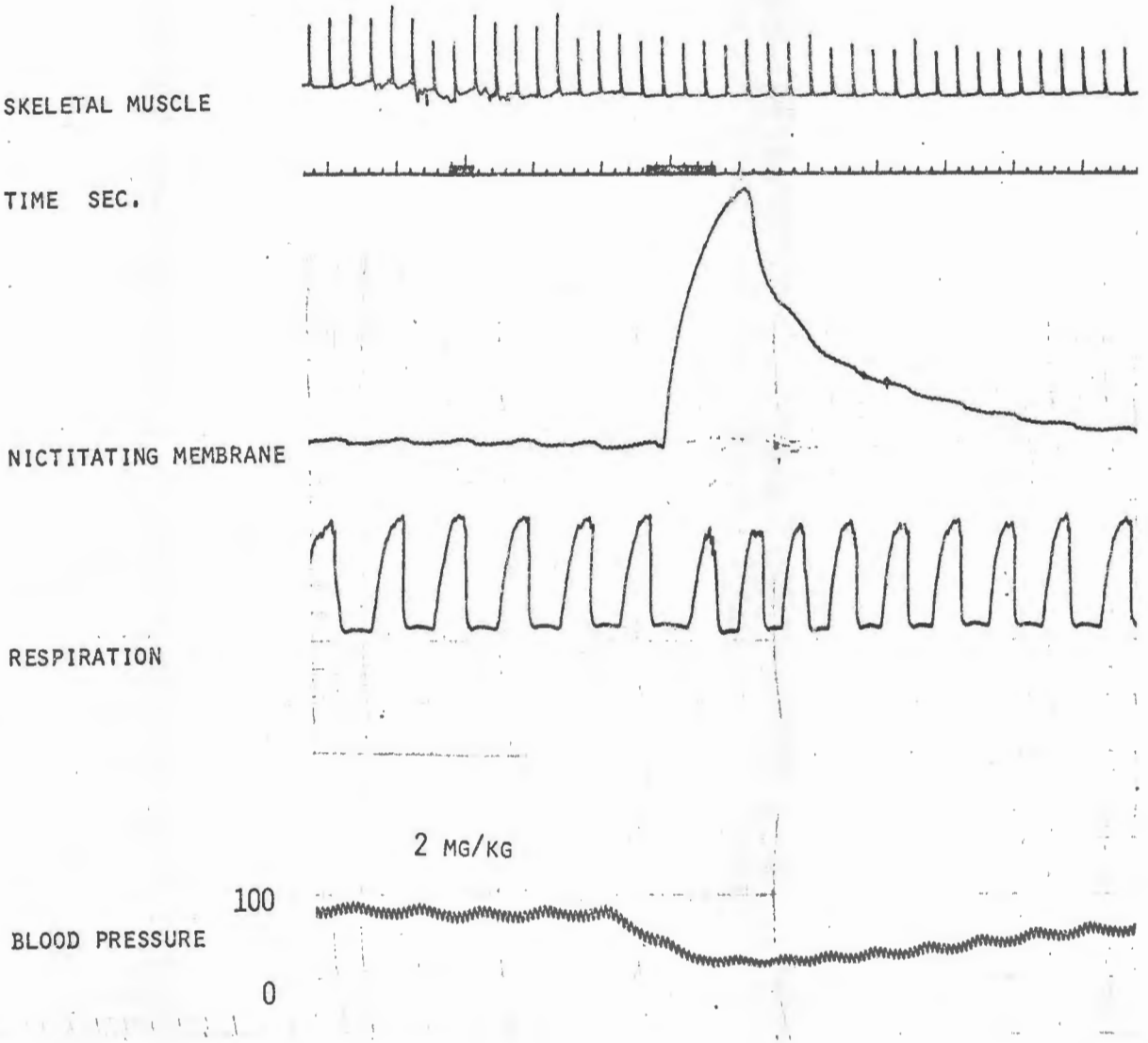

SKELETAL MUSCLE
TIME SEC.
NICTITATING MEMBRANE
RESPIRATION
100
BLOOD PRESSURE
0

striated muscle contracting normally to stimulation. The contraction of the nictitating membrane is not shown but the membrane was contracting normally in response to nerve stimulation.

Figure 4 is a recording made after the intravenous injection of 1.0 mg/kg of asterosaponin. The blood pressure of the cat remained at the pretreatment level of 100 mm Hg for 9 seconds and then began to decline. The lowest level of 50 mm Hg was reached 17 seconds after the injection. The blood pressure returned to a level of between 95 and 100 mm Hg 44 seconds after the injection. The respiration, skeletal muscle contractions and nictitating membrane all functioned normally during the time that the drug was active on the blood pressure.

Figure 5 illustrates the effect of the injection of 2.0 mg/kg of asterosaponin on the monitored physiological functions of the anesthetized cat. There was the usual delay of onset of the action of the asterosaponin, in this case 11 seconds, before there was a drop in blood pressure. The pressure reached its lowest level of 50 mm Hg 20 seconds after the injection, a drop of 42 mm Hg from pre-drug level of 82 mm Hg. The blood pressure did not return to near normal level for 126 seconds. The nictitating membrane functioned normally to the stimulus, but the contraction of the skeletal muscle, appeared to be slightly decreased in amplitude. The respiration was increased by this dose of asterosaponin. The original


SKELETAL MUSCLE
TIME SEC.
NICTITATING MEMBRANE
RESPIRATION
1.0 MG/KG
100
BLOOD PRESSURE
0
GRASS CHART


SKELETAL MUSCLE
TIME SEC.
NICTITATING MEMBRANE
RESPIRATION
2 MG/KG
100
BLOOD PRESSURE
0

respiratory rate was 11.5 breaths per minute. After the asterosaponin the rate increased to 16 breaths per minute for approximately one minute then dropped to 15 per minute. Nine minutes after asterosaponin injection respiration was still at an elevated level of 14 breaths per minute although at this time the asterosaponin had ceased to have an effect on the blood pressure.

The data for the response of the cat's physiological parameters for all of the doses of asterosaponin administered are compiled in Table 9. These data clearly show that the asterosaponin from Asterias forbesi was capable of dose dependently causing a transient lowering of the blood pressure of the anesthetized cat. These data also show that the asterosaponin had no effect on autonomic ganglia as the response of the feline nictitating membrane was not altered throughout the dose range administered when the preparation was stimulated preganglionically. The asterosaponin did not appreciably affect the functioning of the somatic peroneal nerve or the functioning of the skeletal muscle.

J. Rat Blood Pressure, Heart Rate and ECG Response to Asterosaponin

Figure 6 shows the normal recording for blood pressure and th normal ECG pattern of a typical rat before any drug was injected. The blood pressure was 75 mm Hg, there were 270 heart beats per minute and the ECG was regular. A control determination was

TABLE 9. EFFECT OF ASTEROSAPONIN ON CAT

Asterosaponin mg/kg		Blood Pressure[a]	Time[b]	Respiration[c]	Muscle[d]
0	Initial	100		11.5	
	Maximum Effect				X[f]
	Return to Normal				
0.5	Initial	100			
	Maximum Effect	70	19		X
	Return to Normal	100	33		
1.0	Initial	100			
	Maximum Effect	50	17		X
	Return to Normal	95	44		
2.0	Initial	82		11.5	
	Maximum Effect	40	20	16.0	O[g]
	Return to Normal	82	126	540[e]	

[a] mm Hg

[b] Time in seconds from injection of drug to maximal effect or for return to pre-injection level.

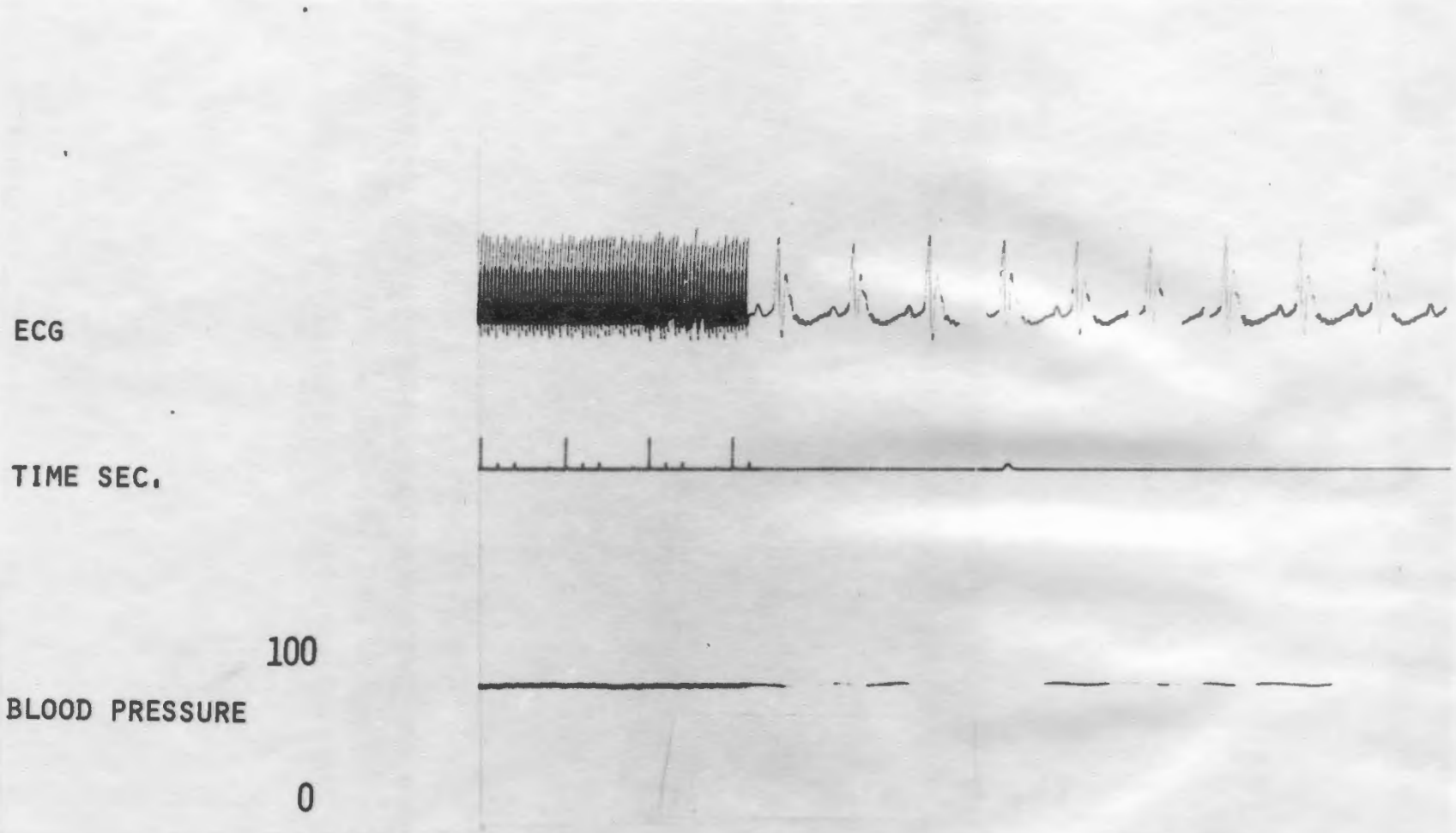
[c] Breaths/min.

[d] Skeletal muscle response to nerve stimulation

[e] Seconds to full recovery

[f] Normal response to nerve stimulation

[g] Slight decrease in amplitude


ECG
TIME SEC.
100
BLOOD PRESSURE
0

made by the injection of 0.5 ml of 0.9% saline into the rat through the venous catheter to evaluate the effect of the injection procedure itself. A slight rise in the blood pressure was noted within 8 seconds. It rose from the 75 mm Hg to a level of 82 mm Hg in 20 seconds and the heightened blood pressure lasted for more then 90 seconds. The heart rate was 280 beats per minute during the time of activity which was interpreted as being not appreciably different from the initial value. The ECG did not change.

The intravenous injection of 1.0 mg/kg of asterosaponin in the rat resulted in a rise in blood pressure from 75 mm Hg to 90 mm Hg in 25 seconds. Heart rate was 300 beats per minute, and the shape of the ECG did not change. The data in Table 10 show the effects of 2 mg/kg of asterosaponin. There was an initial drop in the blood pressure from 85 to 82 mm Hg which lasted only eight seconds and the pressure returned to about the normal level. The heart rate just prior to injection and 40 seconds after administration was 300 beats per minute, and the ECG pattern was not altered.

The administration of 4.0 mg/kg asterosaponin (Table 10) was the lowest dose in the rat where unequivocal alterations in the rat blood pressure occured. Three to four seconds after the drug was administered there was a drop in blood pressure from an initial value of 87 mm Hg to 70 mm Hg in 18 seconds. The blood pressure was back to its normal value 35 seconds after drug administration.

TABLE 10. RAT BLOOD PRESSURE AND ECG REACTIONS TO ASTEROSAPONIN I.

	Blood Pressure (mm Hg)			Heart Rate[c]		
Asterosaponin[a]	Initial	After	Return to Normal[b]	Initial	After	ECG
0	75			270		+[d]
0.5	75	82	90	270	280	+
1.0	75	90		270	300	+
2.0	85	82	8	300	300	+
4.0	87	70	35	312	312	+
8.0	92	75	90	312	312	+
20.0	82	55	330	300	300	+

[a] Dose iv in mg/kg

[b] Time for return to pre-drug pressure in seconds

[c] Beats per minute

[d] + = no change in electrocardiogram recording

The heart rate was 312 beats per minute before and after drug administration and there was no change in the ECG. For the 8.0 and 20.0 mg/kg (Figure 7) doses of asterosaponin the initial blood pressures were 92 and 82 mm Hg respectively and the initial hearts rates were 312 and 300 beats per minute (Table 10). The effect of the drug at both doses was basically the same. After the drug was administered there was an immediate drop in blood pressure which lasted for 25 seconds, while the blood pressure did not completely return to its normal value for minutes. The heart rate and the ECG were not affected.

The preceeding results for the effects of the asterosaponin were obtained by evaluating only the asterosaponin extracted by Les Goldsmith. A similar experiment was performed to compare the asterosaponin extracted by Les Goldsmith and the original extract supplied by Dr. Yuzuru Shimizu. The base line blood pressure was 90 mm Hg while the heart rate was 350 beats per minute. The effect on blood pressure and ECG of the standard 0.5 ml saline injection was similar to that seen for the previous experiment. The heart rate remained between 350 and 360 beats per minute, while the blood pressure rose to a value of 97 mm Hg in 10 seconds.

Table 11 illustrates the effects of the injection of 4.0 mg/kg of the asterosaponin extracted by Dr. Shimizu. These data show that the initial blood pressure was 85 mm Hg and that the heart

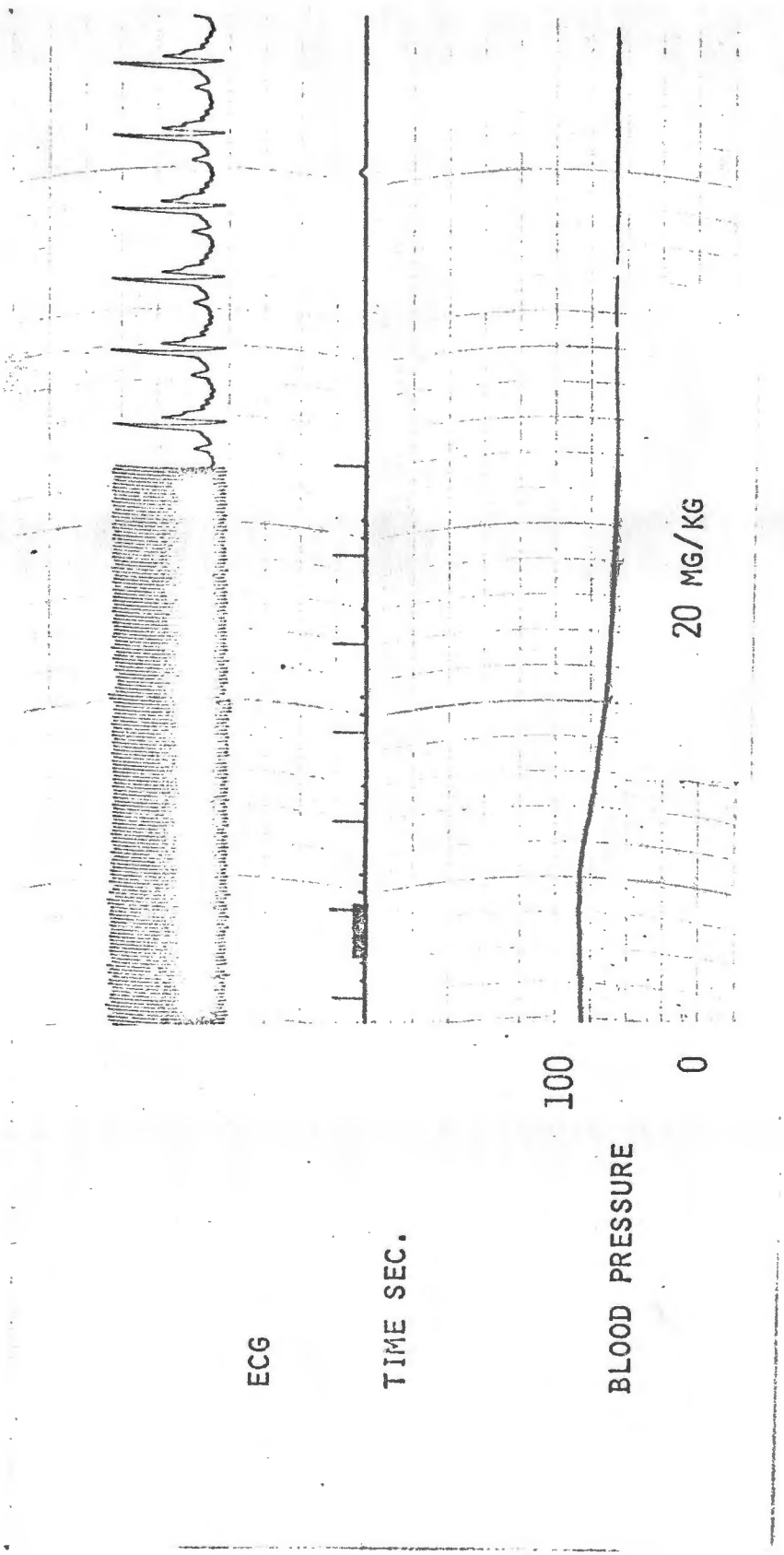

ECG
TIME SEC.
100
0
BLOOD PRESSURE
20 MG/KG

TABLE 11. RAT BLOOD PRESSURE AND ECG REACTIONS TO ASTEROSAPONIN II.

	Blood Pressure (mm Hg)			Heart Rate[c]		
Asterosaponin[a]	Initial	After	Return to Normal[b]	Initial	After	ECG
4.0 (S)[d]	85	57	480	360	285	drop in amplitude
10.0 (S)	62	24	60	285	240	no change
4.0 (S)	85	57	400	360	285	irregular
10.0 (S)	107	15	150	340	276	irregular
20.0 (G)	100	55		390	360	irregular
40.0 (G)	80	55	120	336	324	irregular
10.0 (S)	70	27	85	306	288	drop in amplitude

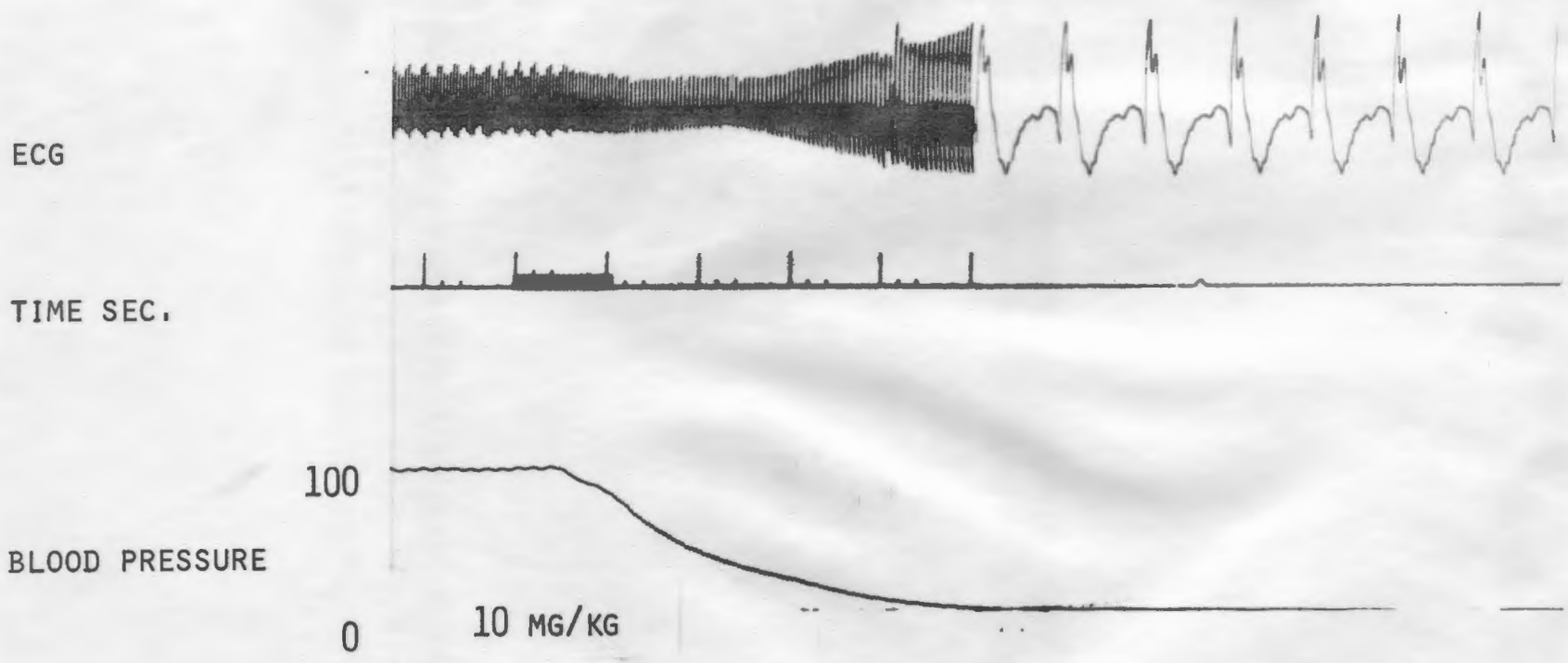
[a] Dose iv in mg/kg

[b] Time for return to pre-drug pressure in seconds

[c] Beats per minute

[d] S represents sample isolated by Shimizu, G represents sample isolated by Goldsmith

rate was 360 beats per minute. Four seconds after the asterosaponin administration a reduction in the blood pressure began. Approximately 60 seconds after injection the lowest blood pressure was reached, a value of 57 mm Hg, which lasted for about five minutes. The blood pressure did not return to normal for more then eight minutes. During these eight minutes the amplitude of the QRS complex of the ECG was decreased and the beats per minute decreased from 360 to a low of 285 beats per minute. Several replications were made for the rat receiving 10 mg/kg of asterosaponin (Table 11). The first 10 mg/kg injection was of Dr. Shimizu's extract. The heart rate at the initiation of the experiment was 285 beats per minute, the blood pressure was 62 mm Hg, and the ECG was normal. Three seconds after drug administration the blood pressure started to drop; 23 seconds after injection the pressure hit its lowest level of 24 mm Hg and the heart rate declined to 240 beats per minute. The effect only lasted one minute, and the rate and pressure returned to the initial levels. Similar alterations were seen following the administration of another 10 mg/kg dose (Figure 8). The blood pressure before the injection was 107 mm Hg and the heart rate was 340 beats per minute, with a normal ECG. After the injection the pressure plummeted to 15 mm Hg in less than 25 seconds, remained at that level for two minutes and then returned to the 100 mm Hg level after another 30 seconds. During this time the ECG became


ECG
TIME SEC.
100
BLOOD PRESSURE
0
10 MG/KG

irregular; the amplitude increased and the form of the pattern changed into many strange and uninterpretable shapes. The heart rate dropped from the 340 to 276 beats per minute. Figure 9 is the recording of the effect of 20 mg/kg of the Les Goldsmith asterosaponin which was administered following the second injection and affect of the 10 mg/kg asterosaponin of Shimizu. The blood pressure prior to injection was 100 mm Hg, and the heart rate was 340 beats per minute. Seven seconds after the intravenous administration of the asterosaponin, the blood pressure began to drop and 25 seconds after the injection the pressure stabilized at 55 mm Hg and remained at 55 mm Hg for more then 3 minutes when the next dose of asterosaponin was administered. Two minutes after the injection the blood pressure was still depressed (75 mm Hg). The ECG had obvious disturbances in its pattern, and the heart rate was depressed.

K. Asterosaponin and the Dog Blood Pressure and Heart Rate

Intravenous asterosaponin injections at various dosages were evaluated in the dog. These data (Table 12) show that the dog was sensitive to asterosaponin at a dose of 2.0 mg/kg and that the dog responded in the characteristic manner to the asterosaponin, i.e., following the asterosaponin injection a drop in blood pressure was seen which was only transient, and the response was dose dependent. The drop in blood pressure was accompanied by an increase in pulse pressure at all dosage levels (Figure 10). The increase in pulse

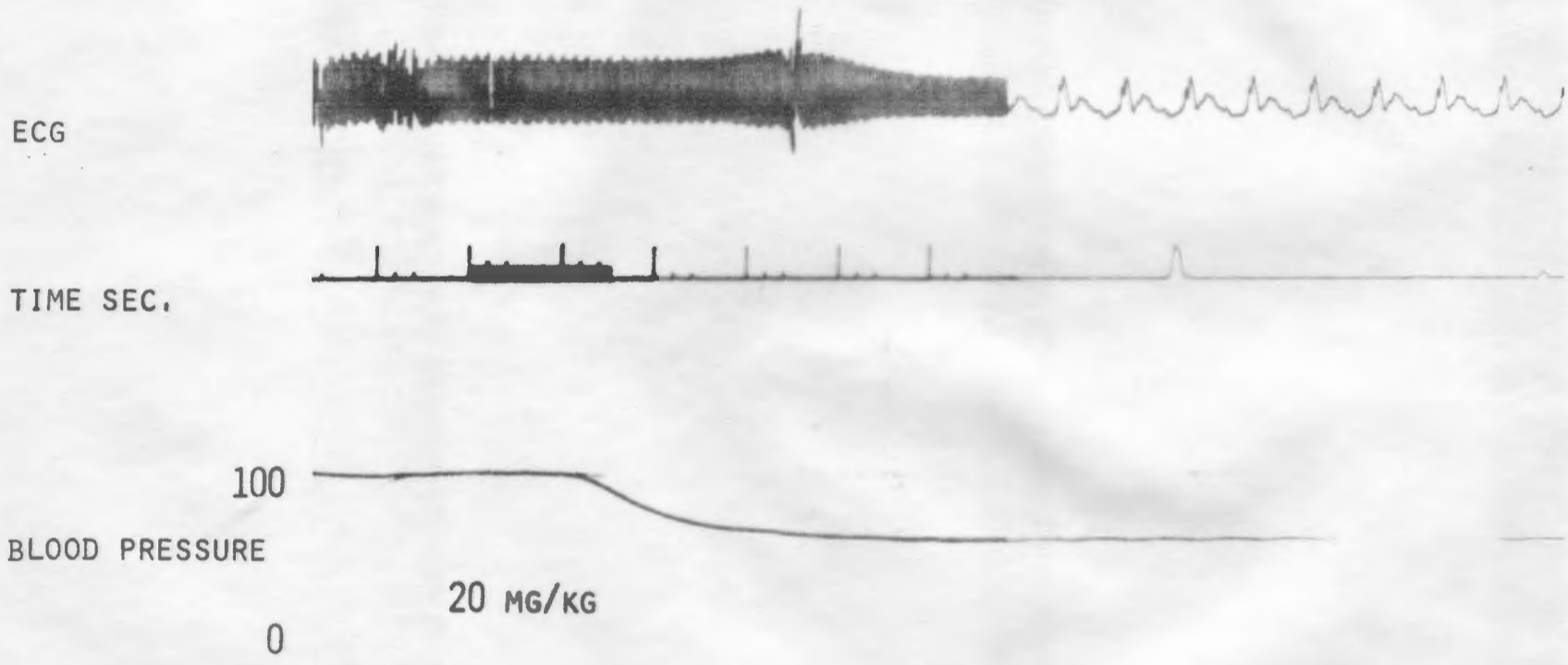

ECG
TIME SEC.
100
BLOOD PRESSURE
0
20 MG/KG

TABLE 12. EFFECTS OF ASTEROSAPONIN ON BLOOD PRESSURE AND HEART RATE IN THE DOG

Preparation	Treatment	Dose[a]	Blood Pressure[b] Before	Blood Pressure[b] After	Pulse Pressure[c]	ΔS/ΔD[d]	Heart Rate[e] Before	Heart Rate[e] After
1	Asterosaponin	2.0	160/100	155/78	60/77	5/22	182	192
1	Asterosaponin	3.0	180/90	162/70	90/92	18/20	192	200
2	Asterosaponin	4.0	175/115	175/105	60/70	0/10	225	232
2	Asterosaponin	10.0	185/120	160/88	65/72	25/32	216	224

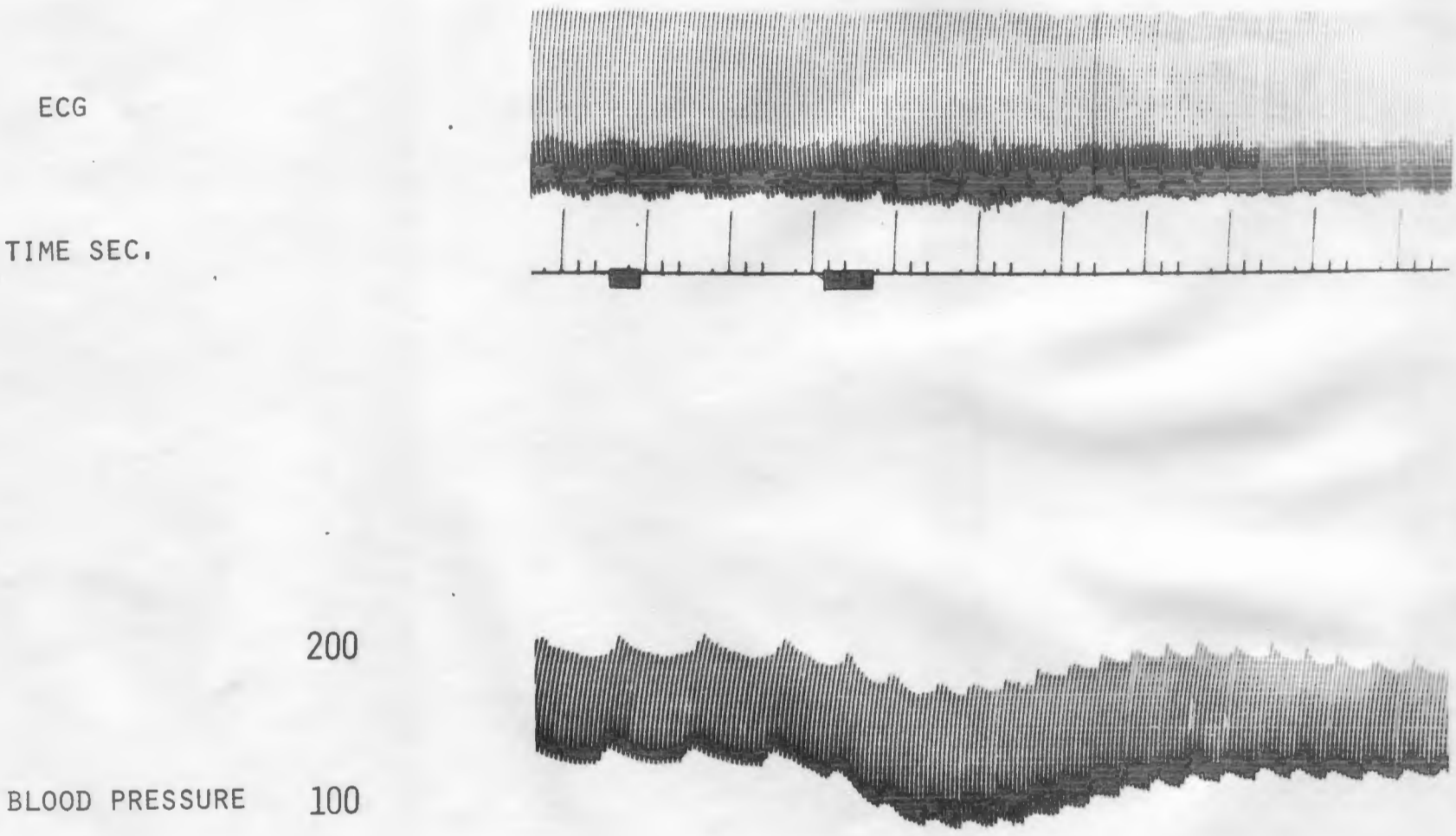
[a] Dose iv in mg/kg

[b] mm Hg: Systolic/Diastolic

[c] Pulse pressure before/Pulse pressure after

[d] Change in systolic pressure/Change in diastolic pressure

[e] Beats per minute


ECG
TIME SEC.
200
BLOOD PRESSURE
100
0
10 MG/KG

pressure was due to a disproportionate drop in the diastolic compared to the systolic pressure, the diastolic pressure always decreased more then the systolic. The heart rate of the dog was increased slightly by asterosaponin injection at all dosage levels.

L. Vagal Influence on the Response of Rat Blood Pressure and Heart Rate to Asterosaponin

Vagal influence was not influential in the creation of the transient hypotension exhibited by rats following asterosaponin administration as indicated by experiments performed on atropinized and vagotomized rats. The results (Table 13) indicate that asterosaponin at a dose of 5 mg/kg was capable of reducing the blood pressure in rats who had been previously treated with atropine at a sufficient level, 0.5 mg/kg to suppress any vagal activity. The results also show that the asterosaponin was capable of producing its effect when both vagi were severed.

M. Histamine Releasing Properties of Asterosaponin in the Rat

The possibility that the asterosaponin was reacting through histamine release was investigated. The data in Table 14 show that when the antihistamine diphenhydramine was administered to a level when exogenously administered histamine no longer produced its hypotensive effects, asterosaponin continued to exhibit its hypotensive action. Histamine when injected at 0.5 mg/kg created a direct hypotension. In this experiment a drop in pressure from 100 to 55

TABLE 13. RAT BLOOD PRESSURE AND HEART RATE;

VAGAL INFLUENCE IN THE ACTION OF ASTEROSAPONIN

Treatment	Dose[a]	Blood Pressure[b] Initial	Blood Pressure[b] After	Heart Rate[c] Initial	Heart Rate[c] After
Asterosaponin	25.0	90	45	276	270
Left Vagus Stimulation[d]		75	70-90	300	
Right Vagus Stimulation[d]		100	75-115		316
Atropine	0.5	75	60-75	402	414
Left Vagus Stimulation		75	75	414	390
Right Vagus Stimulation		75	75	390	390
Asterosaponin	5.0	80	55	414	396
Cut Vagi Asterosaponin	5.0	77	55	384	396

[a] Dose iv in mg/kg

[b] mm Hg

[c] Beats per minute

[d] Vagal stimulation was 0.01 volts, 1.0 Hz for 5 seconds

TABLE 14. HISTAMINE RELEASING PROPERTIES OF ASTEROSAPONIN IN THE RAT

Treatment	Dose[a]	Blood Pressure[b] Initial	After
Diphenhydramine	10.0	100--6[c]---60--4---102--7---75	
Histamine	0.5	100	55
Diphenhydramine	10.0	105--5---70--3---100-13---60	
Histamine	0.5	60	60
Diphenhydramine	10.0	75--6---50--5--- 75-19---40	
Asterosaponin	15.0	40	5

[a] Dose iv in mg/kg

[b] mm Hg

[c] Time in seconds until next extreme pressure was recorded

mm Hg was produced. However, when the histamine was administered to the diphenhydramine pretreated rat the blood pressure did not drop. On the other hand when 15 mg/kg of asterosaponin was administered to the same rat that had received the same antihistamine pretreatment, the asterosaponin (15 mg/kg) did produce the characteristic transient hypotension decreasing the already lowered blood pressure of 40 mm Hg down to 5 mm Hg.

N. Alpha Blocking or Beta Stimulating Properties of Asterosaponin in the Rat

Asterosaponin produces a hypotensive effect when the alpha receptors are blocked and when the beta receptors are blocked as is shown in Table 15. A rat was prepared for the usual blood pressure, ECG evaluation including the venous catheterization. Phenoxybenzamine was then administered through the venous catheter in doses of 0.4 mg/kg until sufficient drug was present so that further doses no longer lowered blood pressure. It was then considered that alpha receptor activity was blocked. Asterosaponin injected at 15 mg/kg produced its transient hypotensive effect dropping the already lowered blood pressure from 48 to 30 mm Hg.

In another rat the possible beta stimulating effect of asterosaponin was evaluated (Table 15). When the rat was pretreated with propranolol to the point where excess propranolol was present in the animal and beta receptor activity was blocked, asterosaponin

TABLE 15. ALPHA BLOCKING AND BETA STIMULATING EFFECTS OF ASTEROSAPONIN

Treatment	Dose[a]	Blood Pressure[b] Initial	After
Phenoxybenzamine	0.4	52[c]	48
Phenoxybenzamine	0.4	48	48
Asterosaponin	15.0	48	30
Propranolol	0.9	78	70-75
Asterosaponin	15.0	70	25

[a] Dose iv in mg/kg

[b] mm Hg

[c] Animal had already received phenoxybenzamine to lower the blood pressure to this level

was intravenously administered at 15 mg/kg. The asterosaponin produced a hypotensive effect, dropping the blood pressure from 70 to 25 mm Hg.

O. The Direct Effect of Asterosaponin on the Vasculature and the Beta Stimulating Effect of Asterosaponin in the Rat -- The Rat Hind Leg Perfusion

The possibility of the asterosaponin producing its hypotensive response through some centrally mediated mechanism was evaluated with the first rat hind leg perfusion preparation. The preparation also evaluated the ability of the asterosaponin to produce hypotension via direct action on the vasculature. The data from Table 16 show that the asterosaponin at a dose of 6.25 mg/kg was capable of dilating the blood vessels of the rat hind leg upon injection and thus increasing the flow of perfusate through that vascular system. Spinal cord transection at the cervical level did not inhibit the vessel dilating properties of the asterosaponin.

The data presented in Table 17 were from a hind leg perfusion preparation that evaluated the reliability of the preparation, the direct effect of asterosaponin on the vasculature, and the beta stimulating effect of the drug. The injection of 4 µg of epinephrine, a predominately alpha stimulant caused a constriction of the vasculature reflected by a drop in the rate of perfusate flow. Isoproterenol (0.1 mg iv) a predominately beta agonist, caused an increase

TABLE 16. EFFECTS OF ASTEROSAPONIN ON THE PERFUSED RAT HIND LIMB I.

Treatment	Dose[a]	Drops/Minute	Percent of Control
Control		38	
Asterosaponin	6.25	52	136
Transection of spinal cord		40	
Asterosaponin	6.25	56	140

[a] mg/kg

TABLE 17. EFFECTS OF ASTEROSAPONIN ON THE PERFUSED RAT HIND LIMB II.

Treatment	Amount Injected	Drops[a]	Percent of Control Rate of Drops/Minute
None	--	167	--
Locke-Ringer	1 ml	188	100
Epinephrine	4 ug	108	57
Locke-Ringer	1 ml	160	100
Isoproterenol	0.1 mg	184	115
None	--	180	100
Propranolol	0.1 mg	136	75
None	--	172	100
Asterosaponin	15 mg	192	111
Locke-Ringer	1 ml	180	100
Asterosaponin	30 mg	216	120
None	--	168	100
Propranolol Isoproterenol	0.1 mg 0.1 mg	152	90
None	-	152	100
Propranolol Asterosaponin	0.1 mg 30 mg	216	142

[a] Drops per minute of perfusate passing through leg.

in the flow of perfusate while the injection of 1 ml of perfusate solution had no effect on the flow. Asterosaponin when injected at 15 or 30 mg iv doses into the preparation caused a substantial increase in the rate of perfusate flow. The beta stimulating properties of the asterosaponin were evaluated with a two-fold experiment. The preparation was pretreated with propanolol (0.1 mg iv) a beta blocker, and then injected with isoproterenol (0.1 mg). The result of the combination was a slight lowering of the flow of perfusate. The animal was again pretreated with propranolol and then treated with asterosaponin (30.0 mg). This time the flow of perfusate was increased in response to the drug treatment indicating that beta blockade failed to alter asterosaponin effect.

V. DISCUSSION

A. Properties Reported in the Literature

The evaluation of the pharmacological properties possessed by the asterosaponin from Asterias forbesi was initiated by employing techniques that would test the compound for pharmacological activities that had been demonstrated by other asterosaponins. Why starfish contain complex asterosaponin compounds has been considered from the time that the first asterosaponins were extracted (Yasumoto et al., 1964). The original researchers who worked with the asterosaponins considered the possibility that the asterosaponins could be compounds used by the starfish to facilitate food procurement via some toxic properties directed toward the starfish's prey, supposedly acting primarily on the adductor muscles of the bivalves on which the starfish often feed (Aldrich, 1957; Nichols, 1964). Some investigators even thought that if the compound was toxic to starfish prey then it was possible that the prey species may be sensitive to the compound and react to it through some type of avoidance response (Pratt and Campbell, 1956). This avoidance response reaction was demonstrated for starfish extracts by Fange (1963), Feder and Lasker (1964), and Mackie et al. (1968).

The work of Friess et al. (1968) and Friess (1970) demonstrated that asterosaponin compounds from starfish did react with cholinergic systems. His experiments showed that some asterosaponins could inhibit the response to stimulation of the cholinergic rat phrenic nerve-diaphram preparation. It was expected then that the asterosaponin from Asterias forbesi might possess some type of reaction on cholinergic systems. The clam heart bioassay (Florey, 1966) was used to evaluate the cholinergic properties of the asterosaponin. The heart of Mercenaria mercenaria was used in these experiments to identify any toxic action by the asterosaponin that would give the starfish any advantage in the predator-prey relationship. The data showed that the assay preparation was very sensitive to acetylcholine, reacting to concentrations as low as 5.5×10^{-13}M. However, the asterosaponin had no cholinergic activity for the dose range of 1.5×10^{-18} to 1.5×10^{-7} g/ml and that there were no abnormal responses of the heart to the asterosaponin. These data support the contention that this asterosaponin from Asterias forbesi does not offer the starfish aid in opening the clam through an action on the heart. Other experiments, discussed below, varify that the asterosaponin had no cholinergic activity.

The investigation of the toxicity of the asterosaponin to clam tissues or the response of mollusk tissues to the asterosaponin was continued with an investigation of the effect of the asterosaponin

on the whole clam using experiments modeled after those of Lavoie (1956) and Yasumoto et al. (1964) where holes were drilled into bivalve shells and the compound injected into the mantle cavity. The data from these experiments show that the asterosaponin did not cause the clam to open and that the compound was not toxic to clams, even at the high concentrations administered. These results correspond with the work of Burnett (1955, 1960) who showed that Asterias forbesi possess sufficient strength to open the bivalve without the use of a toxin.

The possibility remained that even if the asterosaponin was not toxic to the clam that prey species could be sensitive to the compound and initiate an avoidance response to its pressence. The experiments of Mackie et al. (1968) were used as a model for the experiments performed in this research to test this possibility. The data from Table 4 show that the asterosaponin did not initiate an avoidance response by Littorina littorea.

One of the important pharmacological properties reported for the asterosaponins is their blood cell lysing capability (Rio et al., 1963, 1965; Yasumoto et al., 1964; Owellen et al., 1973). The work of Rio et al. (1965) is interesting in that they examined asterosaponins from five different species of starfish, one of which was derived from Asterias forbesi, but is not believed to be the same compound that was used in this investigation. Rio reported hemolytic

activity for four of the starfish species used but not for the asterosaponin from Asterias forbesi. The results show that the asterosaponin from Asterias forbesi used in this investigation was incapable of lysing either erythrocytes or leukocytes.

The asterosaponin was moderately toxic to mice. When various doses of asterosaponin were intraperitoneally injected into mice the asterosaponin, was shown to produce 100 percent lethality with a dose of 200 mg/kg. These results show that the asterosaponin certainly had some pharmacological activity, even though it had not demonstrated avoidance response activity, clam toxicity, or hemolytic action.

B. Activities of Asterosaponin not Previously Reported

The pharmacological testing of the asterosaponin proceeded into an investigation of the compound for activities not previously reported to be present in asterosaponins. The method of Koster (1959) was used to evaluate the asterosaponin for analgesic activity. The asterosaponin did, dose dependently, reduce the number of writhes exhibited by a group of mice that had received an intraperitoneal injection of dilute acetic acid. The presence of analgesic properties on the part of a drug is subjective by definition, and thus is difficult to confirm, but when a 25 mg/kg dose of asterosaponin caused a reduction in the expression of irritation by 99 percent it can at least be said that there is a strong possibility that the

asterosaponin is an analgesic. There is no reference to analgesic properties being exhibited or tested for by any of the other asterosaponins reviewed in the literature.

The initial test used to evaluate the anti-inflammatory capabilities of the asterosaponin was the in vitro method of Grant et al. (1970). The Grant method evaluated the ability of a compound to inhibit the heat denaturation of bovine serum albumin. The ability of a compound to inhibit the heat denaturation of protein may not be a direct indicator of anti-inflammatory activity but compounds that are anti-inflammatory do exhibit this property (Grant et al., 1970; Mizushina and Kobayashi, 1968).

The data show that the asterosaponin inhibited the denaturation of bovine serum albumin as did phenylbutazone, and that the asterosaponin and phenylbutazone produced this inhibition of heat denaturation in a log dose response manner. These results indicate that the asterosaponin has potential as an anti-inflammatory agent, and further investigation into the anti-inflammatory potential of the compound seemed desirable.

Following the positive results obtained for anti-inflammatory activity for the asterosaponin with the bovine serum albumin assay, a more stringent in vivo assay was used to evaluate the asterosaponin. The carrageenin induced rat hind paw edema assay of Winter was

chosen for this purpose, (Winter et al., 1965) with the volume of the paw being measured plethysmographically following the procedure of Van arman et al. (1965). While it is not clear why anti-inflammatory agents inhibit the denaturation of protein it is equally obscure as to why the injection of carrageenin causes such marked inflammation. The carrageenin, which is a high molecular weight polysaccharide is derived from either of two algae Chondrus crispus or Gigartina stellata, and has been hypothesized to create inflammation by many different means. Crunkhorn and Meacock (1971) and DiRosa (1972) concluded that it may be due to: irritant properties, the release of histamine, 5-hydroxytryptamine, or bradykinin, prostaglandins, and even reaction with the blood complement system as a "trigger" for non-immune inflammation. Nevertheless, the rat paw edema assay is a universally accepted method for the in vivo evaluation of anti-inflammatory activity. The assay results showed (Table 6) that both asterosaponin and phenylbutazone restricted the edema formed as a result of carrageenin injection. Phenylbutazone (90 mg/kg) restricted the edema by 52 percent and asterosaponin (75 mg/kg) restricted the edema by 53 percent. The positive results of the bovine serum albumin assay and these results that show that the asterosaponin restricted the edema in rats to the same extent as phenylbutazone, establishes the asterosaponin from Asterias forbesi as an anti-inflammatory agent. The combination

of anti-inflammatory and analgesic activity is advantageous for possible drug compounds. Since the asterosaponin exhibits these properties it is an excellent candidate for further evaluation, however the potent hypotensive properties of the compound could restrict the use of the asterosaponin to certain specific situations. Nevertheless the analgesic and anti-inflammatory properties of the asterosaponin are deserving of more investigation.

C. Cardiovascular Effects

The effect of asterosaponins on the mammalian cardiovascular system had not been investigated previously and the effects of the asterosaponin on respiration, skeletal muscle contraction, and autonomic nervous system functioning had only been studied using the isolated rat phrenic nerve-diaphram preparation (Friess et al., 1968; Friess, 1970). The results obtained by Friess showed that saponin extracts from both sea cucumbers and starfish inhibited the response of the neuronally stimulated rat phrenic nerve-diaphram preparation. The rat phrenic nerve-diaphram preparation is cholinergic in nature and the destruction of its excitability by asterosaponin suggests anti-cholinergic activity. The work of Friess indicated that asterosaponins possess properties that are manifested in mammalian systems, in contrast to the early work that was usually restricted to investigating the effects of starfish extracts on lower species. These studies showed that the asterosaponin did indeed

exhibit true pharmacologic activity which could not be explained by alluding to simple chemoreceptor sensitivity as could be done for avoidance response reactions, or shell gaping activity. The property demonstrated by Friess however was a type of anti-cholinergic activity which is not demonstrated by the asterosaponin from Asterias forbesi, as illustrated below.

The intravenous injection of the asterosaponin from Asterias forbesi caused significant results. In the cat dosages of 0.5 and 1.0 mg/kg (Table 9) of the asterosaponin caused a sudden, evanescent drop in blood pressure with little or no effect on respiration, the contraction of the nictitating membrane or skeletal muscle. The 2.0 mg/kg iv injection of asterosaponin created an even greater drop in blood pressure lasting much longer and caused an increase in the respiratory rate along with a slight decrease in the amplitude of the contractions of the skeletal muscle. Obviously the asterosaponin from Asterias forbesi was not inhibiting the activity of the phrenic nerve of the cat, as the respiratory rate rose and certainly the compound was not exerting any type of anticholinergic activity as the cholinergic fibers of the peroneal nerve innervating the tibialis anterior muscle were functioning almost normally. The lack of anticholinergic activity by the asterosaponin was also shown by the drop in blood pressure, if the compound were exerting anticholinergic activity the blood pressure would be expected to rise,

as a result of the loss of parasympathetic control. The compound did not appear to have any effect on the ganglia or the functioning of adrenergic nerves, as the responses to preganglionic stimulation of the nictitating membrane were normal. The hypotensive activity of the asterosaponin was investigated in rats and dogs after these effects were seen on the cat. The experiments performed on rats (Tables 10 and 11) and dogs (Table 12) show that the asterosaponin could cause a transient hypotensive effect at low dosage, although not as low as in the cat and that the extent of the hypotension and the time which it lasted were both dose dependent.

No effect on heart rate or ECG in response to this asterosaponin injection were seen. These data correspond with the lack of activity the compound had demonstrated on the autonomic nervous system of the cat and suggested that the hypotensive activity seen with the asterosaponin was not due to a depressor activity on the heart. In one rat the asterosaponin did appear to cause a depression in the heart rate. However, it is not believed that this was a direct effect of the drug, but rather a product of a unique rat preparation, or some type of a reflex. This is believed because injections of both the Goldsmith and Shimizu extracted asterosaponin into this rat precipitated ECG irregularities and a drop in heart rate. On all other occasions the asterosaponin did not effect the ECG or the heart rate of the rat. The iv injection of asterosaponin into the

dog created dose dependent hypotension and an increase in the heart rate, probably through a compensatory mechanism. The hypotension produced in the dog was unique in that the pulse pressure increased as a result of asterosaponin administration. Experiments performed by Lederis and Medakovic (1974) with extracts of teleost fish produced similar results in the rat. In the experiments by Lederis and Medakovic pulse pressure was increased following the administration of drug and the increase was due to a more extensive drop in diastolic pressure then in systolic pressure. They concluded that this indicated dilation of resistance vessels. Similar results were obtained with the dog preparation subjected to asterosaponin injections. At all dosages of asterosaponin the pulse pressure increased due to a greater depression in diastolic rather than systolic pressure, implicating some type of vasodilation as being produced by the asterosaponin. Lederis and Medakovic also observed a reflex increase in heart rate due to the hypotension caused by the teleost extract. The results of the dog heart rate and blood pressure experiments indicated that the asterosaponin might have created its hypotensive effect via a direct action on the vasculature. The production of hypotension by the asterosaponin by working directly on the vasculature would also explain the effects of the asterosaponin on the respiratory rate of the cat. Schoop et al. (1957) demonstrated

that the hypotension produced by nitroglycerine caused a reflex hyperpnea in the rat. Thus if the asterosaponin was causing direct vasodilation the respiratory stimulation might be explained.

The transient hypotensive effect of asterosaponin injection was further investigated as to the mechanism of its action. The experiments performed on the cat and dogs had not sufficiently established how the asterosaponin was creating its effect so further experiments were performed on rats whose blood pressure and ECG were monitored, under a variety of conditions.

The possibility of the asterosaponin creating hypotension through vagal stimulation was investigated by testing the effect of asterosaponin on atropinized and vagotomized rats (Table 13). These results show that the asterosaponin continued to exhibit its hypotensive effect even when the vagus nerves were severed. These results in conjunction with the results from the cat experiment that showed no cholinergic activity, and the negative results gained with the clam heart assay for cholinergic activity, discount the possibility of the asterosaponin from Asterias forbesi acting through vagal stimulation.

The asterosaponin was examined for histamine releasing properties in rats (Table 14). These data show that antihistamine pretreatment did not interfere with asterosaponin-induced hypotension. These results correspond with the anti-inflammatory activity of

the asterosaponin so that it can be concluded that the hypotensive action of the asterosaponin is not due to endogenous histamine release.

The data from the cat and dog blood pressure experiments indicate that the asterosaponin worked directly on the vasculature. The lack of effect exhibited by the asterosaponin on the cat nictitating membrane preparation indicated that the drug was not acting through the autonomic nervous system. The possibility that the hypotensive action was due to alpha adrenergic blockade or beta adrenergic stimulation was further evaluated with the rat blood pressure and hind leg experiments. The experiments performed on rat blood pressure and ECG (Table 15) established that the asterosaponin was fully capable of eliciting its characteristic hypotensive response when the alpha or beta receptors were blocked. These results thus add strength to the contention that the asterosaponin is not an alpha blocker or a beta stimulant.

The rat hind leg perfusion preparation supplied much needed information on the mechanism of action of the asterosaponin. The first hind leg perfusion preparation (Table 16) established that the asterosaponin was not acting through any centrally mediated process by showing that the asterosaponin continued to be effective as a hypotensive agent even after cervical transection. The second preparation (Table 17) showed that the asterosaponin was not acting

through beta stimulation, and together with the data obtained from the rat alpha blockade and beta stimulation experiments produced enough evidence to establish that the asterosaponin was not acting through any adrenergic mechanism. The data obtained from the hind leg perfusion experiments, along with all the other data produced in the investigation of the asterosaponin's activity establish that the asterosaponin from Asterias forbesi created its hypotensive effects through direct action on the vasculature.

VI. SUMMARY AND CONCLUSIONS

1. The asterosaponin extracted from the Atlantic starfish Asterias forbesi exhibited no cholinergic activity towards the Mercenaria mercenaria heart bioassay for acetylcholine at concentrations ranging from 1.5×10^{-18} g/ml (1.0×10^{-18} M) to 1.5×10^{-7} g/ml (1.0×10^{-7} M).
2. The asterosaponin produced no observable effects on the whole clam Mercenaria mercenaria when a dose of 1.5×10^{-4} g/ml was injected into the mantle cavity.
3. No blood cell lysing activity was exhibited by the asterosaponin when tested from 0.3 mg/ml to 10.0 mg/ml.
4. The asterosaponin produced no autotomy in Asterias forbesi at a concentration of 1.5×10^{-4} g/ml when injected into the peritoneal cavity.
5. No escape response was elicited in Littorina littorea by asterosaponin when 1 ml of 1.5×10^{-5} g/ml was injected near the head of the snail that was in a dish of sea water. These results along with the results of the clam heart bioassay, and the whole clam assay indicate that the asterosaponin is probably not used by the starfish to facilitate prey procurement.
6. The asterosaponin inhibited the heat denaturation of 1 percent

bovine serum albumin by 89 percent when added to the incubation medium at a concentration of 1000 μg/ml. This inhibition of denaturation was dose dependent and suggestive of possible anti-inflammatory activity.

7. The asterosaponin at 75 mg/kg inhibited rat paw edema formation due to subplanter injection of carrageenin by 53 percent. The results of the in vitro bovine serum albumin assay and those of the in vivo carrageenin induced hind paw edema assay establish the asterosaponin as an anti-inflammatory agent.

8. The LD_{50} of the asterosaponin to mice was between 100 and 200 mg/kg.

9. The blood pressure of the cat dropped in response to 0.5 mg/kg of the intravenously administered asterosaponin. The respiratory rate rose and the blood pressure fell in response to 2.0 mg/kg of the asterosaponin, but there was no effect on the preganglionic stimulation of the nictitating membrane and little effect on the neuronal stimulation of skeletal muscle at the 2.0 mg/kg level.

10. Transient, dose dependent hypotension was produced in rats administered asterosaponin (4.0 to 20.0 mg/kg). The heart rate and ECG were not appreciably directly affected by the compound.

11. The iv administration of asterosaponin (2.0 to 10.0 mg/kg) caused transient hypotension in the dog. This hypotension was indicative of vasodilation as the pulse pressure rose because of a greater drop in diastolic rather than systolic pressure. Heart rate rose following asterosaponin induced hypotension probably as a compensatory mechanism.

12. In the rat, the asterosaponin (5.0 mg/kg) caused hypotension even after the vagus nerves were severed. This along with the lack of cholinergic activity exhibited by the clam heart bioassay for acetylcholine, the lack of effect the asterosaponin exhibited on the neuronally stimulated skeletal muscle of the cat, and the increase in respiratory rate of the cat established that the asterosaponin was not causing hypotension through vagal stimulation or cholinergic activity.

13. The asterosaponin (15 mg/kg) continued to exhibit its characteristic evanescent hypotension following antihistamine (diphenhydramine 10 mg/kg) pretreatment. This indicated that the asterosaponin did not produce hypotension through endogenous histamine release. These conclusions were supported by the anti-inflammatory properties of the asterosaponin.

14. The asterosaponin's (15 mg/kg) hypotensive properties in the rat were not stopped by alpha adrenergic blockade by phenoxybenzamine (0.4 mg/kg). These results along with the lack of

activity the asterosaponin had on the nictitating membrane of the cat established that the asterosaponin was not causing hypotension through alpha blockade.

15. When the asterosaponin (15 mg/kg) was injected iv into the rats pretreated with the beta adrenergic blocking agent propranolol (0.9 mg/kg), the hypotensive activity was still present, indicating that the asterosaponin was not a beta stimulator. The absence of beta-stimulating properties by the asterosaponin was further established by the results of the rat hind leg perfusion experiments, where 30 mg of asterosaponin was effective in creating hypotension after established beta blockade.
16. The fact that the asterosaponin was not causing hypotension through any centrally mediated process was established when cervical transection of the hind leg perfusion preparation failed to diminish the asterosaponin (6.25 mg) induced vasodilation.

VII. REFERENCES

Agarwal, R. A. and Greenberg, M. J.: A comparative study of cardioactive agents from bivalve tissue. Comp. Biochem. Physiol. 31: 835-850, 1969.

Aldrich, F. A.:"On the functional morphology of the alimentary canal of the sea star Asterias forbesi (Desor)." Ph.D. dissertation, Rutgers University, 1954.

Basłow, M. H.: Marine pharmacology--a study of toxins and other biologically active substances of marine origin. Baltimore: The Williams and Wilkins Co., 1969.

Burnett, A. L.: A demonstration of the efficacy of muscular force in the opening of clams by the starfish, Asterias forbesi. Biol. Bull. 109: 355, 1955.

Burnett, A. L.: The mechanism employed by the starfish Asterias forbesi to gain access to the interior of the bivalve Venus mercenaria. Ecology 41: 585-586, 1960.

Chaet, A. B.: A toxin in the coelomic fluid of scalded starfish Asterias forbesi. Proc. Soc. Exp. Bio. Med. 109: 791-794, 1962.

Christensen, A. M.: The feeding behavior of the seastar Evasterias troschelii (Stimpson). Limnol. Oceanogr. 2: 180-197, 1957.

Crunkhorn, P. and Meacock, S. C.: Mediators of the inflammation induced in the rat paw by carrageenin. Br. J. Pharmacol. 42: 392-402, 1971.

DiRosa, M.: Biological properties of carrageenan. J. Pharm. Pharmacol. 24: 89-102, 1972.

Fange, R.: Toxic factors in starfishes. Sarsia 10: 19-21, 1963.

Feder, H. M. and Arvidsson, J.: Studies on a sea-star (Marthasterias glacialis) extract responsible for avoidance reactions in gastropod (Buccinum undatum). Arkiv Zool. 19: 369-379, 1967.

Feder, H. and Christensen, A. "Aspects of Asteroid Biology." In Physiology of Echinodermata, pp. 87-127. Edited by Richard A. Boolootian. New York: Interscience Publishers, 1966.

Feder, H. M. and Lasker, R.: Partial purification of a substance from starfish tube feet which elicits escape responses in gastropod mollusks. Life Sci. 3: 1047-1051, 1964.

Florey, E.: The clam-heart bioassay for acetylcholine. Comp. Biochem. Physiol. 20: 365-377, 1967.

Friess, S. L.: Steroidal saponin esters elaborated by poisonous marine echinoderms. Aldrichimica acta 3: 16-17, 1970.

Friess, S. L. and Durant, R. C.: Blockade phenomena at the mammalian neuromuscular synapse, competition between reversible anticholinesterases and an irreversible toxin. Toxicol. Appl. Pharmacol. 7: 373-381, 1965.

Friess, S. L., Durant, R. C. and Chanley, J. D.: Further studies on biological actions of steroidal saponins produced by poisonous echinoderms. Toxicon 6: 81-92, 1968.

Friess, S. L., Durant, R. C., Chanley, J. D. and Mezzetti, T.: Some structural requirments underlying holothurin A interactions with synaptic chemoreceptors. Biochem. Pharmacol. 14: 1237-1247, 1965.

Friess, S. L., Standaert, F. G., Whitcomb, E. R., Nigrelli, R. F., Chanley, J. D. and Sobotka, H.: Some pharmacologic properties of holothurin, an active neurotoxin from the sea-cucumber. J. Pharmacol. Exp. Ther. 126: 323-329, 1959.

Galtsoff, P. S. and Loosanoff, V. L.: Natural history and method of controlling the starfish (Asterias forbesi, Desor). U. S. Dept. of the Interior, Bur. Fish. Bull. No. 31: 73-132, 1939.

Grant, N. H., Alburn, H. E. and Kryzanauskas, E.: Stabilization of serum albumin by anti-inflammatory drugs. Biochem. Pharmacol. 19: 715-722, 1970.

Greenberg, M. J.: A compendium of responses of bivalve hearts to acetylcholine. Comp. Biochem. Physiol. 14: 513-539, 1965.

Greenberg, M. J. and Jegla, T. C.: The action of 5-hydroxytryptamine and acetylcholine on the rectum of the venus clam Mercenaria mercenaria. Comp. Biochem. Physiol. 9: 275-290, 1963.

Hashimoto, Y. and Yasumoto, T.: Confirmation of saponin as a toxic principle of starfish. Bull. Jap. Soc. Sci. Fish. 26: 1132-1138, 1960.

Koster, R., Anderson, M. and deBeer, E. J.: Acetic acid for analgesia screening. Fed. Proc. 18: 412, 1959.

Lavoie, M. E.: How sea stars open bivalves. Biol. Bull. 111: 114-122, 1956.

Lederis, K. and Medakovic, M.: Pharmacological observations on the hypotensive action of extracts of teleost fish Urophyses (Urotensin I) in the rat. Brit. J. Pharmacol. 51: 315-324, 1974.

Mackie, A. M., Lasker, R. and Grant, P. T.: Avoidance reactions of a mollusc Buccinum udatum to saponin-like surface-active substances in extracts of the starfish Asterias rubens and Marthasterias glacialis. Comp. Biochem. Physiol. 26: 415-428, 1968.

Matsuno, T. and Yamanouchi, T.: A new triterpenoid sapogenin of animal origin (sea cucumber). Nature (London) 191: 75-76, 1961.

Mendes, E. G., Abbud, L. and Umiji, S.: Cholinergic action of homogenates of sea urchin pedicellariae. Science 139: 408-409, 1963.

Mizushima, Y. and Kobayashi, M.: Interaction of anti-inflammatory drugs with serum proteins, especially with some biologically active proteins. J. Pharm. Pharmacol. 20: 169-173, 1968.

Nichols, D.: Echinoderms: experimental and ecological. Oceanogr. Mar. Biol. Ann. Rev. 2: 393-423, 1964.

Nigrelli, R. F.: The effects of holothurin on fish, and mice with sarcoma 180. Zoologica 37: 89-90, 1952.

Nigrelli, R. F., Chanley, J. D., Kohn, S. K. and Sobotka, H.: The chemical nature of holothurin, a toxic principle from the sea cucumber (Echinodermata; Holothurioidea). Zoologica 40: 47-48, 1955.

Owellen, R. J., Owellen, R. G., Gorog, M. A. and Klein, D.: Cytolytic saponin fractions from Asterias vulgaris. Toxicon 11: 319-323, 1973.

Pratt, D. M. and Campbell, D. A.: Enviornmental factors affecting growth in Venus mercenaria. Limnol. Oceanogr. 1: 2-17, 1956.

Prosser, C. L.: Acetylcholine and nervous inhibition of Venus mercenaria. Biol. Bull. 78: 92-102, 1940.

Rio, G. J., Ruggieri, G. D., Stempien, M. F., Jr. and Nigrelli, R. F.: Saponin-like toxin from the giant sunburst starfish, Pycnopodia helianthoides, from the Pacific northwest. Amer. Zool. 3: 554-555, 1963.

Rio, G. J., Stempien, M. F., Jr., Nigrelli, R. F. and Ruggieri, G. D.: Echinoderm toxins. I. Some biochemical and physiological properties of toxins from several species of asteroidea. Toxicon 3: 147-155, 1965.

Ruggieri, G. D.: Echinoderm toxins. II. Animalizing action in sea urchin development. Toxicon 3: 157-162, 1965.

Schopp, R. T., Gilfoil, T. M. and Youmans, W. B.: Mechanisms of respiratory responses to vasodilator drugs Urecholine (carbaminoyl beta-methylcholine) and nitroglycerine. Am. J. Physiol. 189: 123-128, 1957.

Shimizu, Y.: Antiviral substance in starfish. Experientia 27: 1188-1189, 1971.

Shimizu, Y.: Characterization of an acid hydrolysis product of starfish toxins as a 5 pregnane derivative. J. Amer. Chem. Soc. 94: 4051, 1972.

Sullivan, T. D., Laude, D. T. and Nigrelli, R. F.: The effects of holothurin, a steroid saponin of animal origin, on Krebs-2 ascites tumors in swiss mice. Zoologica 40: 49-52, 1955.

Van Arman, C. G., Begany, A. J., Miller, L. M. and Pless, H. H.: Some details of the inflammations caused by yeast and carrageenin (with appendix on kinetics of the reaction). J. Pharmacol. Exp. Ther. 150: 328-334, 1965.

Winter, C. A. and Flataker, L.: Reaction thresholds to pressure in edematous hindpaws of rats and responses to analgesic drugs. J. Pharmacol. Exp. Ther. 150: 165-171, 1965.

Winter, C. A., Risley, E. A. and Nuss, G. W.: Anti-inflammatory and antipyretic activities of Indomethacin, 1-(p-chlorobenzoyl)-5-methoxy-2-methyl-indole-3-acetic acid. J. Pharmacol. 141: 369-376, 1963.

Wolters, B.: The antibiotic action of saponins. II Saponins as plant fungistatic compounds. Planta 79: 77-83, 1968.

Yasumoto, T., Tanaka, M. and Hashimoto, Y.: Distribution of saponin in echinoderms. Bull. Jap. Soc. Sci. Fish. 32: 673-676, 1966.

Yasumoto, T., Watanabe, T. and Hashimoto, Y.: Physiological activities of starfish saponin. Bull. Jap. Soc. Sci. Fish. 30: 357-364, 1964.