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Atromentin

Anticoagulant from *Hydnellum diabolus*

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Gross screening revealed that *Hydnellum diabolus* Banker contained a parenterally effective anticoagulant, while *H. aurantiacum, H. caeruleum,* and *Hydnum laevigatum* lacked activity. A 70 per cent ethanolic extract of *H. diabolus* contained all anticoagulant activity as well as pressor-leiomyotonic principles. The anticoagulant activity grossly resembled that of heparin, except that *in vivo* activity was not reversed by protamine injection. *In vivo,* 1 mg. of the ethanol extract was equivalent to 0.58 u. of heparin. Selective solvent extraction of the mushroom with diethyl ether removed all anticoagulant activity, and from this extract atromentin was isolated. *In vitro,* 1 mg. of atromentin was equivalent to 5.1 u. of heparin and 2.3 mg. of reference 70 per cent ethanol extract. Synthetic dimethylatromentin, 2,5-diphenylbenzoquinone, and polyporic acid were without anticoagulant properties.

HYDNELLUM DIABOLUS Banker is widely distributed from Maine to Alabama and in the Pacific Northwest of the United States (1). While possessing an intense acrid taste and the odor of hickory nuts, it is generally regarded as edible. Until recently (2), pharmacologic activity had not been reported for this species.

EXPERIMENTAL

Collection and Preparation of Plant Material.—Carpophores of *H. diabolus* Banker were collected on Whidbey Island in western Washington on November 1, 1962. After cleaning, they were dried in a forced-air drying oven at 45° for at least 72 hr. A sample of the dried mushroom was pulverized in a ball mill overnight and passed through a 200-mesh sieve. A 70% ethanolic extract was prepared from another sample of this collection (60-mesh powder) using 30 parts of solvent per unit weight of mushroom and shaking on a reciprocal shaker for 20–24 hr. with 3 successive portions of solvent. The ethanolic extracts were separated by suction filtration, combined, and evaporated to dryness under reduced pressure at a temperature not exceeding 45° (37% extractive). This product was termed reference 70% ethanol extract. The marc was freed of solvent and reduced to a 200-mesh powder in a ball mill. The powdered whole carpophores, extract, and marc were coded and submitted for routine pharmacologic examination. Immediately prior to testing, all test materials were dissolved and/or suspended by trituration with 0.25% agar.

Detection of pharmacologic activity in *H. diabolus* suggested the desirability of testing other available members of the *Hydnaceae*. In a similar way, samples representing the powdered whole carpophores, ethanol extracts, and marcs were prepared for *H. aurantiacum* (Fr.) Karst., *H. caeruleum* (Pers.) Karst., and *Hydnum laevigatum* Fr. These mushrooms were collected in western Washington during the autumns of 1962 and 1963. The yields of 70% ethanol extractives were 36, 32, and 50%, respectively. Taxonomic identification of all the species was provided by Dr. D. E. Stuntz, Professor of Botany, University of Washington.

Test Animals.—Albino rats were obtained from E. G. Steinhilber, Oshkosh, Wis., and maintained on an unrestricted diet of laboratory chow (Purina) and water for at least 2 weeks before testing. Unless specified, all intraperitoneal injections were made into the upper left quadrant of the abdomen. Dogs used as blood donors and for pharmacodynamic testing were healthy mongrels purchased from local sources.

Qualitative and Semiquantitative Hippocratic Screening.—Using log-dosages from inactive to lethal, the test materials were injected intraperitoneally into male and female (150–250 Gm.) rats that had not received any previous drug treatment. Animals were not fasted prior to medication, and

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food and water were freely available after injection. The qualitative symptomatology before and after dosage was recorded using the techniques and Hippocratic work-sheet of Malone and Robichaud (3). All test animals and appropriate vehicle controls were sacrificed 7 days after injection and gross necropsy performed.

Determination of Therapeutic Indices.—The median lethal dosage (LD_{50}) and median effective dosage (ED_{50}) were calculated according to the method of Litchfield and Wilcoxon (4). A constant dosage volume of 5 ml./Kg. was used with a log-dosage of 0.25 (1.778×). The *in vivo* anticoagulant effect determined 2 hr. after injection was used as the criterion for determining the ED_{50} dosages. A group of 12 healthy rats and a group of 12 rats receiving 5 ml./Kg. of the 0.25% agar vehicle were decapitated by guillotine and the blood allowed to drain into a glass beaker. The average clotting time for the untreated group was 1.45 min. with a standard deviation (S.D.) of 0.4, while the clotting time for the agar control group was 1.37 (S.D. = 0.38). Considering +2 and +3 S.D., the maximum normal clotting times were calculated as 2.25 and 2.65 min., respectively. In order to leave no doubt, clotting times greater than 2.5 and 3.0 min. after decapitation were used as the two positive criteria for calculating anticoagulant ED_{50} values. The therapeutic index was calculated as LD_{50}/ED_{50}.

In Vivo Antagonism Studies.—The 3-min. ED_{95} dosage of sodium heparin was injected intraperitoneally (upper left quadrant) into three groups of six rats each. Two of these groups were injected simultaneously (upper right quadrant) with 10 and 50 mg./Kg. of protamine sulfate, respectively. Two hours after injection, all animals were decapitated and the clotting time observed. A similar experiment was performed using the ED_{95} dosage of reference 70% ethanol extract. Two more groups received protamine sulfate alone, and one group received 0.25% agar as a vehicle control. A constant dosage volume of 5 ml./Kg. was maintained.

Pharmacodynamic Studies.—A small dog was anesthetized to plane 3 of stage III of anesthesia with a slow infusion of 30% urethan intravenously. After tracheotomy, both vagi were sectioned. Respiration, ECG, blood pressure, and heart rate were recorded continuously as well as the animal's responses to standardized doses of epinephrine, acetylcholine, and histamine and response to electric stimulation of the cardiac end of the cut vagi. The reference 70% ethanol extract was then administered intravenously at a dosage of 1 mg./Kg. and periodically cumulated in the body until death occurred. In between each twofold cumulative dosage of extract, the responses to epinephrine, acetylcholine, histamine, and vagal stimulation were determined. The extract injections were of 10 sec. duration *via* the femoral vein followed by a 10-sec. 3-ml. wash of 0.9% sodium chloride. Control animals were prepared in a like manner to determine the pharmacodynamic effects of heparin and the effects of the 0.25% agar vehicle used to dose both heparin and the extract.

Preliminary Isolated Tissue Studies.—A 3-cm. portion of guinea pig ileum was removed after cranio-vertebral dislocation and placed in a tissue bath filled with oxygenated (95% O_2 and 5% CO_2) Tyrode's solution at 37°. In a like manner, a 3-cm. duodenal segment of rabbit intestine was suspended in a bath containing oxygenated Sollmann-Radamaeker's solution at 37°. The *rectus abdominis* muscle was removed from a pithed frog, divided, and halves suspended in a perfusion bath containing frog Ringer's solution at room temperature. Isotonic lever systems were used to record contraction of all systems.

In Vitro Anticoagulant Evaluation.—Anticoagulant effects were measured by a slight modification of the Lee-White method (5). Whole blood was drawn from trained, nonfasted dogs into dry, heavily siliconized syringes. One milliliter of whole blood was rapidly transferred to each test tube containing 0.1 ml. of a dilution of the substance to be tested. The clotting end point was taken as that time when the blood held a vertical surface with the tube in a horizontal position. Three minutes after recording the end point, the contents were shaken from the tube into water to demonstrate unequivocally the presence of a clot. After 30–60 min., nonclotted samples were also poured into water to establish the absence of a clot. Vehicle controls (0.1 ml. of 0.25% agar) were tested routinely.

Selective Solvent Extraction of H. diabolus.—A quantity of a 60-mesh powder of the whole mushroom was exhausted successively with petroleum ether, diethyl ether, chloroform, an azeotropic mixture of benzene and absolute ethanol, and absolute ethanol using a Soxhlet apparatus. Portions of these extractives equivalent to a 5-Gm. quantity of powdered mushroom and a sample of the marc reduced to a 200-mesh powder were submitted for pharmacologic evaluation (*in vitro* clotting time, pressor, and leiomyotonic screening).

Preparation of Atromentin and Related Compounds.—Atromentin [2,5-dihydroxy-3,6-bis (*p*-hydroxyphenyl)-*p*-benzoquinone] was isolated and identified from the diethyl ether extractive (6), and synthetic dimethylatromentin and atromentin were prepared using the procedure of Kögl *et al.* (7). The related benzoquinone derivatives, 2,5-diphenylbenzoquinone and polyporic acid, were synthesized by the Frank *et al.* modification (8) of the Shildneck and Adams procedure (9). Identities of the synthetic atromentin, dimethylatromentin, and 2,5-diphenylbenzoquinone were confirmed by their infrared spectra and melting points. Quantitative elemental analysis and infrared spectrum were employed to verify the identity of the polyporic acid.

RESULTS

Hippocratic Screening.—The injection of 1 Gm./Kg. of the 200-mesh powdered whole mushroom intraperitoneally caused some abdominal griping with a decrease in spontaneous motor activity noted 5 min. after injection. As depression progressed, there occurred slight analgesia, loss of ability to cling to an inverted screen, and eventually loss of righting reflex just prior to death 133 min. after dosage. The respiratory rate was slightly decreased, while depth slightly increased. The animal became increasingly passive to head-tap and body-grasp challenges (3). Ten minutes prior to death of respiratory arrest, there was a complete loss of skeletal muscle tone, the pupil dilated, ears blanched, and a positive Robichaud test was seen (3)

TABLE I.—DETERMINATION OF MEDIAN EFFECTIVE AND LETHAL DOSAGES

	70% Ethanol Extract		Whole Mushroom	
	mg./Kg.	Slope	mg./Kg.	Slope
LD_{50}	530 (379–742)[a]	1.8 (1.4–2.3)	680 (453–1020)	1.8 (1.4–2.5)
ED_{50}[b]	86 (62–119)	1.5 (1.2–1.9)	275 (200–378)	1.6 (1.3–2.1)
ED_{50}[c]	165 (106–256)	1.9 (1.3–2.8)	362 (276–474)	1.4 (1.2–1.7)
LD_{50}/ED_{50}	3.21–6.16		1.88–2.47	

[a] Figures in parentheses are the 95% confidence limits. [b] Blood clotting time after decapitation equal to or greater than 2.5 min. Determination performed 2 hr. after intraperitoneal injection. [c] Blood clotting time after decapitation ≥ 3.0 min. Determination performed 2 hr. after intraperitoneal injection.

TABLE II.—EFFECT OF INTRAPERITONEAL PROTAMINE ON ANTICOAGULANT EFFECTS OF INTRAPERITONEAL SODIUM HEPARIN AND *Hydnellum* EXTRACT

	Clotting Time, min. (Range, 6 Values)		
	Control	Sodium Heparin, 4.1 mg./Kg.	70% Ethanol Extract, 562 mg./Kg.
0.25% Agar vehicle control	1.4 (1.0–2.5)	≥ 30	(7 – ≥ 30)
Protamine sulfate, 50 mg./Kg.	2.2 (1.5–3.0)	1.1[a] (1.0–1.5)	(15 – ≥ 30)

[a] A dosage of 10 mg./Kg. of protamine sulfate partially blocked the heparin response, with the mean clotting time in three rats = 2.7 min., while the clotting time after decapitation of the remaining three rats ≥ 30 min.

accompanied by slight hypothermia and pilomotor erection. Upon gross necropsy, the thoracic and abdominal cavities were found to be full of blood that would not clot and the spleen, liver, and lungs were blanched. The noted symptoms appeared to be directly correlated with the physical effects of hemorrhage. The injection of 1 Gm./Kg. of the 70% ethanol extract produced similar effects, while the same dosage of the marc produced few acute effects other than slight enopthalmos, miosis, general passivity, and a 3.5° drop in rectal temperature. When 1 Gm./Kg. dosages of the whole mushroom, extract, and marc were administered orally to rats, none of the animals displayed any acute or delayed symptoms. Gross necropsy on the seventh day after injection showed neither changes in blood coagulation nor in appearance of the body organs.

Injection of 562 and 316 mg./Kg. of both the whole mushroom and the extract resulted in hemorrhage terminating in shock and death. The site of hemorrhage in each instance appeared to be the slight wound inflicted by the intraperitoneal injection. The administration of 100 mg./Kg. of the whole mushroom and extract produced a hemorrhage-induced death approximately 15 and 26 hr. after injection, respectively.

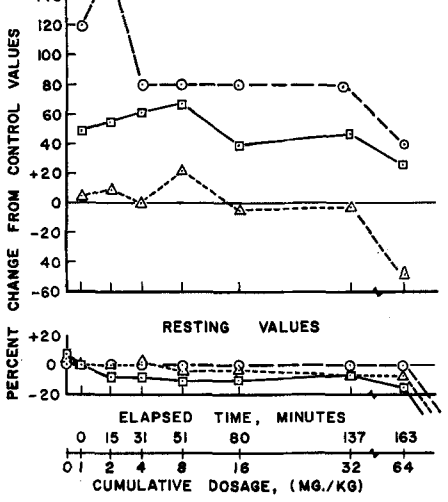

Fig. 1.—Cardiovascular screen of reference 70% ethanol extract of *H. diabolus*. Key: —⊡, blood pressure; - - - - △, heart rate; — — ⊙, respiration rate.

The dosage of 56.2 mg./Kg. was nonlethal. Animals receiving the whole mushroom displayed some persistent respiratory depression and decrease in motor activity. Skin blanching was noted on the fourth through the seventh day after injection. The necropsy on the seventh day showed incomplete clotting of blood and some congestion of both liver and spleen. The symptomatology induced by the extract was similar, with the exception that the clotting time for blood on necropsy was 3 min. and no changes in the spleen were noted.

Hippocratic screening of *H. aurantiacum*, *H. caeruleum*, and *Hydnum laevigatum* revealed that these mushrooms were without activity at maximal dosages of 1 Gm./Kg. Similar screening of sodium heparin (150 u./mg.), bishydroxycoumarin, phenindione, sodium warfarin, and sodium oxalate revealed that an identical symptomatology to that of *H. diabolus* could be induced by sodium heparin intraperitoneally.

Determination of Therapeutic Indices.—Death following intraperitoneal injection of whole powdered mushroom and extract may occur within 10 min. or may take place up to a maximum of 130 hr. after administration. The animals were observed for 7 days. In all cases death was caused by shock resulting from massive hemorrhage into the peritoneal cavity. The calculated LD_{50} and ED_{50} results are summarized in Table I. Only six animals per dosage level were used. More rats per dose would have given more precise values and better estimates of the dose-response slopes, but this was considered wasteful considering the limited amount of the materials available. There was no significant difference between the dose-response slopes of the whole mushroom and the extract.

The 3 min. ED_{50} for sodium heparin (150 u./mg.) was determined in a similar manner: ED_{50} = 1.90 (1.43–2.50) mg./Kg. with a slope of 1.6 (1.3–1.9).

TABLE III.—*In Vitro* ANTICOAGULANT EFFECTS OF *Hydnellum* EXTRACT

Concn. in Whole Blood, %	Mean Clotting Time, min. (Observed Range)
0.091[a]	≥60
0.075	≥30[b] (28–33)
0.062	28 (28–31)
0.051	22 (20–24)
0.029	12 (11–13)
0.016	8 (8–9)
...[c]	5 (4–6)
...	4 (4–6)

[a] One milliliter of dog whole blood was added to 0.1 ml. of 1% *Hydnellum* extract in 0.25% agar vehicle. [b] Incomplete and equivocal clotting responses. [c] One milliliter of dog whole blood was added to 0.1 ml. of 0.25% agar.

Comparing the *in vivo* potency of heparin with the reference extract, 1 u. of heparin = 1.75 mg. of the 70% ethanol extract of *H. diabolus*. The dose-response slopes for heparin and the extract were statistically parallel ($p = 0.05$).

Determination of Lack of Oral Effectiveness.—A group of 16 rats were dosed orally with 1 Gm./Kg. of the 70% ethanol extract. Four each were decapitated at 2 and 4 hr. after dosage while two each were decapitated at 8, 12, 24, and 48 hr. after dosage. The respective times for blood coagulation were well within the normal clotting range, indicating that the anticoagulant principle was essentially inactive orally.

In Vivo Antagonism Studies.—As shown in Table II, the dosage of 50 mg./Kg. of protamine sulfate completely antagonized the anticoagulant effect of the 3 min. ED_{95} dosage (4.1 mg./Kg.) of sodium heparin (150 u./mg.). With 10 mg./Kg. of protamine sulfate, the mean clotting time for two of the six heparin-treated animals was less than 2 min. Protamine sulfate did not reverse the anticoagulant activity of the 3 min. ED_{95} (562 mg./Kg.) of the reference *Hydnellum* extract, thus suggesting that the mechanism of action of the extract may be different than that of heparin.

Pharmacodynamic Studies.—The most striking response of the extract in the dog was a powerful (45% rise) pressor effect of 2.5 min. duration with the initial dosage of 1 mg./Kg. Concurrently, as shown in Fig. 1, there was a marked increase in respiratory rate and a decrease in respiratory depth. The degree and duration of pressor effect was progressively increased with each cumulative injection until maximal pressor effects (67% rise above predrug level) were achieved with the cumulative dose of 8 mg./Kg. At this dose, the duration of pressor activity was 4 min. and was followed by a secondary depressor phase (35% fall) lasting 9 min. After the cumulative dosage of 32 mg./Kg., the pressor effect was decreased (47% rise, 2.5 min. duration) and the depressor response was accentuated (50% fall, 15 min. duration). After the cumulative injection of 64 mg./Kg., the pressor response was decreased further (29% rise, 1.5 min. duration), and the heart stopped during the depressor phase and the animal died. The resting blood pressure (that pressure just before the next cumulative injection of extract), resting heart rate, and respiratory rate were not affected significantly by the extract—an indication of the transient nature of the cardiovascular-respiratory effects.

The pressor response to a standard dose of intravenous epinephrine was unchanged up to a cumulative dosage of 32 mg./Kg. of the extract, after which the pressor effect was decreased by 40%, with no change in the secondary epinephrine depressor response. Also at this cumulative dosage, there was a 35% decrease in the depressor response of the standard dosage of acetylcholine. The animal's response to histamine and to vagal stimulation remained unchanged throughout the entire experiment.

The control experiments carried out with sodium heparin, and agar did not show any dramatic changes —the effect of heparin on mean blood pressure was a mild transient depressor response. It appeared that the *Hydnellum* extract contained a rather powerful musculotropic vasopressor substance that because of its short duration of effect was not identical to the anticoagulant substance.

Preliminary Isolated Tissue Studies.—Extract bath concentrations of 20 mcg./ml. produced marked contraction of guinea pig ileum. The threshold concentration for activity was about 10 mcg./ml. Repeated washing totally reversed extract activity and the standardized response of 20 mcg./ml. of acetylcholine was decreased in the presence of the extract.

A perfusion concentration of 10 mcg./ml. also appeared to be the threshold concentration for rabbit duodenum. The effect of the extract could not be blocked by previous incubation with atropine or reversed by subsequent atropine additions that totally abolish the effects of a standard amount of acetylcholine. It appeared that the reference 70% ethanol extract was able to induce contraction of smooth muscle at rather low concentrations and that this effect was probably musculotropic.

Bath concentrations of 20 mcg./ml. produced marked contraction of the frog *rectus abdominis* equivalent to 100 mcg./ml. of acetylcholine. Repeated washings with the perfusate reversed the activity of the extract.

TABLE IV.—FRACTIONATION OF ACTIVITY BY SUCCESSIVE SOLVENT EXTRACTION OF WHOLE MUSHROOM

Extraction Solvent	*In Vitro* Clotting Time, min.[a]	Duodenum Tonus, mm.[b]	Blood Pressure Change, %[c] Pressor	Blood Pressure Change, %[c] Depressor
Diethyl ether	≥60.0	4	32	0
Chloroform	3.5	36	64	−58[d]
Benzene–ethanol	3.8	30	108	−26[e]
Absolute ethanol	3.7	21	93	−8
Marc	3.7	15	6	0
Agar vehicle control	4.3	0	0	−2
70% ethanol reference extract	≥60.0	20	43	−30[f]

[a] One milliliter of dog whole blood added to 0.1 ml. of test substance (1%) in 0.25% agar vehicle. Each value is the mean of three determinations using a modified Lee-White technique. [b] Contraction of rabbit duodenum at a concentration of 0.092% in oxygenated Sollmann-Radamaeker's solution. [c] Cardiovascular effect upon injection of 1.0 mg./Kg. intravenously into an urethan anesthetized dog. [d] The pressor effect was of 4-min. duration, while the secondary depressor effect was of 21-min. duration. [e] The pressor effect lasted 3.5 min., while the depressor effect persisted for 11 min. [f] Duration of pressor response = 2.5 min.; duration of depressor phase = 11 min.

TABLE V.—*In Vitro* ANTICOAGULANT SCREENING OF ATROMENTIN AND RELATED COMPOUNDS

Test Material	Mean Clotting Time, min. (Observed Range)[a]
Atromentin (natural)	≥60
Atromentin (synthetic)	≥60
Dimethylatromentin	4.5 (4–5)
2,5-Diphenylbenzoquinone	5.4 (4–7)
Polyporic acid	5.7 (4–9)
Agar vehicle control	5.2 (4–6)
70% Ethanol reference extract	≥60

[a] One milliliter of dog whole blood was added to 0.1 ml. of 1% of the test substance in a 0.25% agar vehicle (five to seven determinations).

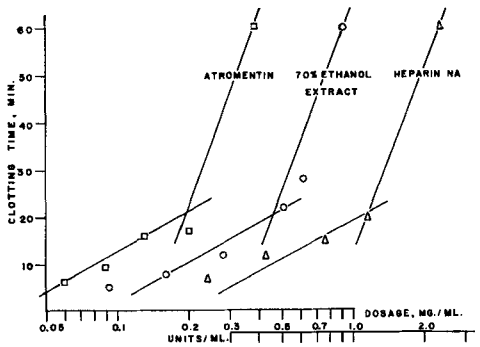

Fig. 2.—*In vitro* concentration-response curves for the anticoagulant activity of the reference 70% ethanol extract of *H. diabolus*, atromentin, and sodium heparin.

In Vitro Anticoagulant Evaluation.—Clotting time was recorded as the mean of three to six runs at a respective concentration, with the mean time rounded off to the nearest whole minute. A typical set of results is illustrated in Table III. A concentration of 0.091% of the reference 70% ethanol extract completely prevented clotting, and dose-response effects were seen at concentrations of 0.016, 0.029, and 0.051%.

Evaluation of Exhaustive Extracts.—The results of screening the extracts produced by successive selective solvent extraction are listed in Table IV. Such extracts were tested at a concentration or dosage that was active unequivocally for the reference 70% ethanol extract as determined by previous testing. The diethyl ether extract appeared to contain quantitatively the anticoagulant activity while possessing relatively little pressor–leiomyotonic properties. The subsequent chloroform extraction removed the major portion of the pressor–leiomyotonic components, but there was a distinct tailing off of activity through the absolute ethanol extract.

Preliminary Pharmacologic Evaluation of Atromentin.—As seen in Table V, atromentin isolated from *H. diabolus* was as active as that prepared by synthetic means. Atromentin appears to account for the majority of the anticoagulant activity previously documented in the reference 70% ethanol extract. Dimethylatromentin, 2,5-diphenylbenzoquinone, and polyporic acid were all inactive at the arbitrary but critical concentration of 0.091%. No pressor–depressor activity (dog) or leiomyotonic activity (rabbit duodenum) was seen at 1.0 mg./Kg. intravenously or at 0.002% bath concentration, respectively, for atromentin.

The total *in vitro* anticoagulant dose-response curve was then determined for atromentin as well as that for sodium heparin (150 u./mg.). The results of this comparison are presented in Fig. 2 along with the data for the reference extract listed in Table III. The relative *in vitro* potencies were calculated graphically. One milligram of the reference 70% ethanol extract was equivalent to 2.2 u. of heparin and 0.43 mg. of atromentin.

SUMMARY

Hippocratic screening in rats revealed an immediately effective anticoagulant principle in *H. diabolus* Banker. The 70% ethanolic extract of the whole mushroom (37% extractive) contained all pharmacologic activity and was less toxic than the whole mushroom as indicated by the therapeutic indices. Like heparin, the extract was ineffective orally; but unlike heparin, the activity was not reversed *in vivo* by protamine sulfate. Pressor–leiomyotonic activity (musculotropic) was another property of the extract. Selective solvent extraction indicated that the anticoagulant activity (confined to the diethyl ether extraction) was totally separate from the pressor–leiomyotonic principle(s). Atromentin was isolated from the diethyl ether extract. *In vivo*, 1 mg. of the 70% ethanol extract was equivalent to 0.58 u. of heparin. *In vitro*, 1 mg. of the extract was equivalent to 2.2 u. of heparin and 0.43 mg. of atromentin.

H. caeruleum and *Hydnum laevigatum* were without anticoagulant activity upon Hippocratic screening. Dimethylatromentin, 2,5-diphenylbenzoquinone, polyporic acid, and preparations of *H. aurantiacum*, a mushroom which has been reported to contain aurantiacin (atromentin-2,5-dibenzoate) (10), were observed to have no anticoagulant activity. No anticoagulant activity (11) was detected with powdered carpophores and an ethanolic extract of *Paxillus atrotomentosus* (Fr.) Fr., but atromentin is known to exist primarily in its leuco form in this agaric (12, 13). The evidence suggests that modification of the oxygen functions in the atromentin molecule destroys the anticoagulant capacity.

Atromentin appears to be a new prototype molecule for parenteral and *in vitro* anticoagulant action.

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