TWO CRYSTALLINE PHARMACOLOGICAL AGENTS OBTAINED FROM THE TROPICAL TOAD, *BUFO AGUA*[1]

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[1] The subject matter of this paper appeared as a preliminary communication entitled: "The Poisons of the Tropical Toad, *Bufo Agua*," in the Journ. Americ. Med. Assoc., May 27, 1911, vol. lvi, pp. 1531-36. Analytical and pharmacological data which could not be incorporated in the preliminary paper are here given.

I. HISTORICAL AND GENERAL

Poisons of animal origin, using this term in its widest sense, have always been of interest to the student of medical science, and of late years they have in several instances become of actual value to the medical practitioner. Among poisons of animal origin[2] may be named the various snake venoms, the poisons of amphibia, of fishes, (e.g., the fugu poison of Japan), of mussels (mytilotoxin), of scorpions, spiders, bees, ants, beetles (e.g., cantharidin), and other insects. Among poisons derived from the organs of mammals may be named those obtained from the thyroid and suprarenal and pituitary glands as having a very high therapeutic value.

While continuing our studies, in the autumn of 1910, on the convulsant action of certain organic dyestuffs,[3] acid fuchsin, betanaphthol, phenolsulphonephthalein, and tropaeolin, we had the opportunity of trying the effect of these substances on a tropical toad, *Bufo agua*. While engaged in this study our interest was aroused by the milky secretion which exudes from its "parotid" glands when the animal is greatly irritated. Scraping off some of the secretion with a knife, we were struck by the bluish-green discoloration which appeared on the blade soon after it had been used. This observation led us to test some of the diluted secretion with ferric chlorid. This reagent was found to develop the characteristic green color of the pyrocatechin reaction. As this reaction is given by the active principle of the suprarenal glands, further tests were at once undertaken with the object of identifying the substance in the toad's secretion which reacts with ferric chlorid as described. It was not a difficult task to demonstrate

[2] Faust, Die Thierischen Gifte. 1906.

[3] Barbour and Abel: This Journ., ii, 167, 1910.

that we were here dealing with a substance which is identical with, or closely allied to, the suprarenal principle. Further work on the secretion of the glands demonstrated the presence of a second body which, in respect to its pharmacologic action, is to be classed with substances belonging to the digitalis group of poisons.

The toad has been regarded from the earliest times as a venomous animal. In the Talmud[4] under the name of *tzab* (from the root meaning to swell or puff up) it is differentiated from the frog and is classed with animals whose touch contaminates. Various peoples have made medicinal use of the toad. The Chinese have long used as a remedy a preparation derived from toadskins which they call *senso*. According to a preliminary notice by Hyashi,[5] *senso* is an impure product similar in its action to digitalis, but fifty to one hundred times more powerful. Western nations also made use of the toad for medicinal purposes during several centuries and many European medical treatises and pharmacopœias of an earlier day give to the dried toad a prominent place among therapeutic agents. Thus, in the "Thesaurus Pharmakologicus" of Johannes Schröder, published in Leyden in 1672, and in the "Pharmacologia" of Samuel Dale, published in London in 1692, powdered toad is highly recommended for dropsy, bleeding of the nose and other ailments. In the "London Dispensatory" of 1702, edited by William Salmon, professor of physick, we read, "They (the toads) are hung up by the neck in the air till they are through dry, and then kept for use. Wierus saith that the poudre of a dry'd toad taken ʒss. at a time or more, cures almost incurable dropsies, carrying away the water by urine. I suppose the ashes of them burned is better." In an abridged text[6] of some of the medical writings of Michael Etmüller (1644–83), professor of medicine at Leipsic, it is stated that "living toads aroused to

[4] Hoffmann, D.: Das Buch Leviticus, Berlin, 1905, i, 342. Babylonian Talmud, Kethuboth 15 a; Mishna, Teharoth, v. 1.

[5] Deutsch. med. Wchnschr., 1911, No. 13, p. 624.

[6] Michaelis Etmulleri Opera in Compendium redacta, Londini, 1701, p. 147. In translating these passages we have omitted a few sentences from the paragraph in which they are found as being of less interest to the reader.

the point of fury are venomous, but found dead they are entirely devoid of poison. Transfixed (alive) in the month of July, dried, powdered (the head and entrails being removed) and administered in doses of twelve grains on alternate days they furnish an excellent cure for dropsy. Others administer this remedy in burning fever at its height. Powdered toad is also an effective remedy against incontinence of urine, and is said to be efficacious because of its anodyne character, while its volatile, penetrating salt acts as a diuretic. From it an anodyne oil is also prepared with the aid of sea-salt and sweet almonds."

Toads as remedial agents are further mentioned in the "Pharmacopeia Universalis" of R. James, M.D., London, 1747, in the "English Dispensatory" of John Quincy, M.D., London, 1749, and as late as 1833, but here in a skeptical way only, by J. A. Paris in his "Pharmacologia."

Further testimony that very powerful principles are found in toads is seen in the fact that primitive peoples have made use of their skin secretion as arrow poisons. Thus, Pagenstecher writes:[7]

> According to reports made by the botanist André, which have been substantiated by Saffray, the Choco Indians of the primeval forests of the Sierra Templada of New Granada at elevations up to 2000 meters employ for this purpose the secretion of a species of pelobates ("spade-footed" toad). The animal is placed in a tube of bamboo, the hands of the operator being protected with leaves, and when some of the poison is desired the tube containing the toad is suspended high over a fire. The toad soon becomes covered with a yellow juice which is allowed to drop into bowls from which it is transferred to small pots in which it gradually acquires the consistence of curari. A further supply of the poisons may later be obtained from the toads thus treated. The poison is smeared on to the tips of arrows which are shot into game from blowing tubes. A small stag is killed by a poisoned arrow in from two to four minutes, a jaguar in from four to eight minutes.

And in regard to the particular toad which is engaging our attention, Filho[8] makes the following statements:

[7] Pagenstecher: Allegemeine Zoologie, as cited by Kobert, Lehrbuch der Intoxikationen, Edition 2, 1906, ii, 470.

[8] Filho, Lacerdo, Algumas Experiencias com o veneno do Bufo ictericus (Spix), Archivos do Mus. Nacion. do Rio Janeiro, 1878, iii, 33.

There exists, more particularly in the regions of the Amazon a species which is a veritable giant among toads and which Spix has described under the name of *Bufo agua*, whose venom it would be worth while to study. It is very probable that this is the species from which the aborigines of the Amazon derive the poison with which they smear the points of their arrows, in place of a sort of curara which certain other tribes use.

Further details in regard to the use of the poison of *Bufo agua* have not yet come to our knowledge, but our own experiments with it give abundant proof that it would be very deadly indeed when used as an arrow poison.

Bufo agua (*horridus, maculiventris, marinus, humeralis, ornatus, ictericus, Lazarus, Rana marina, Bombinator horridus, Neotes, Pseudobufo* and *Docidophryne agua*) is the largest of the *Anura*, or tailless amphibia, attaining a length of 20 or more centimeters and a breadth of 12 cm. It is found in all of the countries and most of the islands of South and Central America, and in the warmer parts of Mexico.[9]

The specmens examined by us were obtained from the neighborhood of Montego Bay, Jamaica, where they are popularly known as bullfrogs.[10] The general color of the animal is light to dark brown with sooty dark patches and its back is covered with warty protuberances. A striking feature is two large oval, so-called "parotid glands" behind each ear. It is from these "glands" that our poison is obtained. According to Seeck,[11] these structures consist of simple, elongated glands closely packed together, the whole bearing a resemblance to a honeycomb. When the glands are hardened in alcohol their lumen is filled with a brownish compact mass.

[9] Brehm, A. C.: Thierleben, Allgemeine Kunde des Thierreichs, 1878, vii, 602; Waite, F. C.: Bufo Agua in the Bermudas, Science, 1901, xiii, 342. Gosse, P. H.: A Naturalist's Sojourn in Jamaica, London, 1851, p. 431.

[10] We are indebted to Prof. E. A. Andrews of this university for much assistance in obtaining a sufficient supply of these animals.

[11] Seeck, Oscar: Ueber die Hautdrüsen einiger Amphibien, Diss. Dorpat, 1891. See also a histological study by G. Calmels: Arch. de Physiol., 3d ser., I, p. 321, 1883.

Bristol and Bartelmez, in a short note in Science,[12] say of the poison glands of *Bufo agua:*

> The poison glands are found only on the upper surface of the body, while mucous glands are found all over the skin, and are crowded together in large parotoid "glands" behind each ear. They are much larger than the mucous glands and extend deep down into the compact corium layer. They are surrounded by a thin layer of loose connective tissue which contains nerve fibers and a dense net-work of capillaries. There is an almost continuous layer of smooth muscle fibers about the gland. The cells of the glandular epithelium develop to an enormous size, and when they mature they disintegrate, their entire plasm becoming the secretion, so that when a poison gland has reached its full development it is simply a reservoir of poison. When the poison is discharged the remains of the glands are resorbed, and at the same time one of the five or six undeveloped glands, grouped around the mouth of the functioning gland, grows down alongside the remains of the discharged gland, pushing it aside to occupy its former place.

When the animal is irritated as by the bite of a dog or by some other sufficiently powerful stimulus, mechanical, chemical or electric, the parotid glands exude a large amount of a creamy secretion having a pungent aromatic odor. The glands are certainly under the control of the central nervous system, as their secretion is discharged in consequence of a peripheral irritation of sufficient strength. Budgett[13] states that "the enormous parotid glands are discharged like squirts when the creature is roughly handled." We have not ourselves observed anything of this kind, though we have repeatedly "milked" as many as sixty of the animals at a time. Under chemical and mechanical stimulation we have only noticed free exudation from the openings of the gland. We obtained the needful amounts of this secretion by the simple device of squeezing (or "milking") the parotid glands with a curved hysterectomy forceps, catching the secretion as it spurted out from the numerous orifices into a large glass bowl held inverted over the toad. The semifluid substance thus

[12] Bristol and Bartelmez: Science, 1908, xxvii, 455.

[13] Quart. Jour. Microsc. Sc., 1899, xlii, 305.

obtained quickly dries in the air to form hard, brittle scales of a yellow color, presenting much similarity in appearance to dried snake venom. When the air-dried scales are brought into contact with water they swell up to gelatinous masses, and in the presence of much water an opalescent, neutral foamy emulsion is obtained of a most nauseating bitter taste and pungent odor. In its physical properties, the secretion presents great similarity to some other animal poisons, such as the Habu snake venom described by Ishizaka.[14]

Experiments made with the crude poison

A little of the emulsified secretion, well diluted with 0.8 per cent solution of sodium chlorid and instilled into the conjunctival sac of the dog or cat, will quickly cause an extreme constriction of its blood-vessels so that the conjunctiva becomes blanched. This action is due mainly to the epinephrin contained in the secretion, but in small part also to the digitalis-like body. When this latter substance, however, is applied to the eye in the form of a solution of the pure crystals it causes at first a momentary constriction of the smaller vessels of the conjunctiva, but this is soon followed by a marked dilatation, so that great injection and irritation result. No dilatation of the vessels of the eye is observed when only very dilute solutions of the crude poison are used, as in this case the action of the epinephrin preponderates.

A little of the emulsion injected into the abdominal lymph-sac of a pithed frog will soon cause slowing of the heart, with apparent prolongation of the diastolic pause and increase in the ventricular contractions. The final effect is complete stand-still of the heart in systole.

Little, if any, effect is noted when only a small quantity of the *dried* venom is given to an animal by mouth. Thus, a cat weighing 2.27 kg., received 0.035 gram concealed in meat. Not the slightest objective symptom was noted during the afternoon on which the animal was under observation. In giving the last

[14] Ishizaka: Ztschr. f. exp. Path. u. Therapie, 1907, iv, 88.

piece of meat with the venom concealed in it, however, a minute piece of the poison came into contact with the animal's mouth. The effect was remarkable in causing a profuse flow of foamy saliva. When the animal had ejected the last traces of the poison from its mouth, which was accomplished in a few minutes, it at once returned to its former state of quiet content. The absence of striking symptoms when even a considerable quantity of the crude venom is given by mouth is explicable when it is recalled that the digitalis-like substance of the venom is only slightly soluble in water and that the hard, dry scales of the crude poison require considerable time for their solution. It will be shown later that this principle is highly toxic when injected by itself, either subcutaneously or intravenously, so small a quantity as 1.5 mg. nearly causing the death of a very large cat.

Injected intravenously into a large cat, a quantity of the emulsion containing less than 0.020 gram of the crude dried venom induced a tremendous rise of blood-pressure followed immediately by a fall due to a sudden and complete stand-still of the heart.

When a larger quantity of the dried crude venom is introduced into the stomach more marked symptoms are produced. Thus, 0.1 gram of the poison enclosed in a capsule was given on an empty stomach to a dog weighing 5.8 kg. In ten minutes a clear bile-stained fluid containing most of the poison was ejected. This was followed by retching and repeated vomiting and profuse salivation, all of which continued at frequent intervals for twenty minutes. For an hour following, during which time the animal was kept under observation, it was much depressed. On the following morning it had quite recovered. In regard to this experiment we would say that the amount of epinephrin present in the venom given to this dog would alone suffice to induce vomiting if given on an empty stomach.

It may further be mentioned that an emulsion of the crude venom is a powerful and rapidly acting agglutinating agent[15] for the red corpuscles of the rabbit, the only animal's blood tested in this connection.

[15] Compare Fr. Pröscher on the haemolytic action of an extract obtained from the skin of two European toads, *Bombinator igneus* and *Bufo cinereus*. Beitr. z. chem. Physiol. u. Pathol., I, 575.

II. ON THE PRESENCE OF DIHYDROXY-METHYL-AMINO-ETHYLOL BENZENE, $C_6H_3(OH)_2.CHOH.CH_2NHCH_3$, IN THE SECRETION OF THE POISON GLANDS

Note by the senior author (A.)

Fortune has thrown in our way an animal secretion which most unexpectedly is found to contain a principle which has hitherto appeared only in the suprarenal glands and in homologous chromaphil structures. In collaboration with A. C. Crawford, I isolated the active principle of the suprarenal glands of sheep and beeves in the form of physiologically active salts which I named salts of epinephrin, for example, epinephrin bisulphate, picrate, etc. The elementary composition of several compounds was established by analysis and for epinephrin bisulphate, for example, was found to be represented by the formula,[16] $C_{17}H_{15}NO_4.H_2SO_4$.

In all of my published papers[17] of that period (1897–1899) it was constantly emphasized by me that epinephrin is an unstable, basic substance, preciptiable from solutions of its active salts by ammonia and capable of being separated from the other constituents of the suprarenal glands by the proper use of benzoyl chlorid.[18] And this at a time, I may perhaps be permitted to say, when von Fürth,[19] another investigator in this field, was upholding the supposition that epinephrin is either tetrahydrodioxypyridin, $C_5H_9NO_2$, or dihydrodioxypyridin, $C_5H_7NO_2$.

Later work[20] of mine showed that what I called epinephrin, and salts and compounds of epinephrin, had each and all retained one of the benzoyl radicles which I had purposely introduced into the molecule when I employed benzoyl chlorid as a precipitant. In consequence of the

[16] Abel and Crawford: Bull. Johns Hopkins Hosp., 1897, viii, 151. Abel: Ibid., 1898, ix, 215; Ztschr. f. physiol. Chem. (Hoppe-Seyler's), 1899, xxviii, 318; Bull. Johns Hopkins Hosp., 1901, xii, 339; Ibid., 1902, xiii, 31; Ber. d. deutsch. chem. Gesellsch., 1903, xxxvi, 1839; Ibid., 1904, xxxvii, 368; Am. Jour. Pharmacy, 1903, lxxv, 301; Contributions to Medical Research dedicated to V. C. Vaughan, 1903, pp. 138, 165; Jour. Biological Chemistry, 1905, i, 1.

[17] References are given in my papers to the discovery of Oliver and Schäfer and Szymonowicz and Cybulski, who first noted the presence of a vasoconstrictor principle in the suprarenal glands, as also to the earlier papers dealing with the chemical constituents of the glands.

[18] See Ztschr. f. physiol. Chem. (Hoppe-Seyler's,) xxviii, 218.

[19] Von Fürth: Ztschr. f. physiol. Chem. (Hoppe-Seyler's), 1898, xxiv, 142; 1898–1899, xxvi, 15.

[20] Abel: Bull. Johns Hopkins Hosp., 1901, xii, 339.

retention of a benzoyl radicle (*an unusual circumstance in work of this kind*) my salts showed a number of so-called alkaloidal reactions in addition to those which belong to the entirely unaltered native compound and which were likewise given by my active salts.

Following this work came the next step, the preparation of an amorphous indigo-colored iron compound of the active principle by von Fürth,[21] for which, however, at this time no analytic data were given from which a molecular formula could be calculated. Very soon after came the work of Takamine[22] and Aldrich[23] who succeeded in precipitating the base from concentrated extracts of the gland by the use of ammonia without the assistance of such agents as benzoyl chlorid or chlorid of iron. The formula $C_9H_{13}NO_3$, first proposed by Aldrich, has proved to be the one which truly represents the composition of our substance, but, as that chemist has remarked, "it is interesting to note in this connection that if we subtract a benzoyl residue from Abel's formula for epinephrin—$C_{17}H_{15}NO_4$—we obtain a formula—$C_{10}H_{10}NO_3$—which is not far removed from that of adrenalin." *Such a result is not due to chance and could have been obtained only from the study and analysis of chemical individuals which were fairly pure and which as was later shown had actually retained a single benzoyl radicle,* $C_6H_5.CO$.

Next came the brilliant researches of the chemists, Dakin, Jowett, Pauly, Friedmann, Stolz and Flächer which have finally culminated in the synthetic production, first of the racemic and now of the laevorotatory form as produced in the animal organism itself.

The Council on Pharmacy and Chemistry of the American Medical Association has lately adopted[24] the name epinephrin for the active principle of the suprarenal glands in preference to using a "protected" name and it is for this reason that we are using the word in this paper.

As already indicated the fine green color produced by the addition of ferric chlorid to a solution of the crude poison of our toad together with the fact that such a solution finally turns pink when exposed to the air and that it exerts a powerful vasoconstricting action sufficed to show that we had discovered a substance closely allied to, if not identical with the vasoconstrictor principle of the

[21] Von Fürth: Ztschr. f. physiol. Chem. (Hoppe-Seyler's), 1899-1900, xxix, 105.
[22] Takamine: Therap. Gaz., 1901, xxv, 221; Am. Jour. Pharm., 1901, lxxiii, 523.
[23] Aldrich: Am. Jour. Physiol., 1901, v, 457.
[24] Proprietary versus Unprotected Names, The Journal A. M. A., March 25, 1911, p. 910.

suprarenal glands. The results of additional tests made with solutions of the crude venom confirmed this conclusion. For example, the addition of ammonia or other alkali brought out a fine pink color which appeared instantly and was intensified by the addition of a trace of iodin or other oxidizing agent, and on boiling with Fehling's solution or with ammoniacal silver nitrate solution reduction of both solutions occurred promptly.

Two methods[25] of isolating the newly found substance were employed, only one of which will here be described. From 6 to 15 grams of the air-dried venom were rubbed up with water until a thin, foamy, opalescent solution, or rather emulsion, was obtained. This was repeatedly shaken with fresh quantities of a mixture of ether and chloroform (1 : 4) by which means the digitalis-like body was removed. The solution was then diluted with much water, whereon the mucoid and other contaminating substances were quickly removed by precipitation with basic lead acetate and the pinkish filtrate was *immediately* treated with hydrogen sulphid, freed from the precipitated sulphid of lead and concentrated to a small bulk under diminished pressure. The above operations from the moment of adding the solution of basic lead acetate to that of introducing the sulphuretted hydrogen must be carried on with great celerity, as otherwise there would be danger of injuring the epinephrin, since the filtrate unavoidably becomes slightly alkaline. Then too, the precipitation with basic lead acetate must be undertaken with highly dilute solutions of the venom only, as otherwise too large an amount of epinephrin will be retained in the precipitate of inert substances. When the filtrate, which has been freed from lead sulphid, has been sufficiently concentrated by distillation under diminished pressure it is only necessary to add ammonia to obtain the epinephrin in crystalline form. By dissolving in acetic acid, adding a small amount of sodium sulphite and again precipitating with ammonia, a very pure, ashless and almost colorless product is obtained.

[25] The other method was that elaborated by Abel for the isolation of epinephrin from the suprarenal glands of beeves. See Ber. d. deutsch. chem. gesell, xxxvi, p. 1841, 1903.

The results of combustion analyses with products obtained in this way are as follows:

I	II	III
Product Analyzed After Second Precipitation; No Sulphite Used	Product Analyzed After Third Precipitation: Sulphite Used	Theoretical Requirements for Formula, $C_9H_{13}NO_3$
C = 58.02	C = 58.68	C = 59.02
H = 6.87	H = 7.56	H = 7.10
N = 7.74; 7.88		N = 7.65

I. 0.1223 gram substance gave 0.2602 gram CO_2 and 0.0751 H_2O, or 58.02 per cent C and 6.87 per cent H.
0.2296 gram substance gave 16.05 cc. N at 20.5° C. under a barometric pressure of 738 mm. from which it is seen N = 7.74 per cent.
0.2082 gram substance gave 14.4 cc. N at 19.5° C under a barometric pressure of 758.5 mm. from which it is seen that N = 7.88 per cent.

II. 0.1060 gram substance gave 0.2282 gram CO_2 and 0.0721 gram H_2O, or 58.68 per cent C and 7.56 per cent H.

It will be seen that the analytical results here given, especially those obtained with the more highly purified preparation, stand in good agreement with the theoretical requirements for the accepted formula, $C_9H_{13}NO_3$, and prove that the elementary composition of the epinephrin from the toad is identical with that found in the suprarenal glands of the higher animals.

Like the principle obtained from the suprarenal glands, *Bufo* epinephrin turns the plane of polarized light to the left. Epinephrin (0.078 gram) obtained from our toads and three times crystallized, when dissolved in 8 cc. of water acidulated with acetic acid containing a trace of sodium sulphite, gave a polarimetric reading of $-1.00°$ in a 2 decimeter tube with sodium light, whence:

$$[\alpha]\frac{20°}{D} - 51.30°, \text{ the specific rotation.}$$

This rotation is in perfect agreement with that obtained by Flächer[26] ($-51.40°$) with the highly purified natural product, and differs from that obtained by Abderhalden and Guggenheim[27]

[26] Ztschr. f. physiol. Chem., 1908–1909, lviii, 189.
[27] Ztschr. f. physiol. Chem., 1908, lvii, 329.

for the synthetic levo-product (*l*-suprarenin) by the error of the instrument only.

Physiological action

In regard to its physiologic activity—we need only state that the *rapid* injection of so small a quantity as 0.000004 gram (1 cc. of a 0.0004 per cent solution) into the femoral vein of a dog weighing 6 kg. caused a rise of blood-pressure amounting to 20 mm. of mercury as measured in the femoral artery and that the injection of 0.000008 gram (2 cc. of a 0.0004 per cent solution) caused a rise of 26 mm. The physiologic activity of the substance is seen, therefore, to equal that[28] of the purest specimens of epinephrin that have been hitherto isolated.

It may also be stated that this epinephrin acts promptly as a vasoconstrictor when applied to the blood-vessels of *Bufo agua*, the toad from which it is derived. In other words, *the animal has not acquired an immunity against this poison;* small hemorrhages in this toad are as easily controlled by a local application as in other animals, and when a pithed toad is prefused with Locke's solution containing a small amount of the new epinephrin, its arterioles are found to be just as responsive to the drug as are those of the frog (*R. clamata or pipiens*).

A protocol of a perfusion experiment (Experiment 1) is here given in which it is shown that 0.0001 gram of bufo-epinephrin very quickly produces a marked constriction of the blood vessels of a pithed toad. It is further to be noted that the vasoconstriction is of considerable duration, the effects of a single injection into the perfusion tube lasting for nineteen minutes. Much smaller quantities of bufo-epinephrin than were used in Experiment 1 were found to be effective, thus so small a quantity as 0.000004 gram injected as above stated was still found to be effective, reducing the outflow from fifteen drops to eight drops in the minute. We have made no quantitative experiments to determine exactly the comparative sensitiveness of *Bufo agua* and *Rana clamata* to bufo-epinephrin but the above data prove that

[28] Schultz, W. H.: Bulls. 55 and 61, U. S Pub. Health and Marine Hosp. Service, Washington, D. C.

this toad is highly sensitive to the action of its vasoconstrictor principle. Fig. 1 gives a part of the tracing in illustration of the protocol of Experiment 1.

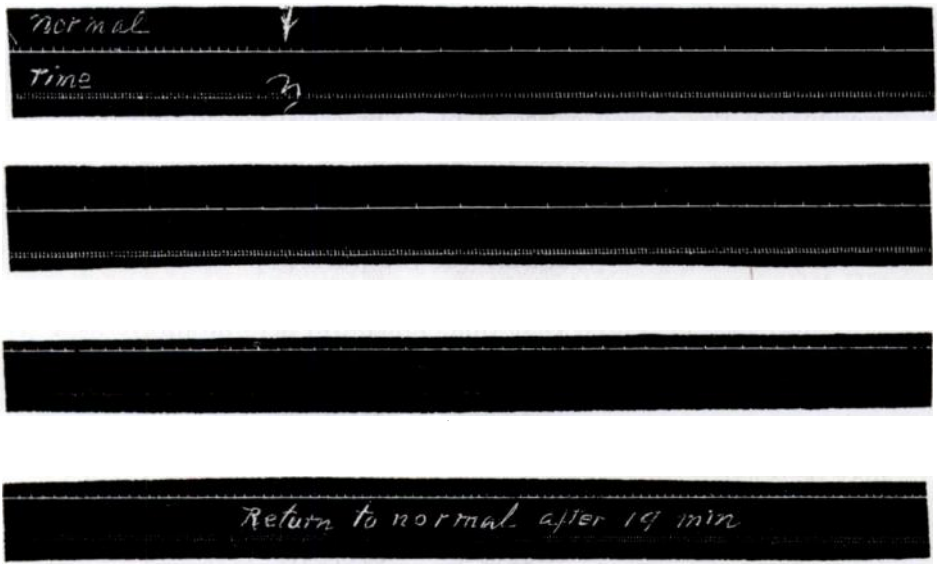


Fig. 1. Perfusion of blood-vessels of *B. agua* with Bufo-epinephrin at a pressure of 30 cm. Salt solution. Drop Record. Single injection of 1 cc. of a solution containing 0.0001 gm. Bufo-epinephrin injected at ↓ through the rubber tube close to the perfusion cannula in the aorta. Lower tracing in each part of the figure is the time curve (seconds).

Experiment 1. Perfusion of a pithed toad, *B. agua*, weighing 200 grams with bufo-epinephrin in 0.8 per cent sodium chloride solution under a pressure of 30 cm. The epinephrin solution (1 cc. = 0.0001 gram) was injected into the perfusion tube close to the aortic cannula. Automatic drop record.

Normal number of drops	14 in 30 seconds
30 seconds after injection	4 in 30 seconds
1 minute after injection	3 in 30 seconds
2 minutes after injection	3 in 30 seconds
3 minutes after injection	3 in 30 seconds
4 minutes after injection	4 in 30 seconds
5 minutes after injection	5 in 30 seconds
9 minutes after injection	8 in 30 seconds

11 minutes after injection	9 in 30 seconds
13 minutes after injection	10 in 30 seconds
15 minutes after injection	10 in 30 seconds
17 minutes after injection	11 in 30 seconds
19 minutes after injection	14 in 30 seconds

So, also, *dilatation of the pupil is as easily produced in the excised eye of the toad as in that of the frog.*

It is seen, therefore, that the results obtained by chemical analysis, by the use of the polarimeter, by quantitative and qualitative physiologic experiments have demonstrated conclusively that the substance isolated by us from the poison glands of Bufo agua is identical with the dihydroxy-methyl-amino-ethylol-benzene, $C_6H_3(OH)_2CHOH\ CH_2NHCH_3$, *produced by the suprarenal glands of the higher animals.*

Percentage amount of epinephrin contained in the venom

It is of interest to learn how large an amount of epinephrin may be isolated from a certain weight of the crude venom. Thus, from 5.42 grams of venom, weighed one hour after it was squeezed out of the glands, we obtained 0.243 gram of crystalline epinephrin, a yield of 4.48 per cent. On the assumption that we obtain by this method only about two-thirds of the epinephrin actually present in the crude venom the true content of epinephrin would be nearly 7 per cent (6.72 per cent). Judging from other experiments in which the yield was somewhat smaller, we conclude that the secretion probably varies in its percentage of epinephrin with the season of the year and other circumstances. But as the method of isolation is not, strictly speaking, a quantitative one, exact statements as to the variability of the epinephrin content cannot be made.

If we assume that the venom as secreted by the animal contains from 20 to 30 per cent of water there would still be present, in accordance with the above analysis, about 5 per cent of epinephrin. In comparison with this we would recall that one[29] of us estimated

[29] Abel: Am. Jour. Physiol., 1903, lxxv, 301. See also Reid Hunt: The Comparative Physiologic Activity of Some Commercial Suprarenal Preparations. The Journal A. M. A., Sept. 8, 1906, p. 790.

that the amount found in the suprarenal glands of beeves is 0.3 per cent.

The chromaphil reaction of the poison glands

The presence of this powerful reducing agent in the poison glands of *B. agua*, makes it almost certain on chemical grounds alone, that their cells will give the histologic reactions of the so-called chromaphil tissue. As is well known, this tissue consists of cell groups which assume a brown color when treated with chromic acid or dichromates in consequence of the reduction of these compounds to brown or reddish brown basic chromates. Such cell groups have hitherto been found only in the medulla of the suprarenal glands, in tissue of similar character lying alongside the abdominal aorta, in the carotid gland and in the sympathetic system.

Mr. E. B. Wood has undertaken the histologic study of the poison glands of *B. agua*, *B. lentiginosus*, and other toads with especial reference to their chromaphil reactions. His results will be published later but it may here be stated that the poison glands of *B. agua* (the only toad whose glands have been examined thus far) give the chromaphil reactions with great intensity. Chemical experiments are also in progress in this laboratory for the purpose of learning whether dihydroxy-methyl-amino-ethylol-benzene is produced in the skin glands of other toads and perhaps also in the skin of other amphibia.

III. ON BUFAGIN, A CRYSTALLINE COMPOUND AND METHODS FOR ITS ISOLATION AND PURIFICATION

It has already been intimated that the venom of *B. agua* owes its efficacy as an arrow poison in a large degree to the presence of a substance whose pharmacological action is similar to that of digitalis, although the large amount of epinephrin present is an important auxiliary in this action. And it is to the former constituent or to substances closely allied to it, such as have been described by Faust,[30] Phisalix and Bertrand[31] that toadskins owe

[30] Arch. f. exp. Pathol. u. Pharmakol., xlvii, 278, 1902; xlix, 1, 1903.

[31] Compt. rend. de l'Acad. des Sciences, Paris, *135*, 47, 1902.

their efficacy as a cure for dropsy, a method of treatment which would perhaps still be in vogue had not William Withering, in 1775, introduced for this very purpose the foxglove, the active constituent of an old wives' remedy. Now that science has begun to study the ancient remedy, toadskin, we may confidently expect further discoveries in this field, with results of undoubted value for practical medicine.

We have isolated the substance here referred to and having obtained it in crystalline form and given proof of its chemical purity and individuality, have named it bufagin in order to use a term sufficiently indicative of its origin.

It is not difficult to isolate bufagin. It is extracted from a solution of the crude venom in water[32] by shaking with a mixture of ether and chloroform as already stated and the solvents are removed by distillation until only a small bulk of fluid remains. This is then poured drop by drop with constant stirring into a large volume of petroleum ether which precipitates the bufagin as a white resin while holding the small amount of fatty substances in solution. The resinous precipitate is taken up in chloroform and again precipitated with much petroleum ether, whereon it may be crystallized from absolute alcohol. As bufagin is fairly soluble in this reagent crystallization can only be effected from hot concentrated solutions.

From this point on further purification is effected by recrystallization from alcohol and by repeated crystallization from hot water as will presently be described in detail.

It may here be remarked that in our earlier work we boiled the resinous precipitate (which is obtained by the use of petroleum ether as above described) for several hours with 2 per cent ammonia, in a flask attached to reflux condenser. The object of this treatment was of course the removal of acids or resinous substances of acid character (such as Faust's bufotalin) that might have been carried down in the petroleum ether precipitate. After pouring off the ammoniacal solution the brittle

[32] By the use of hot water the solution of the crude venom may be greatly facilitated. In this case the aromatic odor which always accompanies solution is greatly intensified. We have not yet succeeded in identifying the odoriferous body.

resin was dissolved in a very small quantity of chloroform, absolute alcohol containing hydrochloric acid was then added until the mixture contained 1 or 2 per cent of the acid when it was poured into a large quantity of water. In a short time the bufagin had settled to the bottom and sides of the beaker as a resinous precipitate which later changed into masses of crystals. This treatment was necessary in order to remove the last traces of ammonia from the resin. After this treatment the substance was repeatedly crystallized from alcohol and water. As it was found on analysis that nothing was gained by boiling either the petroleum-ether precipitate or the first crystals from alcohol with dilute solutions of ammonia as above described, these steps were later omitted.

The method of isolating bufagin above described in which the crude venom was dissolved in water and then shaken with organic solvents was useful as long as it was desired to recover the epinephrin contained in this portion. When it is not desired to isolate the epinephrin one may proceed as follows: The venom is squeezed from the glands as described, allowed to dry in the air, ground to a fine powder in a closed mill and is then extracted with absolute alcohol until this solvent no longer takes up an appreciable amount of the material. The alcoholic extract is then freed of alcohol and water by distillation under diminished pressure, the residue is repeatedly extracted with chloroform, the chloroform extracts are reduced in bulk by evaporation and then poured drop by drop into a larger volume of petroleum ether. The resinous precipitate which now falls out is again taken up in a small quantity of chloroform and this is again poured into an excess of petroleum ether. The nearly white resinous precipitate now obtained often turns into a crystalline mass in the course of a few days if allowed to stand under the precipitating fluid. The substance is then crystallized twice out of hot alcohol, for which concentrated solutions are required. At this stage the compound has already attained a high degree of purity. For analytical and therapeutic purposes we have, however, proceeded further. The substance is again dissolved in absolute alcohol (1 gram to 80 or 100 cc. alcohol) and this solution is poured drop by drop into a litre of water kept close to the boiling point and well stirred.

The clear aqueous solution is set aside to crystallize. Very soon glittering clusters of whetstone shaped or acicular crystals and prisms begin to appear on the sides and bottom of the containing vessel. A second crop of crystals may be obtained by concentrating the filtrate from the first crop. As thus obtained bufagin may be further purified by a repetition of the above process, or it may again be crystallized from alcohol and then again from water. It will thus be seen that it is not difficult to isolate bufagin or to obtain it in any desired degree of purity.

IV. CHEMICAL PROPERTIES OF BUFAGIN

As thus isolated, bufagin is found to contain no nitrogen and to consist only of the elements, carbon, hydrogen and oxygen. Its melting point[23] is 217–218° C. Although bufagin contains neither water nor alcohol of crystallization its crystals lose their lustre after long standing becoming entirely opaque in consequence of a molecular rearrangement. The substance is easily soluble in chloroform and acetone, fairly soluble in absolute alcohol, but only very little soluble in petroleum ether, carbon disulphide or carbon tetrachlorid and also but little soluble in cold benzene. In glacial acetic acid or in acetic anhydride it dissolves with great ease. It is a "neutral" compound insoluble in aqueous solutions of alkalis or acids. It may be sharply differentiated from cholesterin by the use of the following color reactions:

Color reactions

1. Dissolve a small quantity of dry bufagin in a test tube in chlofororm and stratify under this solution an equal volume of concentrated sulphuric acid. At the line of contact between the two fluids a deep red color will soon develop, but only in the sulphuric acid and when the two fluids are well mixed by shaking and again allowed to separate it will be found that the sulphuric acid has taken up all of the color and is highly fluorescent *while the chloroform remains entirely colorless. Cholesterin* (Sal-

[23] By an error the melting point was given as 188° in our preliminary paper. Jour. Med. Assoc., vol. lvi, 1531, 1911.

kowski's Reaction) *as is well known will cause the chloroform to take on a fine red or pink color* while the sulphuric acid behaves as in the reaction just described. A simple way of showing the difference between the two substances is to dissolve equal quantities in separate test tubes in equal volumes of chloroform, add one drop of concentrated sulphuric acid to each tube and shake well. The tube containing the bufagin remains colorless while that containing the cholesterin at once takes on a carmine red color.

2. Dissolve a small quantity of bufagin in a test tube in fluid trichloracetic acid and heat on the water bath for a few seconds. A beautiful green color at once develops. Under the same conditions (Hirschsohn's Reaction[34]) cholesterin gives first a fine pink, then red, then a plum color. Both solutions develop fluorescence on standing for a short time. If the trichloracetic has been freshly prepared so that but little hydrochloric acid has been evolved, it may be well to add a trace of this acid as otherwise the reaction will not develop so promptly as above described.

3. Dissolve a small quantity of bufagin in a test tube in 1 or 2 cc. of acetic anhydride, then add one drop of concentrated sulphuric acid and shake when a pure green color developes immediately. Cholesterin *in the same quantity* treated in the same manner gives immediately *first* a pink or red color, then a blue or purple and only much later a green color. (Liebermann-Burchard Reaction[35]). A cholesterin derivative, dihydro-cholesterin (β-cholestanol, Diels) prepared by us according to the method of Willstaetter and Mayer[36] gives the above reaction in the same manner as bufagin.

4. Dissolve a small quantity of bufagin in a test tube in $\frac{1}{2}$ cc. of glacial acetic acid add $\frac{1}{2}$ or 1 cc. of acetyl chloride and a few granules of fused zinc chloride and warm very gently for a moment over a small flame. In a few seconds color tints appear which pass rapidly into a fine violet which soon gives place to a rich plum color. This again passes into the end stage of the

[34] Pharm. Centralhalle, xliii, 357, cited from W. Glikin in Handb. der Biochemie i, p. 133, 1909.

[35] Ber d. deutsch. chem. Gesellsch., xviii, 1804, 1885.

[36] Ibid., xli, 2199, 1908.

reaction—a deep green with brownish fluorescence, though it may be necessary to apply a little more heat or to add a very little glacial acetic acid according to circumstances, in order to develop this end stage of the reaction. With cholesterin (Tschugaeff's Reaction[37]) in place of bufagin a fine cherry red with an eosin-like fluorescence is obtained. The presence of water in the reagents used in this reaction is detrimental to the success of the reaction but much less so with bufagin than with cholesterin. The reaction is one of the most sensitive of the color reactions described and differentiates bufagin sharply from cholesterin.

5. As might be expected, the reaction of Denigès[38] which is a combination of the reactions of Salkowski and Liebermann gives a negative result with bufagin. As described under 1, so here too with bufagin the chloroform layer remains entirely colorless, while with cholesterin it assumes a brilliant-red color.

Other reactions were not tried, but the above suffice to show how *differently* bufagin behaves from cholesterin when treated as described.

Bufagin differs from cholesterin not only in its response to color reactions, as shown above, but also in its influence on the plane of polarized light, being dextrorotatory while cholesterin is laevorotatory.

A specimen of Product III, the analytic data for which are given below, weighing 0.160 gram, dissolved in 8 cc. of chloroform gave a polarimetric reading of + 0.44° in a 2 decimeter tube with sodium light from which it is seen that the specific rotatory power of the compound is $[\alpha]_D^{24^\circ} = +11^\circ$

Analyses

Combustion analyses show that the elementary composition of bufagin can only be expressed in the formula, $C_9H_{12}O_2$, or in terms of a multiple of this formula, such as $C_{18}H_{24}O_4$. The following tables give some of our analytical results, with specimens dried over sulphuric acid and not heated.

[37] Zt. f. angew. Chem., Nr. 25, 1900. Cited from Hdb. d. Bioch. I. 133.

[38] See, W. Glikin in Oppenheimer's Hdb. d. Biochemie, i, p. 133.

I	II
Obtained directly from venom, but crystallized from dilute solution in petrol ether + chloroform without any other process of purification. C = 70.84 H = 8.67	Product obtained by crystallization from alcohol after a preliminary process of purification. C = 70.98 H = 8.55
III	**IV**
Product same as in II, only was further purified by crystallization from water. C = 71.07 | 70.92 H = 8.28 | 8.25	Theoretical requirements for the formula, $C_9H_{12}O_2$ or for $C_{18}H_{24}O_4 = (C_9H_{12}O_2)_2$ C = 71.01 H = 7.95

It will be seen that our analytical results, especially those obtained with the pure Product III are in perfect agreement with the elementary formula, $C_9H_{12}O_2$ or with some multiple of it as $C_{18}H_{24}O_4$. This Product III was twice crystallized from absolute alcohol and twice from hot water in addition to having undergone the preliminary precipitations with petroleum ether for the removal of fats and cholesterin and also the rather needless treatment with ammonia previously described.

I. 0.0891 gram substance taken gave 0.2311 gram CO_2 and 0.0695 gram H_2O, or 70.74 per cent C. and 8.67 per cent H.

II. 0.1021 gram substance taken gave 0.2668 gram CO_2 and 0.0790 gram H_2O, or 70.98 per cent C and 8.55 per cent H.

III. 0.1812 gram substance taken gave 0.4722 gram CO_2 and 0.1351 gram H_2O, or 71.07 per cent C and 8.28 per cent H.
0.1694 gram substance gave 0.4405 gram CO_2 and 0.1257 gram H_2O, or 70.92 per cent C and 8.25 per cent H.

The material used in the above analyses was dried at room temperature *in vacuo* over sulphuric acid. It appears not to contain either water of crystallization or alcohol of crystallization and can not be dried at high temperatures without undergoing change. When the compound which has been dried to constancy of weight *in vacuo* at room temperature is heated for one hour at a temperature varying from 100–115° C. in a current of dry hydrogen it will lose 4.24 to 4.32 per cent of its weight[39]

[39] The material used in these experiments had been crystallized from alcohol only and had a slightly larger content of hydrogen than is demanded by the formula $C_{18}H_{24}O_4$. It is possible that material crystallized from water would not show this loss.

in this time. In one experiment of this kind an attempt was made to collect the products given off to the current of hydrogen. The drying temperature was maintained at 96–99° C. for an hour except for a few moments when it reached 107°. The products given off were passed through a mixture of solid CO_2 and ether whose temperature was about −80° C. 0.1388 gram substance lost 0.006 gram in the hour, or 4.32 per cent, but of this amount only 0.0016 gram (1.15 per cent) was retained by the cooling mixture. In other experiments carbon dioxide was identified as one of the products given off. It is apparent therefore that bufagin undergoes a slight decomposition when heated to 100° C. or higher in a current of hydrogen.

Behavior of Bufagin toward Bromine

The behavior of bufagin toward bromine is different from that shown by cholesterin, which compound, by virtue of its unsatisfied affinities, readily absorbs bromine, forming with it a colorless crystalline di-bromide. If 50 mgs. of bufagin be dissolved in 1 or 2 cc. of chloroform or carbon disulphide and a solution of bromine in one of these solvents added, not the least diminution in the intensity of the color is to be noted. After standing for an hour, especially if an excess of bromine is present, a part of the bufagin may fall out in the form of a dark brown resin. This resin, which holds bromine in combination, is apparently a substitution product, but we have not yet been able to obtain it in crystalline form. That hydrobromic acid is produced in the reaction above described is easily demonstrated, and we may therefore conclude that bufagin does not contain *an unsaturated carbon linkage* and that bromine acts upon it only to form a brown substitution product of resinous character.

Behavior toward Platinum-black and Hydrogen[40]

Eleven one-hundredths gram of bufagin was dissolved in much ether, about 2 grams of freshly prepared platinum-black were added and a slow current of hydrogen was passed through the ethereal solution for forty-eight hours. The resulting product

[40] See Willstaeter u. Mayer, Ber. d. deutsch. chem. Gesellsch., xli, 2199, 1908.

was a white resin which differed markedly from bufagin in that it could no longer be crystallized from hot water; it now behaved more like cholesterin than bufagin, appearing in water as a colloidal suspension. Its behavior toward such of the color reactions as were tried was also different from that of bufagin. Thus, Hirschsohn's reagent and also acetic anhydride and concentrated sulphuric acid now gave the same play of colors as appear when cholesterin is treated with these reagents. We think it very likely that oxygen was removed in the course of the long reduction and that a substance was thus formed whose properties are more like those of cholesterin than of bufagin. This reduced bufagin was also found to be non-toxic, 0.012 gm. given subcutaneously having no observable effect on a dog weighing 4 kg.

Reaction with Phosphorus-pentachloride

Bufagin reacts as easily with phosphorus-pentachloride as does cholesterin and yields what is apparently a crystalline product corresponding to cholesteryl-chloride and which may be called bufagyl-chloride. A description and analyses of this and other derivatives which we hope to prepare must be reserved for a future paper.

Determination of the molecular weight

Since the publication of our preliminary paper molecular weight determinations of bufagin have been very kindly made for us by Professor Harry Jones of this University and by Dr. George Barger of the Wellcome Research Laboratories. Professor Jones' determination was made by the boiling point method with chloroform as the solvent and the data and results obtained are as follows: 1.5368 grams of bufagin crystallized from water (melting point, 218° C.) raised the boiling point of 90.338 grams of chloroform 0.212° C., from which the molecular weight is calculated to be 294. The molecular weight called for by the formula $C_{18}H_{24}O_4$, which, as has been shown, expresses the results of our elementary analyses, is 304.

Dr. Barger's determinations were made by his microscopical method.[41] A determination in chloroform gave a molecular

[41] Trans. Chem. Soc., lxxxviii, 286, 1904.

weight of 382. Inasmuch as substances which contain one or more hydroxyl groups are associated in this solvent, especially when it is used at room temperature this high figure is explainable. With pyridine in which solvent association never occurs, according to Barger, the determination gave a molecular weight of 288. The data for this determination are as follows: 0.0120 grams bufagin was dissolved in 0.49 gram pyridine, equivalent to a molar concentration of 0.08 to 0.09. The determination gave the molecular weight 288, a result which stands in good agreement with the theoretical requirement 304.

We have here the determinations of two independent observers, made by different methods and with different solvents, which are in close agreement with each other and from which we may conclude that the formula of bufagin is $C_{18}H_{24}O_4$.

Possible relationship to Cholesterin

One is tempted to assume that bufagin is intimately related to cholesterin when one recalls the behavior of the substance toward the cholesterin color reactions as already described. It must be borne in mind, however, that a number of resin acids, terpenes, and unsaturated alcohols of high molecular weight give very similar color reactions, and in this connection it may be stated that cholesterin is declared by Windaus (Arch. der Pharmacie, ccxlvi, 147, 1908) to be without doubt itself a complex terpene. No known derivative of cholesterin has the formula of bufagin, $C_{18}H_{24}O_4$, and none of the numerous oxidation products of cholesterin which have been studied exhibit the pharmacological properties of bufagin. For the present, therefore, no definite statements can be made in regard to the relationship of bufagin and cholesterin.

Faust[42] and his pupil Flury[43] have shown that a number of acidulous oxidation-products of cholesterin, $C_{27}H_{44}O_4$, $C_{27}H_{40}O_5$, and $C_{27}H_{40}O_8$ which were prepared by Windaus are pharmacologically active substances, but the behavior of these acids is such

[42] See works of Faust already cited and monograph, "Ueber das Crotalotoxin," etc., Leipzig, 1911.

[43] Arch. f. Exp. Pathol. u. Pharmakol., lxvi, 221, 1911.

that they must be classed with the biliary acids and the saponines, rather than with bufagin.

Lifschütz[44] maintains that the blood and certain other organs of higher animals contain oxycholesterins, substances which represent the first stages in the physiological oxidation of cholesterin. The liver contains only mere traces of these oxycholesterins and this author assumes that they are changed into bile acids in this organ. The evidence for the existence of these oxycholesterins of Lifschütz in the animal economy rests entirely on spectroscopic observations made upon certain extracts treated with glacial acetic and concentrated sulphuric acids. We may well grant the existence of oxycholesterins in the animal organism *but it should be noted that they have not yet been isolated as chemical individuals of even approximate purity,*[45] and that we are entirely ignorant in regard to their physiological significance.

A brief consideration of the earlier chemical investigations on toad venom

As far as we have been able to learn, ours is the first chemical study to be made of the venom of *Bufo agua;* during the last fifty years, however, repeated attempts have been made to isolate the active principle or principles of the skin secretion of *Bufo vulgaris* and *Bufo viridis,* species widely distributed in Europe. Some account of these earlier investigations will be found in the comprehensive monograph on Animal Poisons of E. S. Faust. This author, Phisalix and Bertrand[46] are the only investigators known to us who have made chemical studies in the past decade of the venom of *Bufo vulgaris,* as obtained either from the whole skin (Faust) or from the secretion of the parotid glands (Phisalix and Bertrand).

According to Faust[47] the chief poison of the common toad is an amorphous, resinous substance—of acidulous character, readily soluble in aqueous solutions of the alkalies, and endorsed with the

[44] Zeitschr. f. physiol. Chem., lviii, 175, 1908; lxiii, 222, 1909. Ber. d. deutsch, chem. Gesellsch., xliv, 252, 1908.

[45] See criticism by A. Windaus, Arch. der Pharmacie, ccxlvi, 148, 1908.

[46] Compt. rend de l'Acad. des Sc., Paris, cxxxv, 46, 1902.

[47] Archiv. f. Exp. Pathol. u. Pharmakol, xlvii, 278, 1902; and xlix 1, 1903.

pharmacological activity of the digitalis group. This resin was named by Faust bufotalin and to it he ascribes the molecular formula $C_{34}H_{46}O_{10}$.

He describes a second substance, pharmacologically similar to bufotalin but weaker in its action, as crystalline and having the formula $C_{34}H_{54}O_2$. He names this second substance bufonin and suggests that it is the mother substance of bufotalin which is derived from it in the body of the toad by oxidative processes. Both substances are thought by him to be derivatives of cholesterin.

Phisalix and Bertrand, who used the expressed contents of the parotid glands, declare that the venom contains but one poison of the digitalis type, not two as maintained by Faust, and they find that it is a noncrystalline resin of neutral properties, not acidulous as is bufotalin. They accepted Faust's name bufotalin for their neutral resin, contending, however, that Faust's form of it was contaminated with substances of acid character derived from the skin of the toad. To their bufotalin they ascribed the formula $C_{119}H_{117}O_{25}$.

As to Faust's second substance bufonin, Bertrand[48] maintains that it is ordinary cholesterin slightly contaminated with the neutral bufotalin of Phisalix and Bertrand, a contamination sufficient to give it a slight degree of digitalis-like action. These authors also assume on physiological grounds the presence of a second poison which they define as a paralysant of the central nervous system. They did not isolate this assumed poison but named it bufoténine. Faust on repeating their work was unable to obtain a substance of this character.

It will therefore be seen that the true nature of the digitalis-like principle of the common-toad of Europe is a matter of controversy. According to Faust it is a resin of acidulous properties according to Phisalix and Bertrand a neutral resin.

In view of our isolation of the crystalline compound bufagin and the possible relationships suggested by the work of the authors above cited, a reëxamination of the venom of *Bufo vulgaris* and *Bufo viridis* would be highly desirable.

[48] Compt. rend. de l'Acad. des Sc., cxxxv, 49, 102.

V. PHARMACOLOGICAL ACTION OF BUFAGIN

It is not our purpose to give a complete account of the numerous pharmacological studies that have been made up to the present time with the venom of the common toad of Europe or with crude extracts and active principles of doubtful purity derived from it. In several particulars the pharmacological investigations of the earlier writers have not been improved upon to this day, as may be seen for example, by reading Fornara's (1877) account of the action of the venom upon the exposed heart in various species of animals. A more complete analysis of the pharmacological action of the venom is given only by investigators of recent date.

The pharmacological studies of E. S. Faust[49] were made with his bufotalin and bufonin and are concerned chiefly with the action of these substances upon the circulatory apparatus. It was shown that these principles act like bodies of the digitalis series and in this respect they reproduce the action of the crude venom and agree with the action of the principle obtained by us from the tropical toad, *Bufo agua*.

From the pharmacological point of view the investigations of the Russian pharmacologist N. P. Kravkov[50] are also of importance.

This investigator made use of an alcoholic extract of the expressed contents of the parotoid glands of *Bufo Viridis* and *Bufo Vulgaris* and his material, therefore, would be much like the crude resin (bufotaline) of Phisalix and Bertrand, and therefore can not claim to represent a single chemical individual. This work is nevertheless interesting and important, and we find that as far as it goes it stands in close agreement with the pharmacological effects noted by us in the use of pure bufagin. Thus, the fatal dose of his poison for a dog, 0.7 mg. per kilo of body weight is practically the same as that of our drug; and his experiments on the action of the venom on the blood pressure of intact and decerebrate dogs, on the isolated kidney and on diuresis all reveal in general the same physiological action as is observed with bufagin.

[49] Arch. f. Exp. Pathol. u. Pharmakol., xlvii, 278, 1902.
[50] Russki. Vrach, iii, 761, 1904.

A. Action of Bufagin on cold-blooded animals.

a. Experiments on the frog and terrapin. Doses of bufagin from varying 0.1 to 0.3 mg. injected into small frogs (*R. clamata*) varying in weight from 20 to 30 grams, produce few if any symptoms. If a dose of 0.5 mg. is injected there is at first no effect. In about an hour after the injection, however, the frog seems to have partially lost its reflexes and moves about as if its legs were stiff. This condition lasts an hour or two, gradually wearing off in this time. The minimum lethal dose for a frog is about 1 mg. per 20 grams of body weight and the effect of such a dose is illustrated by the following protocol.

Experiment 2. Frog, *Rana clamata*, 20 grams.

1.40 p.m. Injected 1 mg. of Bufagin in 2 cc. alcoholic solution (5 per cent alcohol). An equal amount of an alcoholic solution of the same strength was injected under the skin of a control frog.

2.00 p.m. Frog a little stiff in its legs.

2.15 Frog partially paralyzed, lies with its belly touching the table; cannot jump; when placed on its back turns over with great difficulty. Reflexes have disappeared.

2.30 Rests completely flat on table, cannot move or turn over when placed on its back. Slight twitching of abdominal muscles.

The frog is now pithed, its brain and spinal cord destroyed and abdomen opened, the abdominal veins are seen engorged with blood. The heart on being exposed shows the ventricle to be pale, it does not expand much in diastole and the auricles are much dilated and engorged with blood. Heart rate fourteen per minute.

2.40 Pericardium opened, ventricle pale; beats are slow and powerful, twelve a minute.

2.45 Heart beat eight a minute.

4.00 Heart again assumes normal rate, ventricle dilates and becomes well-filled during diastole, heart is recovering. A control experiment made with an equivalent quantity of alcohol gave no such effect.

In the above experiment we see the characteristic action of the drug, namely, a gradual slowing of the heart with an increase of its tonicity and in the force of its beat. In the above case, as far as can be judged by inspection, this increase in tonicity did not go

as far as a final arrest in systole, but a slightly larger dose would have produced that effect.

The same action of the drug may be observed on pithing the frog, opening its pericardium and exposing the heart, and introducing at the same time a few drops of a solution of bufagin into the opened pericardial sac. The action on the excised frog's heart is further illustrated graphically by a suspension experiment (Fig. 2). The tracing shows a marked increase in tonicity, gradual slowing of the beat, and final standstill with systolic contraction.

The effect of bufagin on the terrapin's heart, as far as studied, is exactly the same as on the frog.

We have also studied the action of the drug on the frog's blood-vessels by means of perfusion experiments and find that it has a decided action on the plain muscle of the arteries. Aerated Locke's solution under a pressure of 30 cm. was used as the perfusing fluid and at a given time a small quantity of fluid (2 cc.) containing 0.00044 gram bufagin was injected through the rubber tube close to the perfusing tube. Before the injection the number of drops of outflow from the cannula in the *sinus venosus* was nine in twenty seconds. Twenty seconds after the injection it had fallen to six in twenty seconds, one minute later it was four drops and five minutes later three drops in twenty seconds. This marked vaso-constrictor effect continued undiminished for ten minutes longer.

b. Experiments on the toad, Bufo agua. That the common toad of Europe is relatively immune to its own venom as well as to the various members of the digitalis series of cardiac poisons has been known since the time of Vulpian[51] and has been confirmed by more recent writers.

Fühner,[52] although admitting the high resistance of the common

[51] Vulpian: Compt. rend. Soc. de Biol. 2nd Series 1854, i, 133; 2nd Series 1856, iii, 125; Fornara; Rivista clinica di Bologna, 2nd Series, iii, 299, 1873; Kobert, Arch. f. Exp. Path. u. Pharmakol, xxii, 104, 1887; Honda: Arch. internat. de Pharmacodyn. et de. Thérapie, ix, 431. 1901; Heuser: ibid., x, 483, 1902.

[52] Arch. f. exp. Pathol. exp. u. Pharmakol. lxiii, 374, 1910.

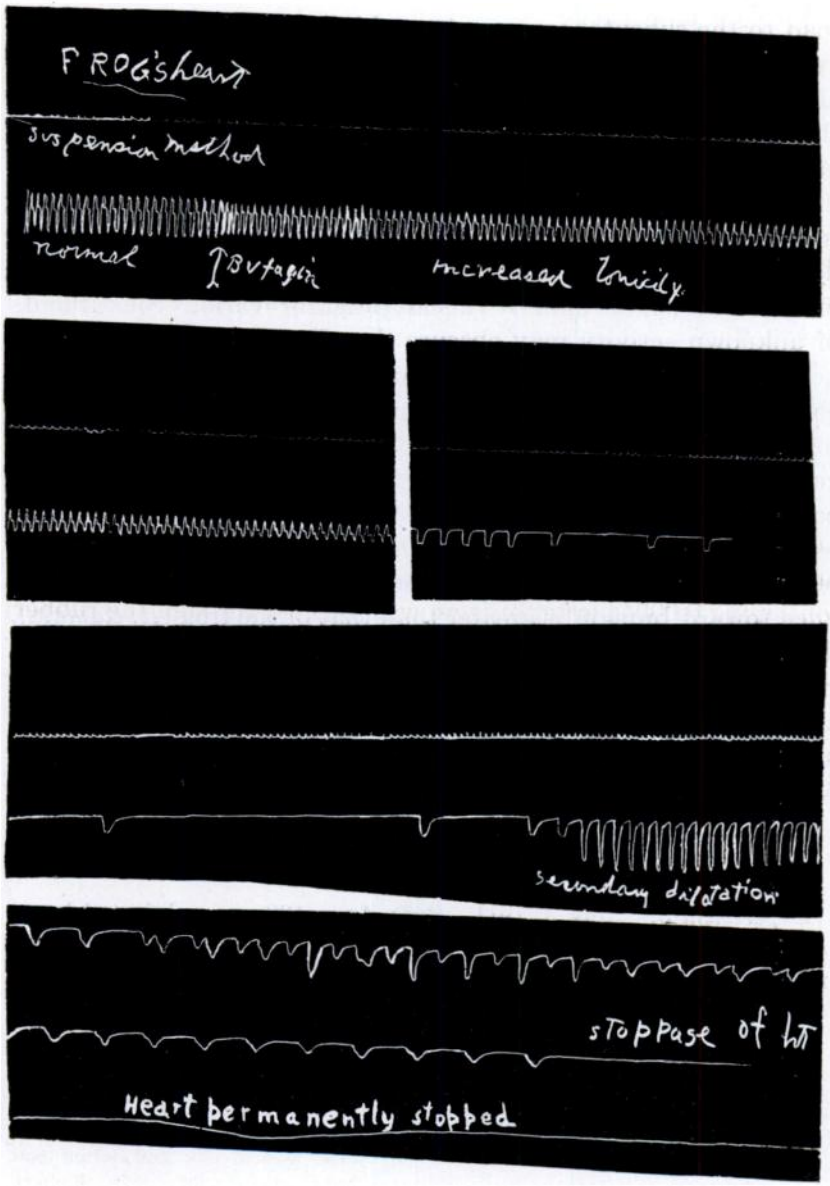


Fig. 2. Action of bufagin on excised frog's heart. Suspension method. Aerated Locke's solution, (at ↑) replaced by Locke's solution, 25 cc., containing about 0.002 gm. bufagin. Upstroke = systole, upper tracing = time in seconds, except in lowermost part of the figure in which the time curve is lacking.

toad to the subcutaneous or stomachic administration of its own venom (Vulpian), concludes from his own experiments made with an aqueous extract of the entire skin of the toad that the heart of this animal is by no means immune to the venom. The extract used by Führer, as is especially pointed out by him *very quickly* induced standstill and systolic contracture of the heart of the frog and of the toad, though the contracture was somewhat less developed in the toad than in the frog. In the use of an aqueous extract such as was used by this investigator various constituents of unknown activity may obscure the effects of the true systolic heart poison. As will be seen from our experiments with bufagin the pure drug does not cause an immediate systolic arrest of the heart of the toad but only slowly alters its rhythm, tonicity and conductivity and finally leads to a perfectly developed systolic contracture.

Our experiments have shown that while *Bufo agua* is not in the least immune to the epinephrin contained in its venom, its resistance toward bufagin is greater than that of the frog (*R. clamata*). That this toad is only relatively immune to this systolic heart poison contained in its venom may be seen from the protocols of the following experiments. An injection of 1.5 mg. has no observable effect but an injection of 8 mg. has a depressant action from which the animal soon recovers.

Effect of Bufagin on Bufo Agua

Experiment 3. May 3, 1911 *Bufa Agua* 100 grams injected intraperitoneally with 2.5 cc. of solution of Bufagin (= 1.5 mg). No effect noted.

May 4 Alive and well; but seems a little sluggish in its movements.
May 7 Alive and normal.

Experiment 4. December 4, 1911, 2.30 p.m. *Bufo Agua* 225 grams. Injected intraperitoneally with 10 cc. = (8 mg. bufagin) of 5 per cent alcohol solution.

2.40 Is partially paralyzed; lies with its belly touching the table.
3.45 Stretches legs out more sluggishly than usual.
4.00 Animal apparently normal. All that can be noted is a little

slowness in movements. Otherwise, it responds to stimuli, turns over when placed on back, and hops as usual. Skin is very *moist*, much more so than at the beginning of the experiment.

December 5 Animal has recovered and is in normal condition. A control experiment was made with an equivalent amount of 5 per cent alcohol. The toad was slightly depressed for half an hour after injection, but soon recovered and was in normal condition.

Experiment 5. March 21, 1911 *Bufo Agua* 120 grams, pithed, chest opened and heart exposed. Rate of heart-beat, fifteen in fifteen seconds.

12.30 Applied to heart 0.1 cc. of bufagin solution (1 cc. = 0.75 mg.).

One minute later rate of heart-beat, six in fifteen seconds; five minutes later rate of heart-beat, ten in fifteen seconds.

2.30 Injected into aorta 0.3 cc. of bufagin solution (1 cc. = 0.75 mg). Rate becomes eight in fifteen seconds; contractions of heart are markedly more powerful.

2.45 Injected into aorta 1 cc. of same solution.

2.50 Rate is eight in fifteen seconds.

3.30 No change.

As far as the action of bufagin on the heart of *Bufo agua* is concerned, our experiments show that the direct application of quantities varying from 0.075 to 0.25 mg. cause a distinct slowing of the heart beat which is, however, of short duration only. When the action of the drug on the excised heart of the toad is studied by the suspension method (ventricle) it is seen that the tonicity of the heart is greatly increased and its rate of beat lessened.

Finally a most interesting phenomenon was observed which was never seen in the frog. Periods of absolute inhibition of the ventricle alternate with periods of action (see Fig. 3). These alternating periods of inhibition and activity of the ventricular rythm are approximately of equal duration. During the periods of inhibition of the ventricle, the auricles are seen to beat ineffectively and slowly and it is for this reason that the condition may be described as one of heart block due to lack of transmission of impulses in the bundle of His. Studies of the ventricular output of the heart of the toad and frog were not undertaken, as it was planned to make such studies on the warm-blooded heart.

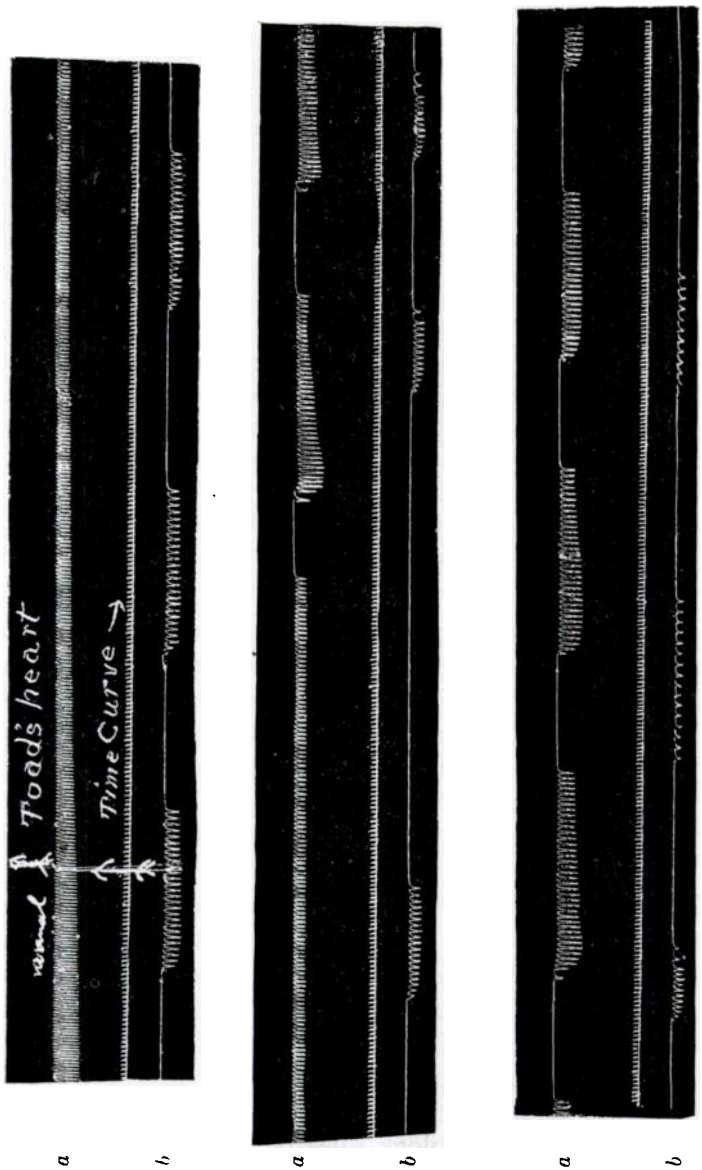


Fig. 3. Action of bufagin on excised heart of *Bufo agua*. Suspension method. Upstroke = systole. Aerated Locke's solution replaced at ↑ by Locke's solution containing 0.002 gram bufagin.

The action of bufagin on the walls of the blood vessels of *B. agua* does not seem to differ in any way from its action on those of the frog, unless it be that the effect of small doses is more marked in the latter. On perfusing the toad with a solution of bufagin, a marked vaso-constrictor effect is observed, as may be seen by a study of the protocol of Experiment 6. Fig. 4 contains a portion of the graphic record of this experiment. The drug exerts a similar action on the plain muscle fibers of the stomach of *B. agua* causing them to contract (Fig. 5).

Experiment 6. January 30 and 31, 1911 Perfusion of *Bufo agua* with bufagin. Perfusing cannula in aorta, outflow cannula in sinus venosus. Locke's solution perfused under a pressure of 35 cm. At stated times solution of bufagin is injected through the wall of the rubber tubing close to the aortic cannula.

January 30, 1910

TIME	NUMBER OF DROPS IN TWENTY SECONDS	REMARKS
3.40–3.50	24	
3.50, injection		Injected 1.5 cc. = 0.00033 gram bufagin. Effect is immediate; fifteen drops in first twenty seconds.
3.51–4.01	9	
4.01–4.15	10	
4.15–4.30	10	
4.30–4.35	10	
4.35, second injection		Injected 1.5 cc. = 0.00033 gram bufagin; immediate effect as before, eight drops in first twenty seconds.
4.36–4.45	9	
4.45–4.53	9	
January 31st		Toad placed on ice until the morning of the 31st, when the perfusion was continued as before, first with normal Locke's solution.
10–10.36	13	
10.37 first injection		1.5 cc. = 0.00033 gram bufagin.
10.37–10.48	9	
10.48–10.53	9	
10.53 second injection		1.5 cc. = 0.00033 gram bufagin.
10.53–10.55	8	
10.55–11.00	7	
11.00–11.03	6	

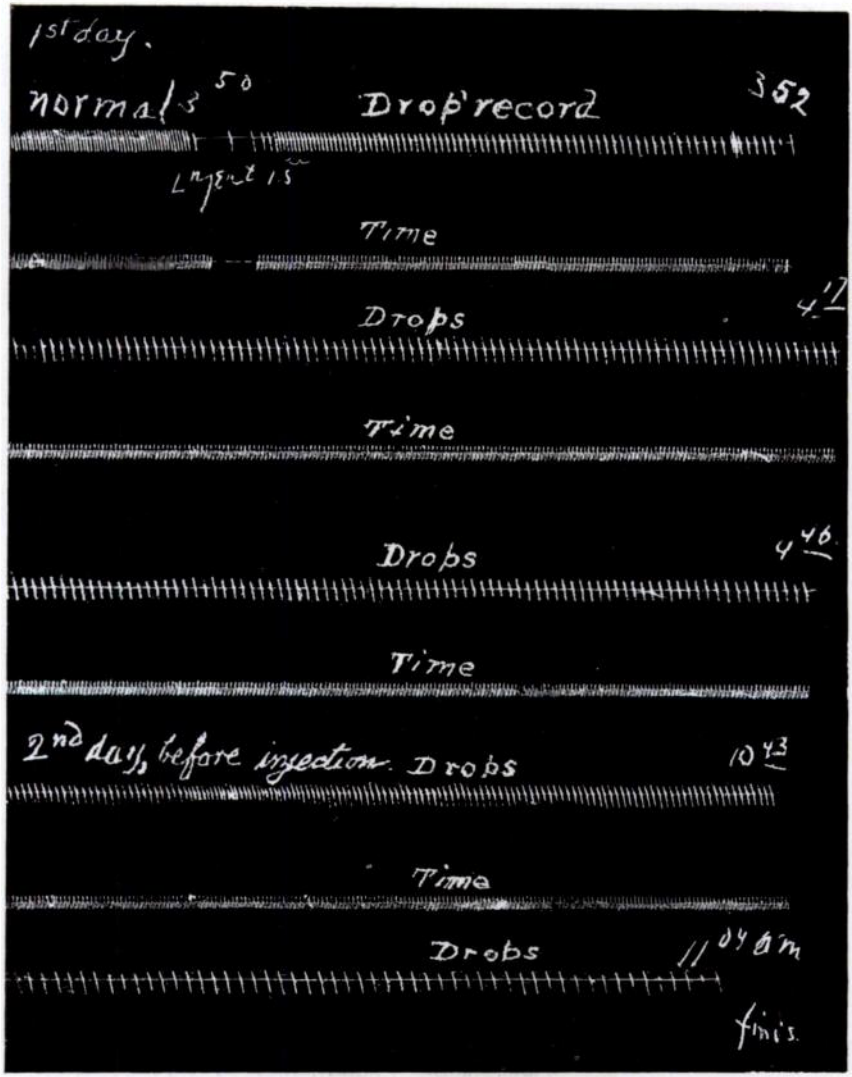


Fig. 4. Perfusion of *B. agua* with bufagin. Locke's solution; perfusion pressure = 35 cm. At intervals as stated in protocol 0.00033 gram bufagin was injected into the perfusion tube.

Fig. 5. Excised stomach of *B. agua*. Suspended in 30 cc. 0.8 per cent aerated salt solution. Piston recorder. At ↑ solution was replaced by one containing 0.2 mg. bufagin.

B. Action of Bufagin on Warm-blooded Animals (Mammalia)

Effects on the intact animal. Our observations were made on white mice, guinea-pigs, rabbits, cats and dogs.

The drug was administered hypodermically, intramuscularly, and by mouth. We have also made some observations on the effect of the drug on man.

We have made experiments with a view of detecting any local irritation or infiltration following hypodermic administration of the drug, and have convinced ourselves that bufagin in the doses used causes no local irritation whatever, unlike digitoxin and other members of the digitalis group of drugs.

As bufagin is but very sparingly soluble in water or in physiological salt solution, (0.2 mg. to 1 cc. water at room temperature), and tends to crystallize out from aqueous solutions containing even this small amount, the solutions in most of our experiments were made up with small quantities of alcohol, the amount of which varied from a fraction of 1 per cent to 5 per cent. Control experiments with equal quantities of alcohol were made, and we have found that the transient local symptoms following injections of bufagin were no greater than those following injections of solutions containing alcohol (1 per cent to 5 per cent) only.

As a result of our observations it was found that the herbivorous animals studied, the guinea-pig and rabbit, are relatively more resistant to the action of bufagin than the carnivorous animals, the cat and dog, the minimal fatal dose for the guinea-pig being over 2.5 mg. and for the rabbit 4.6 mg. per kilo, whereas for the cat it was 1.8 mg. and for the dog 0.76 mg. per kilo.

Special observations were made by us to ascertain the existence of any *cumulative* action following the administration of bufagin. Thus, a rabbit weighing 1200 grams received ten injection of bufagin, varying in strength from 0.25 mg. to 0.75 mg. in the course of *five days*, and was kept under observation for several weeks afterwards. In another case a young dog weighing 3.9 kilos, received daily injections of 1 mg. over a period of two weeks. *In no case was there any evidence of a cumulative effect noted.*

We have numerous protocols of experiments illustrating the

effect of bufagin on the intact animal, but it would take too much space to present them all here.

We will therefore give only a few illustrative examples, and content ourselves with summarizing briefly the general effect of the drug on all of the animals studied.

The systemic effects following administration of *small* doses of bufagin are manifested by the slowing of the pulse-rate, this being sometimes preceded by a short period of acceleration, and by a strengthening of the heart beat, as judged by the stethoscope and by palpation. The respiratory movements show very little change in the first stage of the action of the drug unless there is nausea or vomiting. Later these movements may be somewhat more vigorous and in the last or toxic stage they may be very deep and powerful and of a markedly abdominal type.

The toxic effect of larger doses is manifested in an extreme slowing and irregularity of the heart beat, vomiting and salivation, involuntary passing of urine and feces, incoördination of movements, and finally clonic convulsions which may possibly be due to the insufficiency of the circulation in the last stage. On autopsy the heart in almost all cases is found contracted in systole.

Illustrative protocols

Experiment 7. A white mouse, weighing 12 grams was injected intraperitoneally with 0.25 cc. of bufagin solution (1 cc. = 0.75 mg.). One minute after injection animal falls over in violent convulsions, with very rapid respiration, marked salivation and involuntary flow of urine and feces. Convulsions last two minutes and end in death.

It was found that a much smaller dose 0.05 mg. could induce convulsions in another mouse 12 grams, which were followed by recovery.

Experiment 8. May 30, 1911 Guinea pig 200 grams.

11.20 a.m. Pulse 62 in fifteen seconds. Injected under skin of abdomen 1 cc. of solution of bufagin = 0.25 mg.

11.20 a.m. Pulse 62 in fifteen seconds. Injected under skin of abdomen 1 cc. of solution of bufagin = 0.25 mg.

11.25 Pulse 70 in fifteen seconds. Determined by auscultation.

11.30 Pulse 60 in fifteen seconds.

11.34 Pulse 60 in fifteen seconds. Injected 1 cc. of solution of bufagin = 0.25 mg.

11.37 Pulse 47 in fifteen seconds.

11.43 Pulse 46 in fifteen seconds.

11.49 Pulse 46 in fifteen seconds. Legs give way; lies on its side.

11.53 Pulse 44 in fifteen seconds. On auscultation heart sounds very loud and powerful.

11.56 Pulse 38 in fifteen seconds.

12 m. Pulse 38 in fifteen seconds.

12.03 p.m. Pulse 50 in fifteen seconds.

12.10 Pulse 52 in fifteen seconds.

12.15 Pulse 60 in fifteen seconds.

12.30 Pulse 62 in fifteen seconds. Recovered, eating hay.

Experiment 9. Male rabbit 1740 grams.

May 25. 12.25 p.m. Pulse 45 in fifteen seconds. Injected intraperitoneally 1.5 cc. of solution of bufagin = 1.12 mg.

12.40 Pulse 30 in fifteen seconds. Depressed; lies quietly in corner of cage.

5.00 Pulse 45 in fifteen seconds. Normal again.

May 26. 2.05 Pulse 30 in fifteen seconds. Hypodermic injection of solution of bufagin 4 cc. = 3.0 mg.

2.07 Pulse 60 in fifteen seconds. Passes urine.

2.30 Pulse 30 in fifteen seconds. Lies on its side. Respirations twenty in fifteen seconds.

2.50 Pulse 40 in fifteen seconds.

2.55 Pulse 30 in fifteen seconds. Respirations very shallow.

3.00 Pulse 28 in fifteen seconds.

3.13 Pulse 12 in fifteen seconds. Staggers. Slight convulsion noted. Respirations eight in fifteen seconds.

3.22 Pulse 18 in fifteen seconds. Frequent and violent clonic convulsions.

3.25 Respiration stops. Pupils widely dilated and eyes protruding; heart beat not felt, but on auscultation with stethoscope fibrillary contractions of heart seem to be heard, which soon cease. On autopsy, viscera very pale; ventricles firmly contracted in systole.

Experiment 10. May 29, 1911 Black cat. 2 kg.

12.44 p.m. Injected under skin of abdomen 5 cc. of solution of bufagin = 3.75 mg.

12.48 Vomits. Voids feces.

12.52 Clonic convulsions.
12.53 Pupils widely dilated. Death.

Experiment 11. November 23, 1911 Dog, 3.5 kg.
2.00 p.m. Tube passed into stomach and 4 mg. bufagin in 2½% alcohol solution introduced.
2.15 Animal lies quietly in corner of room.
2.30 Profuse vomiting and salivation. Depressed. One hour later complete recovery.

Experiment 12. June 6, 1911 Dog, 7.9 kg.
10.40 a.m. Pulse 36 in fifteen seconds. Injected 6 cc. solution of bufagin = 3.6 mg.
10.50 Pulse 36 in fifteen seconds.
11.00 Pulse 36 in fifteen seconds.
11.05 Pulse 36 in fifteen seconds. Injected 4 cc. solution of bufagin = 2.4 mg.
11.20 Pulse 24 in fifteen seconds.
11.25 Vomits.
11.35 Pulse 16 in fifteen seconds. Very irregular heart beat. Animal very sick.
11.55 Vomiting and trembling; then convulsions and death.

Experiment 13. Dog, 6 kg.
May 31, 1911. 1.35 p.m. Pulse 36 in fifteen seconds. Injected 10 cc. of solution of bufagin = 2.5 mg.
1.40 Pulse 30 in fifteen seconds.
1.43 Pulse 30 in fifteen seconds.
1.45 Pulse 32 in fifteen seconds. Injected 10 cc. of solution of bufagin = 2.5 mg.
1.48 Pulse 30 in fifteen seconds.
1.50 Pulse 35 in fifteen seconds.
1.55 Pulse 32 in fifteen seconds.
2.00 Pulse 34 in fifteen seconds.
2.05 Pulse 32 in fifteen seconds.
2.15 Pulse 32 in fifteen seconds.
2.30 Clonic convulsions.
June 1. 1.50 Recovered; apparently perfectly normal. Pulse forty in fifteen seconds.

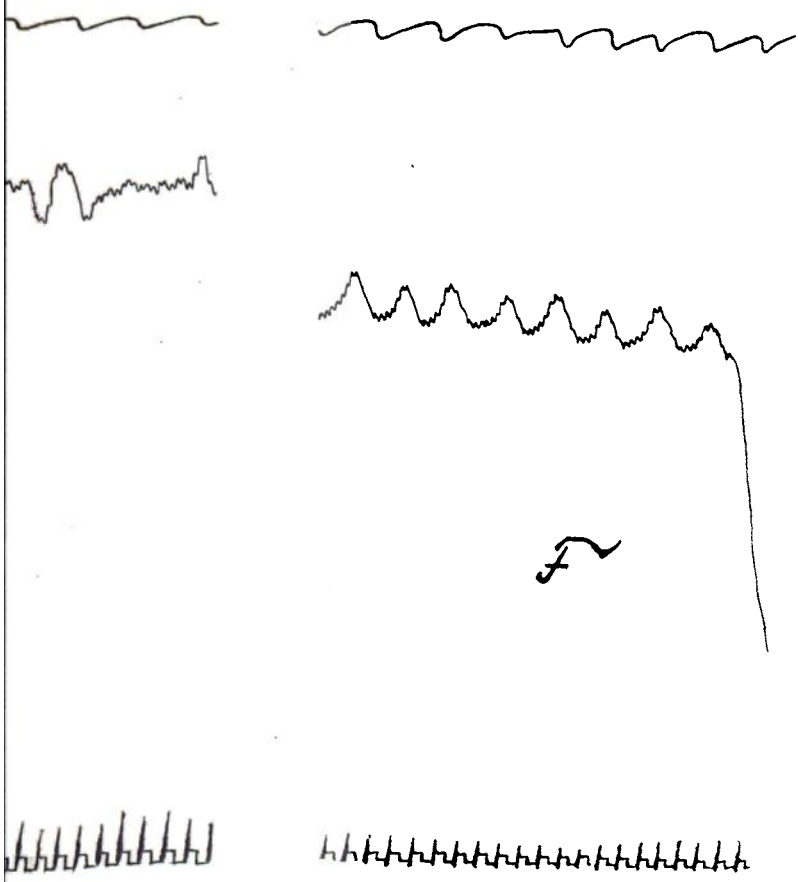
Effect on man. Having studied the effects of bufagin in various doses on the lower animals, we proceeded to try the effect of small

injections of the drug on ourselves and on some of our colleagues. In this place we may merely mention that from injections of 1 mg. doses of bufagin in 5 per cent alcoholic solution (2.5 cc.) on ourselves and other normal subjects, we have noted a definite rise in systolic blood-pressure (from 10 to 25 mm.) and an increase in pulse-pressure (from 10 to 25 mm.) as measured with the Erlanger and Riva-Rocci instruments, and á slowing of the pulse-rate of from four to twelve beats a minute. There were no untoward effects noted, and there was no local irritation except the stinging sensation caused by the alcohol, which was no greater than in control injections of solutions containing the same quantity of alcohol only.

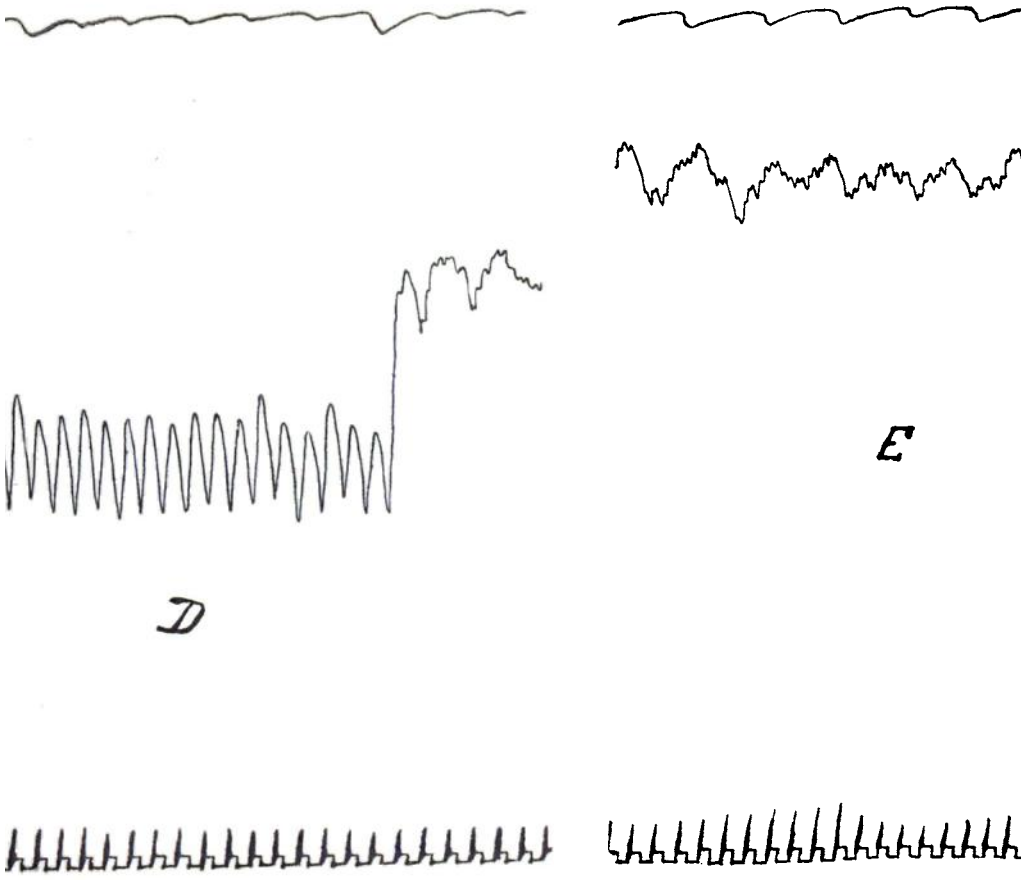
Drs. Macht and Rowntree of this Laboratory are at present engaged in a clinical study of the action of bufagin in pathological cardiac cases in the Johns Hopkins Hospital, and the results of their observations will be published in due time.

Effect of Bufagin on animals under anaesthesia. 1. General action on the circulatory apparatus. If we anesthetize a dog and inject a small non-toxic dose of bufagin (0.2 mg.) into one of its veins the blood-pressure tracing will show the following changes. Immediately or very soon after the injection there occurs a small rise in the blood-pressure with a normal or slightly accelerated pulse rate. This brief preliminary effect soon passes into what we have called the first or therapeutic stage in which we have a blood-pressure above the normal and a distinctly slower pulse rate with the amplitude of the pulse waves distinctly increased. If the dose injected is small the vagus effect gradually wears off and the curve assumes its normal character. If the dose be larger, the first stage passes into the second which may be termed the stage of excessive vagus stimulation. The pulse now becomes very slow, thirty or even twenty beats in the minute and the pulse waves are enormously increased in amplitude. The blood-pressure still remains high. The slowing of the heart beat may, however, be so extreme that the pressure falls below the level of the first stage in spite of the peripheral asterial constriction.

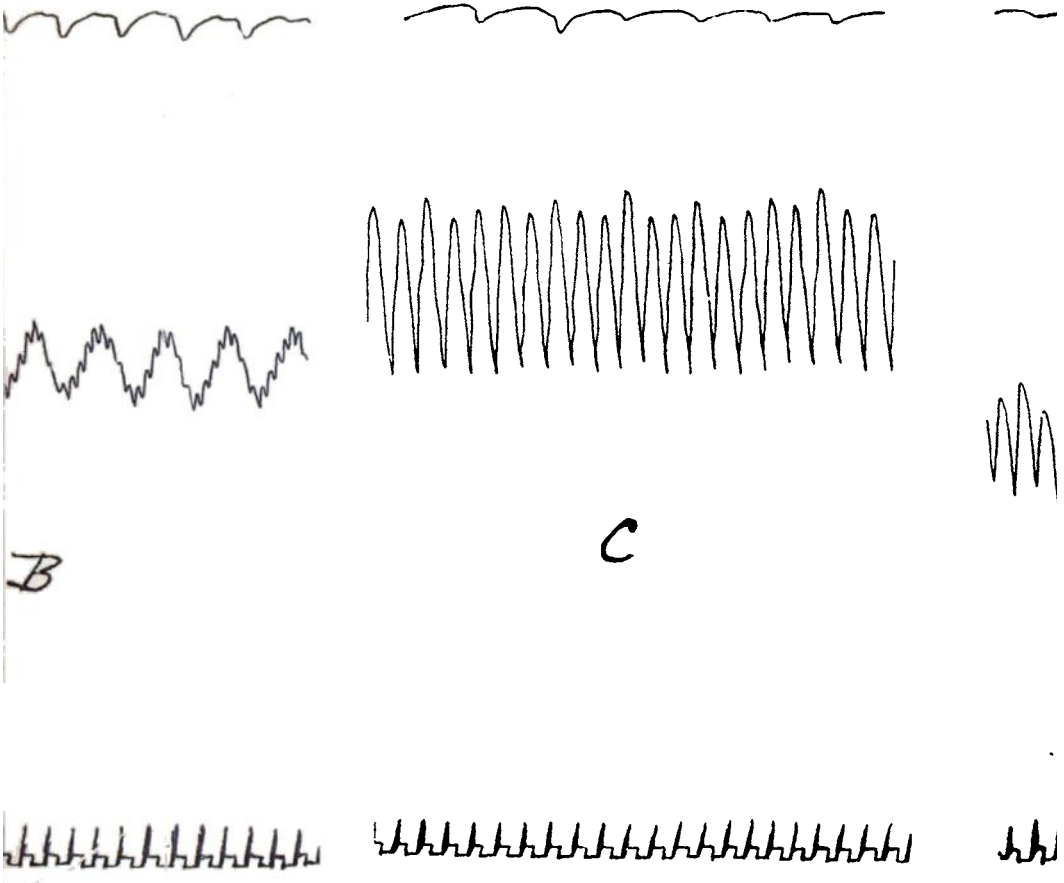
If during this stage the heart be exposed as was done in our



D = spontaneous escape from vagal inhibition, E = toxic stage, F = sudden



arterial pressure curve. A = normal, B = first effect, C = stage of extreme slowing of heart beat



tracing is of the respiration, lower is base line and time curve (seconds). The middle tracing is th

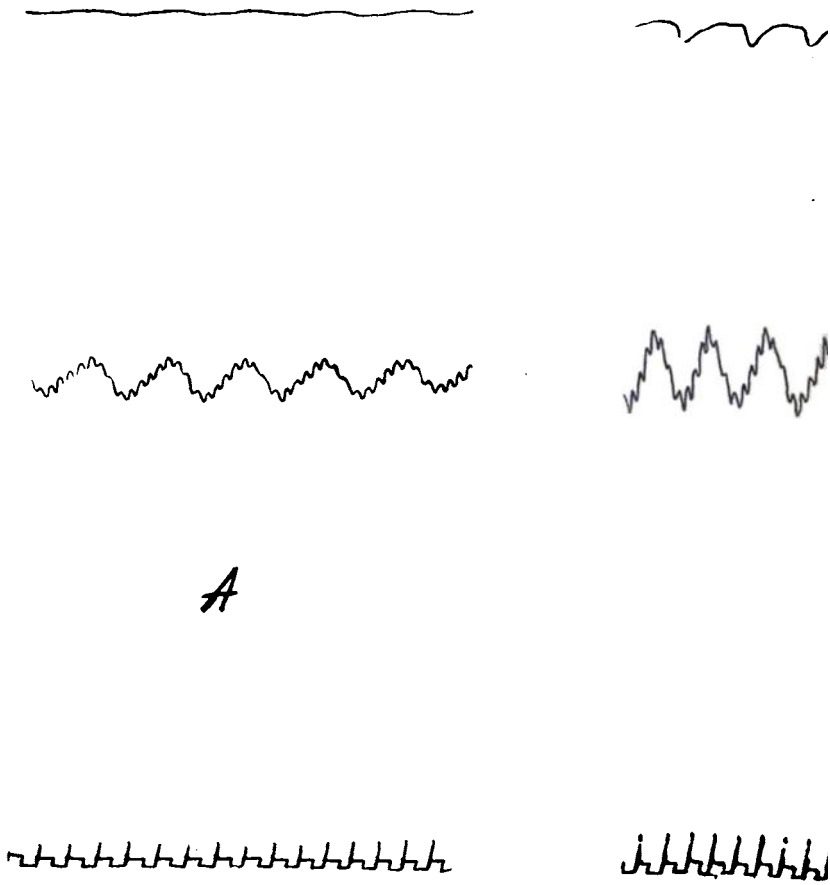


Fig. 6. Parts of a graphic record illustrating action on circulation. Upp
drop of pressure preceding systolic standstill of the heart.

experiments with artificial respiration and curare, a true heart block may occasionally be observed, the auricles and ventricles beating in the ratio of 2 : 1, 3 : 1, or even 4 : 1. In exceptional cases, the heart may be permanently arrested in this stage, in which event, the ventricle will usually be found in diastole. More usually, however, and especially if more bufagin be injected, the action passes on to the third or toxic stage. The pulse now becomes very rapid and the blood-pressure rises to a higher level. Soon irregularities of the heart beat are noted and the blood pressure begins to fluctuate. The heart muscle is now seriously poisoned. Presently the pressure drops suddenly to near the zero level and the heart comes to a standstill. After such a fatal drop in the arterial pressure the left ventricle of the heart is almost invariably found to be firmly contracted in systole. The action of bufagin as above described coincides with that of the digitalis series whose action on the circulatory apparatus was first clearly classified in this manner by Cushny.

We have found that the same course of events follows the administration of bufagin hypodermically, intramuscularly and by mouth except that in the last case a slightly larger dosage is required to produce a given effect in a given time on account of the slowness of absorption.

The following protocols will illustrate the course of events above described. Fig. 6 contains parts of a kymograph tracing illustrative of the various stages in the action of bufagin on the circulation.

Experiment 14. March 20, 1911 Dog, 7.8 kg. Manometer connected to right carotid artery; injection cannula in left femoral vein; ether anaesthesia; curare; tracheal cannula; artificial respiration.

TIME	PULSE RATE	BLOOD PRESSURE IN MM.	REMARKS
	per min.		
10.00 a.m			Experiment begins.
10.01	84	120	
10.02	84	126	Injected bufagin 1 cc.= 0.000224 gram.
10.02½	84	126	Injection ends.
10.03	84	140	
10.10	90	116	Injected bufagin 2 cc. = 0.000448 gram.
10.10½	90	116	Injection ends.
10.11	90	124	
10.12	94	126	Respirations increased in rate and amplitude.
10.18	88	114	
10.18½	88	114	Injected bufagin 1 cc. = 0.000756 gram. (Stronger solution).
10.19	88	120	Injection ends.
10.19½	64	128	Vagus effect begins, slowing of rate, and greater amplitude.
10.20	64	126	
10.20½	32	126	Full vagus effect, pulse waves of great amplitude (20 mm.), extreme slowing,
10.21	32	128	
10.22	32	142	Respiration rapid.
10.23	32	144	
10.24	32	144	
10.30	32	112	
10.31	32	100	
10.33	32	104	
10.34	32	108	
10.35	110	140	Vagus effect ends, pulse waves of small amplitude, pulse very rapid.
10.36	118	118	
10.37	118	120	
10.42	122	140	Heart beat irregular.
10.42½	122	150	
10.43	122	130	
10.44	122	120	
10.45	122	120	
10.49	122	96	
10.50	122	96	Slow injection of bufagin 2 cc. = 0.001512 gram.
10.51	120	100	
10.51½	120	132	
10.52	120	150	
10.52½	120	164	Injection ends.
10.53	136	180	
10.55	136	190	
11.00			Sudden drop, stoppage of heart, death.

Experiment 15. Dog, 8.0 kg. Manometer connected to left carotid artery; injecting cannula in it femoral vein; ether; tracheal tube.

TIME	PULSE RATE	BLOOD PRESSURE IN MM.	REMARKS
	per min.		
11.00a.m			Experiment begins.
11.01	80	104	Respirations sixteen to one minute.
11.05	80	104	Injection of bufagin 0.5 cc. = 0.000112 gram.
11.05½	84	106	
11.06	84	124	
11.07	88	124	
11.10	88	126	
11.11	88	128	
11.12	88	130	
11.13	88	132	
11.19	88	136	
11.20	76	120	Vagus effect noticeable, slowing of rate. Respirations deeper.
11.21	66	112	
11.22	68	106	
11.23	68	96	
11.25	68	84	
11.29	68	90	
11.37	68	86	Injections of bufagin 1 cc. = 0.000224 gram.
11.38	76	100	Blood pressure rising.
11.38¾	44	110	Marked vagus effect, pulse waves of great amplitude.
11.40	44	112	
11.42	32	106	
11.54	54	110	
11.55½	54	130	Injections of bufagin 1. cc = 0.000224 gram.
12.00	30	130	
12.01p.m.	30	130	
12.10	30	122	
12.10¼			Injection of bufagin 10 cc. = 0.002240 gram begins.
12.11	Pulse too small and too rapid to count.	132	
12.11½		144	
12.12		164	
12.12¾			Injection ends.
12.13		144	
12.15		110	Pulse waves too small and too rapid to be counted. Respirations powerful and abdominal in type.
12.16		140	
12.16½			Sudden drop of pressure. Death.

Experiment 16. Dog, 3.9 kg. Manometer connected with rt. carotid; injection cannula in left femoral vein; ether anaesthesia; tracheal tube.

TIME	PULSE RATE	BLOOD PRESSURE IN MM.	REMARKS
	per min.		
4.00p.m.	90	112	Respiration thirty in one minute.
4.02	90	112	
4.05	90	112	Injection of bufagin 2 cc. = 0.0015 gram, very slowly.
4.08½			Injection ends.
4.08	90	112	Respirations, thirty.
4.09¼	90	132	
4.08½	92	148	
4.10½	40	150	Very fleeting vagus effect, lasting about thirty seconds.
4.11			Pulse gets very rapid, small, and *irregular*.
4.12	124	126	
4.13	132	144	
4.14	126	134	
4.15¼	126	160	
4.16			Sudden drop of blood pressure. Death.

2. Analysis of the action on the circulatory apparatus. The vagus effect. The slowing of the pulse rate and the increase in the amplitude of the pulse wave noted in the latter part of the first stage and more especially throughout the second stage of the action of bufagin are consequences of the stimulating action of the drug on the cardio-inhibitory center as may be easily demonstrated by cutting the vagi, see Protocol of Experiment 17, or by administering atropine. In either case the inhibition is at once removed and the pulse curve assumes the well known characteristics of lack of vagus control. The transition from the first stage, or stage of moderate slowing, to the second stage of excessive vagus action is usually abrupt, and (see A, Fig. 7) the decrease in pulse rate is then *exactly one-half* of what it was just prior to the transition.

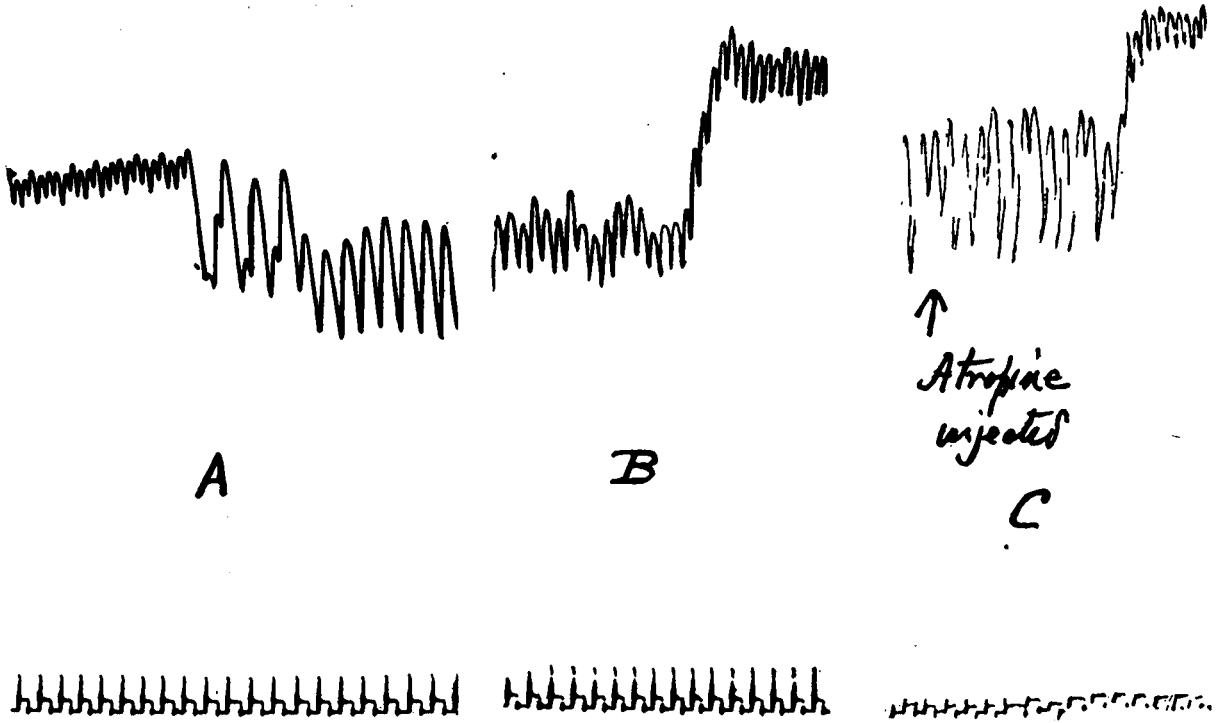


Fig. 7. Dog (7 kilos). Ether. Showing effect of 2.1 mg. of bufagin in divided doses intravenously. At *A* abrupt transition from first to second stage, at *B* five minutes later, abrupt transition to a condition similar to the first stage of the action of bufagin. *C* is part of a graphic record obtained from another dog showing the effect of 2 mgs. of atropine in abolishing the stage of extreme inhibition.

Experiment 17. March 23, 1911 Dog, 8.56 kg. Manometer connected to right carotid artery; injection cannula in left femoral vein; ether anaesthesia; tracheal tube; curare; artificial respiration.

TIME	PULSE RATE	BLOOD PRESSURE IN MM.	REMARKS
	per min.		
3.00p.m.			Experiment begins.
3.01	80	116	Respiration forty to the minute.
3.05	80	32	Curare injected in slightly excessive dose.
3.20	90	52	
3.21	90	62	Injection of bufagin 2 cc. = 0.001512 gram *begins.*
3.22	110	112	
3.23	108	144	
3.23½	104	176	Injection ends.
3.24	84	190	
3.26	84	190	Vagus effects noticeable.
3.30	52	206	Marked vagus effect, very long and slow sweeps of the writing pen.
3.34	52	156	
3.35	68	156	Right vagus cut.
3.36	130	206	Left vagus cut.
3.37	130	200	

The period of marked inhibition (2nd stage) sooner or later gives way to a stage of fast pulse and higher arterial pressure, the transitions to this stage being generally as abrupt as was the change from stage one to that of the slow pulse. (See *D*, Fig. 6 and *B* Fig. 7.) The events as here described are seen when considerable doses of bufagin are injected *intravenously.* The stages are crowded together, so to speak, the stage of marked inhibition may continue for only fifteen or twenty minutes, while in the unaesthetized animal which has received the drug by intramuscular injection it may last for hours. The cause of the abrupt change from the second to the third stage may be assumed, as in the case of the members of the digitalis series, to be due not to a paralysis of the mechanism of the vagus, but to be indicative rather of an increased state of irritability of the musculature of the heart. The irritability of the heart reaches such a degree that the normal impulses of the vagus are inadequate to produce their usual

effect. This explanation was first offered by Cushny[53] for the analogous phenomenon seen in the digitalis series and was further elaborated later by Lhotak von Lhota.[54] Like other observers[55] who have studied digitalis-like bodies we have also found that, in the third stage of poisoning with bufagin, that is, at the time when the vagus mechanism is apparently paralysed, electrical stimulation of the vagus will, after a brief refractory period, cause marked inhibition of the heart with a fall of blood-pressure.

Action on the blood-vessels and other structures containing plain muscle. The marked rise of blood-pressure after injections of solutions of bufagin is due in some measure to the peripheral vaso-constrictor action of the drug. This action was well illustrated in the perfusion experiments with frogs and toads described in an earlier section of our paper. It is also illustrated in the action of the drug on the blood vessels of a loop of the dog's jejunum as measured with the intestinal plethysmograph (see Fig. 8). It may also be mentioned here that mere inspection of the contracted blood-vessels of the intestines in the stage of high blood pressure convinces us that vaso-constriction is a marked element in the rise of blood-pressure. The vaso-constriction was also noted in experiments made with the kidney enclosed in the oncometer, when it was seen that the *first* or immediate effect of an injection of bufagin is to cause a diminution in the volume of that organ.

In further illustration of the peripheral vaso-constrictor action of our drug we may cite the results of an experiment on a decerebrate cat with pithed cord (see Experiment 18). Through a misunderstanding the animal had received an excessive amount of morphine prior to the anaesthesia with ether and this together with a considerable loss of blood during the operation gave us rather a poor subject for the experiment. Under the conditions of the experiment, even though the arterial pressure did not rise to as high a level as might be expected (from 42 to 72 mm.) we may still believe that the observed rise was in a large measure due to an action on the peripheral vessels.

[53] Journ. of Exp. Medicine, ii, 233, 1897.

[54] Arch. f. Exp. Pathol. u. Pharmakol, lviii, 350, 1908.

[55] See Dale and Laidlaw. Action of Apocynum, Heart, i, 154 (1909-10).

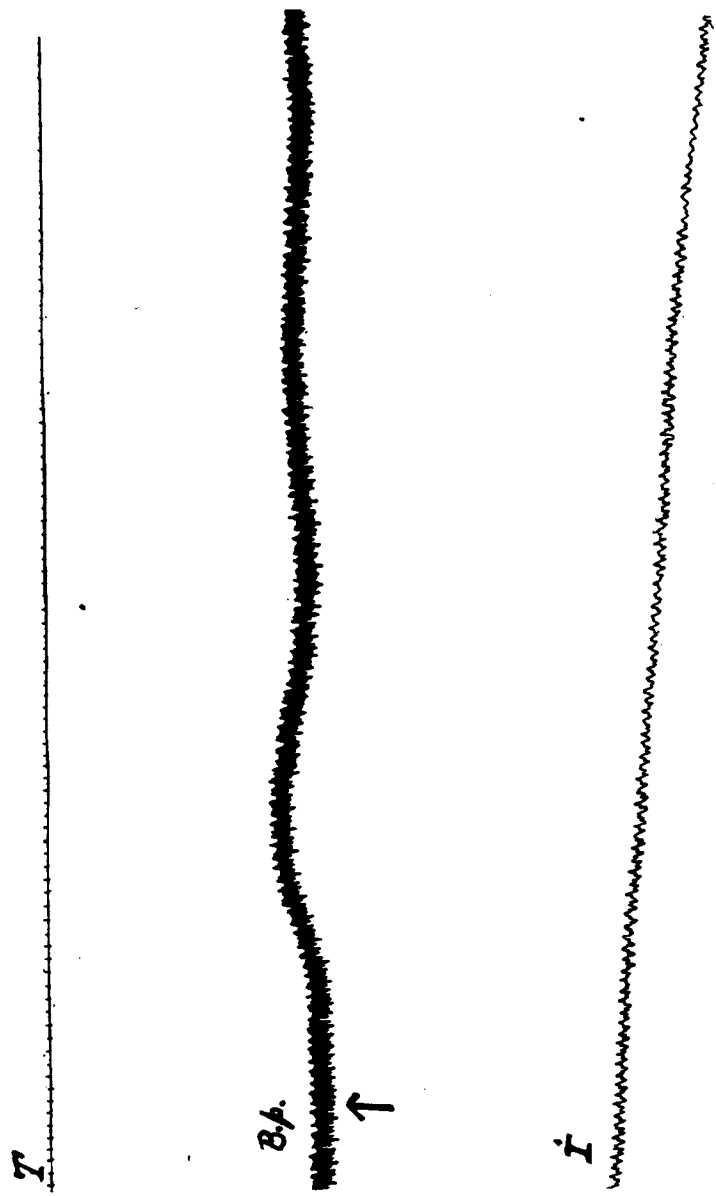


Fig. 8. Dog (7 kilos). Ether. T = time in seconds, $B.\ p$ = carotid blood-pressure, I = intestinal plethysmograph. At ↑ 1 cc. = 0.34 mg. bufagin injected into right femoral vein.

Experiment 18. March 31, 1911 Cat, 2.8 kg. Ether and morphine; carotids and vertebral arteries tied off and animal decerebrated; spinal cord destroyed; manometer in right carotid artery; injections through left femoral vein; curare; artificial respiration.

TIME	PULSE RATE	BLOOD PRESSURE IN MM.	REMARKS
	per min.		
11.00a.m	84	44	Experiment begins. Head removed and cord destroyed at 10.50.
11.03	84	44	
11.05	84	44	
11.07	84	40	
11.10	84	40	
11.13	84	42	
11.15	84	40	
11.15½			Injections of bufagin 1 cc. = 0.0007 gram begins.
11.16	90	46	
11.17½	108	54	Injection ends.
11.18	100	64	
11.18½	100	66	
11.22	92	50	
11.22½	92	44	Injection of bufagin 2 cc. = 0.0014 gram.
11.23	114	64	
11.23¾	114	72	
11.24	114	72	
11.24½			Sudden drop. Exitus.

The question as to whether the local vaso-constricting action of bufagin is exerted on the arterial musculature itself or on a nervous mechanism distal to the spinal cord was studied by Dale's[56] method. After the injection of a quantity of ergotoxine sufficient to abolish entirely the vaso-constrictor effect of epinephrin so that an injection of this substance gave the paradoxical fall of pressure, it was found that small doses of bufagin produced their usual effect in raising the arterial pressure. From this we concluded as was done by Dale and Laidlaw[57] under similar circumstances in their study of Apocynum, that bufagin acts

[56] Biochemical Journ., ii, 246, 1907.
[57] Heart, i, 138, 1909-10.

directly upon the plain muscle and not upon the nervous elements of the blood vessels.

The plain muscle of the rabbit's jejunum, of the pig's jejunum and of the isolated horn of the rabbit's uterus was in each case found to be stimulated to contraction by adequate quantities of bufagin. See Fig. 9.

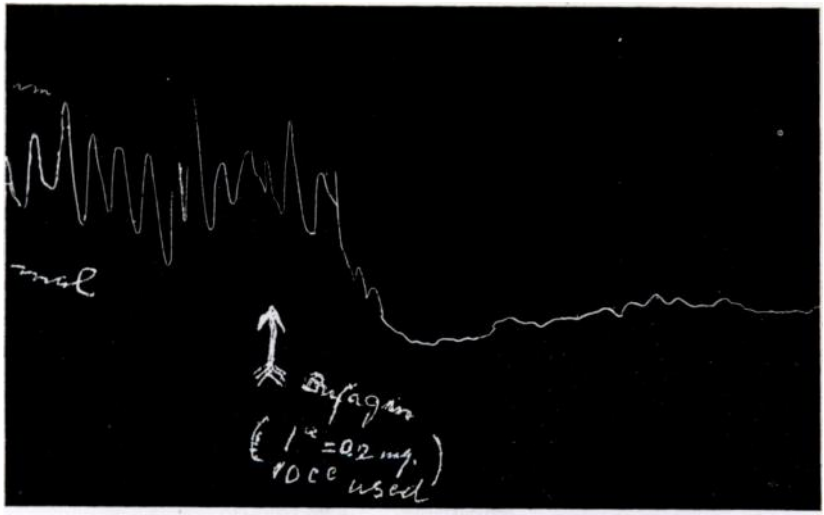


Fig. 9. Effect of bufagin on plain muscle. Isolated loop of rabbit's jejunum. Downstroke of lever indicates contraction. At ↑ 2 mg. 10 cc. of solution were added to the warm oxygenated Locke's solution 200 cc. in which the loop was suspended.

The effect on the heart. Our experiments with the excised hearts of frogs, terrapins and toads have already indicated that bufagin has a powerful action directly on the heart itself. In those experiments we noted the great increase in tonicity and contractility, as well as the slowing of the heart beat, and, in the case of the toad, *Bufo Agua*, we found evidence of an effect on the bundle of His, causing a disturbance in conductivity and resulting in a heart-block.

The action of bufagin on the mammalian heart was studied by observation of the exposed heart in situ, by an analysis of blood-

pressure curves, and by means of tracings obtained with Cushny's modification of the Roy-Adami myo-cardiagraph,[58] with the cardiac plethysmograph, and with Henderson's[59] cardiameter, an instrument well adapted to the study of ventricular volume change.

The effect on the heart after moderate doses of bufagin which result in the first or therapeutic stage, may be briefly summarized as follows:

There is a distinct increase in the tonicity of the heart-muscle (see Fig. 10), a slightly heightened irritability, and a decided increase in its contractility. The ventricular out-put is very much augmented, and this is due in part to greater tonicity and stronger contractions of the heart-muscle, and in part to a more complete relaxation in diastole (see Figs. 11 and 12[60]).

The most striking effect on the heart-muscle in the second stage of the action of bufagin is the change in its conductivity, which finally leads to heart-block as already noted. This stage, as was pointed out previously is characterized by a marked action on the cardio-inhibitory centre. The contractile power of the heart is still unimpaired, but from the point of view of therapeutic use, this stage must be regarded as one fraught with danger.

In the toxic stage, the heart-muscle is poisoned, as may be seen from the disturbance of all its functions. The normal impulses from the vagus are inoperative, the rhythm of the heart-beat is impaired and its excitability is greatly increased, as may be seen from the extremely rapid and irregular pulse, with extra systoles. *Pulsus alternans* appears, as an indication of weakened contractile power. The contractions are small and irregular. The ventricular output is diminished and there is a relaxation in the tone of the muscle.

[58] Heart, ii, 1, 1910.

[59] Americ, Jour. Physiol., xvi, p. 352, 1906.

[60] We are indebted to Dr. G. S. Bond for kind assistance in performing this experiment.

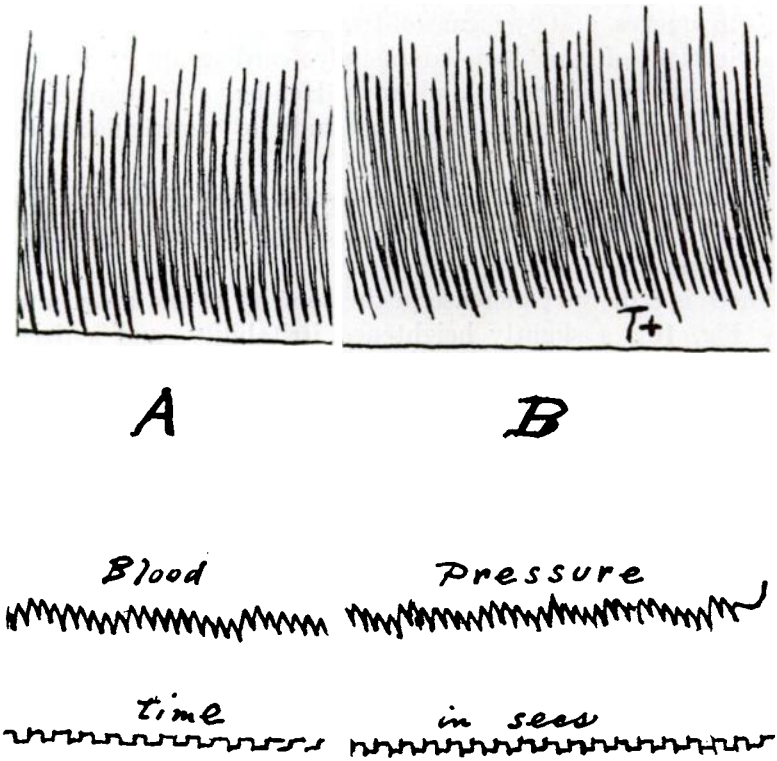


Fig. 10 Dog (9.15 kilos). Trichlor-tertiary-butyl alcohol. Ventricular tracing with Henderson's cardiometer, showing increase in tonicity in *B*. Upstroke = systole, *A*, normal; *B*, thirty seconds after injection of 0.2 mg. of bufagin. Early in the first stage of the action of the drug.

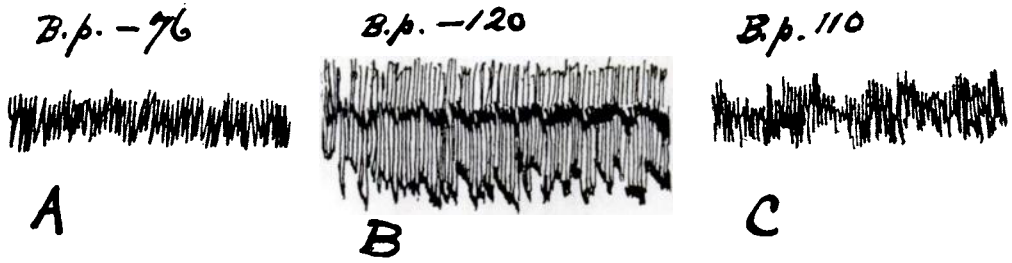


Fig. 11. Dog (9.5 kilos). Ether and Trichlor-tertiary-butyl alcohol. Myocardiographic Record. *A*, normal; *B*, two minutes after intravenous injection of 1.5 mg. bufagin; *C*, Toxic stage after further injections totaling 3.5 mgs.

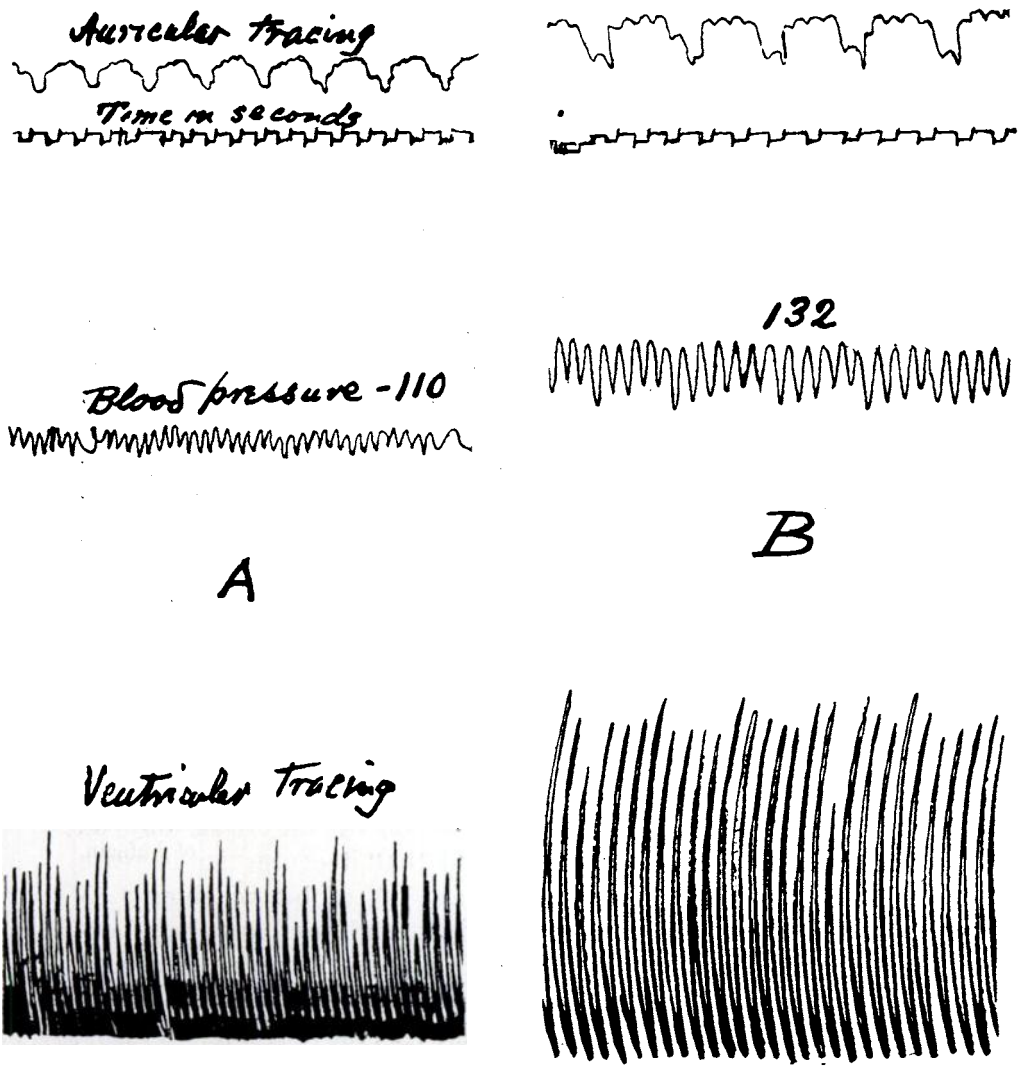


Fig. 12. Dog (6 kilos). Anaesthetized with Trichlor-Tertiary-Butylolcohol Upper tracing gives the auricular contractions, the lower tracing is the cardiometer-record (Henderson) of the ventricular volume. Downstroke = systole. *A*, Normal. *B*, three minutes after injection into left femoral vein of solution containing 0.4 mg. of bufagin. Bloodpressure recorded from the left carotid artery.

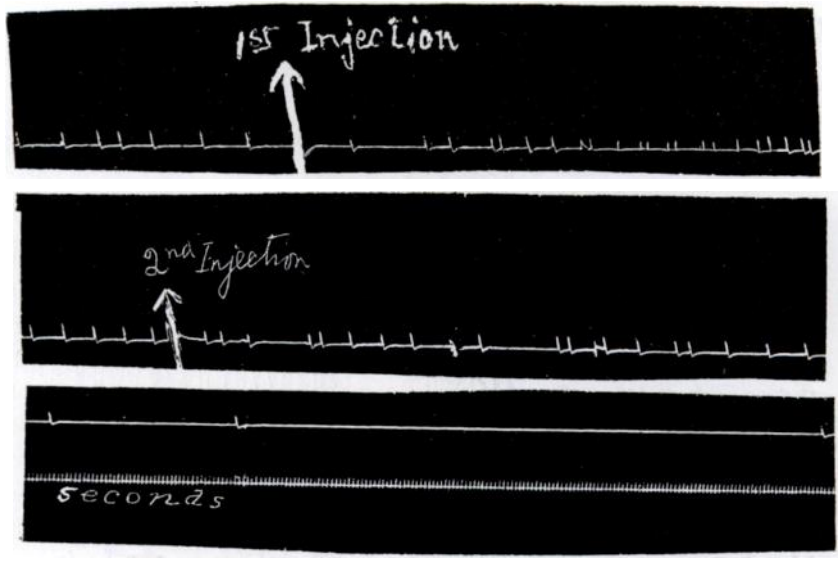


Fig. 13. Cat (1.6 kilos). Anesthesia with Trichlor-tertiary-butyl alcohol. Drop record of flow of urine. 1st injection into left jugular = 0.1 mg. Second injection five minutes later = 0.1 mg. Third line of drop record shows partial suppression of urine following repeated injections aggregating 0.6 mg. Last line record in seconds.

Diuretic action.

We have studied the diuretic action of the drug on the rabbit and the cat and have found that it causes an increase in the flow of urine in both animals. A rabbit weighing 1.85 kilos, anæsthetized with paraldehyde and urethane had normally a urinary flow of seventeen drops per minute as measured by a drop record from a bladder cannula. After the injection of 0.5 mg. of bufagin into the left jugular vein the rate of flow quickly increased to twenty-nine drops per minute which rate was maintained for nearly five minutes. The carotid blood pressure during this time increased from 80 mm. to 100 mm.

In the cat small doses of the drug quickly induce a free flow of urine, as may be seen from a study of the protocol of Experiment 17 and of Fig 13, with this animal as with the rabbit, toxic doses

cause a diminution of the flow which finally passes into complete suppression. These positive results in the way of diuresis may be gained with therapeutic doses.

Oncometer and blood pressure experiments on the cat during diuresis after bufagin have yielded the following results: After small doses there is seen at first, though not invariably, a diminution in the volume of the kidney. This is soon followed by a dilatation of the renal blood vessels, as is shown by an increase in the volume of the kidney, which goes hand in hand with a rise of blood-pressure and a marked increase in the flow of urine. After the administration of toxic doses the volume of the kidney decreases coincidently with the period of very high blood pressure and marked vaso-constriction, the flow of urine is lessened and soon complete suppression occurs (see Fig. 14).

It is evident from the above that small doses of bufagin cause a vaso-dilatation in the kidney at a time of constant or higher blood pressure and vaso-constriction elsewhere in the body. In this respect the drug acts like other members of the digitalis series whose behavior in this regard has been studied by Jonescu and Loewi.[61]

Experiment 17. Diuresis. Cat (1.6 kilos). Anaesthetized with trichlor-tertiary-butylalcohol. Injection cannula in left jugular vein. Cannula with funnel shaped end in bladder, so that lumen of bladder is reduced to the size of the cannula. Urethra ligated. Normal rate of dropping of urine = six per minute. Thirty seconds after injection of 0.1 mg. bufagin number of drops = twelve per minute. Three minutes later still twelve per minute. Five minutes later second injection of 0.1 mg. One minute later flow = nine drops per minute, three minutes later ten drops per minute, five minutes later six per minute. Then repeated injections at intervals of about one minute until the whole quantity from the beginning totaled 0.8 mg. The result of this toxic dose was gradual suppression of the urinary flow terminating in complete anuria.

[61] Arch. f. Exp., Pathol. u. Pharmakol, lix, 71, 1908.

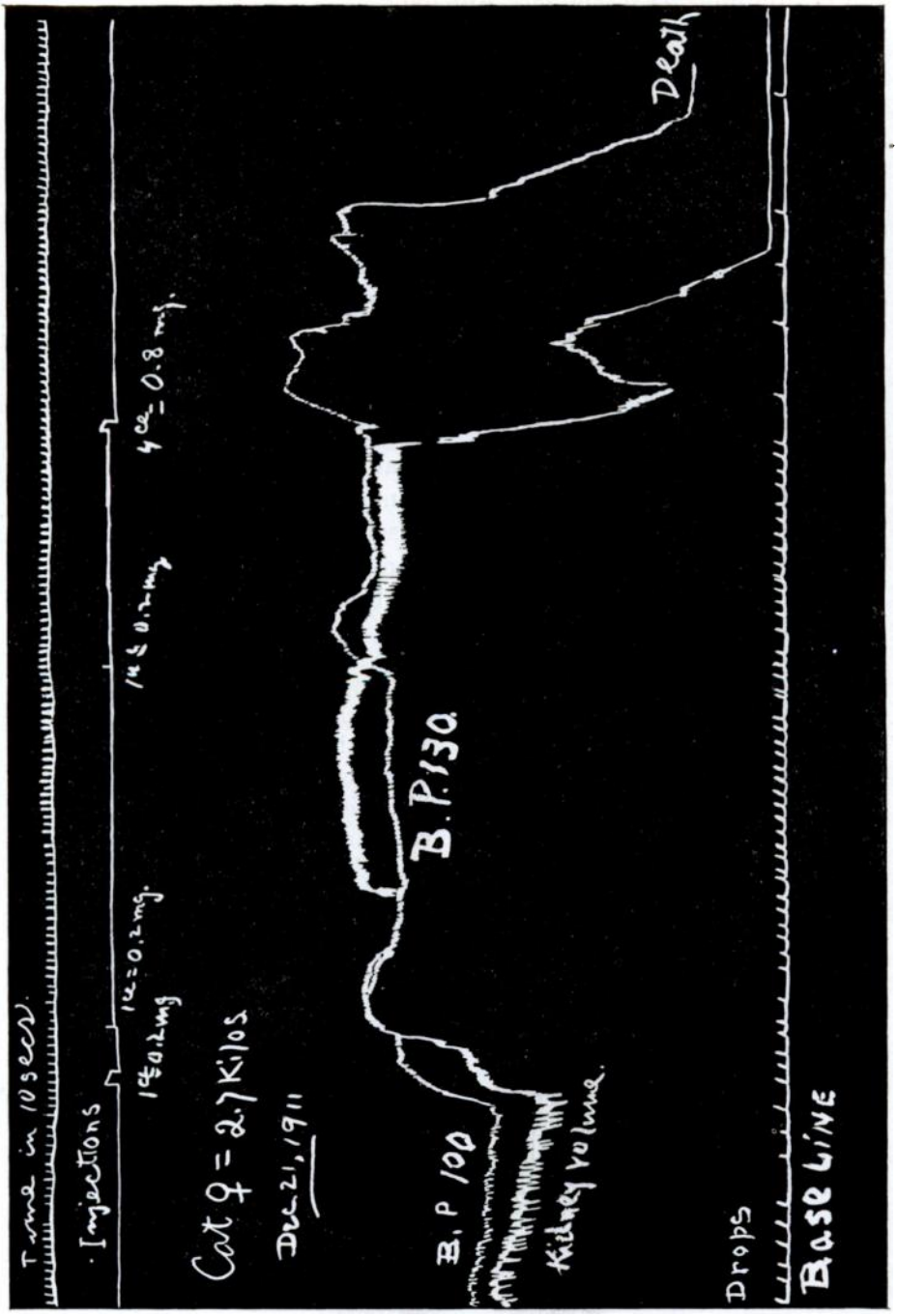


Fig. 14. Cat (2.7 kilos). Trichlor-tertiary-butyalcohol. Carotid blood pressure, injection cannula in left femoral vein. Urinary cannula in bladder. Left kidney in oncometer. Piston recorder. Drop record for urinary flow is base line of blood pressure. Time intervals = ten seconds. Dose of bufagin and times of injection indicated on second line.

VI. SUMMARY

1. From the secretion of the parotoid glands of the tropical toad, *Bufo agua*, we have isolated two distinct, physiologically active crystalline principles.

2. It has been shown by chemical reactions and by analyses, by polarimetric observations, and by qualitative and quantitative physiological experiments that one of these substances is identical with dihydroxy-methyl-amino-ethylol-benzene (epinephrin, adrenalin, suprarenin).

3. It is calculated that the crude venom contains nearly seven per cent (6.72 per cent) of this amino alcohol.

4. By means of chemical reactions and analyses it has been shown that the venom also contains a crystalline principle which we have named bufagin.

5. Bufagin is dextrorotatory (+11°), "neutral" in character, slightly soluble in water and readily soluble in a number of organic solvents. Its melting point is 217–218° C. Its elementary composition and molecular weight are represented by the formula $C_{18}H_{24}O_4$. Its behavior toward bromine shows that it does not contain an unsaturated carbon linkage of cholesterin.

6. The marked action of bufagin on the heart, cardio-inhibitory centre and musculature of the blood-vessels compel us to class this drug with the most efficient members of the digitalis series. Its action on the heart muscle is especially noteworthy. It increases its tonicity, the strength of its contractions and the ventricular volume output. Small doses of the drug cause a marked diuresis.

7. Bufagin does not appear to have a cumulative action and it may be administered hypodermically.

8. The toad, *Bufo agua* is not at all immune to dihydroxy-methyl-amino-ethylol-benzene, but is relatively immune to bufagin.

9. The pharmacological properties of bufagin, its keeping qualities, its chemical purity and the consequent ease with which it lends itself to exact dosage are facts that urge a trial of this substance in therapeutics. The successful use of toad skins in the medicine of an earlier day also lends support to our suggestion.