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The Venom of the Colombian Arrow Poison Frog *Phyllobates bicolor*

By F. MÄRKI* and B. WITKOP**

I. *Introduction.* The use of arrow poisons—once the most sophisticated weapon in the arsenal of man—is becoming obsolete. It is only in inaccessible jungle areas which have not yet been touched by civilization, that the use of these poisons and the often secret art of their preparation are still practiced. One of the little-known arrow poisons is the kokoi, a substance of unusually high toxicity which is used by the Indians of the Chocó in Colombia (South America). This article summarizes the present knowledge of the kokoi venom and describes recent chemical and pharmacological studies carried out by the authors and their colleagues.

II. *Geographical and Ethnological Background.* The jungle area of the Chocó is situated in the northwest of Colombia, between the Pacific coast and the *Cordillera Occidental* and stretches from about 4 to 8° north of the equator (Figure 1). It is isolated from the rest of the country by the western chains of the Andes, the highest peaks of which, like the Cerro Tatamá, tower to an altitude of over 4000 m. It is a very rare event when, between the frequent thunderstorms, their forest-clad tops can be seen through a break in the heavy cloud cover. An annual precipitation of 11 m and high temperatures all year around are favorable to the growth of an almost impenetrable jungle. The only practical routes of transportation are the rivers, which often flood wide areas.

The life of the native population of Chocó Indians has barely changed since the time of the conquistadores. Although a penetration of Negroes—former slaves of the Spaniards—has advanced during the centuries along the lower parts of the big rivers, the mountain areas are still the undisputed property of the Indians. They live in many different tribes which speak the Noanamá language as do the Cholos on the upper Rio San Juan or the somewhat related language of Emperá as do the Cunas on the Rio Atráto. We are fairly well informed about their life and culture by several ethnological studies, for instance the one by WASSÉN[1] on the Southern group of Chocó Indians (on the lower Rio San Juan, Figure 2).

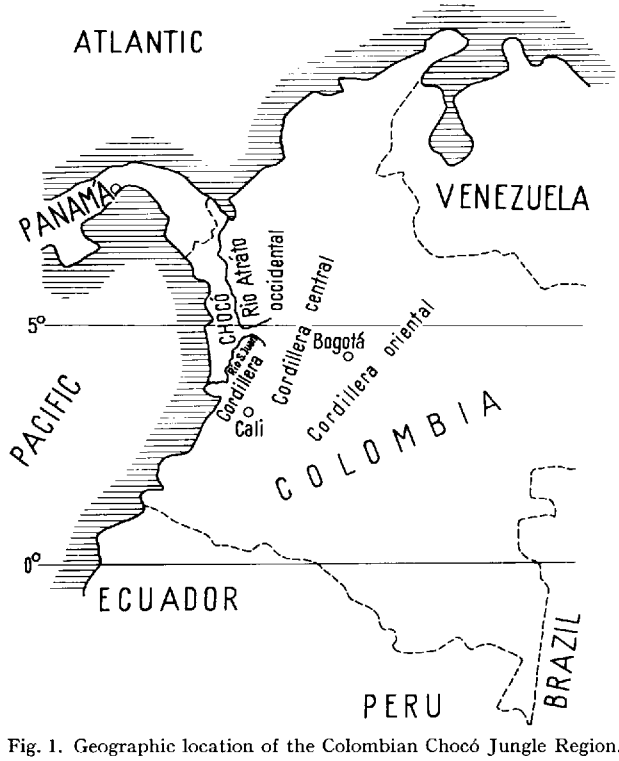

Fig. 1. Geographic location of the Colombian Chocó Jungle Region.

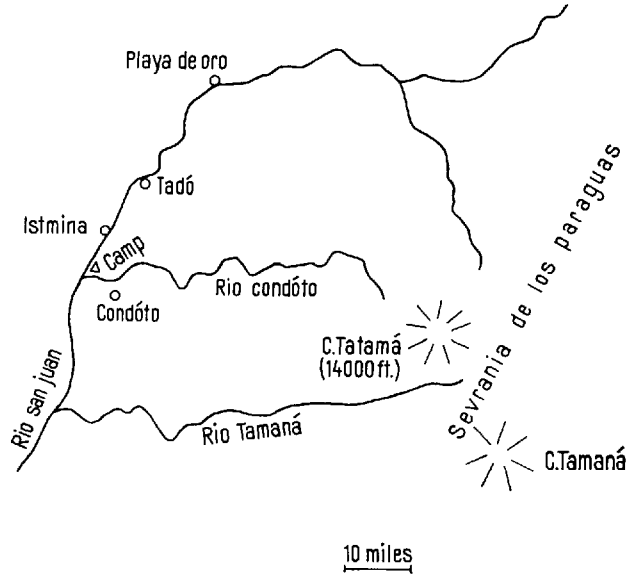

Fig. 2. The frog material was collected in the upper region of the Rio San Juan with Playa de Oro as advanced base and the camp at Andagoya laboratory and working base.

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[1] H. WASSÉN, *Etnologiska Studier* (Göteborg Museum, 1935), p. 35.

It seems that in the Chocó (and in many jungle areas of South America as well) the blowgun, together with poisoned arrows, is still the preferred weapon for game hunting. Several kinds of arrow poison are used for this purpose; they are usually prepared from poisonous plants or trees such as the pakurú (called kierátchi in the Noanamá language)[1], a powerful cardiac glucoside which has been studied by URIBE PIEDRAHITA[2], SANTESSON[3] and MEZEY[4]. The Cholo Indians, however, who live in the mountains near the headwaters of the Rio San Juan and its tributaries (Rio Agüita, Rio Tadocito), poison the tips of their arrows with the skin secretion of a small black frog, which they call Kokói in their Noanamá language. The same name is also used by them for the venom obtained from the frog.

The earliest report on this interesting substance, which exceeds curare in its toxicity, has been published as far back as 1869 by the Colombian POSADA ARANGO[5]. Later detailed reports by the same author[6], supplemented in this century by studies of WASSÉN[7], give us a well-rounded picture of the preparation of the kokoi poison, with a few sidelights also on some of its pharmacological effects. However, the great difficulties to obtain a sufficient amount of the venom for scientific studies have until very recently frustrated any attempt to isolate the toxic principle and to gain some insight into its chemical nature.

III. *Preparation, Use and some Pharmacological Properties of the Kokoi Venom.* The kokoi frog is very small in size; an average adult animal has a body length of 2–3 cm and weighs slightly over 1 g. The skin is black with either two small yellow stripes along the back or two broad bands of a deep reddish yellow, with sprinkles of the same color in between these bands. One animal of each of these two varieties is shown in Figure 3. Common to both are tiny spots of a greenish dark blue color on the legs and also on the lower part of the abdomen.

There exists some controversy in the literature about the correct nomenclature. POSADA, noting the close similarity with *Phyllobates bicolor*, introduced the term *Phyllobates chocoensis*[6]. SAFFRAY[8] uses the name *Phyllobates melanorrhinus*; whereas WASSÉN and SANTESSON[7], after consulting the Swedish zoologist L. G. ANDERSSON, prefer *Dendrobates tinctorius* Schneider. According to a personal communication from Dr. DORIS M. COCHRAN[9] the kokoi frog should be designated as *Phyllobates bicolor*.

The kokoi frog lives in the dense, shady forests of the mountains near the headwaters of the Rio San Juan and its tributaries. It has also been spotted occasionally on the upper Rio Atráto[10], but never in the lower regions (for instance below Playa de Oro on the Rio San Juan), as we have been assured by several reliable persons who live in that area and are quite familiar with the wildlife of the region. POSADA ARANGO[6] mentions that in his time the kokoi was quite abundant, especially around the pueblo of Chamí near the source of the Rio San Juan. Our own experience shows, however, that nowadays it is quite difficult to obtain a large number of the frogs.

The Indians, when collecting frogs, use a little trick[6]. They imitate the frog's peeping, which sounds like fiú-fiú-fiú, with great skill, by whistling and at the same time beating their cheek with the fingers. Their imitation is so perfect that a frog present not too far away usually answers the call and thus can be located. Trying to find these small frogs, which live well hidden among the plants near the ground, by any other means, would seem hopeless.

The Indians are quite reluctant to touch the frogs without protecting their hands with leaves. We have found that the poisonous secretion of the frog has no effect whatsoever on the intact skin. However, in contact with even the smallest scratch it causes a long-lasting, pungent pain not unlike a bee's sting.

The way of poisoning the arrows with the skin secretion has been described in detail by POSADA[6], LEWIN[11] and WASSÉN[7]. The frogs, carried back from the forest in a piece of bamboo tube, are pierced with a specially cut stick called *siuru kida* ('bamboo tooth') through the mouth and the whole body and then held above

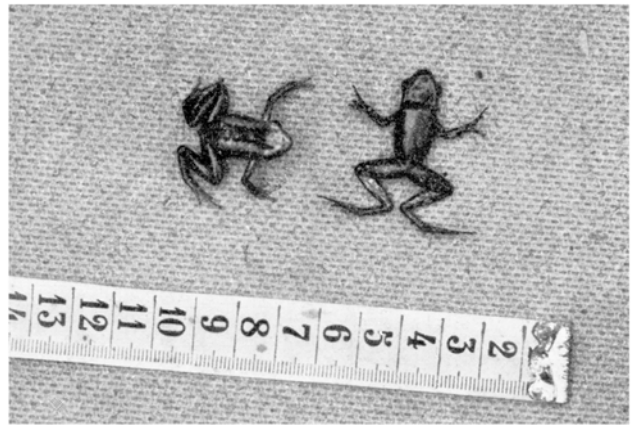


Fig. 3. The two species of kokoi frogs (*Phyllobates bicolor*) used in this investigation. The scale is in centimeters.

[2] C. URIBE PIEDRAHITA, *Apuntaciones sobre la geografía médica de la región de Urabá*, Thesis, Medellín (1920).
[3] C. G. SANTESSON, Skand. Arch. Physiol. *55*, 230 (1929). – *Comparative Ethnographical Studies* (ed. by E. NORDENSKIÖLD, 1931), vol. 9, p. 155.
[4] K. MEZEY, Rev. Acad. colomb. Bogotá *7*, 319 (1947).
[5] A. POSADA ARANGO, *Pubellón Médico de Madrid*, año IX (1869). – Archives de médicine navale XVI (1871). – Revue et magasin de zoologie (2), XXIII (1869). Cited from [6].
[6] A. POSADA ARANGO, Ann. Acad. med. Medellín *1*, 69 (1888).
[7] S. H. WASSÉN, Etnografiska Museet Göteborg, Årstryck 1955–1956 (1957), p. 73.
[8] C. SAFFRAY, Le tour du monde, nouveau journal des voyages *26*, 2 (Paris 1872). Cited from [7].
[9] Smithsonian Institution, Washington (D.C.).
[10] Personal communication by Mr. ALLMENDINGER, Andagoya.
[11] L. LEWIN, *Die Pfeilgifte* (Leipzig 1923), p. 422.

an open fire. Both the pain and the heat provoke an abundant production of a milky secretion, especially on the back of the animal. The tips of the arrows are then dipped into this secretion and afterwards allowed to dry in the shade. One tiny frog produces enough poison to prepare up to 50 arrows.

The arrows are made of the leaf-ribs of the chonta palm and are 20–25 cm long[7]. A spiral incision is cut around the tip so that the poison may stick better, and the tail end is wrapped with a wad of cotton-like plant fibres in order to provide a close fit of the arrow in the tube of the blowgun.

The pacific Cholos of today use the poisoned arrows only for hunting game, such as jaguar, tiger, deer, agouti, monkey and birds like the partridge and the toucan[1]. From the old Chocó myths collected by WASSÉN[1] it is evident, however, that in earlier times the poisoned arrows were also used as deadly weapons in the fight against hostile tribes.

An animal struck by a poisoned arrow becomes paralyzed almost immediately and dies within a short time. The Indians then cut out the arrow from the flesh together with a small piece of meat immediately surrounding it. This is only done as a precaution, since the kokoi venom—like curare—is usually completely harmless when taken orally. A small scratch in the mouth, however, or an ulcer in the digestive tract of a person eating such meat may, quite obviously, cause a dangerous situation.

There are several reports in the literature describing the pharmacological effects of the kokoi venom. Before they are discussed, it should be emphasized that almost all of these studies were done with arrows which had been poisoned up to 15 years before they were used. This may well explain some of the controversial results reported. The symptoms of poisoning, as observed after *subcutaneous* injection of about 15 μg skin extract into a mouse, may be summarized as follows: Within about 1 min after the injection the animal appears to be almost immobilized. It is still able to move, if forced to do so, but only with difficulty. The equilibrium and the coordination of movements are impaired. Dyspnea and violent intermittent convulsions (especially of the neck) follow at about 3 min. The animal falls on its side or on its back, gasping for air. Cyanosis becomes evident and at about 8 min the last violent convulsions of the legs occur. If the thorax is opened immediately, the heart may still be found beating, but it stops within half a minute.

SANTESSON[12], who studied the effect of the kokoi venom in mice, frogs and rabbits, came to the conclusion that it acts by paralyzing the muscles and the central nervous system, especially the respiratory center, but also the heart. He found that in a lethally poisoned frog electrical stimulation of a motor nerve did not produce muscle contraction, whereas direct electrical stimulation of the muscle itself produced twitches. A frog gastrocnemius, immersed in a 1% poison solution and stimulated electrically, responded with twitches of rapidly decreasing amplitude, followed by a strong contracture. Essentially the same effect was noted when a few drops of a poison solution were applied to the exposed heart of a frog; the heart soon stopped in systolic contraction. The venom was found to be devoid of any hemolytic action and could thus be easily differentiated from a toxic material with many similar properties from the skin secretion of the common water frog, *Rana esculenta*, studied by FLURY[13].

While the results of SANTESSON are in good agreement with the much earlier work by POSADA[6], they differ in some aspects from the findings by MEZEY[4]. The latter states that in the nerve-muscle preparation of the toad as well as in the lethally poisoned intact dog there is no change in the direct or indirect excitability of the skeletal muscles. Noting a marked effect on the blood pressure at rather low doses he concluded that the apparent muscular paralysis is probably the result of a reduced circulatory blood volume caused by the arterial hypotension. It should perhaps be mentioned here that the poison used by him was at least 15 years old.

The studies on the toxicity of the kokoi venom showed that rabbits or dogs are about 100 times more sensitive than mice, whereas frogs or toads are affected only at extremely high dosage levels. The minimal lethal dose for mice was found to be about 10–15 mg/kg[12,14], for rabbits about 0.1 mg/kg[4] and for toads or frogs over 200 mg/kg[12]. This holds only for subcutaneous or intravenous injection, since even 100-fold higher doses had no effect at all when administered orally[4,6]. The Indians do not know of any antidote against the kokoi venom. Plant preparations like 'guaco' or extracts from *Aristolochia pilosa* which they use against snake bites, are ineffective[6].

IV. *Isolation of the Active Principle.* So far only one attempt to isolate the active principle of the kokoi venom has been reported in the literature. The results of this early study by the chemist J. ARONHSON are briefly mentioned in the papers published by POSADA in 1871 and 1888[5,6]. According to ARONHSON the active principle is insoluble in ether, chloroform and pure water, slightly soluble in a solution of sodium chloride and in CO_2-saturated water, and well soluble in alcohol. Adsorption to charcoal, followed by elution with alcohol, gave an almost colorless material which seemed to appear crystalline under the microscope, and which he considered to be pure. It had properties of an alkaloid, rich in carbon, nitrogen and phosphorus, but free of oxygen.

[12] C. G. SANTESSON, *Etnologiska Studier* (Göteborg Museum, 1936), vol. 2, 15.
[13] F. FLURY, Arch. exp. Path. Pharmak. *81*, 319 (1917).
[14] We found a LD_{50} of 0.57 mg/kg (subcutaneous), see below.

SANTESSON[12] was later unable to confirm the presence of an alcohol-soluble alkaloid in the kokoi venom.

In the following we will report the results of our own recent study which led us to the isolation of a highly active, homogeneous compound with all the pharmacological properties characteristic of the kokoi venom.

(a) *Preparation of a Crude Extract.* Because of the special nature of the poison material it was not easy to secure an amount sufficient for the isolation of the toxic component. An attempt to ship live kokoi frogs, collected in the Chocó by Mrs. M. LATHAM, failed because the delicate animals did not survive the trip from Colombia to Washington (by air freight). It was therefore decided to prepare extracts of the venom on the spot in the Chocó and to process these to a stage where they could be brought back to the laboratory without loss of activity. This was carried out in August 1962 by one of us (F.M.) at the mining camp of the Cia. Minéra Chocó Pacifico, S.A.[15], at Andagoya, Chocó.

The frogs used for the extraction were all of the species *Phyllobates bicolor*, of the two varieties shown in Figure 3[16]. They were collected by Mrs. M. Latham in the San Juan drainage (in the mountains above Playa de Oro) and on the upper Rio Tadocito. Through her efforts in more than 4 weeks of collecting, a total of 330 kokoi frogs became available for the preparation of the venom extract. The procedure which was finally adopted was as follows: The frogs (in batches of about 10 at a time) were killed with ether. The skins[17] were removed immediately, cut into small pieces with scissors and extracted at room temperature (35–40°C) with 70% aqueous methanol for 2-3 h. The liquid was decanted and the residue extracted again with fresh solvent overnight. The extracts were combined, filtered and concentrated *in vacuo* on a Rinco rotary evaporator. In order to remove traces of moisture, absolute alcohol was added to the residue and the evaporation repeated. The dry, tan residue was then stored in a refrigerator. It did not lose any activity over a period of 6 months, provided it was kept dry.

The combined residue obtained from the 330 frogs had a weight of 910 mg. The toxicity of the material was determined for white mice (subcutaneous injection), using the modified 'Sequential Test' method[18]. The value found for the LD_{50} of 570 ($\pm$40) μg/kg body weight is about 25 times lower than the minimal lethal dose determined by SANTESSON for poison eluted from arrow tips[12].

Most of the residue of the crude extract dispersed easily when mixed with 0.9% sodium chloride solution. The resulting greenish-brown, opalescent solution had a neutral pH and could be heated for 5 min in a boiling water bath without a significant decrease in toxicity.

(b) *Assay.* A highly sensitive, quantitative assay was needed to evaluate different purification procedures in the course of the isolation of the active compound. Taking advantage of the high toxicity of the kokoi venom, it was possible to develop a reliable and convenient method which required only a few micrograms of the toxic material. The method is based on the fact that there is—within certain limits and under controlled conditions—a definite relationship between the amount of poison injected into a test animal and the survival time. This relationship is plotted in Figure 4. Each point represents the average of six individual determinations. Varying amounts of kokoi venom, dissolved in 100 μl of 0.9%NaCl, were injected *subcutaneously* into male white mice of 10 g weight and the elapsed time between the injection and the death of the animal measured. If all animals were injected in exactly the same way, individual determinations of the survival times differed by no more than 10%. The amount of venom which kills a mouse under the specified conditions 8 min after the injection, is (arbitrarily) defined as 'one standard dose'.

To determine the venom concentration of an unknown solution, an aliquot is injected and the survival time measured. The number of standard doses is then determined by using the curve of Figure 4. If the survival time does not fall within the useful range of the curve (between 4 and *ca.* 12 min), the concentration of the injected sample is appropriately adjusted. The average of 3 to 6 individual determinations is usually accurate enough for routine purposes.

Since all purification procedures were carried out in organic solvents (see below), the aliquots used for the assay could not be injected directly. In this case, the samples were instead placed into a small tube and the

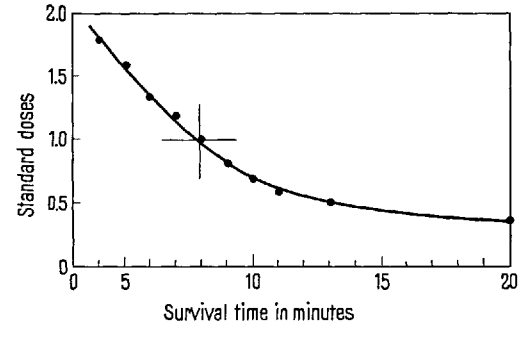


Fig. 4. Assay of the venom: Relationship between dose (subcutaneous injection) and survival time.

[15] The camp is on a peninsula formed by the confluence of the Rio San Juan and the Rio Condóto. – We would like to thank the president and the manager of the Mining Company for permission to stay at the camp. The generous cooperation of Mr. STEWART and Dr. GRANADOS (and many others) was most important for the successful outcome of our project.

[16] Several other, unidentified species of frogs which were collected near Andagoya, were also examined. They were similar in size to the kokoi frog, but quite different in color. Their skin secretion was not – or only very slightly – toxic.

[17] About 90% of the venom extractable from a homogenate of a *whole* frog is found in the skin.

[18] A. W. KIMBALL, W. T. BURNETT JR., and D. G. DOHERTY, Radiation Res. *7*, 1 (1957).

solvent removed in a stream of nitrogen at ca. 35°C. The residue was then dissolved in 0.025 ml acetone, 0.015 ml Tween 80 (Polyoxyethylene [20] monooleate) in water and, in a separate consecutive step, 0.060 ml 0.9% NaCl was added, and the mixture heated to 50°C for about 30 sec. The sample was allowed to cool to room temperature and then injected subcutaneously. This procedure to dissolve the residue was found very satisfactory, whereas the use of NaCl-solution alone did not dissolve the venom in the presence of lipophilic material. The amounts of acetone and Tween 80 indicated above are not toxic by themselves.

One standard dose is equivalent to about 12 μg crude extract or ca. 0.2 μg of the pure toxic component. From an extrapolation of the curve (Figure 4) it can be seen that the minimal lethal dose under these conditions is approximately 0.25 standard doses, or equivalent to ca. 0.05 μg of the pure compound.

Still higher activities are observed on intravenous application. The LD_{50} of a not completely homogeneous sample was determined by Dr. S. L. FRIESS as 2.7 ± 0.2 μg/kg mouse. This is a minimal figure. The kokoi venom ranks about third or fourth place among the most active toxic compounds (Table I) and is the most active venom sofar known.

(c) *Purification.* Two different methods of purification have been developed. Both yield a product which, by the criteria of thin-layer chromatography, is homogeneous and considered to be the pure active principle of the kokoi venom. Since the amounts obtained were so small (less than 1 mg from 100 frogs), no criteria of purity other than those of thin-layer chromatography are available at the moment. The main reason for the low yield is the unpredictable and irrational *lability of the compound.* Although in the crude extract—and in some stages during the purification as well—the activity appears to be rather stable, considerable losses of activity could never be avoided in certain other steps or sometimes even on storage of the partially purified material under dry nitrogen in the refrigerator. A similar behavior has been reported for some of the most active curare alkaloids, for instance the alkaloids E and G (see [19]).

The second of the two purification procedures is much simpler than the first one. Since the second procedure was not elaborated until most of the crude extract had already been processed by the first method, both of them will be described here.

Procedure I consists of the following steps: (1) Preparation of a neutral, aqueous solution of the crude extract, (2) extraction of the activity into chloroform, (3) chromatography on a column of silicic acid, (4) counter-current distribution and (5) thin-layer chromatography. In procedure II the time-consuming steps (3) and (4) are eliminated; after a modified, differential extraction there follows immediately thin-layer chromatography.

Procedure I. Step 1: A neutral solution of the brittle, crude extract is prepared by trituration of the residue with 0.9% sodium chloride solution in a mortar.

Step 2: This solution (concentration of venom 1%) is extracted twice with the two-fold volume of freshly distilled chloroform. Centrifugation is usually required to separate the phases. The chloroform extracts are combined, washed with $^1/_{10}$ volume of water and evaporated *in vacuo*, to leave a brown, oily residue.

Step 3: The residue is dissolved in a small volume of freshly distilled chloroform (containing 1% ethanol as preservative) and adsorbed on a column of silicic acid, prepared in chloroform. The bulk of the inactive, colored material can be removed by washing with chloroform and a mixture of chloroform and methanol 4:1. The active principle is then eluted with a chloroform-methanol mixture 2:1.

Step 4: For the countercurrent distribution (32–50 transfers, volume of each phase 10 ml) in a commercially available Craig apparatus[20], two different solvent

Table I. Toxicity of the most active naturally-occurring poisons

Venom, toxin or poison	Toxicity MLD/μg of cpd.	Animal	Reference
Botulinus toxin crystalline type A	1200[a]	Guinea-pig	C. LAMANNA et al. Science *103*, 613 (1946).
Tetanus toxin	1200[a]	Guinea-pig	W. VAN HEYNINGEN *Bacterial Toxins* (Thomas, Ill. 1950), p. 6.
Diphtheria toxin	3.5	Guinea-pig	
Kokoi venom	20–30	Mouse	This paper.
Calabash curare alkaloid E	0.95–8	Mouse	J. KEBRLE, H. SCHMID, P. WASER, and P. KARRER, Helv. chim. Acta *36*, 116 (1953).
Calabash curare alkaloid G	0.7–12	Mouse	
Paralytic shell fish poison	5–6	Mouse	E. J. SCHANTZ et al., J. Amer. chem. Soc. *79*, 5230 (1957).
Tarichatoxin (eggs)	7	Mouse	M. S. BROWN and H. S. MOSHER, Science *140*, 295 (1963).
Poison from toxic puffer or globe fish	3–5	Mouse	E. F. MURTA, Ann. N.Y. Acad. Sci. *90*, 821 (1960).
Gonyaulax catenella poison (purif.)	5	Mouse	E. J. SCHANTZ et al., Amer. chem. Soc. Meeting (Sept. 1962).
Cobra venom neurotoxin	0.9	Mouse	*Venoms*, edited by E. E. BUCKLEY and N. PORGES, Amer. Assoc. Adv. of Science (Washington, D.C. 1956).

[a] Strictly on a formal weight basis this would correspond to 30,000 mouse units/μg of toxin.

[19] P. G. WASER, in *Curare and Curare-like Agents* (Ed. by D. BOVET et al., Elsevier, 1959), p. 244.

[20] L. C. CRAIG and D. CRAIG, in *Technique of Organic Chemistry* (Ed. by A. WEISSBERGER (Interscience Publ., N.Y., 1956), vol. III, p. 149.

systems can be used. System A is made up of chloroform, carbon tetrachloride, methanol and water in the volume ratio 3:3:4:2; system B has the composition methylene dichloride, cyclohexane, methanol and water 10:1:8:3 (all solvents should be distilled before use).

The results of a representative countercurrent distribution in system A are shown in Figure 5. It is evident that an excellent purification can be achieved, but there is a tendency to form emulsions[21] with this system, unless the solute concentration is below 0.02%. Distributions of more than 3 mg are therefore carried out in solvent system B. In this system the exact position of the peak fractions has to be determined after each distribution experiment, because in the course of the operation a small amount of methylene dichloride evaporates and thus slightly changes the composition of the solvent mixture.

Step 5: The final purification by thin-layer chromatography is as outlined in procedure II (step 3), but the first crude separation can be omitted.

Table IIa presents the data obtained in a typical purification experiment carried out according to the method just described.

Procedure II. When the active component was recognized as a base with an apparent pK of 4.8 in a water–chloroform system, the purification procedure was considerably simplified. The bulk of neutral, lipophilic material, which previously had to be removed by chromatography on silicic acid and by countercurrent distribution, was now separated from the activity by a simple differential extraction as follows:

Step 1: As in Procedure I.

Step 2: The pH of the extract is adjusted to 2 by the addition of hydrochloric acid and this acidic solution extracted twice with the two-fold volume of chloroform. The aqueous phase is then brought to pH 8.5 by the addition of 2.0*N* ammonia. The active principle is now extracted into chloroform (two extractions with the two-fold volume). The organic layers are combined, washed with 1/10 volume of water and evaporated to dryness *in vacuo*.

Step 3: The residue, which contains only the chloroform-soluble, basic components of the crude extract, is chromatographed on thin layer plates (silica gel G, Merck) in the solvent system chloroform–methanol 6:1. For the first, crude separation up to several milligrams may be applied to one square plate. Although the Rf values are somewhat variable at such heavy loads, the location of the active component presents no problem; when the plate (after drying at room temperature) is inspected under a UV-lamp, at least a dozen fluorescent bands are visible and may serve as 'markers'. Figure 6 (left) shows a section of such a plate, photographed under UV-light. As a rule the active material moves immediately behind the second, intensely fluorescent double band near the front. Since there is no direct way of locating the exact position of the (non-fluorescent) venom on the plate, a small vertical strip of the silica gel layer is cut into small fractions, which are separately eluted with a mixture of methanol and

Table II

(a) Procedure I

Step	Fraction	Weight mg	Total activity Standard doses	Specific activity Standard doses/mg
1	Crude extract	100	8400	84
2	Chloroform extract	18	6000	330
3	Silicic acid chromatography	8	4600	580
4	Counter-current distribution	1.5	4000	2700
5	Thin-layer chromatography	ca. 0.3	1500	5000

(b) Procedure II

Step	Fraction	Weight mg	Total activity Standard doses	Specific activity Standard doses/mg
1	Crude extract	100	8500	85
2	(Differential) chloroform extr.	4	5100	1300
3	Thin-layer chromatography	ca. 0.3	1500	5000

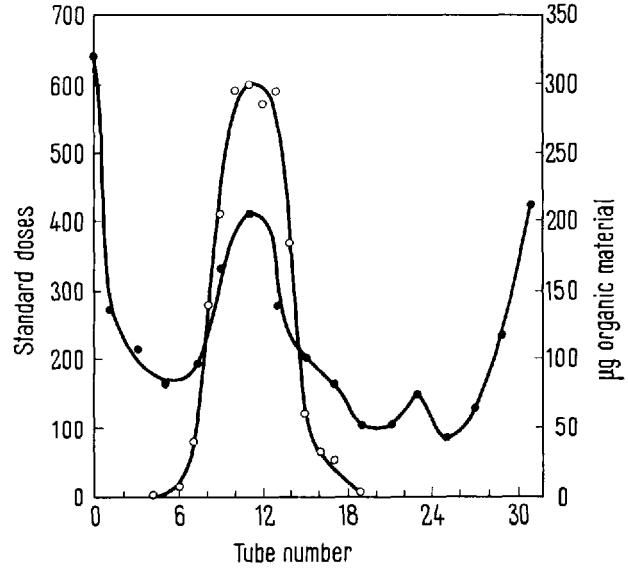


Fig. 5. Countercurrent distribution of the venom. Solvent system A (composition see text), 32 transfers. Curve 1 (open circles): activity (measured by the standard assay procedure). Curve 2 (solid circles): total organic material (determined by a microadaptation of the bichromate method of JOHNSON[22]). Partition coefficient of the active principle: 0.56.

[21] When this is the case, the activity is usually lost completely.
[22] M. J. JOHNSON, J. biol. Chem. *181*, 707 (1949).

chloroform (1:1). Aliquots of the eluates are then assayed for toxicity according to standard procedure. The area indicated between the arrows (Figure 6) contains the venom. The right half of Figure 6 shows a section of the same plate after exposure to iodine vapor. This sensitive, unspecific method provides a rough estimate of the distribution of organic material on the plate.

Rechromatography of the active material in the same solvent system, resolves the enriched venom into four spots (made visible by iodine vapor, see Figure 7, Ia). The activity is located in the main spot (Rf ca. 0.55). When sprayed with iodoplatinic acid, only the two spots in the middle give a positive reaction (Figure 7, Ib).

A third chromatography of the substance with an Rf between 0.50 and 0.62 (indicated by arrow in Figure 7, I) reveals that the material is now homogeneous: only one spot is found after treatment with iodine vapor (Figure 7, IIa) or on spraying with iodoplatinic acid (Figure 7, IIb). No fluorescence is detectable under UV-light. If finally a strip of the chromatogram is cut into small segments and these assayed for activity, the distribution of the latter coincides with the only detectable spot on the plate (Figure 7, III).

The results of a purification according to this procedure are summarized in Table IIb.

(d) *Chemical properties*. The active material obtained by either one of the two purification procedures had a specific activity of 5000 standard doses/mg and behaved as a single, homogeneous compound when examined by thin-layer chromatography. Since not more than a few hundred micrograms of this material were available, no attempt was made to crystallize it. The melting point or the elemental composition were therefore not determined. These and other properties commonly used to characterize an unknown compound will have to be determined in the future, if more material should become available.

In order to obtain some information about the functional groups of the molecule, the behavior relating to different types of ion exchange resins was studied. The active principle (dissolved in 90% aqueous methanol) was not adsorbed on a column of Dowex 1-X8 (strongly basic anion exchange resin, OH^--form) and, therefore, has no anionic groups. On the other hand, it was very strongly bound on a column of Aminex MS (cation exchange resin, sulfonated styrene type, NH_4^+-form). The activity was recovered (although not quantitatively) by elution with 0.1*N* barium acetate. This behaviour indicates the presence of a basic (cationic) group in the molecule.

The pK of this basic group was determined in the following way: Samples of 3.0 ml of stock solutions of purified venom either in chloroform or in a mixture of 10% benzene in cyclohexane were extracted with equal volumes of 0.1*M* aqueous buffer solutions, ranging in pH from 3 to 10.5 at room temperature. After equilibration and centrifugation an aliquot of each organic phase was withdrawn, evaporated to dryness and the activity of the residue measured by the standard assay procedure.

Starting at low pH, it was found that by increasing the pH of the aqueous phase increasing amounts of the venom moved to the organic phase until a maximal value was reached. The partition coefficient in the system 10% benzene in cyclohexane/water was found to be 1.3. Exact determinations in the system chloroform/water were not possible, because of $K \geq 200$. For a system of this kind a plot of the percent of maximal organic-extractable activity, which for all practical purposes substitutes well for the expression $N'(1+1/K)$, *versus* $pH+\log(1+K)$ will give a titration curve for the group (or groups) involved in the solubilization of the venom in the organic phase.

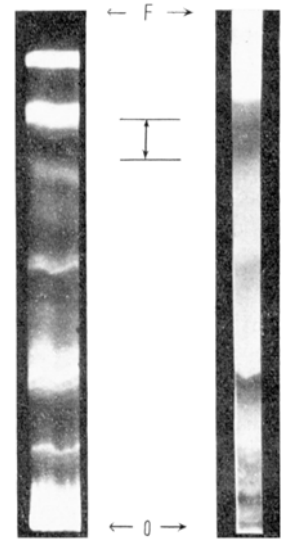


Fig. 6. Thin-layer chromatography of the chloroform-soluble, basic components of the crude extract. The picture on the left shows the fluorescence under UV-light; the picture on the right was photographed (in daylight) after exposure to iodine vapor. O = origin, F = solvent front. The activity is found in the area indicated by the arrow.

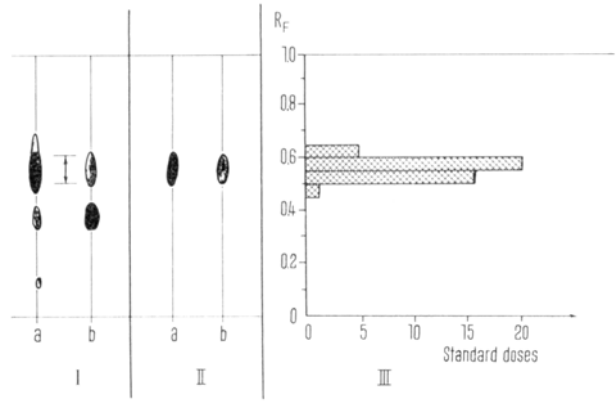


Fig. 7. Rechromatography on thin-layer plates. Explanation in the text.

Figure 8 indicates a pK of 7.1 and 8.0 for the systems water–chloroform, and water-benzene-cyclohexane, respectively. Basic groups within this pK range would include imidazole and carbinolamines containing the element $>N-\overset{|}{\underset{|}{C}}-O-$. The Pauly reaction with diazotized sulfanilic acid under alkaline conditions was negative.

The UV-spectrum (in methanol or $N/100$ HCl) is of the 'end absorption' type, with shoulders at 220, 230 and 260 mμ. Assuming a molecular weight of 400 from preliminary mass-spectrometric data, the ε-values at 200 and 260 mμ are approximately 10^4 and 1.5×10^3, respectively.

The IR-spectrum is shown in Figure 9. A sharp absorption band at 5.9 μ may indicate the presence of an amide carbonyl group. Evidence for amino groups is found from the peaks near 3 μ.

Work in progress by Dr. K. BIEMANN (M.I.T.) on the behaviour of the material in the mass spectrometer may lead to further insights into the chemical nature of the kokoi venom.

V. *Recent Pharmacological Studies with Purified Venom.* The pharmacology of the kokoi venom which has been reported in the literature so far, was studied with crude material. In those instances where the material had been eluted from poisoned arrows, which were many years old, many of the effects observed are not characteristic of the pure venom.

A series of experiments was done by Drs. FRIESS and DURANT[23] to study the effect of the purified venom on the isolated nerve-muscle preparation (rat diaphragm, phrenic nerve in Ringer solution at 37°). Their results are shown in Figure 10. The diaphragm responded to electrical stimulation (applied in 10-sec-intervals, either directly to the muscle or indirectly via the phrenic nerve) with contractions of constant amplitude (left-hand side). When purified venom was added (arrow, I) to the preparation to give a concentration of 0.15 standard doses/ml (ca. 0.03 μg/ml), a contracture began to develop after a lag of about 90 sec. After an additional 3 min the amplitude of the nerve-stimulated contractions began to decrease rapidly and at $6^1/_2$ min after addition of the venom a total block had occurred. The amplitude of the contractions induced by direct stimulation of the muscle was only moderately reduced at this stage, but decreased steadily to zero (III) in the course of 10 min. Evidently the venom acts irreversibly, since washing the muscle preparation with venom-free Ringer solution (indicated by the arrows II and III) did not reverse or even delay the effect of the venom. The last stage (after complete blockade of the contractions) was a powerful contracture. In a parallel experiment, in which the venom concentration used was almost twice as high, the same effects were observed, except that the final stage was reached after a much shorter time.

D-Tubocurarine, which at a concentration of $10^{-5}M$ caused a total, but fully reversible, block of neuromuscular transmission, did not prevent or delay the myotropic effects of the frog venom.

Another series of experiments was undertaken by Dr. I. TASAKI[24] to study the effect of the venom on the electrophysiological properties of the toad nerve-muscle preparation. In these experiments sciatic-sartorius preparations of the toad *Bufo marinus* were mounted on a plexiglass platform equipped with three pairs of silver electrodes. The first pair, making contact with the proximal portion of the nerve, was used to deliver stimulating pulses to the preparation. The other two pairs served to record the action potentials from the nerve (trunk) and from the muscle. The preparations were kept immersed in Ringer solution at room temperature (ca. 22°), except when the platform was raised from the bath for the measurements. Figure 11,A shows a typical recording of the action potentials (nerve and muscle) of a normal sciatic-sartorius preparation. After addition of the venom (0.5–1 standard

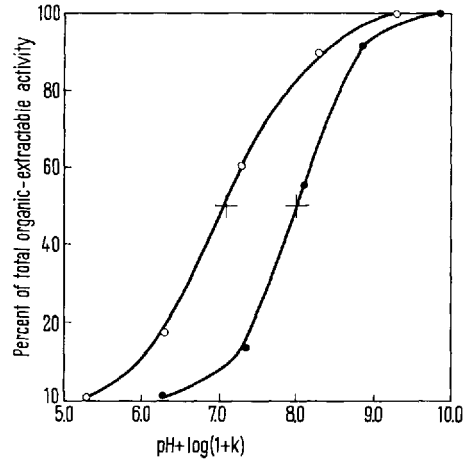


Fig. 8. Determination of the pK values by measuring the organic-extractable activity of the venom in two different solvent systems. The open circles (o-o-o) are for the system water/chloroform and lead to a pK of 7.1. The solid circles (●–●–●) are for the system water/10% benzene-cyclohexane and lead to a pK of 8.0.

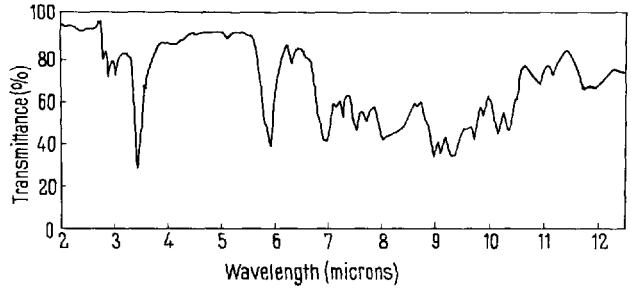


Fig. 9. Infrared spectrum of the active, highly purified venom in $CHCl_3$ solution.

[23] U.S. Naval Medical Research Institute, Bethesda (Md.).
[24] Laboratory of Neurobiology, National Institute of Mental Health, N.I.H.

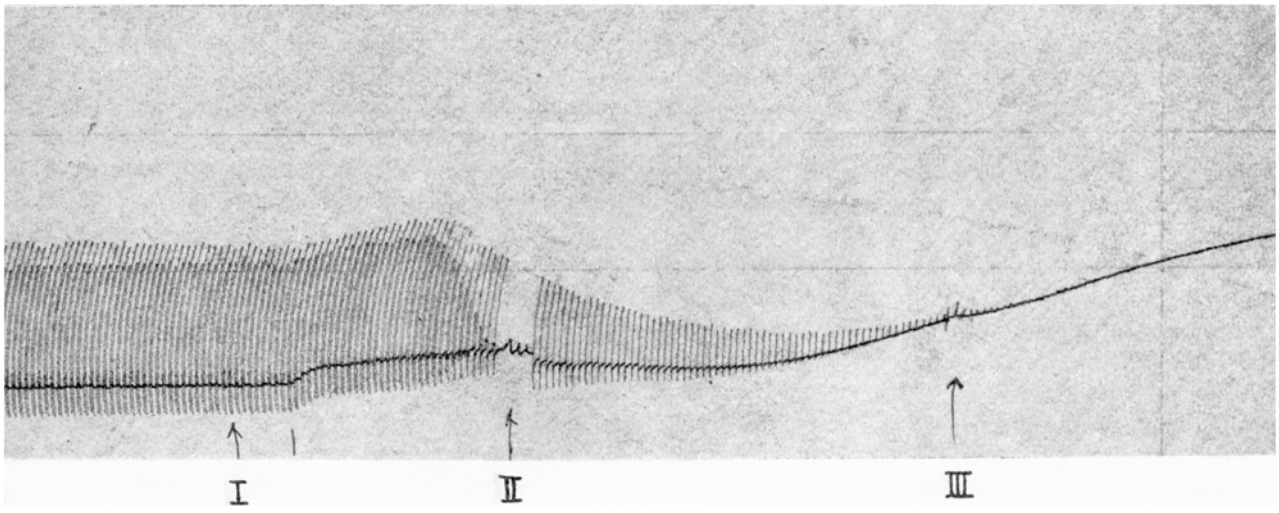


Fig. 10. Effect of purified kokoi venom on the isolated rat diaphragm-phrenic nerve preparation (Ringer solution at 37°). Contractions were induced by direct electric stimulation of the muscle (high amplitude) or indirectly *via* phrenic nerve. The intervals between successive stimulations were 10 sec. I: Addition of venom (circa 0,03 µg/ml). II, III: Preparation washed repeatedly with venom-free Ringer solution.

dose/ml) a decrease of the action potential of the muscle (but not of the nerve), and spontaneous, repetitive discharges in the muscle were observed (Figure 11,B). Later on, spontaneous repetitive firing of impulses (in the absence of external stimulation) occurred in the muscle fiber (Figure 11,C). Associated with this phenomenon was the appearance of small, frequent twitches of the muscle which could be seen with the naked eye. A stage was finally reached where the muscle no longer produced an action potential upon stimulation, although the action potential of the nerve was still unchanged (Figure 11,D). However, when the electrical stimulation was applied directly to the muscle, large propagated action potentials, associated with strong twitches, were observed.

Due to the low sensitivity of the toad to the poison, and probably also because of the low temperature, at

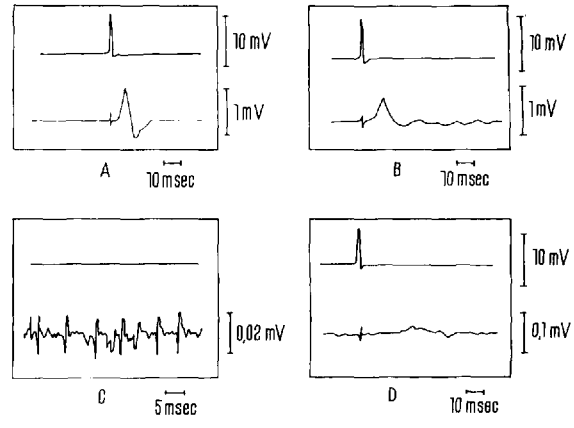


Fig. 11. Recordings of action potentials of sciatic-sartorius preparation (*Bufo marinus*). Top trace: nerve. Bottom trace: muscle. Time sequence is from left to right. Explanation in the text.

Table III. Effects of kokoi venom at the sublethal and lethal dose level in cats
[observed by Dr. S. L. Friess (U.S. Naval Medical Research Institute, Bethesda, Md.)]

Time after intravenous injection (min)	Sublethal dose: 3 µg/kg in 3.8 kg cat	Time after injection	Lethal dose: 5 µg/kg in 3 kg cat
0.5–2	Strong muscle contraction, powerful gag reflex, salivation, urination, powerful tonic convulsions, gut contraction, nictitating membrane not affected	0.5	Back muscles pulled taut, gut tight, mydriasis
2–4	Cat was able to walk off, strong salivation continued	2	On side, nictitating membrane about $^1/_4$ closed (i.e. superior cervical ganglion affected), powerful salivation
4–5	Breathing difficulties, rocking respiration	3	Fasciculations along side, stiffening and extension of limbs
5–10	Choking on saliva, vomiting attempts. Breathing shallow and rapid	6	Strong salivation and lacrimation continue, tongue out, thrashing, respiration strongly affected, cat able to right and walk
10–30	Salivation unabated, lacrimation, very rapid panting, no response to outside stimuli, trembling in head region	8	Chewing, tongue biting, frothing through mouth and nose, muscle contracture, anoxic convulsions body completely tight and doubled up
	Complete recovery after 8 h	9	Exitus

which the experiments were done, the block of the neuromuscular transmission occurred only after about 2 h (as compared to a few minutes in the rat preparation at 37°).

The resting potential of the muscle as studied by the microelectrode technique[25], appeared to be normal, even after complete blockade of neuromuscular transmission.

The lethal effect of the kokoi venom is caused by a multiplicity of events of which the irreversible block of neuromuscular transmission, probably at the neuromuscular junction, is the only property which it has in common with curare. In addition there must be a strong central action to judge from the powerful tonic convulsions observed in mice over a large dose range. At high intravenous dose levels death, which occurs within seconds, is accompanied by complete muscular rigidity. The toxic syndrome besides convulsions, involves powerful contractures over the entire body in line with the observations on the myotropic effects in the nerve-muscle preparations. Observations on cats (Table III) confirm these views.

A possible role of the venom in the transport of ions across the frog skin membrane (sodium pump action)[26] is only an interesting speculation at this stage.

The storage of the venom in a physiologically active form in the frog skin poses an interesting problem. It is possible that after death the venom is released into the tissue where it is destroyed by enzymes[27].

Zusammenfassung. Ein hochaktives Gift mit einer LD_{50} von mindestens 2.7 ± 0.2 μg/kg Maus wurde aus der Haut des columbischen Pfeilgiftfrosches *Phyllobates bicolor* isoliert. Nach 60facher Anreicherung erwies sich das Produkt in der Dünnschichtchromatographie als einheitlich. Es ist löslich in organischen Lösungsmitteln wie Chloroform und Methylenchlorid und lässt sich aus solchen Lösungen mit wässriger Säure extrahieren. Aus dem Verteilungsverhalten der Aktivität bei verschiedenen pH-Werten wurden pK-Werte von 7.1 und 8.0 ermittelt, die auf die Anwesenheit eines basischen Strukturelementes hinweisen. Das UV-Spektrum zeigt Endabsorption mit Schultern bei 220, 230 und 260 mμ. Eine ausgeprägte Absorptionsbande im IR-Spektrum ($CHCl_3$) bei 1690 cm^{-1} deutet auf eine Amidcarbonylgruppe hin. Das Gift bewirkt im Nerv-Muskelpräparat zunächst eine irreversible Blockierung der Nervenendplatten; später folgen myotrope Effekte (Kontraktur). *In vivo* wird neben der Atemlähmung eine starke, wahrscheinlich zentral ausgelöste Krampfwirkung neben anderen Effekten beobachtet, die das Kokoigift deutlich vom Curare abhebt. Das Kokoigift ist das stärkste bis jetzt bekannte Gift animalischen Ursprungs.

[25] I. TASAKI and S. HAGIWARA, Amer. J. Physiol. *188*, 423 (1957).

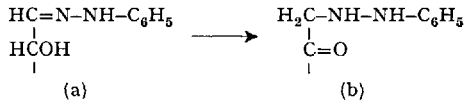
[26] H. H. USSING, *Biochemistry of the Central Nervous System*, Proceedings of the Fourth International Congress of Biochemistry, Vienna, September (1958), (Ed. by F. BRICHE, Pergamon Press, New York 1959), p. 1.

[27] *Acknowledgments.* We are greatly indebted to Dr. S. L. FRIESS, Naval Medical Research Institute, and to Dr. and Mrs. I. TASAKI for the toxicological, pharmacological and neurophysiological data. Mrs. M. LATHAM, Member of the National Academy of Sciences, has made this investigation possible by leading an expedition into the Chocó Jungle and by the skilful organization of the frog collection and processing under adverse conditions.

Brèves communications – Kurze Mitteilungen – Brevi comunicazioni – Brief Reports

Über den Mechanismus der Osazonreaktion

Die im Jahre 1884 von FISCHER[1] gefundene Osazonreaktion der Aldosen und Ketosen soll nach Untersuchungen von WEYGAND[2] und SIMON[3] über die Amadoriumlagerung des primär gebildeten Phenylhydrazons (a) zu 1-Desoxy-1-phenylhydrazino-D-fructose (b) verlaufen.



(a) (b)

Diese Deutung ist nicht befriedigend, da es bisher nicht gelang, eine Amadoriverbindung mit Phenylhydrazin als N-Komponente am C-1-Atom darzustellen. Im Rahmen unserer Untersuchungen über den Mechanismus der Amadoriumlagerung[4] suchten wir die Frage zu klären, ob sich aus einer Aldose mit Phenylhydrazin die von WEYGAND als Zwischenprodukt angenommene Amadoriverbindung bilden kann.

4,6-Benzal-D-glucose (I) gibt mit überschüssigem Phenylhydrazin in hoher Ausbeute 1,1-Bis-phenylhydrazino-4,6-benzal-D-glucose (II). 1,1-Bis-N-acetale von aliphatischen oder aromatischen Aminen sind, wie wir zeigen konnten, Zwischenprodukte beim Übergang in die Derivate der 1-Desoxy-1-amino-D-fructose[4]. Unterwirft man jedoch II den Bedingungen dieser Umlagerung, dann entsteht *nicht* das erwartete Amadoriprodukt (b). Dass der

[1] E. FISCHER, Ber. dtsch. chem. Ges. *17*, 579 (1884); *20*, 821 (1887).

[2] F. WEYGAND, Ber. dtsch. chem. Ges. *73*, 1284 (1940). – F. WEYGAND, H. SIMON und J. F. KLEBE, Chem. Ber. *91*, 1567 (1958).

[3] H. SIMON, K. D. KEIL und F. WEYGAND, Chem. Ber. *95*, 17 (1962).

[4] F. MICHEEL und I. DIJONG, Tetrahedron Letters *1962*, 21; Liebigs Ann. *658*, 120 (1962).