case of eledoisin-physalaemin and other peptides[2-4], in the analysis of the fragments obtained by enzymatic degradation of phyllomedusin.

Upon digestion with chymotrypsin it was soon evident that the C-terminal half of the molecule had the same composition and sequence as that of eledoisin.

Pyr-Asn-Pro-Asn-Arg-Phe-Ile-Gly-Leu-Met-NH_2
|— Chym-1 —| |Chym-2| |–Chym-3 | |Chym-4|

Three peptide bonds were, in fact, hydrolyzed with the release of 4 fragments, 2 of which were readily identified as methionine-amide (Chym-4) and as the tripeptide H-Ile-Gly-Leu-OH (Chym-3). Furthermore, the specificity of the chymotryptic attack allowed the precise localization of the phenylalanine residue adjacent to that of isoleucine, as in the C-terminal pentapeptide of eledoisin: -Phe-Ile-Gly-Leu-Met-NH_2. The chymotryptic fragment Chym-2 migrated electrophoretically as a distinctly basic spot ($E_{1.2} = 1.5$ Glu, and $E_{5.8} = 0.84$ His). It was positive to the Sakaguchi reagent and on total acid hydrolysis contained only the arginine and phenylalanine residues. To this fragment the dipeptide structure H-Arg-Phe-OH could be confidently assigned. The last spot (Chym-1) was positive only to chlorine, indicating the lack of free amino groups, it was steady at pH 1.2 and migrated anodically at neutral pH ($E_{5.8} = 0.45$ Glu) indicating an acidic character. Its acid hydrolysate contained 1 mole of glutamic acid, one mole of proline and 2 moles of aspartic acid. Hence, it was clearly the N-terminal tetrapeptide with the composition Pyr-[Pro, 2 Asp]-OH.

Upon tryptic digestion of phyllomedusin only the Arg-Phe bond was hydrolyzed, producing 2 pentapeptides Tryp-1 and Tryp-2.

Pyr-Asn-Pro-Asn-Arg-Phe-Ile-Gly-Leu-Met-NH_2
|—— Tryp-1 ——| |—— Tryp-2 ——|

One of the 2 pentapeptides was the N-terminal half-chain bearing the arginine residue in its C-terminal position, as deduced by the specificity of trypsin. The position of arginine was also indicated by the analysis of Chym-2 and was definitely confirmed by digesting Tryp-1 with carboxypeptidase B which readily liberated this amino acid from the fragment.

The N-terminal tetrapeptide (Chym-1) resulting from the chymotryptic digestion of phyllomedusin, or from digestion of Tryp-1 with carboxypeptidase B, was, on the other hand, completely resistant to the attack of the carboxypeptidases A and B and its sequence had to be deduced by other means.

The electrophoretic migration of phyllomedusin itself (basic in both neutral and acidic media) and of the peptide Tryp-1 (basic at pH 1.2 and neutral at pH 5.8) demonstrated that both the aspartic acid residues were present in the amide form. Furthermore, the chymotryptic attack between Chym-1 and Chym-2 indicated that one of the asparagines had to be bound to the amino group of arginine. This was confirmed by submitting Chym-1 to hydrazinolysis which produced the β-hydrazide of the aspartic acid. The uncertainty of the N-terminal part of the chain, thus restricted to the relative positions of one of the asparagines and of the proline residue, was clorified by partial acid hydrolysis of Chym-1. In the acid hydrolysates obtained by heating the fragment with 0.1 *N* HCl at 100 °C for 1 or 2 h, besides the individual free amino acids, the 2 dipeptides Pyr-Asp-OH and H-Pro-Asp-OH were in fact clearly identified.

Riassunto. Viene descritto l'isolamento e il chiarimento della struttura della phyllomedusina, decapeptide attivo della pelle dell'anfibio sudamericano *Phyllomedusa bicolor*. La phyllomedusina è apparsa strutturalmente assai vicina alla eledoisina e alla fisalemina.

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Cardiotonic Action of 3-Deoxydigitoxigenin

Extending our serial studies on the structure-activity relationship of cardiotonic steroids[1-3], the cardiac action of 3-deoxydigitoxigenin was studied in comparison with that of digitoxigenin.

Experiments were performed on the isolated frog's heart (Straub's preparation). The Straub's cannula contained 2.0 ml of Ringer's solution, the composition of which was as follows: NaCl 111 m*M*; KCl 2.7 m*M*; $CaCl_2$ 1.8 m*M*; $NaHCO_3$ 1.2 m*M*; glucose 2.7 m*M*. The bathing medium was aerated via a fine polyethylene tubing inserted into the cannula. All the experiments were conducted at room temperature (20–25 °C).

3-Deoxydigitoxigenin used was synthesized and kindly supplied by Dr. M. Okada of Tokyo Biochemical Research Institute, Tokyo[4]. Digitoxigenin was obtained from the same source. Stock solutions of these two compounds were prepared, by dissolving them in 70% ethanol in a concentration of 1 mg/ml. Immediately before use, the stock solution of digitoxigenin was diluted to a desired concentration with distilled water. Because of the low water solubility of 3-deoxydigitoxigenin, the stock solution of this compound was first diluted with 70% ethanol to make a solution of 100 µg/ml, from which a solution of a desired concentration was prepared by dilution with distilled water.

Prior to the administration of the drugs, an impairment of the contractile force was induced by reducing the calcium concentration of Ringer's solution to 0.6 m*M*, $^1/_3$ of the normal. Then a small volume of the test solu-

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tions (usually 0.02–0.06 ml) was added to the bathing medium. Every 10–15 min, another small volume of the test solution was added to the bathing medium to produce a step-wise increase in the concentration of the test compounds, until the heart went into a systolic arrest.

The results are summarized in the Table. As can be seen from this table, despite the absence of 3β-hydroxy group, 3-deoxydigitoxigenin produced almost as strong a cardiotonic action as digitoxigenin in this preparation. Very recently, ZÜRCHER et al.[5] have reported that 3-deoxydigitoxigenin could induce a marked inhibition of a transport ATPase prepared from the heart muscle of the guinea-pig, a finding which is in good accordance with ours.

Actions of digitoxigenin and 3-deoxydigitoxigenin on the isolated frog's heart (STRAUB's preparation)

Concentration (g/ml)	10^{-8}	3×10^{-8}	10^{-7}	3×10^{-7}
Digitoxigenin	—	—	+	×
	—	+	+	×
	—	+	+	×
	—	+	+	×
	—	+	+	×
3-Deoxydigitoxigenin	—	—	+	×
	—	—	+	×
	—	—	+	×
	—	+	+	×
	—	+	+	×

—, no effect; +, improvement of contractility without a tendency to systolic arrest; ×, systolic arrest.

In a previous communication[3], we reported that 14-deoxy-14βH-uzarigenin retains cardiotonic activity, despite the absence of 14β-OH. Thus, it is now clear that, against the long-standing belief on the structure-activity relationship of the cardiotonic steroids, neither 3β-OH nor 14β-OH is indispensable for the cardiotonic action of the compounds.

A preliminary report of the present study was read at the 42nd Annual Meeting of the Japanese Pharmacological Society held in Tokyo on the 2nd of April, 1969[6].

Zusammenfassung. Die Herzwirksamkeit von 3-Deoxydigitoxigenin am isolierten Froschherzen wurde geprüft. Im Gegensatz zur bisherigen Auffassung (3β-Hydroxygruppe notwendig für kardiotonische Wirkung der Digitalis-Verbindungen) zeigte 3-Deoxydigitoxigenin eine starke kardiotonische Aktivität.

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The Red Impurity in Trypan Blue

Dyes such as trypan blue, Evans blue and Niagara blue 2B, prepared by coupling benzidine or a derivative with an aminonaphtholdisulphonic acid, were introduced into biology as vital stains and later used in the measurement of blood volume. Publications on these biological uses contain numerous references to complications arising from the invariable presence of contaminating red compounds in samples of the dyes. The first systematic study, of the red impurity in trypan blue, was made by KELLY[1], whose paper also includes a summary of the earlier literature. Further interest in the red impurities followed the discovery that trypan blue was both teratogenic[2] and carcinogenic[3], and subsequent reports of variable responses to different commercial samples of trypan blue led to suggestions that the red impurity might be the active principle of the whole dye. Experiments on fractions isolated from commercial trypan blue suggest that this is not so: the teratogenic activity of trypan blue is probably due to its major blue component[4,5], whereas the carcinogenic activity appears to reside in a further 'purple' impurity present in some samples[6]. However, it is not certain whether the red impurity contributes to the teratogenic or carcinogenic potency of the whole dye. An investigation of this question requires more of the compound than can conveniently be prepared by extraction from the whole dye. We have therefore determined the structure of the red impurities of Niagara blue 2B (Colour Index no. 22610) and trypan blue (Colour Index no. 23850) with a view to preparing synthetic samples in quantity for metabolic and toxicological studies.

A small quantity of the red impurity of Niagara blue 2B, extracted from the whole dye as described by BECK and LLOYD[4], was reduced with dithionite, acidified and the decolourized solution passed through a column of Dowex 1 (chloride form) to remove anionic materials. The eluate gave an absorption spectrum in 0.1 *N* HCl and 0.1 *N* NaOH identical with that of 4-aminobiphenyl[7]. Paper electrophoresis of the reduction products by the method of LLOYD and BECK[8] was consistent with these

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