in both the preparations, and simultaneously part of these samples were made alkaline (pH 12.0) boiled and again acidified to pH 5.0 lost acetylcholine-like activity when assayed on these preparations. Standard acetylcholine solutions were made with acidified cerebrospinal fluid used for perfusion.

In a total of 285 samples, acetylcholine was found in varying amounts obtained in 25 conscious dogs. The Table shows the frequency of acetylcholine release in half-hour samples. Usually the output was between 0.1 to 1 ng/min and the next frequent range was 1 to 5 ng/min. Wide variation is seen in the ramaining samples ranging from 0.0 to 0.1 and upto 120 ng/min. Acetylcholine output was nil in 23 samples. The output varied in different dogs and from day to day in the same dog. The high values in between 20 to 120 ng/min were found in dogs on the 3rd to 4th days of perfusion. The reduced output in some dogs were found to be in a state of drowziness and during relaxation. These results are plotted in the form of a histogram in the Figure.

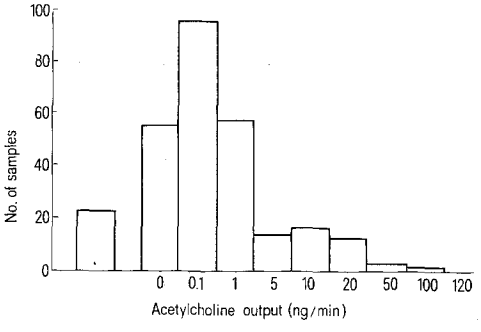
There is also a bearing on the size leading to high output of acetylcholine as seen by the values from 5 ng to 20 ng/min. Moreover, the maximum activity of the animals also showed an increase, usually from 10 to 50 ng/min and sometimes more than 100 ng/min.

This study indicates that the acetylcholine output into the liquor spaces in unanaesthetized dogs is large, since no anticholinesterase has been included in the perfusate. Another alternative is that the liquor spaces of brain have a low concentration of cholinesterase enzyme to hydrolize acetylcholine released into the perfusate. In an earlier study, we reported[6] that even 10 µg/ml eserine in the perfusate did not induce any change in the acetylcholine output into the liquor spaces in unanaesthetized dogs. The continuous perfusion might have further reduced even if low concentration of acetylcholine-esterase was present in the liquor spaces.

The source of acetylcholine present in the perfusate was from the cerebral ventricles, subarachnoid spaces around the brain stem and the upper cervical cord, since these areas are included in the perfusion system. More acetylcholine could have come from the large areas of grey matter, such as the caudate nucleus, hypothalamus and 4th ventricle, as BELESLIN et al.[12] suggested that much of acetylcholine release is from grey matter when the different parts of lateral and 3rd ventricles were perfused in cats under chloralose anaesthesia.

The frequency range of acetylcholine release into the liquor spaces of unanaesthetized dogs in 30 min ventricular perfusate samples

Serial No.	Acetylcholine output (ng/min)	Total	%
1	Nil samples	23	8.070
2	0–0.1	57	20.000
3	0.11–1.0	97	34.035
4	1.1–5.0	59	20.700
5	5.1–10.0	15	5.263
6	10.1–20.0	17	5.970
7	20.1–50.0	13	4.560
8	50.1–100.0	3	1.052
9	100.1–120.0	1	0.350



The frequency distribution of acetylcholine output in 285 half hourly perfusate samples during perfusion of liquor spaces with artificial cerebrospinal fluid in unanaesthetized dogs. The columns show number of samples in each range and the concentration of acetylcholine (ng/min) is given at its base.

Zusammenfassung. Acetylcholin kann im Liquor von wachen Hunden gemessen werden, wobei die gemessene Menge dem wachen Zustand entspricht.

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[12] D. BELESLIN, E. A. CARMICHAEL and W. FELDBERG, J. Physiol., Lond. *173*, 368 (1964).

[13] This investigation was financed by the Indian Council of Medical Research, New Delhi 110016 and has been carried out at Department of Pharmacology, Kurnool Medical College, Kurnool 518002 (A.P.) India. I am thankful to Professor P.S.R.K. HARANATH for advice and helpful suggestions during the period of study.

Structure-Activity Relationship of the Cardenolides Derived from Digoxigenin and Digitoxigenin, with Special Reference to the Configuration at C-5

In connection with the structure assigned to a new cardiac aglycone, syriogenin[1], 5α-digoxigenin (Ia)[2] was prepared from digoxigenin (IIa) via 3-oxodigoxigenin (IIc) and 3-oxo-Δ^4-digoxigenin (IIIa). In the course of this synthetic work, 3-epi-digoxigenin (IIe) and 3-oxo-$\Delta^{1,4}$-digoxigenin (IVa) were also obtained. Thus, the cardiotonic activities of these 6 compounds were tested by using the Straub's preparation, and compared with those of the corresponding compounds derived from digitoxigenin (IIb), namely uzarigenin (Ib), digitoxigenone (IId), Δ^4-digitoxigenone (IIIb), 3-epi-digitoxigenin (IIf), and $\Delta^{1,4}$-digitoxigenone (IVb).

[1] L. MASLER, Š. BAUER, O. BAUEROVÁ and D. ŠIKL. Colln. Czech. chem. Commun. *27*, 895 (1962).

[2] M. OKADA and T. ANJYO, Chem. Pharm. Bull. Tokyo, to be published.

Final concentrations and the relative potencies of the compounds used

Digoxigenin-Series			Digitoxigenin-Series		
Compound (12β:OH)	C-contr. (g/ml)	RP (IIa = 1.0)	Compound (12β:H)	C-contr. (g/ml)	RP (IIb = 1.0)
Ia (5α-digoxigenin)	10^{-5}	0.1	Ib (uzarigenin)	3×10^{-6}	0.1
IIa (digoxigenin)	10^{-6}	1.0	IIb (digitoxigenin)	3×10^{-7}	1.0
IIc	3×10^{-6}	0.3	IId	3×10^{-7} — 10^{-6}	0.3–1.0
IIe	$(1–3)\times10^{-5}$	0.03–0.1	IIf	3×10^{-6} — 10^{-5}	0.03–0.1
IIIa	3×10^{-6} — 10^{-5}	0.1–0.3	IIIb	$(1–3)\times10^{-6}$	0.1–0.3
IVa	3×10^{-6}	0.3	IVb	$(1–3)\times10^{-6}$	0.1–0.3

C-contr., concentration of the drug reached when systolic contracture of the heart took place; RP, relative potency.

The method of assay is the same as described in the previous papers[3–5]. Frogs, *Rana nigromaculata*, were used. The Straub's cannula contained 2 ml of Ringer's solution (NaCl 111 m*M*, KCl 2.7 m*M*, $CaCl_2$ 1.8 m*M*, $NaHCO_3$ 15 m*M*, glucose 2.7 m*M*), aerated with 95% O_2 + 5% CO_2. The contraction of the heart was recorded isotonically on smoked drums. The heart was first made hypodynamic by reducing the concentration of calcium to 0.6 m*M*, $^1/_3$ of the normal, and then the effect of one of the compounds was tested in the following way.

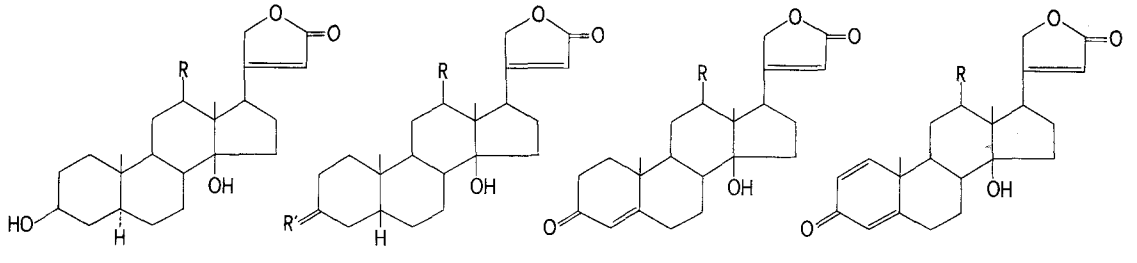
Stock solutions were prepared by dissolving each compound in 95% ethanol in a concentration of 1 mg/ml. Before experiment, these solutions were diluted with the low Ca Ringer to desired concentrations. Starting from a subthreshold dose, a small amount (0.02–0.14 ml) of a diluted solution was added to the cannula every 15 min, so that a stepwise increase in the cumulative concentration of the test compound was achieved, until the heart went into systolic contracture. The way of increase in the cumulative concentration was: 10^{-n}, 3×10^{-n}, $10^{-(n-1)}$, $3\times10^{-(n-1)}$, $10^{-(n-2)}$. The relative potencies were obtained on the basis of the concentration of each compound in which systolic contracture of the heart was brought about (C-contr. in the Table).

Four frogs were allocated to each compound, and to either digoxigenin (IIa) or digitoxigenin (IIb) which was used as the standard in the same lot of animals. In experiments with Ia, Ib, IIe, IIf, the stock solutions were diluted with 30% ethanol, because of their low water solubility, and 0.02 ml of a test solution was added into the cannula. In these cases, the standard compound (IIa or IIb) was also treated in the same way. The experiments were carried out at room temperature of 22–25 °C.

The results are summarized in the Table. C-contr. of digoxigenin (IIa) and digitoxigenin (IIb) were 10^{-6} and 3×10^{-7} (g/ml), respectively. As for the values of relative potency (RP), IIa is taken as unity in the digoxigenin-series, and IIb in the digitoxigenin-series. The relative potency of 5α-cardenolides (Ia, Ib) was $^1/_{10}$ of the corresponding 5β-cardenolides (IIa, IIb). As a whole, the values for the 6 compounds in digoxigenin-series coincide well with those of the corresponding compounds in digitoxigenin-series, indicating that the presence of 12β-hydroxyl group makes the compounds in the former group about 3 times less active than their corresponding ones in the latter.

Among a great number of cardenolide aglycones found in nature, there are several instances where both type (*cis*- and *trans*-A/B) of compounds isomeric only at the C-5 position are known: digitoxigenin (IIb) and uzarigenin

[3] T. Shigei and S. Mineshita, Experientia *24*, 466 (1968).
[4] K. Takeda, T. Shigei and S. Imai, Experientia *26*, 867 (1970).
[5] T. Shigei, H. Tsuru, Y. Saito and M. Okada, Experientia *29*, 449 (1973).



Ia: R=OH
Ib: R=H

IIa: R=OH, R'=β-OH, α-H
IIb: R=H, R'=β-OH, α-H
IIc: R=OH, R'=O
IId: R=H, R'=O
IIe: R=OH, R'=α-OH, β-H
IIf: R=H, R'=α-OH, β-H

IIIa: R=OH
IIIb: R=H

IVa: R=OH
IVb: R=H

(Ib); cannogenin and corotoxigenin; cannogenol and coroglaucigenin, etc. [6]. So far as we know, however, cardiotonic activities were determined and compared by the same bioassay only in the first pair, digitoxigenin and uzarigenin[7–9]. Principally based on these comparisons, it is generally accepted that the A/B *cis*-cardenolides are much more potent than the A/B *trans*-cardenolides[10,11], although this is not always true with the cardenolide glycosides[12]. The present results with digoxigenin (IIa) and 5α-digoxigenin (Ia) thus provide the second instance which supports the above view concerning the structure-activity relationship of cardenolide aglycones[13].

Zusammenfassung. Es wurden die kardiotonischen Wirkungen des Digoxigenins, sowie deren 5 Derivate, einschliesslich des 5α-Digoxigenins, auf das isolierte Froschherz untersucht und Vergleiche mit den entsprechenden Aglykonen der Digitoxigenin-Reihe gezogen.

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[6] T. REICHSTEIN, Naturwissenschaften *54*, 53 (1967).
[7] K. K. CHEN, A. Rev. Physiol. *7*, 677 (1945). – K. K. CHEN and F. G. HENDERSON, Archs int. Pharmacodyn. Thér. *140*, 8 (1962).
[8] K. REPKE, Proc. 2nd Int. Pharmac. Meeting (Pergamon Press, New York 1965), vol. 5, p. 65.
[9] A. L. ABELES, J. gen. Physiol. *52*, 268 (1969).
[10] CH. TAMM, Proc. 1st Int. Pharmac. Meeting (Pergamon Press, New York 1963), vol. 3, p. 11.
[11] K. H. R. REPKE and H. J. PORTIUS, Planta med. Suppl. *4*, 66 (1971).
[12] K. D. ROBERTS, EK. WEISS and T. REICHSTEIN, Helv. chim. Acta *49*, 316 (1966).
[13] This study was supported in part by a research grant No. 787012 from the Ministry of Education, Japan.
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Rifampicin and Cysteamine Protect against the Mushroom Toxin Phalloidin

Phalloidin, a cyclic heptapeptide, is quantitatively the most important representant of the rapidly acting phallotoxins isolated from the poisonous mushroom *Amanita phalloides*[1]. In an earlier series of investigations, mice could be protected against lethal doses of phalloidin by prior treatment with hepatotoxic agents such as carbon tetrachloride, sodium-cinchophen (sodium salt of 2-phenylcinchoninic acid) and thioacetanide[2,3]. Subsequent studies disclosed that marked protection against phalloidin was also provided by rifampicin and phenylbutazone[4]. The present work reports, in addition to further characteristics of the rifampicin activity, the antagonistic efficacy of cysteamine. Moreover, it was tested whether other drugs with hepatotoxic potencies and whether antidotes to the slowly acting mushroom poison α-amanitin, would also affect phalloidin toxicity.

Methods. Female NMRI mice (S. Ivanovas, 7964 Kisslegg, West-Germany), weighing 18–22 g were used throughout the experiment. Phalloidin was dissolved in distilled water and applied in a volume of 0.1 ml/10 g body weight by the i.p. route. Death after lethal doses of phalloidin occurred in general within 2–5 h after the application. As no deaths were observed later than 24 h, survival was scored at this time.

The agents to be tested were dissolved immediately before use in distilled water and applied in 0.1 or 0.2 ml/10 g body weight in single doses at the times indicated in Tables II and III. The control groups received the respective volumes of distilled water only by the corresponding route. The chemicals used included: Rifampicin ('Rimactan'), Cysteamine (Fluka AG, CH 9470 Buchs), Chlorpromazine ('Largactil'), Penicillin-G ('Specilline G'), Aureomycin (Lederle, suspended in tap water), Erythromycine ('Erythrocin'), Cytochrome C ('Cyto-Mack'), Reserpine ('Serpasil'), Phenylbutazone ('Butazolidine'), 'Synthalin A' (Decamethylenediguanidine 2 HCl) and activated charcoal (Carbo adsorbens, suspended in tap water).

Table I. Toxicity of phalloidin in NMRI mice

Dose of phalloidin (mg/kg, i.p.)	Proportion of mice surviving at 24 h	Survival(%)
1.0	6/6	100
2.0	4/6	67
3.0	4/39	10

Results. As seen from Table I, the LD_{50} of phalloidin amounted to ~2.5 mg/kg and the LD_{90} to 3 mg/kg. The drugs were tested against this latter dose. Rifampicin provided again a complete protection at both doses tested of 100 and 300 mg/kg (Table II). In addition, it was demonstrated that rifampicin is still active if applied 24 or even 48 h prior to the phalloidin. The protection afforded by phenylbutazone, after intervals of 8 or 24 h, was only marginal. Significant protection was caused by cysteamine and, to a lesser degree, by chlorpromazine. No significant activity was demonstrated by the agents entered in Table III.

Discussion. Fiume had observed that newborn rats were resistant to lethal doses of phalloidin[5]. He ascribed this phenomenon to the immaturity of drug metabolizing enzymes in newborns. Based on this and other[6] observations, it was held likely that hepatotoxic agents such as carbon tetrachloride exert their protective effect by damaging drug-metabolizing enzymes and thus preventing the transformation of phalloidin to toxic metabolites[2,3].

[1] TH. WIELAND, Science *159*, 946 (1968).
[2] G. L. FLOERSHEIM, Biochem. Pharmac. *15*, 1589 (1966).
[3] G. L. FLOERSHEIM, Helv. physiol. pharmacol. Acta *24*, 219 (1966).
[4] G. L. FLOERSHEIM, Agents Actions *2*, 142 (1971).
[5] L. FIUME, Lancet *1*, 1284 (1965).
[6] A. VON DER DECKEN, H. LÖW and T. HULTIN, Biochem. Z. *332*, 503 (1960).