INHIBITION OF TRANSPORT (Na^+, K^+)-ATPase BY CARDENOLIDES OF THE STROPHANTHIDIN GROUP AND A STUDY OF THE STRUCTURE–ACTIVITY RELATIONSHIP

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The investigation of the activity of cardiac glycosides is based on the determination of their toxicity to the animal organism as a whole. Usually, such experiments are performed on frogs or cats and give a more or less correct idea of the suitability of the substances under investigation as medicinal preparations. However, the size of the lethal dose cannot fully characterize the therapeutic effectiveness of the drug. A number of compounds exist (Veratrum alkaloids, caffeine, nicotine, saponins, etc.) which, it would appear, possess an action on the cardiac muscle similar to that of Digitalis but which on further testing have shown that their influence is nonspecific and from the point of view of pharmacodynamics is completely different from the effect shown by cardiac glycosides.

In recent years, a new approach to the evaluation of the activity of cardiotonic substances has been planned, which has become possible because of advances in the study of the mechanism of the action of the cardiac glycosides at the molecular level (see the reviews [1-3]). The question has arisen of whether the process of inhibiting the transport (Na^+,K^+)-ATPase of surface membranes cannot be used for the qualitative evaluation of biological activity and for the preliminary screening of new compounds.

The enzymatic method, based on measuring the inhibiting action on ATPase, has a number of advantages:

1. When this method is used, it is exclusively the specific activity that is determined, undistorted by secondary factors that may be superimposed on the substance under investigation in its transport by the blood or lymph from the site of introduction to the point of contact with the cells of the heart muscle (addition to proteins, nonuniform distribution in the organism, destruction under the action of blood enzymes, etc.).

2. The direct possibility arises of analyzing the structure–effect relationship, which reveals promise for the creation of new cardiac drugs. This is important, in particular, in relation to synthetic derivatives differing from the cardenolides and the bufadienolides. The dependence of the action of the latter on their structure has been studied in fairly great detail.

3. The comparative study of the inhibiting effect on a large number of compounds structurally differing from one another permits a deeper insight into the mechanism of the functioning of the enzyme system of the sodium pump which is responsible for the therapeutic effect of cardiotonic agents.

4. Very small doses (1-2 μmole) of the compounds being tested are sufficient to determine the enzyme activity.

It stands to reason that the enzymatic method is also associated with some difficulties and limitations, but in combination with the traditional methods of test on the isolated heart or on intact animals it broadens the possibilities of an objective evaluation of the inotropic action of cardiotonic drugs.

Repke et al. [4, 5] were the first to draw attention to the fact that a definite interrelationship exists between the structures of cardenolides and bufadienolides and their in-

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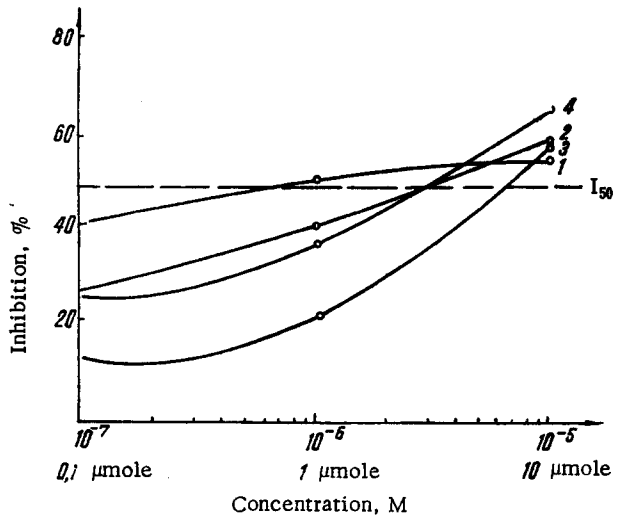

Fig. 1. Curves of the dependence of the inhibiting action of derivatives of strophanthidin 3-β-D-glucuronopyranoside on the concentration: 1) k-strophanthin-β; 2) methyl ester of S-GlcUA; 3) triacetate of the methyl ester of S-GlcUA; 4) amide of S-GlcUA.

hibiting action on transport ATPase (review [6]). Of the cardenolides, compounds comprising the Digitalis group have been studied, in the main. The results obtained have proved completely comparable with those existing previously on the dependence of the lethal doses of cardiac glycosides on their chemical structures.

In various animals, the sensitivity to cardioactive substances is different. It is natural that the ATPase from different organs (heart, brain, etc.) of one and the same animal will be inhibited differently. Furthermore, the degree of the suppressive effect of cardiac glycosides depends on their concentration and the time of incubation with the enzyme. So far, no one accepted standard for the quantitative evaluation of the inhibiting effect has been chosen. Repke et al. have adopted as the basis of the parameter of comparison the concentration of the substance in micromoles which causes 50% inhibition of transport ATPase (I_{50}). To obtain comparable results, we have made use of Repke's proposal, although it has been observed that for different substances the degree of suppressing action does not fall strictly proportionally to a decrease in concentration (dilution). In other words, in a graphical illustration of the dependence of the inhibiting effect on the concentration the curves of comparable compounds may not have the same angle of slope to the axis of abscissas (compare, for example, the curves of the inhibition due to k-strophanthin-β and of the glucuronosides of strophanthidin in Fig. 1).

We have investigated strophanthidin, its glycoside, and some synthetic derivatives. In spite of some arbitrariness of the evaluation, the interrelationship between the inhibiting activity and the lethal dose of the compounds investigated can be traced fairly clearly (Table 1). Mono- and diglycosides of strophanthidin, which are known as highly active cardiotonic drugs, show an inhibiting effect at concentrations of 0.5 μM or less. The suppressing action on ATPase of strophanthidin itself is less pronounced, which is in harmony with old observations relating to the low activity of cardenolide aglycones as compared with their glycosides.

The acetylation of strophanthidin at the C_3 hydroxy group raises both its toxicity and its inhibiting effect. Strophanthidin 3-O-acetate has an effect which approximates to that of the glycosides and the recommendation for the use of this compound as a drug is, it must be assumed, justified. Conversely, the acetylation of erysimoside lowers its activity index.

These compounds stand out: 17α-strophanthidin acetate, strophanthidinic acid, and isostrophanthidin. For the first two, the two indices obtained by independent methods are fairly low, but they correlate well with one another. Isostrophanthidin is known as a compound having no inotropic action. All three substances show a pronounced (i.e., more than 50%) suppressive influence on transport ATPase only at concentrations not less than $1 \cdot 10^{-4}$ M, while for truly cardioactive compounds (k-strophanthidin-β, olitoriside, convallatoxin, etc.) the inhibiting effect is fairly large at dilutions two orders of magnitude greater, i.e., at a concentration of $1 \cdot 10^{-6}$ M (Table 2).

We also investigated some derivatives of strophanthidin 3-β-D-glucuronopyranoside (see Fig. 1). Cardenolides glycosylated with uronic acids at the C_3 hydroxyl have not been found in plants. They are of interest as possible products of the detoxification of cardiac agly-

TABLE 1. Dependence of the Biological Activity (at the molecular level and in experiments on animals) on the Structures of Glycosides and Other Derivatives of Strophanthidin

Cardenolide	Concentration (μM) causing 50% inhibition of the ATPase from rat brain	Lethal dose for cats	
		mg/kg	μmole/kg
Diglycosides			
k-strophanthin-β	0,60	0,128*	0,180
olitoriside	0,50	0,110	0,158
erysimoside	0,18	0,100	0,144
erysimoside pentaacetate	1,6	†	
Monoglycosides			
convallatoxin	0,22	0,079*	0,143
corchoroside A	0,36	0,091	0,170
erysimin (helveticoside)	0,16	0,080*	0,150
The aglycone and its nonglycoside derivatives			
strophanthidin	0,65	0,324*	0,801
strophanthidin acetate	0,32	0,187*	0,419
17α-strophanthidin acetate	30	2,08 [8]	4,660
strophanthidinic acid	53	2,649	6,307
isostrophanthidin (14,21-epoxy-strophanthidin)	71	Inactive	
Derivatives of strophanthidin 3-β-D glucuronopyranoside (S-GlcUA) [9]			
methyl ester	3,0	‡	
triacetate of the methyl ester	6,3	Not determined	
amide	2,8	**	
Rhamnosides of other aglycones			
evomonoside	0,23	0,278*	0,534
periplorhamnoside	0,26	Not determined	
Lokundjoside (cuspidoside)	0,26	0,100	0,181
ouabain	0,30	0,116*	0,198

*Results taken from the literature [7]. The other glycosides were isolated from natural sources or were partially synthesized in the laboratory of glycoside chemistry, and their activities on animals were determined in the pharmacology aboratory of the Institute of Chemistry of Plant Substances of the Academy of Sciences of the Uzbek SSR.
†The activity on cats was not determined. In the tests on frogs, erysimoside pentaacetate was 2.5 times less active than erysimoside: 25,000 and 625,000 frog activity units (FAU), respectively.
‡Activity 5000 FAU.
**Activity 2000 FAU.

cones by the liver [10]. This idea has not been strictly confirmed, and therefore we have specially synthesized [9] several compounds of this type. Their activity in tests on frogs was low and was considerably inferior to the activity of the usual cardiac drugs. In agreement with this, for these compounds again the concentrations necessary for 50% inhibition proved to be high (see Table 1).

The examples given permit a deeper understanding of the role of the sugar moiety of the glycosides in inhibition processes. The fairly strict correlation between the enzyme test and the lethal dose shows that cardioactive substances reach the cells of the cardiac muscle, at least in acute experiments for determining toxicity, without appreciable destruction of the sugar components. Application to the surface membrane takes place without the detachment of the carbohydrate moiety, otherwise the glycosides and aglycones would possess the same inhibiting activity. The aglycone moiety has a direct connection with transport ATPase by a mechanism which is still unknown to us, but the role of the sugars is so considerable that in individual cases they may bring the inhibiting effect to nought. The replacement of hydroxy groups by acetate groups should apparently facilitate the penetration of the glycosides through the lipoid cell membrane, but in actual fact this has proved to be valid only for strophanthidin acetate. Glycosides acetylated in the carbohydrate moiety (pentaacetylgitoxin [6], pentaacetylerysimoside) proved to be only slightly active in the enzyme test. Apparently, here steric factors acquire first-degree significance.

TABLE 2. Dependence of the Inhibiting Action of Glycosides and Other Derivatives of Strophanthidin on Their Concentration (% inhibition of transport ATPase)

Cardenolide	Concentration, M			
	$1 \cdot 10^{-4}$	$1 \cdot 10^{-5}$	$1 \cdot 10^{-6}$	$1 \cdot 10^{-7}$
Olitoriside	84,5	76,3	56,4	43,6
Convallatoxin	83,9	80,7	72,4	34,6
Strophanthidin acetate	76,9	75,6	61,4	39,7
17α-Strophanthidin acetate	64,1	34,6	26,1	15,8
Strophanthidinic acid	61,5	33,7	28,0	17,6
Isostrophanthidin	56,4	28,2	12,6	0,9
Methyl ester of strophanthidin 3-β-D-glucuronopyranoside	68,4	60,0	42,0	27,3

Glycosides containing 6-deoxyaldohexoses (rhamnose, fucose, gulomethylose) are highly active compounds, as a rule. In particular, convallatoxin, i.e., strophanthidin rhamnopyranoside, is a drug with a pronounced inotropic action. On the basis of the enzyme test, we have made a comparative investigation of the rhamnosides of digitoxigenin, periplogenin, bipindogenin, and ouabagenin (see Table 1). The inhibiting doses of the five (including convallatoxin) rhannosides compared were small and were fully comparable with one another.

EXPERIMENTAL METHOD

The microsomal fraction of the cells of the rat cerebral cortex were isolated and incubated by Skou's method [11]. The brain was dissected out 15-20 min after the decapitation of the rat. All the operations up to the preparation of the membrane were performed at 0 to +3°C under strictly standardized conditions.

The gray matter of the brain was separated from the membrane and the blood vessels, weighed, and homogenized in a Potter homogenizer in a 10-fold volume of isolation medium. The medium contained 25 mM tri(hydroxymethyl)methylamine hydrochloride (tris-HCl, pH 7.4), 0.5 M sucrose, 5 mM disodium salt of ethylenediaminetetraacetic acid (Na_2EDTA), and 0.1% sodium deoxycholate (DOCh). To eliminate the nuclei, the cell fragments, and the mitochondria, the supernatant was first centrifuged in a TsVR-1 centrifuge at 12,000g for 30 min and then, to obtain the microsomes, at 40,000g for 60 min. The microsomal fraction was dissolved in a 1 mM solution of tris-EDTA (pH 7.1) in an amount of 7 mg of protein in 1 ml.

The ATPase activity of the enzyme preparations was determined from the increase in inorganic phosphorus after incubation in a medium containing 30 mM tris-HCl (pH 7.4), 2 mM $MgCl_2$, 135 mM NaCl, and 5 mM KCl. The final volume of the reaction mixture was 1.0 ml, and the amount of membrane protein 10 μg. After preincubation with the cardiotonic compound in the absence of ATP (15 min at 37°C), the reaction was begun by the addition of 2 mmole of ATP and the mixture was incubated at the same temperature for 20 min. The reaction was stopped by the addition of trichloroacetic acid (final concentration 5%). The inorganic phosphate was determined by the Fiske—Subbarow method [12], and the protein by Lowry's method [13]. The specific activity of the preparations was between 150 and 250 μmole P_{inorg}/mg of protein per 1 h.

The substances tested had no action on Ca^{2+} ATPase isolated from the sarcoplasmatic reticulum [14].

The concentrations corresponding to 50% inhibition (I_{50}) were calculated on a logarithmic scale, as shown in Fig. 1.

SUMMARY

A comparative study of the inhibiting action on transport (Na^+,K^+)-ATPase of 15 derivatives of strophanthidin and four rhamnosides of other aglycones have been made on the microsomal fraction of the cells of the rat cerebral cortex.

It has been shown that the method of determining the activity of cardiotonic substances by determining the lethal dose and the enzyme test give completely comparable results. Both biological methods objectively reflect the characteristic features of the chemical structure of the cardenolides.

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ALKALOIDS OF *Doronicum macrophyllum*

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The genus *Doronicum* (family Compositae) has not previously been studied chemically. The roots of *Doronicum macrophyllum* collected in the stage of the withering of the epigeal part in October, 1974, in the Nakhichevan ASSR contained 0.03% of total alkaloids. These were separated on a column of silica gel, and three bases were isolated. Two of them were identified as otosenine (II) and floridanine (III) [1], and the third proved to be new, and we have called it *doronine* (I). Substance (I) crystallized from a mixture of benzene and cyclohexane with mp 113-114°C (decomp.), $[\alpha]_D$ +45.4° (c 1.1; chloroform); picrate, mp 235°C (decomp.). The IR spectrum of (I) has absorption bands of active hydrogen in the 2800-3500-cm^{-1} region and of carbonyl groups at 1750 and 1620 cm^{-1}, the latter having the position and shape that is characteristic for otonecine esters [2]. The presence in the mass spectrum of (I) of the peaks of ions with m/e 168, 151, 150, 122, and 110 confirms that this base belongs to the group of otonecine diesters. The molecular ion of the substance has the form of a doublet with m/e 459/461 in a ratio of 3:1, which shows the presence of one chlorine atom in the molecule. Ions with m/e 424 and 423 (M − Cl and M − HCl, respectively) confirm the presence of the halogen.

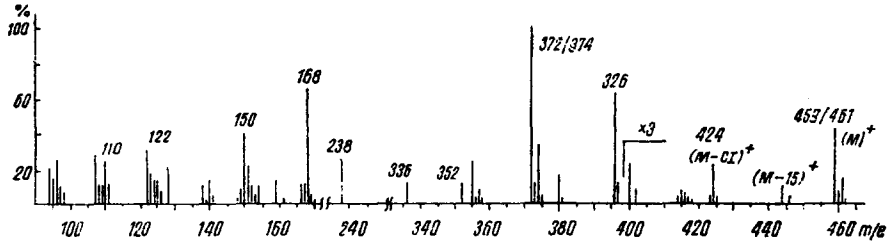

Fig. 1

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