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The thermodynamic essence of the reversible inactivation of Na^+/K^+-transporting ATPase by various digitalis derivatives is relaxation of enzyme conformational energy

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This paper reports on the kinetic and thermodynamic parameters describing the interaction of selected digitalis derivatives with hog and guinea-pig cardiac ($Na^+ + K^+$)-ATPase (Na^+/K^+-transporting ATPase EC 3.6.1.37). 32 digitalis derivatives were characterized as to the values of the $\Delta G^{\circ\prime}$, $\Delta \vec{G}^{\neq}$, and $\Delta \bar{G}^{\neq}$ quantities in their interaction with ($Na^+ + K^+$)-ATPase from hog cardiac muscle in the presence of ATP, Mg^{2+}, Na^+ and K^+. Nine derivatives were additionally characterized as to the values of the $\Delta H^{\circ\prime}$, $\Delta S^{\circ\prime}$, $\Delta \vec{H}^{\neq}$, $\Delta \vec{S}^{\neq}$, $\Delta \bar{H}^{\neq}$, and $\Delta \bar{S}^{\neq}$ quantities in their interaction with the hog enzyme promoted by ATP, Mg^{2+} and Na^+ in the presence or absence of K^+. The formation of the inhibitory complexes is in any case an endothermic, entropically driven process. The Gibbs energy barriers in the formation and dissociation of the complexes, $\Delta \vec{G}^{\neq}$ and $\Delta \bar{G}^{\neq}$, are imposed by large, unfavourable $\Delta H^{\neq}$ values. K^+ decreases the $\Delta G^{\circ\prime}$ value by increasing the $\Delta \vec{G}^{\neq}$ value more than the $\Delta \bar{G}^{\neq}$ value. In comparison with hog ($Na^+ + K^+$)-ATPase, the interaction of three derivatives with guinea-pig cardiac enzyme in the presence of ATP, Mg^{2+}, Na^+ and K^+ is characterized by lower $\Delta G^{\circ\prime}$ values caused by lower favourable $\Delta S^{\circ\prime}$ values, and is accompanied by lower $\Delta \bar{G}^{\neq}$ values. The magnitude of the kinetic parameters and the characteristic of the thermodynamic quantities describing the interaction between various digitalis derivatives and ($Na^+ + K^+$)-ATPase, indicate the induction of substantial conformational changes in the enzyme protein. A large entropy gain in the enzyme protein, observed irrespective of enzyme origin and ligation, appears to be the common denominator of the inhibitory action of all digitalis derivatives studied, suggesting that the digitalis-elicited relaxation of high conformational energy (negentropy strain) of the enzyme protein is the thermodynamic essence of the reversible inactivation of ($Na^+ + K^+$)-ATPase.

Introduction

The recognition of ($Na^+ + K^+$)-ATPase as the digitalis receptor [1–4] has allowed the relationship between chemical structure and activity of digitalis compounds to be studied at the molecular level. An increasing body of information has been gathered since the first analysis in 1964 [5] on the nature of the digitalis-enzyme interaction [6,7]. In 1985, Schönfeld et al. [8] characterized 73 structurally highly different, but digitalis-like acting steroids as to their apparent Gibbs energy changes

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of interaction with human cardiac $(Na^+ + K^+)$-ATPase.

Since Gibbs energy changes are relatively insensitive to variation in the molecular details of ligand-protein interactions, knowledge of the interplaying contributions from enthalpy and entropy changes forms a less ambiguous basis for the molecular interpretation of the thermodynamic parameters than the Gibbs energy data alone [9].

The purpose of the present paper is to understand the characteristic of the inhibited digitalis-$(Na^+ + K^+)$-ATPase complex more fully by determining the equilibrium and activation thermodynamic quantities which define the interaction of selected digitalis derivatives with the cycling enzyme. No information is as yet available about the equilibrium and transition state thermodynamics of the interactions between a variety of digitalis derivatives and cycling $(Na^+ + K^+)$-ATPase, and no thermodynamic study of the influence of K^+ or the species-origin of the enzyme on these interactions has been reported so far. A small part of the present data was digested in a preliminary manner in the context of a survey [10].

Materials and Methods

Materials

The digitalis compounds studied are listed in Table I, which also shows their structure (using the nomenclature given in Ref. 11) and numbering for easy reference in the text. The naturally occurring representatives were obtained from various commercial suppliers (1, 4, 5, 6, 8, 11, 15, 20) or isolated in the Institute in Tashkent (10, 12, 14, 21). By appling established procedures the derivatives were prepared in the Institute in Berlin by R. Megges (7, 9, 17, 23, 26, 28, 30) and C. Lindig (3, 16, 18, 22, 24, 27, 32) or in the Institute in Tashkent (13, 19, 25, 29, 31: cf. references [12,13]). 2 was a gift of K. Meyer, Basle. All compounds proved pure in chromatographic analysis.

The tritium-labelled compounds were obtained from Isocommerz, Berlin G.D.R. (1, 5–7, 9, 15, 18, 20) and the New England Nuclear Corp. (4, 8). Thin-layer chromatography showed them to be free of labelled and unlabelled contaminants.

All chemicals and biochemicals were of analytic grade.

$(Na^+ + K^+)$-ATPase preparations and activities

The enzymes were little purified on purpose to get undeteriorated, stable preparations of non-vesicular membrane fragments. They were obtained from the ventricle muscle of hog and guinea-pig by the procedure of Matsui and Schwartz [14]. The portion of ATPase activity suppressed by 0.1 mM ouabain (> 95%) was taken as $(Na^+ + K^+)$-ATPase activity which amounted to 15–25 or 5–10 μmol P_i produced per mg protein per h by hog or guinea-pig enzyme, respectively. Protein was determined according to Lowry et al. [15]. The activity of the $(Na^+ + K^+)$-ATPase preparations was stable during the time of the experiments under the conditions specified below. The digitalis derivatives were dissolved and transferred in dimethylformamide into the assay medium so that the concentration of the solvent was 1% (v/v) which did not impair enzyme activity.

In the following presentation of procedure and data, the symbolism and terminology in enzyme kinetics as recommended in Ref. 16 were used.

Basics of data calculation

In accordance with the generally applied procedure [17] we used the bimolecular interaction model

$$D + E \underset{\bar{k}}{\overset{\bar{k}}{\rightleftharpoons}} D' \cdot E' \qquad (1)$$

in which: D is free digitalis, E enzyme in receptive state, $D' \cdot E'$ the inhibited complex with D and E in altered conformational states (cf. Discussion), and $\bar{k}$ and $\bar{k}$ the forward and backward rate constants. The inhibitory action of digitalis compounds on, and their binding to, $(Na^+ + K^+)$-ATPase were studied under the conditions of maximized enzyme turnover to favour the occurrence of a single digitalis-receptive enzyme state. The apparent inhibition and dissociation constant could be described thus:

$$K_i = K_D = \frac{[D][E]}{[D' \cdot E']} = \frac{\bar{k}}{\bar{k}} \qquad (2)$$

The validity of this model was corroborated by the following eight pieces of evidence.

The Hill coefficient was not significantly different from unity, indicating that the digitalis bind-

TABLE I

KINETIC AND THERMODYNAMIC PARAMETERS CHARACTERIZING THE INTERACTION BETWEEN 32 5β,14β-ANDROSTANE-3β,14-DIOL DERIVATIVES AND ($Na^+ + K^+$)-ATPase FROM HOG CARDIAC MUSCLE ESTIMATED IN THE PRESENCE OF ATP, Mg^{2+}, Na^+ AND K^+

All data are for 37 °C. The parameters for compounds 6, 7, 9, 18, 20 were derived from binding studies, all others from inhibition studies.

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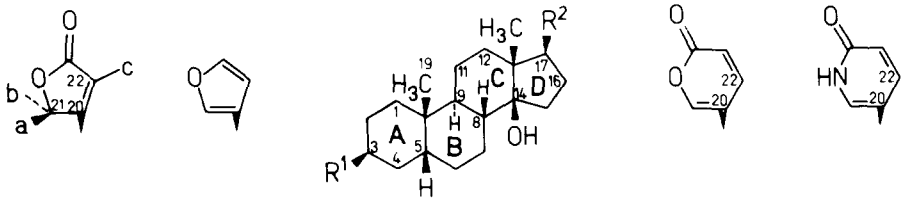

Compound No.	Structural variation			$\bar{k}(\times 10^{-4})$ ($M^{-1} \cdot s^{-1}$)	$\bar{k}(\times 10^{3})$ (s^{-1})	K'_D (μM)	$\Delta\bar{G}^{\neq}$ (kJ/mol)	$\Delta\bar{G}^{\neq}$ (kJ/mol)	$-\Delta G^{\circ\prime}$ (kJ/mol)
	R^1	R^2	others						
1	O-rhamnosyl	III	Δ4	11	0.16	0.0015	46.2	98.6	52.4
2	OH	III	–	18	0.49	0.0026	44.8	95.7	50.9
3	O-tridigitoxosyl	I a, b: H; c: CH_3	–	4.1	0.70	0.017	48.7	94.8	46.1
4	O-tridigitoxosyl	I a, b, c: H	–	1.2	0.29	0.024	51.9	97.1	45.2
5	O-rhamnosyl	I a, b, c: H	C1β-OH, C5-OH, C11β-OH, C19-OH	0.82	0.30	0.036	52.8	97.0	44.2
6	O-tridigitoxosyl	I a, b, c: H	C12β-OH	0.50	0.21	0.042	54.1	97.9	43.8
7	O-tridigitoxosyl	I a, b, c: H	C16α-OCH_3	1.7	0.77	0.046	51.0	94.6	43.6
8	OH	I a, b, c: H	–	9.8	5.1	0.053	46.4	89.7	43.3
9	O-tridigitoxosyl	I a, b, c: H	C16α-$OCOCH_3$	0.32	0.17	0.053	55.3	98.5	43.2
10	O-[*O*-glucosyl]-digitoxosyl	I a, b, c: H	C5-OH, C19 = O	2.1	1.2	0.059	50.5	93.4	42.9
11	O-tridigitoxosyl	I a, b, c: H	C16β-OH	1.1	0.65	0.062	52.2	95.0	42.8
12	OH	I a, b, c: H	C5-OH, C19 = O	3.9	2.8	0.071	48.8	91.1	42.3
13	O-ribosyl	I a, b, c: H	–	2.1	1.7	0.083	50.4	92.4	42.0
14	O-[*O*-glucosyl]-cymarosyl	I a, b, c: H	C5-OH, C19 = O	0.31	0.32	0.11	55.4	96.8	41.4
15	OH	I a, b, c: H	C12β-OH	2.9	3.5	0.12	49.6	90.7	41.1
16	O-tridigitoxosyl	II	–	2.6	3.2	0.12	49.8	90.9	41.1
17	O-rhamnosyl	IV	Δ4	2.2	4.3	0.20	50.3	90.1	39.8
18	O-tridigitoxosyl	I a, b, c: H	C16α-OH	1.2	3.2	0.27	51.9	90.9	39.0
19	O-ribosyl	I a, b, c: H	C5-OH, C19 = O	0.71	2.2	0.30	53.2	91.9	38.7
20	OH	I a, b, c: H	C16β-OH	16	51	0.32	45.1	83.7	38.6
21	O-[*O*-glucosyl]-boivinosyl	I a, b, c: H	C5-OH, C19 = O	0.72	2.3	0.31	53.2	91.8	38.6
22	OH	I a, b: H; c: CH_3	–	3.8	12	0.32	48.9	87.5	38.6
23	O-[tetra-*O*-acetyl]-tridigitoxosyl	I a, b, c: H	C16β-$OCOCH_3$	0.22	1.9	0.83	56.1	92.3	36.1
24	O-tridigitoxosyl	II	C16α-OH	0.68	9.9	1.5	53.3	88.0	34.7
25	O-rhamnosyl	I a, b, c: H	C5-O-rhamnosyl, C19 = O	0.20	3.1	1.5	56.4	91.0	34.6
26	OH	I a, b, c: H	C12β-O-NO_2	0.77	14	1.8	53.0	87.1	34.1
27	O-$COCH_3$	I a: CH_3; b, c: H	–	0.56	20	3.6	53.8	86.2	32.3
28	O-tridigitoxosyl	I a, b, c: H	C12β-$OCOCH_3$	0.18	6.9	3.9	56.8	88.9	32.1
29	O-$COCH_3$	I a, b, c: H	C5-O-rhamnosyl, C19 = O	0.20	13	6.3	56.4	87.3	30.9
30	O-tridigitoxosyl	I a(b): OCH_3 * (a) b, c: H	C16β-OH	0.29	19	6.7	55.5	86.3	30.8
31	OH	I a, b, c: H	C5-O-rhamnosyl, C19 = O	0.13	8.3	6.7	57.7	88.5	30.8
32	O-tridigitoxosyl	C17 = O	–	0.07	31	45	59.2	85.0	25.8

* 'a' denotes the likely configuration.

ing sites were all identical and independent as follows at once from simple mass law considerations [18]. The $\vec{k}$ value was independent of the digitalis concentration. The value of $\bar{k}$, directly determined by following up the complex dissociation, was independent of the digitalis occupancy of the enzyme population and not significantly different from the value of $\bar{k}$ calculated according to Eqn. 2 from the rate of complex formation at different digitalis concentrations. Mathematical curve fitting revealed an excellent fit of the observed and theoretic time course of digitalis interaction with the enzyme. In simultaneous determinations the extent of inhibition of $(Na^+ + K^+)$-ATPase activity by digitalis compounds correlated well with their binding to the enzyme and the kinetic constants determined by binding or inhibition studies were equal within the limits of error. The computation of K_i, K_D, $\vec{k}$ and $\bar{k}$ by means of different algorithms described below yielded similar quantities. The van't Hoff plots of the dependence of the K_i and K_D values on temperature, and the Arrhenius plots of the dependence of the $\vec{k}$ and $\bar{k}$ values on temperature showed linear regression lines over the temperature range studied (in general 17–37 °C, in suitable cases extended down to 3 °C), apparently excluding a change in the digitalis receptive enzyme state as a function of temperature. The above findings confirmed earlier experimental data (reviewed in Refs. 6 and 7) and are therefore not shown here.

The concentration of the digitalis derivatives was in large excess over the enzyme concentration so that only a negligible digitalis fraction was bound to the enzyme molecules and the concentration of free digitalis molecules was approximately equal to the digitalis concentration applied.

Determination of kinetic parameters

Inhibition studies. The inhibitory digitalis action on $(Na^+ + K^+)$-ATPase activity was monitored in the optical test by NADH dehydrogenation through measuring the decrease of absorbance at 334 nm. The assay medium contained 1.7 mM ATP, 4 mM $MgCl_2$, 5 mM KCl, 130 mM NaCl, 5 mM $(NH_4)_2SO_4$, 25 mM imidazole-HCl-buffer (pH 7 at incubation temperature), 0.4 mM phospho*enol*pyruvate, 0.2 mM NADH, 10 I.U. pyruvate kinase, 10 I.U. lactate dehydrogenase and 0.15–0.3 mg protein of the hog enzyme preparation, all in a volume of 3 ml. Slowly or rapidly inhibiting digitalis derivatives were present in the assay medium before or after the reaction was started by enzyme addition, respectively.

Calculation of $\vec{k}$ and $\bar{k}$. According to Eqn. 2, the time course of the inhibitory digitalis action is expressed [19] by

$$\gamma_t = \gamma_\infty(1 - e^{-kt}) \tag{3}$$

in which γ_t and γ_∞ denote the degree of inhibition of enzyme activity at time t and at equilibrium, respectively. The time constant k therein is defined by

$$k = \vec{k}[D] + \bar{k} \tag{4}$$

Taking Eqn. 3 as a basis, the data points of γ_t were analyzed by the non-linear regression programme MRKR 10 [20] which gives the most probable values of γ_∞ and k. From at least duplicate determinations of k at five digitalis concentrations, $\vec{k}$ and $\bar{k}$ were calculated by linear regression taking Eqn. 4 as a basis. In addition, MRKR 10 served to compute the initial rate of inhibitory digitalis action, v_i, which is defined by

$$v_i = \vec{k}[E]_0[D]_0 \tag{5}$$

where $[E]_0$ and $[D]_0$ denote concentrations at the start of interaction. $\vec{k}$ was also directly calculated from v_i and $[D]_0$. The determination of v_i was repeated at least five times.

Determination of K'_D. If not computed from the $\bar{k}/\vec{k}$ ratio according to Eqn. 2, K'_D was calculated from the γ_∞ values (at least twice determined at five concentrations of D) by linear regression using the logarithmic form of the Hill equation

$$\log \frac{\gamma_\infty}{100 - \gamma_\infty} = n_H \log[D] - \log K'_D \tag{6}$$

in which γ_∞ denotes percentage of inhibition at equilibrium. The Hill coefficient n_H showed, as usual, some experimental scattering, but more profound experiments then revealed that it was not different from unity. Because of the great influence of the slope of the regression line on

K'_D, its value was computed from the intercept of the regression line with the abscissa. When not directly determinable in the optical test due to slow attainment, γ_∞ was calculated by Eqn. 3.

Binding studies. The formation of the digitalis-enzyme complexes was monitored by incubating the tritium-labelled compounds in a medium containing 2 mM ATP, 4 mM $MgCl_2$, 130 mM NaCl, 5 mM KCl (if need be), 25 mM imidazole-HCl buffer (pH 7 at incubation temperature) and the hog or guinea-pig enzyme preparation (0.04–0.1 mg protein/ml). Complex formation was started by enzyme addition. Nonspecific binding was assessed in parallel by omitting ATP, $MgCl_2$ and KCl from the medium. After progressive time intervals, 5-ml aliquots were removed to separate enzyme-bound and free tritiated compounds by filtering the membranous enzyme suspension through glass fibre filters (Whatman GF/C, 2.3 cm in diameter). Accelerated by suction, the filtration and the washing of the membrane film on the filter (twice 2.5 ml ice-cold solution of 130 mM NaCl and 50 mM imidazole-HCl buffer (pH 8.4)) took about 20 s. Either the filter with the membrane film or (when high fitler binding) only the membrane film (removed after drying) was transferred into 10 ml scintillation fluid containing 1 ml water [21]. From the radioactivity measured in a scintillation spectrophotometer, the enzyme-bound digitalis quantity was calculated by making allowance for counting efficiency, protein quantity on filter and nonspecific binding.

The kinetics of the dissociation of tritiated compounds from the complex with the enzyme variants were revealed by adding an excess (at least 100-fold) of unlabelled compound preventing the rebinding of tritiated compound (for further details see above and Ref. 22).

Calculation of $\vec{k}$, $\overleftarrow{k}$ and K'_D. In principle these parameters were computed as detailed above for the calculation of the corresponding parameters of enzyme inhibition. The $\overleftarrow{k}$ value was also calculated from the data points of first-order complex dissociation by linear regression analysis.

Estimate of thermodynamic quantities

In the presentation of the procedure and data, the thermodynamic terms and symbols as well as the SI units and symbols as recommended in Ref. 23 were used. Accordingly, for the equilibrium state in the reversible formation of an inhibited digitalis-enzyme complex, $\Delta G^{\circ\prime}$ denotes the apparent standard Gibbs energy change, $\Delta H^{\circ\prime}$ the apparent standard enthalpy change, and $\Delta S^{\circ\prime}$ the apparent standard entropy change. The term 'apparent' means that the components of the complex formation may include distinct molecular species. For the transition state in forming or dissociating of an inhibited complex, $\Delta\vec{G}^{\neq}$ and $\Delta\overleftarrow{G}^{\neq}$ denote the activation Gibbs energy barriers, $\Delta\vec{H}^{\neq}$ and $\Delta\overleftarrow{H}^{\neq}$ the activation enthalpy changes, and $\Delta\vec{S}^{\neq}$ and $\Delta\overleftarrow{S}^{\neq}$ the activation entropy changes related to the forward and backward process, respectively.

Equilibrium quantities. The apparent Gibbs energy change in the inhibitory action of a given digitalis derivative on, or in its binding to, the enzyme variants was calculated by

$$\Delta G^{\circ\prime} = RT \ln K'_i = RT \ln K'_D \tag{7}$$

in which R refers to the gas constant and T to the absolute temperature. Changes in the $\Delta G^{\circ\prime}$ value as a function of digitalis structure or incubation temperature may reflect varying values of both the apparent enthalpy change and the apparent entropy change as

$$\Delta G^{\circ\prime} = \Delta H^{\circ\prime} - T\Delta S^{\circ\prime} \tag{8}$$

Because of the low temperature dependence of the K'_i and K'_D values, the $\Delta H^{\circ\prime}$ and $\Delta S^{\circ\prime}$ values for only two compounds (26, 32) were estimated from the $\Delta G^{\circ\prime}$ values on the basis of Eqn. 8. On account of the higher temperature dependence and hence higher precision of the $\vec{k}$ and $\overleftarrow{k}$ values, the $\Delta H^{\circ\prime}$ and $\Delta S^{\circ\prime}$ values were usually obtained by

$$\Delta H^{\circ\prime} = \Delta\vec{H}^{\#} - \Delta\overleftarrow{H}^{\#} \tag{9}$$

and

$$\Delta S^{\circ\prime} = \Delta\vec{S}^{\neq} - \Delta\overleftarrow{S}^{\neq} \tag{10}$$

Transition state quantities. The estimate of the Gibbs energy barrier of activation, $\Delta G^{\neq}$, based on the Eyring theory of absolute reaction rates [24], was for the forward reaction obtained using

$$\Delta\vec{G}^{\neq} = \left(\ln\frac{k_B \cdot T}{h} - \ln\vec{k}\right) \cdot RT \tag{11}$$

in which k_B and h denote Boltzmann's and Planck's constants, respectively. For the calculation of the value of the $\Delta\bar{G}^{\neq}$ quantity an equation analogous to Eqn. 11 was applied. The values of the activation enthalpy quantity for the forward and reverse reaction were calculated from the values of Arrhenius' activation energy, E_A, using

$$\Delta H^{\neq} = E_A - RT \tag{12}$$

The E_A values were derived from the Arrhenius plots of $\vec{k}$ or v_i and $\bar{k}$. The $\Delta S^{\neq}$ values for the forward and reverse reaction were computed from the corresponding $\Delta G^{\neq}$ and $\Delta H^{\neq}$ values using

$$\Delta S^{\neq} = \frac{\Delta H^{\neq} - \Delta G^{\neq}}{T} \tag{13}$$

Error evaluation

The confidence intervals ($\alpha = 0.05$) for the K'_D, $\vec{k}$ and $\bar{k}$ values were determined to be $\pm 30\%$ which is consistent with general experience [25]. It follows that the confidence intervals for the $\Delta G^{\circ\prime}$ and $\Delta G^{\neq}$ values directly calculated from the above kinetic values, lay in the range of ± 1 kJ/mol.

The confidence intervals ($\alpha = 0.05$) for the $\Delta H^{\circ\prime}$ and $\Delta H^{\neq}$ values were estimated to lie in the range of $\pm 10\%$. In effect, the kinetic approach to the estimation of these quantities meets with severe limitations arising from the limited temperature range which can be covered [26].

The confidence intervals for the $\Delta S^{\circ\prime}$ and $\Delta S^{\neq}$ values could have reached $\pm 30\%$ because these values were derived from the $\Delta G^{\circ\prime}$ and $\Delta H^{\circ\prime}$ or $\Delta G^{\neq}$ and $\Delta H^{\neq}$ values, and were thus burdened with the errors of their estimates.

Since the estimates of the values of enthalpy and entropy changes could only be said to have a relative accuracy, the comparative interpretation of these values was confined to the delineation of major quantitative and qualitative differences characterizing the interaction between different digitalis derivatives and $(Na^+ + K^+)$-ATPase of different ligation or origin.

Results

The values of the kinetic and thermodynamic quantities characterizing the interaction between digitalis compounds of variant structure and $(Na^+ + K^+)$-ATPase are gathered in Tables I and II, and in part represented in Fig. 1. Table I indicates the chemical structures of the compounds and the numbers assigned to them that for convenient reference are often used in the text.

In the following, the terms 'increase' or 'decrease' of the thermodynamic quantities apply to their numerical values without change of sign. As the inactivating interaction is an endothermic, entropically driven process (see below), a decrease of the $\Delta H^{\circ\prime}$ value (with positive sign) and an increase of the $\Delta S^{\circ\prime}$ value (with positive sign) raise both the $\Delta G^{\circ\prime}$ value (cf. Eqn. 8) meaning that the underlying variation of enzyme ligation is favourable for complex formation.

A decrease of the $\Delta\vec{H}^{\neq}$ value and an increase of the $\Delta\vec{S}^{\neq}$ value reduce both the $\Delta\vec{G}^{\neq}$ value (cf. Eqn. 13) meaning that the underlying variation of

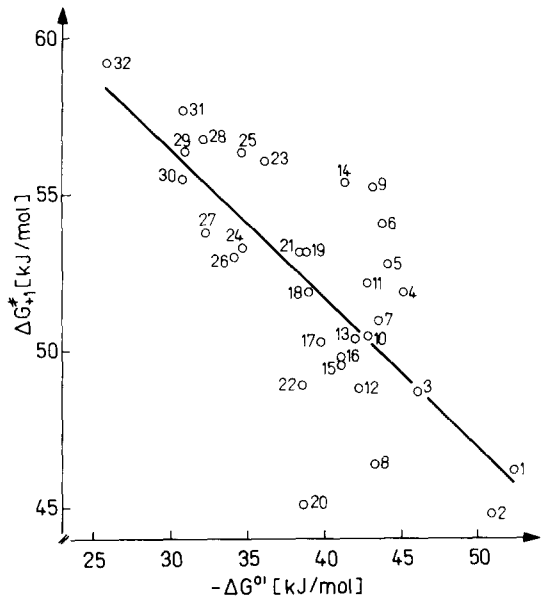

Fig. 1. Test on the occurrence of 'linear free energy relationships' by plotting the $\Delta\vec{G}^{\neq}$ values versus the $\Delta G^{\circ\prime}$ values characterizing the interaction between 32 5β,14β-androstane-3β,14-diol derivatives and $(Na^+ + K^+)$-ATPase from hog cardiac muscle estimated in the presence of ATP, Mg^{2+}, Na^+ and K^+ at 37 °C. The numbering of the data points refers to the compounds, the structures of which are specified in Table I. The underlying kinetic data was determined in binding studies (6, 7, 9, 18, 20) or inhibition studies (all others). The regression line for the data points derived from the latter primary data is described by $\Delta\vec{G}^{\neq} = 0.50\ \Delta G^{\circ\prime} + 71.8$ kJ/mol ($r = 0.92$).

enzyme ligation lowers the activation Gibbs energy barrier and is thus favourable for forming a complex.

An increase of the $\Delta \bar{H}^{\neq}$ value and a decrease of the $\Delta \bar{S}^{\neq}$ value raise both the $\Delta \bar{G}^{\neq}$ value (cf. Eqn. 13) meaning that the underlying variation of enzyme ligation enhances the activation Gibbs energy barrier and is thus favourable for the stability of a complex.

(1) Hog cardiac ($Na^+ + K^+$)-ATPase

Equilibrium Gibbs energy changes

32 digitalis derivatives were characterized as to the $\Delta G°'$ values of their interaction with the enzyme (Table I). These values allowed the extrathermodynamic derivation of quantitative structure-activity relationships (cf. Refs. 27 and 28). On the basis of the additivity postulate [29], the integral $\Delta G°'$ value of a given compound was expressed as being the resultant of favourable and unfavourable energetic contributions of the substructural variables to be defined. Their contributions were calculated from the difference between the $\Delta G°'$ quantities of a pair of compounds which differed in a single substructural feature; the structural difference in question will be exemplified in the following by an arrow between the numbers of the paired compounds whose structures are shown in Table I. This well established procedure [8] afforded the following major results.

Favourable (e.g. 22 → 3) or unfavourable (e.g. 12 → 31) influences of glycosidation depended upon the character of the sugar, the structure of the aglycone and the location of sugar attachment on the steroid skeleton. All other substitutions on the steroid backbone of the digitalis prototypes, digitoxigenin (8), digitoxin (4) or digoxin (6), reduced the interaction Gibbs energy change of the precursor compounds (e.g. 6 → 28). In conclusion, the digitalis interaction matrix of ($Na^+ + K^+$)-ATPase appeared to be tailored to provide a snug, three-dimensional fit for only the unsubstituted steroid nucleus present in bufalin (2), digitoxigenin (8) and digitoxin (4), i.e., 5β,14β-androstane-3β,14-diol, which was identified by use of human cardiac ($Na^+ + K^+$)-ATPase as the lead structure in cardiac glycosides [8]. The steroid lead, filling in its binding subsite on the enzyme, delineated then also in hog cardiac ($Na^+ + K^+$)-ATPase the interactive energy surface [25], whose tight occupancy by a digitalis compound was required for the attainment of maximum interaction energy.

The various modifications of the butenolide or pentadienolide side chains at C-17 of steroid nucleus decreased the $\Delta G°'$ value of the precursor compounds (e.g. 1 → 17). The removal of the butenolide side chain from digitoxin (4 → 32) reduced the $\Delta G°'$ value from −45.2 to −25.8 kJ/mol. Despite this large reduction of interaction energy, the residual steroid glycoside still showed complete ($Na^+ + K^+$)-ATPase inhibition. The fact that digitoxose, still present in 32, is alone unable to inhibit [6], implied that quite simply the steroid nucleus in digitoxin, i.e., 5β,14β-androstane-3β,14-diol, contained sufficient information for the recognition of the receptor enzyme. This confirmed the same conclusion derived from studies with human cardiac ($Na^+ + K^+$)-ATPase [8].

Component equilibrium entropy and enthalpy changes

As shown in Table II (presence of K^+), the formation of the inhibited complexes between the digitalis derivatives and ($Na^+ + K^+$)-ATPase was always an endothermic, entropically driven process. Remarkably, the inherent entropic disadvantage that renders more difficult binding of a flexible ligand to a macromolecule [25], amounts at 37 °C to −2.9 kJ/mol for the loss of each internal degree of conformational freedom and −61 kJ/mol for the loss of overall rotational and translational entropy [30]. Hence, these entropy losses were here still more than counterbalanced by even larger favourable entropy gains. That largely entropy-driven protein ligations mask entropy losses upon ligand binding is a more general experience [31].

Contrary to our intuitive expectation, the reduction of internal conformational entropies by removal of the tridigitoxose side chain (4 → 8) or of the butenolide side chain (4 → 32) did not increase, but considerably reduced the entropy gains. This indicated that the observed $\Delta S°'$ values were governed by the interaction mode of the digitalis derivatives with the interactive energy surface of ($Na^+ + K^+$)-ATPase so that the bind-

TABLE II

INFLUENCE OF K^+ ON THE KINETIC AND THERMODYNAMIC PARAMETERS CHARACTERIZING THE INTERACTION BETWEEN NINE 5β,14β-ANDROSTANE-3β,14-DIOL DERIVATIVES AND $(Na^+ + K^+)$-ATPase FROM HOG CARDIAC MUSCLE ESTIMATED IN THE PRESENCE OF ATP, Mg^{2+}, Na^+ AND ALSO K^+ WHEN INDICATED

The kinetic data as well as the Gibbs energy and the enthalpy quantities are for 37 °C. The numbers refer to the compounds, the structures of which are specified in Table I. The parameters for compounds 26 and 32 are derived from inhibition studies, all others from binding studies.

Compound No.	KCl presence	$\vec{k}(\times 10^{-4})$ ($M^{-1} \cdot s^{-1}$)	$\bar{k}(\times 10^{3})$ (s^{-1})	K'_D (μM)	$\Delta\vec{G}^{\neq}$ (kJ/mol)	$\Delta\vec{H}^{\neq}$ (kJ/mol)	$\Delta\vec{S}^{\neq}$ (J/mol per K)	$\Delta\bar{G}^{\neq}$ (kJ/mol)	$\Delta\bar{H}^{\neq}$ (kJ/mol)	$\Delta\bar{S}^{\neq}$ (J/mol per K)	$-\Delta G^{\circ\prime}$ (kJ/mol)	$\Delta H^{\circ\prime}$ (kJ/mol)	$\Delta S^{\circ\prime}$ (J/mol per K)
1	yes	19	0.73	0.0040	44.7	357	1010	94.7	111	50.9	49.9	246	956
4	yes	2.7	0.24	0.0093	49.8	136	281	97.6	48.2	−159	47.7	88.3	439
4	no	19	1.2	0.0066	44.8	97.6	170	93.4	70.8	−73.3	48.6	19.3	243
5	yes	1.2	0.09	0.0066	51.9	114	201	100	71.2	−96.0	48.6	43.1	296
5	no	7.0	0.15	0.0021	47.3	72.9	83.7	98.8	26.0	−234	51.5	46.9	318
8	yes	19	5.5	0.030	44.8	117	234	89.5	109	63.6	44.7	8.0	171
8	no	130	16	0.013	39.8	89.2	159	86.7	106	59.9	46.9	−16.3	219
15	yes	4.3	5.7	0.13	48.5	153	337	89.4	114	79.1	40.9	39.1	258
18	yes	1.2 [a]	3.2 [a]	0.27 [a]	51.9 [a]	122	226	90.9 [a]	105	45.1	39.0 [a]	17.2	181
18	no	7.0	4.5	0.065	47.3	102	176	90.0	90.0	0.8	42.7	11.7	176
20	yes	16 [a]	51 [a]	0.32 [a]	45.1 [a]	129	271	83.7 [a]	101	55.4	38.6 [a]	28.3	216
26	yes	0.77 [a]	14 [a]	1.8 [a]	53.0 [a]	88.2	114	87.1 [a]	68.5	−59.4	34.1 [a]	19.7	173
32	yes	0.07 [a]	31 [a]	45 [a]	59.2 [a]	83.5	78.3	85.0 [a]	59.9	−81.0	25.8 [a]	23.4	159

[a] Data from Table I.

ing of the tridigitoxose or the butenolide side chains elicited additional entropy gains. Clearly, the loss of translational, external and internal rotational entropies, which the compounds experienced in the process of binding to the enzyme, had greatly mingled with the entropy gains in the enzyme protein that had thus been correspondingly larger than the observed net $\Delta S^{\circ\prime}$ values.

As also shown in Table II (presence of K^+), ouabain (5), digitoxin (4) and proscillaridin A (1) greatly differed in eliciting favourable entropy values in binding to the enzyme. However, these differences were essentially compensated for by unfavourable enthalpy values so that the interaction Gibbs energy changes with the three glycosides were not much different. On the other hand, the $\Delta G^{\circ\prime}$ values of most derivatives (especially 32, 26, 18, 8) differed greatly because large differences in unfavourable $\Delta H^{\circ\prime}$ values were not accompanied by compensating variations of favourable $\Delta S^{\circ\prime}$ values.

Activation quantity changes

The value of the activation Gibbs energy barrier in the interaction between 32 digitalis derivatives and $(Na^+ + K^+)$-ATPase varied between 45 and 59 kJ/mol in complex formation, and between 84 and 99 kJ/mol in complex dissociation (Table I). In both directions the energy barrier was due to large unfavourable activation enthalpy changes, which were but partially compensated for by favourable activation entropy changes, whereas in the reverse direction even unfavourable activation entropy changes occurred (Table II, presence of K^+).

As shown in Fig. 1, a number of digitalis derivatives showed an almost linear correlation between the values of activation and equilibrium Gibbs energy quantities in forming the inhibited complexes with $(Na^+ + K^+)$-ATPase. Their recognition by the receptor enzyme thus appeared to be governed by the close-range forces and geometrical limitations exerted by the interactive energy surface. However, in the derivatives whose data points lay below or above the regression line, receptor-induced favourable or unfavourable orientation mechanisms, respectively, appeared to have been the dominant part of the recognition

process operating over distances where molecular contact had not yet (or no longer) occurred. Such long-range orientation forces could thus have significantly increased or decreased the frequency of 'productive' encounters of the digitalis derivatives with the recognition and binding matrix of the enzyme in the receptive state to form the inhibited complex.

K^+ influence on the thermodynamic quantities

The significance of K^+ was estimated by comparing the values for the thermodynamic quantities of the interaction between digitoxin (4), ouabain (5), digitoxigenin (8) or 16α-gitoxin (18) and $(Na^+ + K^+)$-ATPase promoted by ATP, Mg^{2+} and Na^+ either in the absence or presence of K^+ (Table II). Most remarkably, the complex formation was an endothermic (except 8), entropically driven process also in the absence of K^+. However, K^+ whose binding to the enzyme induces a low-entropy, low-enthalpy, i.e., highly structured conformer [32], altered the size of the thermodynamic parameters. So, K^+ decreased the $\Delta G^{\circ\prime}$ value at an average of -2 kJ/mol, mostly through an increase of an unfavourable $\Delta H^{\circ\prime}$ value (4, 8, 18) or a decrease of a favourable $\Delta S^{\circ\prime}$ value (5).

K^+ increased the $\Delta \vec{G}^{\neq}$ value for the formation of the complexes of the various digitalis compounds with the enzyme at an average of 4.8 kJ/mol. However, through K^+-ligation of the enzyme the $\Delta \overleftarrow{G}^{\neq}$ value for the dissociation of the complexes with the various compounds was very differently increased (between 0.9 and 4.2 kJ/mol) because of divergent effects on the $\Delta \overleftarrow{H}^{\neq}$ and $\Delta \overleftarrow{S}^{\neq}$ quantities.

(2) Guinea-pig $(Na^+ + K^+)$-ATPase

As in hog $(Na^+ + K^+)$-ATPase, also in the guinea-pig enzyme the formation of the inhibited complex of cardiac glycosides with ATP, Mg^{2+}, Na^+ and K^+ was an endothermic, entropically driven process (not shown). Compared with the hog enzyme, the equilibrium Gibbs energy changes in the interaction between the guinea-pig enzyme and digitoxin (4), ouabain (5) or 16α-gitoxin (18) were smaller by 5.6, 11.5 or 10.7 kJ/mol, respectively. The reduction of the $\Delta G^{\circ\prime}$ value resulted from the decrease of the component favourable $\Delta S^{\circ\prime}$ value which was incompletely compensated for by the decrease of the component unfavourable $\Delta H^{\circ\prime}$ value. Judged from the difference in the $\Delta G^{\circ\prime}$ values found with the two enzymes for the three glycosides, and from the diversity in the component $\Delta S^{\circ\prime}$ and $\Delta H^{\circ\prime}$ values, the interactive energy surface in the two enzymes appeared to differ so that the favourable points of interaction were less or weaker in the guinea-pig enzyme.

In corroboration of this conclusion, the values for the transition-state thermodynamic quantities characterizing the formation and dissociation of the inhibited enzyme complexes with digitoxin (4), ouabain (5) or 16α-gitoxin (18) differed in the hog and guinea-pig cardiac $(Na^+ + K^+)$-ATPase as a function of the glycoside structure. In the guinea-pig enzyme, the $\Delta \vec{G}^{\neq}$ values were a little lower (4, 5) or higher (18), and the component $\Delta \vec{H}^{\neq}$ and $\Delta \vec{S}^{\neq}$ values were, as far as they were estimated, also variable. Although the $\Delta \overleftarrow{G}^{\neq}$ values were consistently considerably lower with this enzyme, the $\Delta \overleftarrow{H}^{\neq}$ and $\Delta \overleftarrow{S}^{\neq}$ values likewise nevertheless varied as a function of glycoside structure.

Discussion

Molecular mechanism of digitalis $(Na^+ + K^+)$-ATPase interaction

The highest values for $\Delta G^{\circ\prime}$, $\Delta H^{\circ\prime}$ and $\Delta S^{\circ\prime}$, as observed in the interaction betwen proscillaridin A (1) and hog cardiac $(Na^+ + K^+)$-ATPase, were -49.9 kJ/mol, 246 kJ/mol and 956 J/mol per K, respectively (Table II). Rather similar values for the thermodynamic quantities were found in the reaction of nicotine with solubilized acetylcholine receptor, in which the natural lipid environment of the receptor was replaced by detergent [33]. Hence, the magnitude of the values for the thermodynamic quantities observed in the present study strongly suggests that substantial conformational modifications of the enzyme protein accompany the formation of the inhibited digitalis-$(Na^+ + K^+)$-ATPase complex. Actually, a significant ouabain-induced structural change of $(Na^+ + K^+)$-ATPase was independently derived from an increase of heat stability and a decrease of transition width in the apparent heat capacity versus temperature profile of the ouabain-enzyme

complex [34], and from the change of α,α-subunit interaction in the $\alpha_2\beta_2$ oligomer as revealed by chemical crosslinking [35].

The chemical nature of the digitalis-receptive intermediary state of cycling $(Na^+ + K^+)$-ATPase has not yet been identified [17]. However, our knowledge that a large entropy gain in the enzyme protein is, irrespective of enzyme origin and ligation, the common denominator of the inhibitory action of all digitalis derivatives (this paper), suggests that the thermodynamic essence of enzyme inactivation is a relaxation of 'conformational energy' (negentropy strain [36]) of the enzyme protein, which is the sum of the potential energy for all intrapolypeptide interactions and the Gibbs energy for all interactions involving structured water [37]. Reciprocally, the common denominator of the digitalis-binding conformational state of the phospho- and dephosphoenzyme [38] is then a 'high-energy conformation' built up through phosphorylation from ATP, and Mg^{2+}- or Mn^{2+}-complex formation with the enzyme.

The knowledge that diffusion-controlled interactions of certain drugs with their receptors are characterized by loss of entropy and no change of enthalpy [39,40], but that the formation of the inhibited complex of digitalis derivatives with $(Na^+ + K^+)$-ATPase is associated with a great gain of entropy and a large increment of enthalpy (Table II) independently supports the conclusion that the energetic parameters estimated reflect digitalis-induced conformational changes of enzyme protein.

In addition to this reason, understanding of the striking slowness in the development of the inhibitory digitalis action on $(Na^+ + K^+)$-ATPase [7] also requires an extension of the simple bimolecular model in Eqn. 1. In the amended model it is assumed that the reversible formation of inhibited digitalis-enzyme complexes proceeds in two steps (like the reversible formation of productive substrate-enzyme complexes [41]) according to

$$D + E \underset{\vec{k}_d}{\overset{\vec{k}}{\rightleftharpoons}} D\text{- -}E \underset{\vec{k}_{is}}{\overset{\vec{k}_{is}}{\rightleftharpoons}} D' \cdot E' \qquad (14)$$

in which: D is free digitalis, E enzyme in receptive state, D- -E non-inhibited diffusive complex, $D' \cdot E'$ inhibited isomerized complex, $\vec{k}_d$ and $\overleftarrow{k}_d$ diffusion rate constants, $\vec{k}_{is}$ and $\overleftarrow{k}_{is}$ isomerization rate constants, and $\vec{k}$ and $\overleftarrow{k}$ observed forward or reverse rate constants.

During the several minute long time period when our measurements were made, the observed kinetic parameters, $\vec{k}$ and $\overleftarrow{k}$ (gathered in Table I) appear to be largely dominated by the isomerization constants, $\vec{k}_{is}$ and $\overleftarrow{k}_i$, through the conditions: $\vec{k}_{is} \ll \vec{k}_d$ and $\overleftarrow{k}_{is} \ll \overleftarrow{k}_d$, so that $\vec{k} \approx \vec{k}_{is}$ and $\overleftarrow{k} \approx \overleftarrow{k}_{is}$. Compared with the diffusion rate constant of slowly diffusing reactants lying near $10^9\ M^{-1} \cdot s^{-1}$ [41], the $\vec{k}$ values were by 4 to 6 orders of ten smaller, and the $\overleftarrow{k}$ values by 5 to 7 orders smaller, but pretty close to the isomerization constant characterizing the conformational change in substrate-enzyme interactions ranging from $10^2\ s^{-1}$ to $10^4\ s^{-1}$ [39]. After all, the slow rate of the formation of the inhibited digitalis-$(Na^+ + K^+)$-ATPase complex appears to result partly also from the rare formation of a 'productive' diffusive complex with the enzyme in the receptive intermediary state which traps the digitalis molecule in the appropriate rotational state and spatial position upon the binding matrix to isomerize into the inhibited complex.

The interactive energy surface in $(Na^+ + K^+)$-ATPase protein appears to be a cleft, approx. 20 Å deep between lobes of the catalytic α-chain, that becomes locked on cardiac glycoside binding so as to envelope the butenolide side chain, lying at the bottom of the cleft, the steroid nucleus, and the sugar next to the steroid nucleus lying near the mouth of the cleft [8]. The movement of large protein domains involves a negligible entropy gain [31]. Hence, the cleft closing on cardiac glycoside interaction cannot essentially contribute to the large entropy and enthalpy changes observed, but rather appears to act as a 'switching device' in eliciting the underlying peptide chain rearrangements in the enzyme protein.

The digitalis-binding site cleft in $(Na^+ + K^+)$-ATPase is known to be exposed on the external face of the membrane, and the catalytic centre, phosphorylatable from ATP, is known to be exposed on the cytoplasmic side. The two sites, both residing in the α-peptide, are about 74 Å apart [42]. The digitalis-binding site cleft is connected to the phosphorylation site by an amino acid se-

quence which appears to be an important component of the energy transducing system [43,44]. Via transduction of digitalis-elicited relaxation of the negentropy strain along this pathway, a geometric distortion of catalytic centre appears to result that would explain the lowered affinity for ATP [17] and the suppression of phosphorylation from ATP [45,46]. The resulting inhibition of Na^+/K^+ pumping is the initial event in the chain of events leading to the inotropic digitalis action (reviewed in Ref. 4).

Cycling versus non-cycling ($Na^+ + K^+$)-ATPase

The complex formation between all compounds, including ouabain (5), and cycling hog and guinea-pig cardiac ($Na^+ + K^+$)-ATPase, initiated in the presence of ATP, Mg^{2+}, Na^+ and K^+, is an endothermic, entropically driven process as shown in the present paper. However, the complex formation between ouabain and noncycling beef cardiac ($Na^+ + K^+$)-ATPase, initiated in the presence of Mg^{2+} and orthophosphate, is an exothermic, but also largerly entropically driven process [47]. Apparently, caused by different ligations, the conformational changes of the enzyme upon formation of the inhibited complex are somewhat different.

Six of the digitalis representatives studied here (4, 5, 8, 12, 14) as to their interaction with cycling hog cardiac ($Na^+ + K^+$)-ATPase in the presence of ATP, Mg^{2+}, Na^+ and K^+ (Table I) have also been examined with regard to their interaction with non-cycling bovine brain ($Na^+ + K^+$)-ATPase in the presence of Mg^{2+} and orthophosphate [48]. Under the latter conditions, the $\Delta G^{\circ\prime}$ values were on an average by -2.4 kJ/mol greater because either the $\Delta \vec{G}^{\neq}$ values were smaller or the $\Delta \overleftarrow{G}^{\neq}$ values were greater.

Conclusions

Taken together, the available evidence suggests that the basic thermodynamic characteristics in the interaction of digitalis derivatives and ($Na^+ + K^+$)-ATPase, i.e., a dominant entropy gain in the enzyme protein linked with a high activation Gibbs energy barrier, are independent of species and organ origin of the enzyme, although the nature of ligation (especially concerning ATP, K^+ or orthophosphate) strongly modulates the thermodynamic quantities. Remarkably, the binding of ouabain to erythrocytes was shown to be associated with a large positive (i.e. unfavourable) value of $\Delta H^{\circ\prime}$ and an even larger positive (i.e. favourable) value of $\Delta S^{\circ\prime}$ [49]. In accordance with the above conclusions, the latter data was interpreted to reflect a highly ordered configuration of the ($Na^+ + K^+$)-ATPase molecule that is lost upon ouabain binding and that 'drives' the reaction despite the positive value of $\Delta H^{\circ\prime}$. This knowledge allows for the generalization of present findings, but also calls for the choice of cycling enzyme in studies aiming at the extrapolation of data to in situ conditions. Actually the receptor thermodynamics, reported here for the first time, appear to dominate some peculiar pharmacological properties of the digitalis derivatives as will be shown elsewhere.

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