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Effects of Sodium Iodide, Lithium Bromide, and Deoxycholate on Dissociation of [^{3}H]Ouabain–Na,K-ATPase Complex during Enzyme Isolation

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SUMMARY

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In an attempt to resolve the current controversy regarding whether or not myocardial Na,K-ATPase remains inhibited following washout of digitalis inotropy in isolated hearts, the effects of various protein extracting agents used in enzyme isolation procedures were investigated on dissociation of the drug-enzyme complex. The drug–enzyme complex, [^{3}H]ouabain–Na,K-ATPase, was isolated both after *in vitro* incorporation of [^{3}H]ouabain to crude rabbit heart Na,K-ATPase fraction and after *ex vivo* binding of [^{3}H]ouabain in isolated Lagendorff rabbit hearts. The drug–enzyme complex was then treated at 0–4°C with 2 M NaI, 0.1% deoxycholate (DOC), or 0.1% DOC plus 2 M NaI, the protein extracting agents used in many laboratories, and compared against treatment with LiBr, the extracting agent used in our laboratory. It was found that a sequential treatment of 0.1% DOC followed by 2 M NaI resulted in over 95% of the [^{3}H]ouabain dissociating from both *ex vivo* and *in vitro* bound drug–enzyme complexes in comparison to control. For *ex vivo* bound drug–enzyme complex, 2 M NaI treatment alone removed approximately 90% of the [^{3}H]ouabain activity and 0.1% DOC removed approximately 45% of the drug. In contrast to these results, treatment of the *ex vivo* and *in vitro* labeled drug–enzyme complexes with 1 M LiBr caused no detectable difference in drug dissociation in comparison to control complex treated with 1 mM Tris–HCl. Therefore, these findings may account for the inability of some investigators to demonstrate inhibition of Na,K-ATPase following enzyme isolation from inotropically stimulated Lagendorff rabbit hearts as well as from noninotropic hearts of various species following drug washout due to dissociation of the drug–enzyme complex by NaI and DOC. Since LiBr does not appreciably dissociate the drug–enzyme complex, in comparison to 1 mM Tris–HCl control, the present findings support our original observation that inhibition of Na,K-ATPase is not responsible for the inotropic action of digitalis, because enzyme inhibition is still present in noninotropic rabbit hearts following drug washout.

INTRODUCTION

At present one of the postulated mechanisms for the positive inotropic action of digitalis is believed to be due to inhibition of the myocardial cation transport enzyme, Mg^{2+}-dependent (Na^+,K^+)-activated ATP phosphohydrolase, EC J.6.1.3 (Na,K-ATPase) (1–5). Inhibition of this enzyme by digitalis is believed to cause an increase in intracellular sodium which in turn is believed to increase intracellular Ca^{2+} either by competition for intracellular Ca^{2+} sites or by a sodium-induced augmentation of Ca^{2+} influx. The indirect increase in intracellular Ca^{2+} is then considered to augment myocardial contractility. However, recent findings from our laboratory (6–9) as well as those of other laboratories (10–16) do not support the postulation that the mechanism of the positive inotropic action of digitalis is due to inhibition of Na,K-ATPase. Based on drug washout studies in isolated Langendorff rabbit hearts, we have previously reported (7) that Na,K-ATPase isolated from such preparations is still significantly inhibited, whereas the positive inotropic effect is no longer present. Also, based on kinetic considerations the half-life of digitalis inotropy is comparatively very short, whereas the half-life of enzyme inhibition is

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very long. Therefore, these findings indicate a dissociation of Na,K-ATPase inhibition from the inotropic action of digitalis.

Recently, however, both Schwartz *et al.* (17) and Akera *et al.* (18) have been unable to confirm our findings. Both laboratories reported that they were unable to demonstrate inhibition of Na,K-ATPase obtained from nonwashed ouabain-treated hearts of rabbits or guinea pigs, a relatively digitalis-insensitive species, as well as from cats, a digitalis-sensitive species, following drug washout in the absence of inotropy (17). Since these investigators used NaI and/or deoxycholate (DOC) as the protein extracting agents in their enzyme isolation procedure, whereas LiBr was used in our laboratory, the possibility that differences in enzyme extraction procedures may account for the differences in experimental findings was investigated. Therefore, the effects of DOC and NaI were compared against LiBr on the dissociation of the [^{3}H]-ouabain–Na,K-ATPase complex formed under both *in vitro* and *ex vivo* (isolated heart) conditions.

METHODS

Crude Na,K-ATPase isolation. Albino rabbits weighing 2–3 kg were killed by decapitation. The heart was quickly removed, atria and vessels excised, the ventricles cut into small pieces, and homogenized with 10 vol of 0.25 M sucrose plus 1 mM EDTA, pH 6.8, at 0–4°C in a Sorvall Omni Mixer at medium speed for 1 min. The suspension was squeezed through four layers of cheesecloth and then centrifuged at 10,000*g* for 15 min. The pellet was homogenized with 8 vol of 0.25 M sucrose + 1 mM EDTA, pH 6.8, in a glass homogenizer with 10 strokes, the suspension centrifuged at 10,000*g* for 15 min, and the pellet resuspended uniformly by homogenization in a glass homogenizer with 12 ml of 1 mM Tris–HCl buffer, pH 7.4.

In vitro binding of [^{3}H]ouabain to Na,K-ATPase. Both Type 1 and Type 2 drug–enzyme complexes were formed using the following incubation mixtures in a final volume of 10 ml in centrifuge tubes: 50 mM Tris–HCl at pH 7.4, 1×10^{-6} M [^{3}H]ouabain, 25–35 mg Na,K-ATPase fraction, and either 50 mM NaCl, 5 mM $MgCl_2$, and 2.0 mM ATP for Type 1 binding or 1 mM $MgCl_2$ and 1 mM H_3PO_4 for Type 2 binding. Controls were run without ATP or inorganic phosphate in order to determine the extent of nonspecific binding. After equilibration at 37°C for 5 min with stirring in a Dubnoff metabolic shaking incubator, the enzyme was added to the incubation mixture and the incubation continued for another 20 min. The reaction mixture was cooled and then centrifuged at 47,000*g* for 20 min. The pellet was resuspended in 10 ml of 0.25 M sucrose by homogenizing with three strokes in a glass homogenizer and recentrifuged at 47,000*g* for 20 min. The rinsing procedure was repeated a second time in order to remove unbound [^{3}H]ouabain. The pellet was finally suspended in 2–3 ml of 1 mM Tris–HCl, pH 7.4, by homogenizing in a glass homogenizer with five strokes. An aliquot of the suspended [^{3}H]ouabain–Na,K-ATPase complex obtained in the presence or absence of ATP or inorganic phosphate was assayed for protein content by the method of Lowry *et al.* (19). A second aliquot was assayed for ^{3}H radioactivity by overnight solubilization in 1 ml of NCS tissue solubilizer (Amersham/Searle) followed by the addition of 10 ml of Liquifluor (New England Nuclear) and counted in a liquid scintillation counter. Internal ^{3}H standards were used to determine counting efficiency and absolute dpm.

For the subsequent treatment of the drug–enzyme complex with various protein extracting agents, a larger batch of the labeled enzyme was prepared in the presence of ATP or inorganic phosphate. The [^{3}H]ouabain–Na,K-ATPase preparation was then stored in 1 mM Tris–HCl and used as the common *in vitro* labeled drug–enzyme complex pool.

Ex vivo binding of [^{3}H]ouabain to Na,K-ATPase. Albino rabbits weighing 2–3 kg were killed by decapitation 15–25 min after the intravenous administration of 1000 units of heparin. The heart was quickly removed, the aortic root cannulated, and the heart perfused at 30°C using a modified Kreb's solution containing 145 mM Na^+, 5.6 mM K^+, 2.2 mM Ca^{2+}, 2.1 mM Mg^{2+}, 25 mM HCO_3^-, and 10 mM glucose. The perfusion pressure was adjusted to 50 cm of water pressure and the flow rate varied between 7 and 12 ml/min. The perfusate was continously gassed with 95% O_2–5% CO_2. The heart was electrically stimulated at 120 beats/min, isometric contractile force measured by a force-displacement transducer, and a resting tension of 4 g maintained. After an equilibration period of 90 min, the perfusate was changed to one containing 7.5×10^{-7} M [^{3}H]ouabain and the heart perfused for 40 min or until the first evidence of arrhythmia. The heart was then frozen in a dry ice–acetone bath and stored at −70°C or immediately processed for the isolation of the [^{3}H]ouabain–Na,K-ATPase complex in exactly the same manner described previously for the isolation of the crude enzyme fraction. The drug–enzyme complex in 1 mM Tris–HCl was then used as the common *ex vivo* labeled drug–enzyme pool from which aliquots were obtained for subsequent treatment with the various protein extracting agents.

Treatment of in vitro and ex vivo bound [^{3}H]ouabain–Na,K-ATPase complex with various protein extracting agents. The effects of 2 M NaI, 0.1% deoxycholate (DOC), and 0.1% DOC + 2 M NaI were compared against 1 M LiBr and a control solution of 1 mM Tris–HCl, pH 7.4, on the dissociation of the [^{3}H]ouabain–Na,K-ATPase complex formed under *in vitro* and *ex vivo* conditions. The duration and temperature of exposure of the drug–enzyme complex to LiBr were similar to those used in the Na,K-ATPase isolation procedure employed by Okita *et al.* (7), and for treatment with DOC and NaI they were similar to those used in the enzyme isolation employed by Matsui and Schwartz (20) and are detailed as follows. *(1) Treatment with 1 M LiBr*: 1–3 ml of the [^{3}H]ouabain–enzyme complex suspension in 1 mM Tris–HCl, pH 7.4, from the common drug–enzyme pool was treated with an equal volume of 2 M LiBr and incubated for 1 h at 0–4°C. The suspension was then diluted with an equal volume of 1 mM Tris–HCl, pH 7.4, and centrifuged at 105,000*g* for 60 min. The pellet was next resuspended in 2–3 ml of Tris–HCl by homogenization in a glass homogenizer. The volume of the suspension was then measured and an aliquot assayed for protein content, ouabain-sensitive Na,K-ATPase activity, and ^{3}H radioactivity. *(2) Treat-*

ment with 2 M NaI: An aliquot of the drug–enzyme complex was mixed with half of its volume of a 6 M NaI solution containing 15 mM EDTA, 7.5 mM $MgCl_2$, and 120 mM Tris (pH of the mixture is about 8.3 at 4°C) and stirred gently in an ice bath for 30 min. The protein solution was then diluted with Tris–HCl to 0.8 M NaI, centrifuged for 60 min at 105,000*g*, resuspended in Tris–HCl, and assayed for protein, enzyme activity, and radioactivity. *(3) Treatment with 0.1% DOC*: An aliquot of the drug–enzyme complex was mixed with 10 vol of 0.1% DOC for 3 min in a Sorvall Omni Mixer at medium speed at 0–4°C. The suspension was then centrifuged for 60 min at 105,000*g* and processed in the same manner described previously following the centrifugation step. *(4) Treatment with DOC plus NaI*: After the DOC treatment and centrifugation of the drug–enzyme complex as described previously, the pellet was suspended in 1 ml Tris–HCl and treated with a final concentration of 2 M NaI as described previously for the NaI treatment procedure. The pellet obtained after centrifugation was suspended in 1 mM Tris–HCl and assayed for protein, enzyme activity, and radioactivity. *(5) Control*: 1 mM Tris–HCl was selected as the control solution because this is the medium used to store the *in vitro* and *ex vivo* labeled drug–enzyme complexes prior to its treatment with the various protein extracting agents. An aliquot of the drug–enzyme complex suspension in 1 mM Tris–HCl, pH 7.4, from the common drug–enzyme pool was incubated for 1 h at 0–4°C, centrifuged at 105,000*g* for 60 min, resuspended in 1 mM Tris–HCl, and assayed as previously.

A separate series of experiment also was conducted to determine the effects of the various protein extracting agents on the recovery of protein and ouabain-sensitive Na,K-ATPase activity from drug-free and drug-bound pools of crude Na,K-ATPase suspension in 1 mM Tris–HCl, pH 7.4.

All of the extraction methods employed, except for the DOC plus NaI treatment, were found not to significantly denature the enzyme since apparent total recovery of enzyme activity was accounted for in the pellet. Five independent experiments were conducted for each extracting agent treatment procedure. The results are not expressed in absolute values due to variability from animal to animal in Na,K-ATPase activity and, therefore, the amount of [³H]ouabain bound to the enzyme. Since the complete series of enzyme extracting agents was used for each independent set of experiments and the treated drug–enzyme complexes were compared as a paired experiment against the control drug–enzyme complex within an independent set of experiments, the results are expressed as a percentage change from control values in order to minimize experimental variability. The results from the five independent experiments are expressed as mean values ± standard errors of the means. Student's *t* test was utilized to determine significant differences between control and treated groups.

RESULTS

Effects of protein extracting agents on recovery of protein and ouabain-sensitive Na,K-ATPase activity from drug-free crude enzyme preparations. Prior to determining the effects of various protein extracting agents on dissociation of the ouabain–Na,K-ATPase complex, the effects of the extracting agents on recovery of total ouabain-sensitive Na,K-ATPase activity were determined from a common enzyme pool of crude drug-free rabbit enzyme preparations in order to rule out possible denaturation of the enzyme by the extracting agents. The extent of non-Na,K-ATPase protein extraction and specific enzyme activities were also compared following treatment with the various extracting agents.

As shown in Table 1, a slight but statistically insignificant loss of ouabain-sensitive Na,K-ATPase activity was observed following treatment of nonlabeled enzyme with 1 M LiBr, 2 M NaI, and 0.1% DOC in comparison to 1 mM Tris control values. Following sequential treatment of the enzyme with DOC and NaI, significant enzyme activity was lost, with only 48% recovery in the pellet fraction and 15% recovery in the supernatant fraction. Therefore, treatment with DOC + NaI appears to cause some denaturation of the ouabain-sensitive Na,K-ATPase.

Protein recovery of the crude enzyme preparation was lowest (10%) for DOC + NaI treatment, but also gave the highest purification as indicated by comparison of enzyme specific activities. LiBr treatment gave 53% protein recovery, while NaI gave only 28% recovery, but with reasonably high specific activity. DOC treatment gave high protein recovery (83%), but was not very selective in removing non-Na,K-ATPase protein as indicated by its low specific activity. It should be noted that all enzyme specific activities are lower than those usually published in the literature since the complete enzyme extraction procedure was not employed.

Specificity of binding of [³H]ouabain to Na,K-ATPase. Specific vs nonspecific binding *in vitro* of [³H]ouabain to the transport enzyme was determined in the presence and absence of ATP for Type 1 binding and inorganic phosphate for Type 2 binding. As shown in Table 2, approximately 8 to 10% nonspecific binding was observed in the absence of ATP for Type 1 binding. Maximum *in vitro* binding of the drug to the enzyme was achieved within 5 min, whereas nonspecific binding increased approximately 30% after 15 min of incubation in comparison to 5 min. About 8–10% nonspecific binding

TABLE 1

Effects of enzyme extracting agents on recovery of Na^+,K^+-ATPase and protein from crude enzyme preparations

Crude rabbit heart enzyme preparations from a common enzyme pool treated with extracting agents as described in Methods. Values presented are the means ± SE of five experiments.

Treatment	Na,K-ATPase specific activity	Enzyme units recovered		Protein recovered
		Pellet	Supernate	
	μmol P_i/mg/h	*% of control*		*% of control*
Control, 1 mM Tris–HCl	2 ± 0.3	100	ND[a]	100
1 M LiBr	3.8 ± 0.4*	89 ± 6	ND	53 ± 5*
2 M NaI	6.6 ± 0.4*	90 ± 6	ND	28 ± 4*
0.1% DOC	2.3 ± 0.3	86 ± 7	ND	83 ± 5
0.1% DOC + 2 M NaI	7.3 ± 0.4*	48 ± 4*	15 ± 2*	10 ± 3*

[a] ND = not detectable.

* $P < 0.01$ from control values.

TABLE 2

Specificity of in vitro binding (Type 1) of [^{3}H]ouabain to Na^+,K^+ ATPase fraction

Standard Type 1 binding for the drug-enzyme complex from a common enzyme pool was performed as described in Methods. Binding of [^{3}H]ouabain to Na^+,K^+-ATPase in the absence of ATP represents nonspecific binding. Binding in the presence of ATP represents the sum of specific and nonspecific binding. Values presented are the means of duplicate assays.

Expt	Time of incubation	−ATP		+ATP		Net specific binding[a]
	min	*dpm/mg protein*	*picomol/mg protein*	*dpm/mg protein*	*picomol/mg protein*	*picomol/mg protein*
1	5	645	0.44	7920	5.46	5.01
2	5	667	0.45	7900	5.45	5.00
3	15	865	0.59	7900	5.44	4.85
4	15	800	0.55	7770	5.35	4.80

[a] The relatively low specific binding of approximately 5 pmol of [^{3}H]ouabain/mg protein is due to the use of crude Na,K-ATPase fractions which were not treated with protein extracting agents.

was also observed for Type 2 binding. Specific radioactivity (dpm/mg protein) was usually 54% higher for Type 2 bound drug-enzyme complexes than for those formed under Type 1 conditions (data not shown). It is possible that the lower specific activity obtained for Type 1 complexes may be due to the fact that the ATP concentration of 2 mM was inadequate for the amount of crude enzyme preparation used. Nevertheless, the binding of 5 pmol of [^{3}H]ouabain/mg protein of the Type 1 complex was considered adequate for the purposes of our drug-enzyme complex dissociation experiments.

Effect of protein extracting agents on dissociation of in vitro bound [^{3}H]ouabain-enzyme complex. As indicated in Tables 3 and 4, for results expressed as the amount of total dpm in the treated fraction, there is 100% recovery of [^{3}H]ouabain radioactivity for both Type 1 and Type 2 drug-enzyme complexes treated with 1 M LiBr, as compared to control complex treated with 1 mM Tris-HCl. Thus, LiBr does not cause detectable dissociation of the drug-enzyme complex in comparison to controls. However, the combination of 0.1% DOC + 2 M NaI caused greater than 95% dissociation of the drug-enzyme complex for both Type 1 and Type 2 complexes in comparison to control and LiBr treatment. For 2 M NaI treatment alone, approximately 75–80% dissociation was noted for both types of complexes, while 0.1% DOC alone gave about 70% dissociation for Type 1 complex and 50% dissociation for Type 2 complex. The amount of [^{3}H]-ouabain dissociation from *in vitro* and *ex vivo* labeled complexes following control treatment with 1 mM Tris-HCl at 0.4°C was less than 10%.

When the drug-enzyme complex is expressed as specific radioactivity (dpm/mg protein), DOC + NaI caused approximately 64–73% drug loss for both types of complexes, whereas DOC alone caused 28–41% loss, and NaI alone 18–32% loss in comparison to control values. The higher specific activity of the LiBr-treated complex in comparison to the Tris-HCl-treated control complex is due to the decrease in nontransport enzyme proteins

TABLE 3

Effects of enzyme extracting agents on dissociation of in vitro bound (Type I) [^{3}H]ouabain-Na^+,K^+-ATPase complex

Dissociation of the drug-enzyme complex from a common pool was performed using conditions as described in Methods. Values presented are the means ± SE of five experiments. Complexes labeled in the absence of ATP served as controls for nonspecific binding and were subtracted from complexes labeled in the presence of ATP in order to obtain net specific binding.

Treatment	dpm in treated fractions	dpm/mg protein	Protein recovered	Enzyme units recovered
	% of control	*% of control*	*% of control*	*% of control*
Control, 1 mM Tris-HCl[a]	100	100	100	100
1 M LiBr	100.0 ± 2.5	200 ± 6.0*	52 ± 5*	127 ± 17
2 M NaI	21.9 ± 5.6*	68.4 ± 12*	29 ± 4*	145 ± 10*
0.1% DOC	31.3 ± 3.9*	59.0 ± 16*	85 ± 5	115 ± 16
0.1% DOC + 2 M NaI	3.2 ± 1.4*	27.3 ± 6.0*	11 ± 3*	121 ± 20

[a] Dissociation of [^{3}H]ouabain from the control drug-enzyme complex was less than 10% following treatment with 1 mM Tris-HCl at 0–4°C.
* $P < 0.01$ from control values.

removed by LiBr extraction as indicated by the loss of approximately 50% of the protein from the recovered crude drug-enzyme complex. For this reason the data expressed as total radioactivity remaining in the treated fraction are considered to be a better index of dissociation of the drug-enzyme complex since the specific radioactivity data may give misleading values due to further purification of the drug-enzyme complex following treatment with the various protein extracting agents.

The extents of protein recovery for both Type 1 and Type 2 complexes are approximately similar to the values obtained in Table 1 for drug-free crude enzyme prepa-

TABLE 4

Effects of Enzyme extracting agents on dissociation of in vitro bound (Type II) [^{3}H]ouabain-Na^+,K^+-ATpase complex

Dissociation of the drug-enzyme complex from a common enzyme pool was performed using conditions as described in Methods. Values presented are the means ± SE of five experiments. Complexes labeled in the absence of P_i served as control for nonspecific binding and were subtracted from complexes labeled in the presence of P_i in order to obtain net specific binding.

Treatment	dpm in treated fractions	dpm/mg protein	Protein recovered	Enzyme units recovered
	% of control	*% of control*	*% of control*	*% of control*
Control, 1 mM Tris-HCl[a]	100	100	100	100
1 M LiBr	100 ± 2.8	200 ± 5.0*	50 ± 2*	117 ± 28
2 M NaI	23.0 ± 3.5*	81.6 ± 8.6	25 ± 3*	127 ± 18
0.1% DOC	50.7 ± 6.9*	72.0 ± 16	90 ± 5	109 ± 12
0.1% DOC + 2 M NaI	4.1 ± 0.3*	36.0 ± 0.8*	10 ± 2*	129 ± 26

[a] Dissociation of [^{3}H]ouabain from the control drug-enzyme complex was less than 10% following treatment with 1 mM Tris-HCl at 0–4°C.
* $P < 0.01$ from control values.

rations treated with the extracting agents. In general, there is a correlation between the loss in protein recovery and the specificity of the extracting agents in removing non-Na,K-ATPase protein. Thus, it is not surprising that DOC + NaI caused an approximately 90% loss of protein from the crude drug–enzyme preparation and also gave the highest specific activity for the crude Na,K-ATPase preparation (see Table 1). Likewise, treatment with DOC alone caused a minimal loss of protein, but also gave the lowest specific enzyme activity.

In an attempt to determine the extent of enzyme reactivation and/or enzyme denaturation caused by the extracting agents on Types 1 and 2 drug–enzyme complexes, recovery of total units of enzyme activity also was determined. As indicated in both Table 3 and Table 4, recovery of enzyme activity for all treated drug–enzyme complexes was greater than 100%. However, only the NaI-treated Type 1 complex was significantly greater than control values, indicating partial reactivation of the ouabain-inhibited enzyme following partial dissociation of the drug–enzyme complex. Although P values for the other treated complexes were not statistically significant, it is possible that slight enzyme reactivation may have occurred since all mean values were consistently greater than 100%. For Types 1 and 2 complexes treated with DOC + NaI, it is very likely that partial enzyme reactivation may have occurred since data from Table 1 indicated partial denaturation of the drug-free crude enzyme preparation after treatment with the combined extracting agents. Considering that the combined treatment dissociates over 95% of the drug–enzyme complex and also removes nonenzyme proteins, enzyme specific activities similar to control values may indicate combined effects of enzyme reactivation, denaturation, and enzyme purification.

Effect of protein extracting agents on dissociation of ex vivo bound [^{3}H]ouabain–enzyme complex. The more relevant data on the effects of protein extracting agents on dissociation of the drug–enzyme complex are the demonstration of such effects on complexes formed under *in vivo* or *ex vivo* conditions. As shown in Table 5, for *ex vivo* bound drug–enzyme complexes treated with 0.1% DOC + 2 M NaI, approximately 97% of the [^{3}H]ouabain was dissociated from the radioactive enzyme fraction in comparison to control samples treated with 1 mM Tris–HCl. However, as demonstrated for *in vitro* bound drug–enzyme complexes, LiBr treatment did not cause any greater dissociation of the *ex vivo* bound complex than that observed for Tris–HCl-treated controls, thus indicating stability of the drug–enzyme complex during isolation of Na,K-ATPase by LiBr extraction. For NaI treatment alone about 90% of the drug–enzyme complex was dissociated, and for DOC alone approximately 44% was dissociated.

In terms of specific radioactivity, the combination of DOC + NaI lowered the specific activity of the drug–enzyme complex 87%, while for NaI-treated complex the specific activity was reduced 74% and for DOC treatment 47% from control complexes treated with Tris–HCl. Reduced specific radioactivity from control values reflect partial dissociation of [^{3}H]ouabain from the drug–enzyme complex. The higher specific radioactivity values for

TABLE 5

Effects of enzyme extracting agents on dissociation of ex vivo bound [^{3}H]ouabain–Na$^+$,K$^+$-ATPase complex

Dissociation of the drug–enzyme complex from a common enzyme pool was performed using conditions as described in Methods. Values presented are the means ± SE of five experiments.

Treatment	dpm in treated fraction (total radioactivity)	dpm/mg protein	Protein recovered in the pellet	Enzyme units recovered in the pellet
	% of control	*% of control*	*% of control*	*% of control*
Control, 1 mM Tris–HCl[a]	100	100	100	100
1 M LiBr	100 ± 2.8	151 ± 3.5*	50 ± 2*	98 ± 3
2 M NaI	9.6 ± 1.5*	25.7 ± 6.0*	32 ± 3*	98 ± 2
0.1% DOC	55.5 ± 8.5*	53.0 ± 7.9*	85 ± 5**	97 ± 5
0.1% DOC + 2 M NaI	2.5 ± 1.4*	12.7 ± 3.6*	10 ± 2*	61 ± 5*

[a] Dissociation of [^{3}H]ouabain from the control drug–enzyme complex was less than 10% following treatment with 1 mM Tris–HCl at 0–4°C.

* $P < 0.01$ from control values.

** $P < 0.05$ from control values.

LiBr-, NaI-, and DOC + NaI-treated complexes than the corresponding values for radioactivity remaining in the treated fraction (column 2, Table 5) may be due to further purification of the crude complexes by the extracting agents. This is further indicated by the reduction in protein recovery following treatment of the crude complexes with the various extracting agents. As in the *in vitro* bound drug–enzyme complex experiment, the relative recoveries of protein are similar following treatment with the various extracting agents. For recovery of Na,K-ATPase activity, only the DOC + NaI-treated complex showed a 39% loss of enzyme activity, which may in part be due to partial denaturation of the enzyme. Since the loss in enzyme activity was less than that observed for drug-free enzyme preparations (52%, Table 1) following DOC + NaI treatment, it is possible that the difference may also reflect partial reactivation of the drug-inhibited enzyme. For complexes treated by the other extracting agents, no loss in enzyme activity was observed. The inability to demonstrate reactivation of the enzyme may be due to the low absolute enzyme activities of the crude preparations and the moderate degree of inhibition of the enzyme.

DISCUSSION

The purpose of this study was to account for the inability of various investigators to demonstrate inhibition of rabbit and guinea pig Na,K-ATPase by digitalis following its isolation from digitalis-treated hearts. On the possibility that various protein extracting agents used in Na,K-ATPase isolation procedures may dissociate the drug–enzyme complex during enzyme fractionation, the effects of 2 M NaI, 0.1% DOC, and the sequential treatment of DOC + NaI were investigated on the dissociation of both *ex vivo* and *in vitro* bound [^{3}H]ouabain–Na,K-ATPase complex. The most relevant findings from the present study are those based upon the effects of the

various extracting agents on dissociation of *ex vivo* bound drug–enzyme complex.

For *ex vivo* bound [^{3}H]ouabain–Na,K-ATPase complex, the sequential treatment of 0.1% DOC and 2 M NaI (the procedure used by many investigators for Na,K-ATPase isolation) removed 97% of the drug from the drug–enzyme fraction and reduced the specific radioactivity of the complex by 87% in comparison to 1 mM Tris–HCl control values (see Table 5). In contrast to these results, treatment of *ex vivo* bound drug–enzyme complex with LiBr (the extracting agent used by our laboratory for cardiac, Na,K-ATPase isolation) caused no loss of [^{3}H]ouabain from the radioactive fraction containing the drug–enzyme complex and increased specific radioactivity 50% over Tris–HCl-treated control values. The latter increase is due to further purification of the crude enzyme preparation with no difference in dissociation of the drug–enzyme complex following treatment with LiBr in comparison to control. Based upon this consideration, the actual loss of [^{3}H]ouabain radioactivity from the total dpm of the treated fraction is considered a better index of dissociation of the drug-enzyme complex than specific radioactivity values. For 2 M NaI alone 90% of the complex was dissociated, and for 0.1% DOC alone 44% drug loss was observed. Similarities in total dpm for the drug–enzyme fractions for both 1 mM Tris–HCl control and 1 M LiBr-treated samples were surprising and indicate the mild nature of LiBr on dissociation of the drug–enzyme complex.

Our findings, therefore, indicate that the inability of Schwartz *et al.* (17) and Akera *et al.* (18) to demonstrate inhibition of Na,K-ATPase isolated from inotropic rabbit or guinea pig hearts perfused with ouabain is due to their use of DOC or DOC + NaI in their enzyme extraction procedure which causes dissociation of the ouabain–enzyme complex. Although Akera *et al.* (21) did not use both DOC and NaI in their enzyme extraction procedure, the 50% higher concentration of 0.15% DOC used by these investigators may account for their observed dissociation of the drug–enzyme complex from ouabain treated guinea pig hearts. The present findings also confirm the validity of extracting the drug–enzyme complex with 1 M LiBr for 1 h, the procedure used in our laboratory (7), since no increase in dissociation of the drug–enzyme complex was noted in comparison to 1 mM Tris–HCl controls. These findings are further substantiated by our earlier observations (7) that [^{3}H]ouabain remains bound to rabbit heart Na,K-ATPase when LiBr is used as the enzyme extracting agent. For example, we obtained specific radioactivity of 23 pmol of [^{3}H]ouabain/mg protein for drug–enzyme complex isolated from inotropically stimulated rabbit hearts (7). Therefore, contrary to the opinion of other investigators (17, 18), rabbit and, presumably, guinea pigs are adequate species to use for *in vivo* and *ex vivo* Na,K-ATPase inhibition studies provided LiBr is used as the enzyme extracting agent instead of DOC and NaI.

The inability of Schwartz and his colleagues (17) to demonstrate inhibition of Na,K-ATPase in noninotropic isolated cat hearts following drug washout, whereas enzyme from nonwashed inotropic cat hearts showed inhibition, may be explained on the following basis. Since Na,K-ATPase from digitalis-sensitive species such as the cat are known to bind digitalis drugs more tightly than enzyme from less sensitive species such as rabbits and guinea pigs (3, 17, 18), only partial dissociation of the drug–enzyme complex was effected by DOC plus NaI. Partial dissociation of the cat drug–enzyme complex is indicated by the fact that Schwartz and his colleagues (16) found only 16 pmol of [^{3}H]ouabain/mg protein bound to their cat heart enzyme when isolated from inotropically stimulated cat hearts. As mentioned previously, using LiBr, we obtained specific radioactivity of 23 pmol of [^{3}H]ouabain/mg protein for rabbit heart Na,K-ATPase isolated from inotropically stimulated rabbit hearts (7). The inability of Schwartz and his colleagues to demonstrate inhibition of Na,K-ATPase in noninotropic cat hearts following drug washout may be due to partial dissociation of the drug–enzyme complex during the long drug washout period required for complete washout of positive inotropy with further dissociation during enzyme isolation with DOC plus NaI. Therefore, the present findings support our original observation (6, 7) that the positive inotropic action of digitalis is not due to inhibition of Na,K-ATPase because enzyme inhibition can be demonstrated in washout experiments in the absence of positive inotropy provided LiBr is used as the enzyme extracting agent.

The effects of the various protein extracting agents on *in vitro* bound drug–enzyme complex were also investigated in order to compare the extent of drug dissociation of *in vitro* vs *ex vivo* bound complexes, as well as between Type 1 and Type 2 complexes. Surprisingly, greater than 95% dissociation of the drug–enzyme complex was observed with DOC plus NaI treatment for all three types of complexes (Type 1, Type 2, and *ex vivo* complexes) based upon total dpm in the treated fractions. For complexes expressed in terms of specific radioactivity, DOC plus NaI treatment of Type 2 complex and NaI treatment for both Type 1 and Type 2 complexes caused slightly less dissociation of the drug–enzyme complex than corresponding treatment of *ex vivo* bound complexes. This may be due in part to the fact that the *in vitro* bound complexes did not undergo a preliminary crude Na,K-ATPase isolation procedure required for all *ex vivo* bound complexes prior to treatment with the protein extracting agents. This additional preliminary isolation procedure for *ex vivo* bound complexes may have altered the binding affinities of [^{3}H]ouabain to Na,K-ATPase so that following treatment with the protein extracting agents, a slightly higher dissociation of the drug–enzyme complex was obtained than would have otherwise occurred. There is also the possibility that there may be differences in the binding affinities of [^{3}H]ouabain to the transport enzyme under *in vitro* versus *ex vivo* binding conditions (i.e., the presence of K^+ and Ca^{2+} and other ligands under *ex vivo* conditions) which may in part account for the observed differences in dissociation of the drug–enzyme complexes. A priori it was also expected that Type 2 complexes may dissociate to a lesser extent than Type 1 complexes since they are considered to have higher binding affinities (22–25). However, only the 0.1% DOC treatment of the Type 2 drug–enzyme complex demonstrated higher drug concentration than the corre-

sponding treatment of the Type 1 complex (Table 2).

For both *ex vivo* and *in vitro* bound drug–enzyme complexes (Tables 3–5), it was also noted that for all extracting agents used, the percentage radioactivity values for complexes expressed as specific radioactivity were higher than for the same sample expressed as total dpm in the treated fraction. This probably is due to the fact that these extracting agents are further purifying the crude Na,K-ATPase fraction so that specific radioactivity values will increase for the same amount of [^{3}H]-ouabain radioactivity in the treated fraction.

It is also of interest to note that with the exception of the DOC plus NaI treatment, the three extracting agents, when used alone, caused an insignificant or minimal loss of ouabain-sensitive Na,K-ATPase activity for drug-free crude enzyme preparations (Table 1). However, following sequential treatment of the enzyme with 0.1% DOC plus 2 M NaI, approximately 48% of the enzyme activity could be recovered in the pellet fraction and 15% in the supernatant fraction. Thus, under the conditions employed, DOC plus NaI caused a 37% loss in enzyme activity. In addition, DOC plus NaI caused an approximately 90% loss in protein recovery in comparison to Tris–HCl-treated controls. Therefore, the combination of loss in enzyme activity and poor protein recovery may account, at least in part, for the low yield of Na,K-ATPase when DOC plus NaI are utilized in enzyme isolation procedures.

Finally, it should be pointed out that when LiBr is used as the protein extracting agent for isolation of drug–enzyme complex, one should not expect high enzyme activity if one also wishes to avoid dissociation of the drug enzyme complex. Other investigators (17) have criticized the level of our Na,K-ATPase activity because it is approximately 25% lower than that obtained by their enzyme extraction procedure (20). However, for recovery of drug–enzyme complex and demonstration of enzyme inhibition following drug washout of digitalis-treated Lagendorff hearts, sacrifice of enzyme activity is preferable to dissociation of the drug–enzyme complex.

In conclusion, studies on the effects of various protein extracting agents on the dissociation of both *ex vivo* and *in vitro* bound rabbit heart [^{3}H]ouabain-Na,K-ATPase complex revealed the following. A sequential treatment of the complex with 0.1% DOC and 2 M NaI (common extracting agents used by many laboratories for Na,K-ATPase isolation) resulted in over 95% of the [^{3}H]ouabain dissociating from both *ex vivo* and *in vitro* bound drug–enzyme complexes in comparison to 1 mM Tris–HCl control values. For NaI treatment alone, 90% of the *ex vivo* bound complex was dissociated, and for 0.1% DOC, 44% drug removal was observed. In contrast to these results, treatment of the *ex vivo* and *in vitro* drug–enzyme complexes with 1 M LiBr (the extracting agent used in our laboratory) caused no loss of [^{3}H]ouabain from the radioactive fraction in comparison to control complex treated with 1 mM Tris–HCl. These findings, therefore, may account for the inability of some investigators (17, 18) to demonstrate inhibition of Na,K-ATPase following isolation of the enzyme from ouabain-stimulated rabbit and guinea pig hearts since NaI and/or DOC was used in their enzyme extraction procedure. Present findings also verify our original observation that Na,K-ATPase isolated from noninotropic digitalis-treated rabbit hearts following drug washout remains inhibited since LiBr was used as the enzyme extracting agent. Therefore, these results support our earlier postulation (7, 9) that Na,K-ATPase inhibition is not responsible for the positive inotropic action of digitalis steroids, but may in part by responsible for its cardiotoxic effects.

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