BBA 75977

# SPECIES AND TISSUE DIFFERENCES IN THE RATE OF DISSOCIATION OF OUABAIN FROM ($Na^+ + K^+$)-ATPase

THOMAS TOBIN*, RUTH HENDERSON AND AMAR K. SEN**

*Department of Pharmacology, University of Toronto, Toronto 181, Ontario (Canada)*

(Received March 6th, 1972)

## SUMMARY

The rates of the association and dissociation reactions of ouabain with ($Na^+ + K^+$)-ATPase (ATP phosphohydrolase EC 3.6.1.3) were compared. The enzymes were prepared from kidney, heart and brain tissues obtained from guinea pig, dog and cat. The rate of dissociation of ouabain from ($Na^+ + K^+$)-ATPase varied about 60-fold between different species. The dissociation rate also differs between tissues within a given species. The rate of association of ouabain with these enzyme preparations did nog differ appreciably. The results suggest that the species differences in sensitivity to the cardiac glycosides are principally related to the different rates of dissociation of the drug–enzyme complex.

## INTRODUCTION

Cardiac glycosides are specific inhibitors of the ($Na^+ + K^+$)-ATPase[1]. Evidence is accumulating in favour of the hypothesis that these glycosides exert their cardiotonic effect by inhibiting the membrane bound ($Na^+ + K^+$)-ATPase[2,3]. There is, however, a remarkable species difference in *in vivo* effects of these glycosides[4]. Similar species differences have also been observed with isolated ($Na^+ + K^+$)-ATPase preparations[5]. A relatively stable and specific binding of ouabain and digoxin to various preparations of ($Na^+ + K^+$)-ATPase has been demonstrated[6–10]. Allen and Schwartz[11,12], observing a stable binding to dog and beef heart enzymes and a "loose" binding to rat heart preparations, suggested that the well documented species differences in sensitivity to the cardiac glycosides might be related to differences in stability of the glycoside–enzyme complex in these different species. In this communication, we report the rates of both association and dissociation reaction of ouabain with ($Na^+ + K^+$)-ATPase obtained from different species and also from different tissues of the same species.

## EXPERIMENTAL

Enzymes were prepared and ($Na^+ + K^+$)-ATPase activity was measured by the method of Post and Sen[13]. Where "non-urea treated" is indicated, the top layer of the

* Present address: Department of Pharmacology, Michigan State University, East Lansing, Mich. 48823, U.S.A.

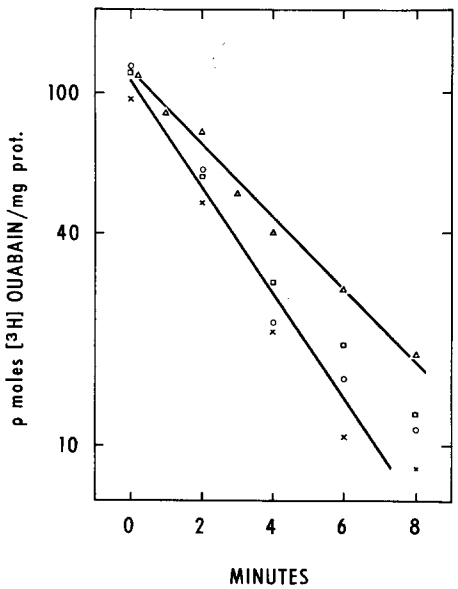
** Reprint requests should be addressed to Dr A. K. Sen, Room 4342, Medical Science Building, University of Toronto, Toronto 181, Ontario, Canada.

pellet after heparin treatment was suspended in buffer containing 10 mM imidazole, 5 mM HCl and 0.1 mM $H_4EDTA$ (pH 7.0 $\pm$ 0.1) to give the enzyme preparation. Protein was determined by the method of Lowry *et al.*[14]. [$^3$H]Ouabain binding was estimated by the method of Matsui and Schwartz[6] as modified by Tobin and Sen[10]. For accurate time resolution (Fig. 3) the binding reactions were stopped by adding 6 ml of ice cold 10 mM imidazole–glycylglycine, pH 7.4, and $5 \cdot 10^{-4}$M unlabelled ouabain. This rapidly reduced the temperature of the system to 10 °C, stabilizing the enzyme–ouabain bond. Subsequent procedures were as in ref. 10. Rates of dissociation were measured by allowing the enzyme to bind [$^3$H]ouabain in the presence of 4 mM $Mg^{2+}$ and 1 mM $P_i$ at 37 °C for 20 min[10]. Enzyme–ouabain complex was then cooled to 0 °C and washed twice by alternate centrifugation (48000 $\times$ *g*, 10 min, 0 °C) and resuspension in the same volume of buffer. Dissociation was started by adding 0.2 ml of enzyme–ouabain to 0.8 ml of 10 mM imidazole–glycylglycine buffer, pH 7.4, at 37 °C, containing $2.5 \cdot 10^{-4}$ M unlabelled ouabain. The reaction was stopped by centrifugation at the required times and subsequent treatment was as in ref. 10. Ouabain binding was calculated as pmoles [$^3$H]ouabain per mg protein. [$^3$H]ouabain was from New England Nuclear, (Boston, Mass.) and diluted with carrier ouabain (Sigma Chemical Co., St. Louis, Mo.) to give 100 Ci/mole. Other chemicals and reagents were obtained or prepared as described previously[10].

## RESULTS AND DISCUSSION

There is good evidence for non-covalent binding of ouabain to inhibited ($Na^+ + K^+$)-ATPase[6,9,15], which dissociates with a half-life of about 2.5 min in guinea pig kidney preparations[10,16]. Fig. 1 shows that this exchange occurs at about the same rate into media containing unlabelled ouabain and different ligands of this enzyme. In other experiments the enzyme–ouabain complex dissociated at the same rate into unlabelled ouabain or 5 mM EDTA[16]. It appears that in these preparations when the enzyme–ouabain complex is formed in the presence of $Mg^{2+}$ and $P_i$ the rate of dissociation of the enzyme–ouabain complex is essentially constant, independent of the medium into which it dissociates. Urea treatment appears to produce a small acceleration of the dissociation of the enzyme–ouabain complex. Since detergent treatment does not increase specific glycoside binding[17], subsequent experiments were done on non-urea treated enzymes unless otherwise indicated.

Fig. 2 shows the rates of dissociation of the enzyme–ouabain complex in different tissues and species. The fastest rate observed was in guinea pig heart with a half-life of about 2.0 min. It is interesting to note that a half-life of about 3.5 min for the inotropic effect of $\kappa$-strophanthidin on guinea pig heart has recently been reported by Fricke and Klaus[18]. The slowest dissociation observed was in dog kidney where the initial decay had an apparent half-life of about 2 h. Except for the brain, the plotted dissociations are approximately exponential. However, there appears to be a residual pool of stable binding in the guinea pig heart preparation. In other experiments the amounts of tritiated ouabain remaining at 4- and 8-h time periods to dog kidney preparations were higher than those expected from a simple exponential dissociation. The dissociation of ouabain from the guinea pig brain preparation appears to be multiexponential. The dissociation of ouabain from the enzyme is known to be associated with recovery of phosphorylation and ATP-hydrolyzing activity[19,20].



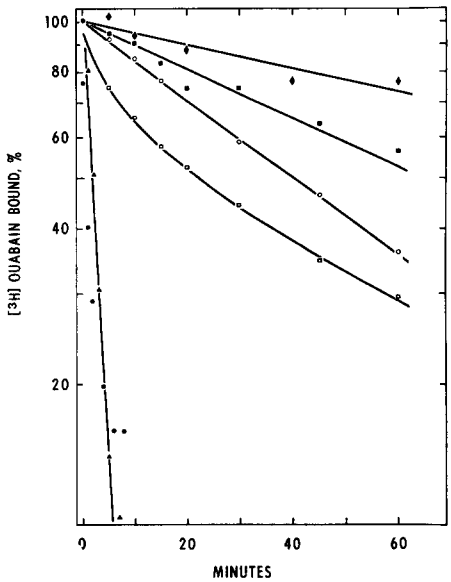




Fig. 1. Dissociation of enzyme–ouabain complex in the presence of different ions. Guinea pig kidney $(Na^+ + K^+)$-ATPase was allowed to bind ouabain in the presence of 4 mM $Mg^{2+}$ and 1 mM $P_i$. [$^3$H]Ouabain–enzyme was then dissociated into $2.5 \cdot 10^{-4}$ unlabelled ouabain *plus* 2 mM ATP (×), *plus* 16 mM KCl (O), or *plus* 16 mM $Na^+$ (□). The open triangles (△) show the rate of exchange of label in a non-urea treated guinea pig kidney enzyme.

Fig. 2. Dissociation of enzyme–ouabain complex in different tissues and species: [$^3$H]Ouabain–enzyme was formed and its dissociation followed as indicated in Experimental. The symbols indicate the percentage of the binding at zero time in a given tissue remaining at the times indicated. The enzymes prepared from different tissues were as follows: ▲, guinea pig kidney; ●, guinea pig heart; □, guinea pig brain; ■ dog heart; ♦, dog kidney; ○, cat heart.

The order of decreasing rates of dissociation, guinea pig>cat>dog is associated with increasing glycoside sensitivity of the cardiac $(Na^+ + K^+)$-ATPases in these species[4,21]. However, different rates of association would alter inhibitory effectiveness[22]. Therefore the rates of formation of the enzyme–ouabain complex in different tissues and species was investigated. It was found experimentally convenient[16] to use $2.5 \cdot 10^{-7}$ M ouabain and this concentration was used in all experiments. Fig. 3 shows that under these conditions the initial rates of the enzyme–ouabain complex formation varied directly with the maximal number of binding sites filled. When these curves were replotted as a per cent of the sites occupied at 200 s the time course for occupation of the binding sites was relatively constant. The experiment suggests that the rate of association of ouabain with the enzyme does not differ greatly between different tissues or species.

The experiments support the suggestion of Allen and Schwartz[11,12] that the species differences in effectiveness of the glycosides is due to a relatively unstable binding in the insensitive species. They support the observation of Albers *et al.*[8] that binding is "essentially irreversible" in the more sensitive species but indicate reversibility in some tissues in the less sensitive species. The species most resistant to glycosides, *i.e.* rat [4,21] was not investigated as reproducible specific binding to rat kidney enzyme could not be demonstrated.

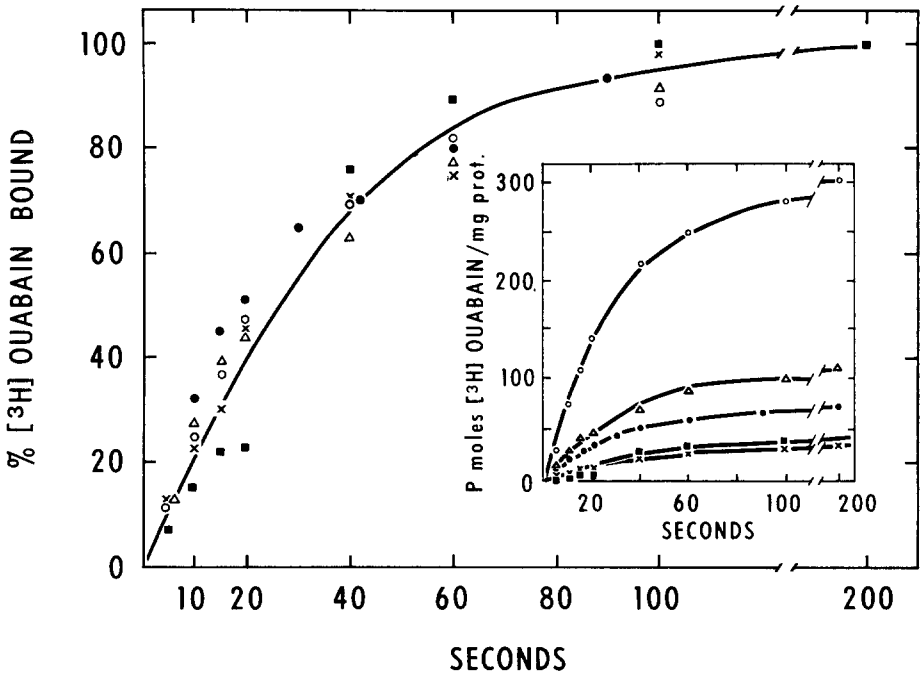



Fig. 3. Rates of formation of enzyme–ouabain complex in different tissues. The inset shows the time course of the labelling of 5 different enzyme preparations in the presence of $Na^+$ 200 mM, Mg–ATP 4 mM and $2.5 \cdot 10^{-7}$ M ouabain. The enzyme preparations were from: ○, guinea pig brain; ●, guinea pig kidney (non-urea treated); ×, guinea pig heart; △, dog kidney; ■, dog heart. The main figure shows these curves replotted as a per cent of the binding observed at 200 s.

The experiments show that the differences in the stability of the enzyme–glycoside complexes are principally due to differences in the rate of dissociation of the enzyme–ouabain complex. This is markedly different from the sulphonamide–carbonic anhydrase complex where the rate of dissociation of different sulphonamides from the enzyme varies much less than the rates of association[22]. Lastly, the experiments suggest differences between tissues in a given species. The reason for these differences is unknown. It could either be due to difference in the enzyme itself or to the composition of the matrix in which the enzyme is imbedded. Further experimentation is needed to arrive at the proper answer.

ACKNOWLEDGEMENT

This work was supported by grant No. MT2485 from the Medical Research Council of Canada.

REFERENCES

1 I. M. Glynn, *Pharmacol. Rev.*, 16 (1964) 381.
2 A. Schwartz, J. C. Allen, S. Harigaya, *J. Pharmacol. Exp. Ther.*, 168 (1969) 31.
3 P. F. Baker, M. P. Blaustein, A. L. Hodgkin, R. A. Steinhardt, *J. Physiol.*, 200 (1969) 431.
4 K. Repke, M. Est and J. H. Portius, *Biochem. Pharmacol.*, 14 (1965) 1785.

5 J. C. Skou, *Physiol. Rev.*, 45 (1965) 596.
6 H. Matsui and A. Schwartz, *Biochim. Biophys. Acta*, 157 (1968) 655.
7 A. Schwartz, H. Matsui and A. Laughter, *Science*, 159 (1968) 323.
8 R. W. Albers, G. J. Koval and G. J. Siegel, *Mol. Pharmacol.*, 4 (1968) 324.
9 J. F. Hoffman, *J. Gen. Physiol.*, 54 (1969) 343S.
10 T. Tobin and A. K. Sen, *Biochim. Biophys. Acta*, 198 (1970) 120.
11 J. C. Allen and A. Schwartz, *J. Pharmacol. Exp. Ther.*, 168 (1969) 43
12 J. C. Allen and A. Schwartz, *Fed. Proc.*, 29 (1970) 850.
13 R. L. Post and A. K. Sen, in S. P. Colowick and N. O. Kaplan, *Methods in Enzymology*, Vol. 10, Academic Press, New York, 1967, p. 762.
14 O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. Biol. Chem.*, 193 (1951) 265.
15 I. M. Glynn, *J. Physiol.*, 136 (1957) 148.
16 T. Tobin, Ph. D. Thesis, University, of Toronto, 1970.
17 J. C. Ellory and M. W. Smith, *Biochim. Biophys. Acta*, 193 (1969) 137.
18 U. Fricke and W. Klaus, *Arch. Pharmacol.*, 268 (1971) 192.
19 A. K. Sen, T. Tobin and R. L. Post, *J. Biol. Chem.*, 244 (1969) 6596.
20 S. P. Banerjee, V. K. Khanna and A. K. Sen, *Biochem. Pharmacol.*, 20 (1971) 1649.
21 T. Akera, F. S. Larsen and T. M. Brody, *J. Pharmacol. Exp. Ther.*, 170 (1969) 17.
22 R. W. King, P. W. Taylor and A. S. V. Burgen, *Proc. Int. Congr. Pharmacol., Basel*, (1969) 413.