Biochemical Pharmacology, Vol. 29, pp. 1995–2000
© Pergamon Press Ltd. 1980. Printed in Great Britain.
0006-2952/80/0715–1995 $02.00/0

# ENZYME MODIFICATIONS THAT ALTER INTERACTIONS OF $K^+$ AND CARDIOACTIVE STEROIDS WITH ($Na^+ + K^+$)-DEPENDENT ATPase*

JOSEPH D. ROBINSON

Department of Pharmacology, State University of New York, Upstate Medical Center, Syracuse, NY 13210, U.S.A.

(*Received* 6 *December* 1979; *accepted* 30 *January* 1980)

**Abstract**—Treatment of a purified ($Na^+ + K^+$)-dependent ATPase preparation from dog kidney medulla with acetic anhydride or trinitrobenzene sulfonate (TNBS) produced a dose-dependent irreversible inactivation of the ($Na^+ + K^+$)-ATPase and $K^+$-phosphatase reactions catalyzed by the enzyme. Both $K^+$ and the cardioactive steroid strophanthidin protected against inactivation. Prior treatment with concentrations of acetic anhydride or TNBS that produced only partial inactivation also modified the residual activity of the enzyme, as manifested by an increase in the $K_{0.5}$ for $K^+$ as activator of the phosphatase reaction (mediated through the moderate-affinity $\alpha$-sites for $K^+$) but not in the $K_{0.5}$ for $K^+$ as activator of the ATPase reaction (mediated through the high-affinity $\beta$-sites for $K^+$); correspondingly, the $I_{50}$ values (concentrations required to produce 50 per cent inhibition) for ouabain and strophanthidin as inhibitors of the ATPase reaction were increased, and the binding of [$^3$H]ouabain was decreased, by such treatment. Ouabain activated the phosphatase reaction in the absence of $K^+$ and, after similar treatment, the apparent affinity for ouabain as activator was also decreased. These experiments demonstrate interactions between cardioactive steroids at their sites on the extracellular face of this transmembrane enzyme and $K^+$ at its moderate-affinity $\alpha$-sites on the intracellular face, and further indicate that $K^+$ can modulate enzyme–drug interactions at such sites, as well as through $K^+$-sites on the extracellular face of the enzyme.

The ($Na^+ + K^+$)-dependent ATPase is the enzymatic correlate of the plasma membrane sodium pump, and both enzyme activity and cation transport are inhibited by the pharmacologically important cardioactive steroids such as ouabain, which may exert their therapeutic effects through this interaction [1–5]. Inhibition of enzyme and pump and binding of the steroids to the enzyme are sensitive to a variety of ligands, most notably (i) an increased inhibition and binding in the presence of $Mg^{2+}$ plus inorganic phosphate or of $Mg^{2+}$ plus $Na^+$ plus ATP, and (ii) a decreased inhibition and binding in the presence of $K^+$ [6–11]. A further indication of the intimate interrelationships among enzyme, $K^+$, and cardioactive steroids is demonstrated in studies of the $K^+$-dependent phosphatase reaction that is also catalyzed by this enzyme. Pitts and Askari [12, 13] showed that, although ouabain inhibits the phosphatase reaction in the presence of $K^+$, it can activate it in the absence of $K^+$ (presence of $Mg^{2+}$ and substrate).

Recently, we showed that treatment of the ATPase with acetic anhydride or TNBS† can lead to specific modifications of enzymatic activity [14]. Thus, at concentrations of either reagent that produce only partial inactivation of the ($Na^+ + K^+$)-ATPase or $K^+$-phosphatase reactions, the residual enzymatic activity displays a decreased apparent affinity for $K^+$ as activator of the phosphatase reaction, an activation mediated through moderate-affinity $\alpha$-sites for $K^+$ [15], but displays no decrease in apparent affinity for $K^+$ as activator of the ATPase reaction, an activation mediated through high-affinity $\beta$-sites for $K^+$ [15].

Such modifications thus present the opportunity for exploring further the relationships among the therapeutically important cardioactive steroids, the ($Na^+ + K^+$)-ATPase which may be their receptors, and the modulating cation $K^+$. In this paper we describe experiments examining (i) the effects of acetic anhydride and TNBS on cardioactive steroid binding to, and inhibition of, the enzyme as well as on $K^+$-activation of enzymatic activity, (ii) the effects of both cardioactive steroids and $K^+$ on inactivation by those reagents, and (iii) the effects of those reagents on cardioactive steroid-activation of the phosphatase reaction.

## METHODS

The enzyme preparation was obtained from the medullae of frozen canine kidneys by a modification [16] of the procedure of Jørgensen [17]; purity, estimated by sodium dodecylsulfate gel electrophoresis, was approximately 85 per cent.

($Na^+ + K^+$)-ATPase activity was measured at 37° in terms of the production of inorganic phosphate, as described previously [18]; the standard medium contained 30 mM histidine · HCl–Tris (pH 7.8), 3 mM

* Supported by U.S. Public Health Service Research Grant NS-05430.

† Abbreviations used: TNBS, trinitrobenzene sulfonate; EDTA, ethylenediamine tetracetate; $K^+$-phosphatase, $K^+$-dependent phosphatase; and ($Na^+ + K^+$)-ATPase, ($Na^+ + K^+$)-dependent ATPase.

ATP, 3 mM $MgCl_2$, 90 mM NaCl and 10 mM KCl. K-phosphatase activity was routinely measured in terms of the production of *p*-nitrophenol from *p*-nitrophenyl phosphate during incubations at 37°, as described previously [19]; the standard medium contained 30 mM histidine·HCl–Tris (pH 7.8), 3 mM nitrophenyl phosphate, 3 mM $MgCl_2$ and 10 mM KCl. In experiments measuring ouabain-activated phosphatase activity, 0.2 mM umbelliferone phosphate was substituted for nitrophenyl phospate as the substrate, and KCl was omitted from the medium; production of umbelliferone was measured spectrophotofluorometrically, as described previously [20]. [$^3$H]Ouabain binding was measured according to Van Winkle *et al.* [21]: the standard medium contained 30 mM histidine·HCl–Tris (pH 7.8), 2 mM $MgCl_2$, 1 mM EDTA, and, routinely, 1 μM [$^3$H]ouabain, plus either (i) 2 mM ATP and 50 mM NaCl, or (ii) 2 mM inorganic phosphate; after incubation with the enzyme preparation for 5 min at 37°, 1-ml portions of the mixture were filtered through Gelman membrane filters (pore size 0.45 μm), which were then washed four times with ice-cold water. The radioactivity remaining on the filters was measured by liquid scintillation counting. Non-specific binding under each experimental condition was estimated in parallel incubations containing, in addition, 250 μM unlabeled ouabain.

For reaction with acetic anhydride, the enzyme preparation was first equilibrated at 0° in a medium containing 30 mM imidazole·HCl (pH 7.8) plus other reagents as noted, and the inactivation incubation was then begun by adding, routinely, 0.15 μl acetic anhydride/ml of medium. After incubation for 10 min at 0°, the mixture was diluted with 10 vol. of 0.25 M sucrose; the enzyme preparation was then collected by centrifugation and resuspended in 0.25 M sucrose for assay [14]. For reaction with TNBS, the enzyme preparation was first equilibrated at 25° in a medium containing 30 mM imidazole·HCl (pH 7.8) plus other reagents as noted, and the inactivation incubation was then begun by adding, routinely, 0.03% (w/v) TNBS. After incubation in the dark for 15 min at 25°, the mixture was diluted with sucrose and the enzyme was collected as described above, except that the preparation was shielded from light until completion of the assay [14]. With both reagents, parallel control incubations were performed with identical treatment except for the omission of acetic anhydride and TNBS.

Data presented are averages of four experiments, each performed in duplicate or triplicate, ± standard error of the mean where appropriate.

## RESULTS

As demonstrated previously with a partially purified enzyme preparation from brain [14], acetic anhydride and TNBS inhibited, in a dose-dependent fashion, the ($Na^+ + K^+$)-ATPase activity of a purified kidney enzyme preparation. Total inactivation occured after incubation of the enzyme with 0.03% (v/v) acetic anhydride for 10 min at 0°, although after treatment with 0.015% acetic anhydride, one-fourth of the ($Na^+ + K^+$)-ATPase activity remained. Analogously, incubation of the enzyme for 15 min at 25°

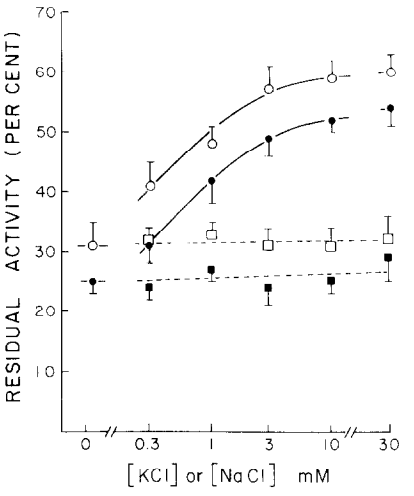


Fig. 1. Protection by KCl against acetic anhydride-induced loss of activity. The enzyme preparation was incubated for 10 min at 0° in media containing 30 mM imidazole·HCl, 0.015% acetic anhydride, and the concentrations of KCl (● or ○) or NaCl (■ or □) indicated. After dilution, collection by centrifugation, and resuspension, as described under Methods, the residual ($Na^+ + K^+$)-ATPase activity (● or ■) and [$^3$H]ouabain binding activity in the presence of $Mg^{2+}$ plus $P_i$ (○ or □) were measured, as described under Methods. Data are presented as per cent of the values for concurrent controls in which acetic anhydride was omitted.

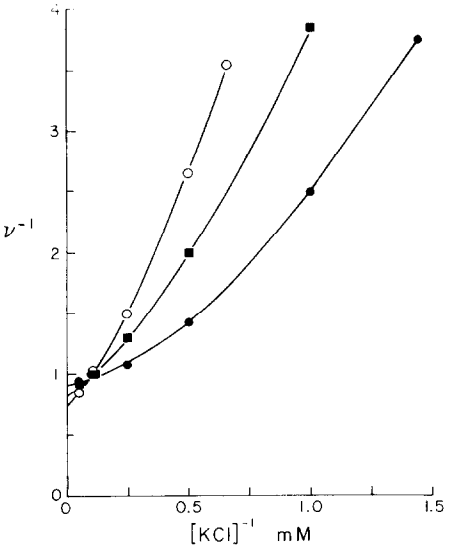


Fig. 2. Effects of acetic anhydride and TNBS on $K^+$-activation of the phosphatase reaction. The enzyme preparation was first treated with 0.015% acetic anhydride (○), 0.03% TNBS (■) or neither reagent (●), as described under Methods, and then the nitrophenyl phosphatase activity was measured in the standard medium, but with the concentrations of KCl indicated. Data are presented in double-reciprocal form; for ease of comparison the velocity with 10 mM KCl was, in each case, set at 1.0, although after treatment with acetic anhydride this velocity was 26 per cent of control values, and after treatment with TNBS it was 54 per cent. The $K_{0.5}$ values were, for control preparations, 1.4 mM; for acetic anhydride-treated, 4.0 mM; and for TNBS-treated, 2.7 mM.

with 0.2% (w/v) TNBS totally inactivated the enzyme, whereas incubation with 0.03% TNBS reduced the $(Na^+ + K^+)$-ATPase activity only by half. With both reagents, at both concentrations, the effects were irreversible: the loss of activity persisted after dilution and successive washing of the treated enzyme preparations.

Inclusion of KCl in the inactivation medium with either acetic anhydride (Fig. 1) or TNBS (data not shown) protected against inactivation by these reagents; NaCl was ineffective. Moreover, the concentration of KCl required for half-maximal protection (Fig. 1) was, with both reagents, near 1 mM. This value corresponds to the $K_D$ for $K^+$ at the moderate-affinity $\alpha$-sites of the enzyme [15]. (Although acetic anhydride and TNBS decrease the apparent affinity for $K^+$ at the $\alpha$-sites [14], such an effect would be minimal here if occupancy of the $\alpha$-sites by $K^+$ markedly decreases the likelihood of inactivation by acetic anhydride, whereas enzyme that previously reacted with acetic anhydride is afforded negligible protection against further inactivation by $K^+$ binding to $\alpha$-sites of diminished affinity. On the other hand, although the $K_{0.5}$ for $K^+$-activation of the $(Na^+ + K^+)$-ATPase reaction, an effect mediated through the high-affinity $\beta$-sites [15], is frequently reported as near 1 mM (e.g. Ref. 18), such measurements are made in the presence of high concentrations of $Na^+$, and $Na^+$ acts as a competitor toward $K^+$ at the $\beta$-sites; when the $K_{0.5}$ for $K^+$ is corrected to the absence of $Na^+$ [22], the $K_{0.5}$ for $K^+$ is then 0.1 mM or lower. Moreover, treatment with acetic anhydride and TNBS did not increase the $K_{0.5}$ for $K^+$ at the $\beta$-sites (see below)).

Treatment of the enzyme preparation with 0.015% acetic anhydride or 0.03% TNBS also affected the kinetic properties of the residual $K^+$-phosphatase activity, most notably causing an increase in the $K_{0.5}$ for $K^+$-activation (Fig. 2), an activation also mediated through the moderate-affinity $\alpha$-sites. By contrast, the $K_{0.5}$ values for $K^+$- and $Na^+$-activation of the residual $(Na^+ + K^+)$-ATPase reaction catalyzed by the treated enzyme preparation were not increased (Figs. 3 and 4). These findings thus confirm earlier studies [14] with the partially purified enzyme preparation from brain.

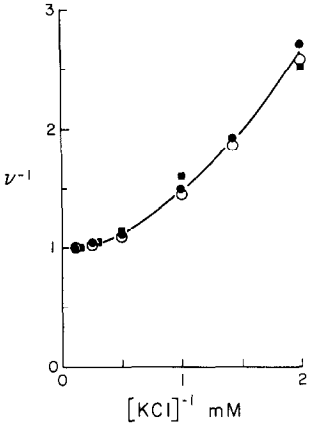


Fig. 3. Effects of acetic anhydride and TNBS on $K^+$-activation of the $(Na^+ + K^+)$-ATPase reaction. The enzyme preparation was first treated with 0.015% acetic anhydride (○), 0.03% TNBS (■) or neither reagent (●), as described under Methods, and then the $(Na^+ + K^+)$-ATPase activity was measured in the standard medium but with the concentrations of KCl indicated. Data are presented in double-reciprocal form; for ease of comparison the velocity with 10 mM KCl was, in each case, set at 1.0, although after treatment with acetic anhydride, the velocity was 24 per cent of control values, and after treatment with TNBS it was 59 per cent.

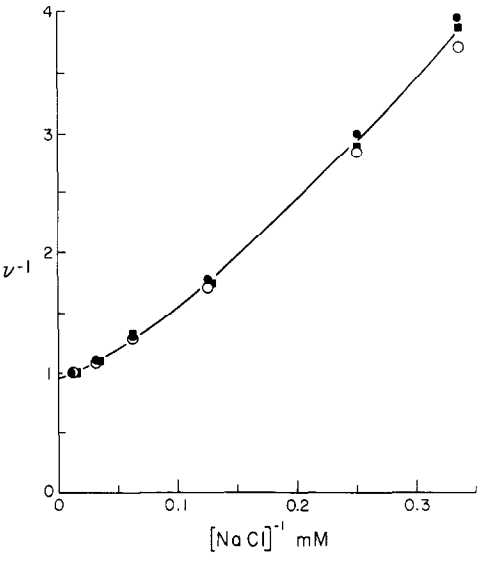


Fig. 4. Effects of acetic anhydride and TNBS on $Na^+$-activation of the $(Na^+ + K^+)$-ATPase reaction. Experiments were performed and the data are presented as in Fig. 3, except that the NaCl concentration in the assay incubation was varied as indicated. Key: preparation treated with 0.015% acetic anhydride (○), or 0.03% TNBS (■), or neither reagent (●).

Since interactions betwen cardioactive steroid and the enzyme are sensitive to $K^+$ [1–11], modification of the enzyme with acetic anhydride and TNBS (which, as shown above, is both sensitive to the presence of $K^+$ at the $\alpha$-sites and influences activation at the $\alpha$-sites) might alter such interactions as well. This was the case, as shown in Table 1 and Figs. 1 and 5. Prior treatment of the enzyme preparation with 0.015% acetic anhydride or 0.03% TNBS increased the $I_{50}$ (concentration required to produce 50 per cent inhibition) for the cardiac glycoside ouabain and the aglycone strophanthidin as inhibitors of the residual enzymatic activity (Table 1). Similarly, maximal binding of [$^3$H]ouabain measured in the presence of $Mg^{2+}$ plus $Na^+$ plus ATP was reduced, and the apparent $K_D$ increased, by prior treatment of the enzyme with acetic anhydride or TNBS (Fig. 5). With both reagents the increases in $K_D$ correspond to the increases in $I_{50}$ values noted above (Table 1); however, the absolute $I_{50}$ values are about 3-fold higher, probably reflecting the different experimental conditions in binding and kinetic experiments. Treatment with these reagents produced similar changes in [$^3$H]ouabain binding measured in the presence of $Mg^{2+}$ plus $P_i$ as well (data not presented). The presence of $K^+$ in the inacti-

Table 1. Effect of chemical modification of the enzyme on the $I_{50}$ for cardioactive steroids*

| Treatment | $I_{50}$ for ouabain ($\mu$M) | $I_{50}$ for strophanthidin ($\mu$M) |
|---|---|---|
| None | 1.2 ± 0.1 | 0.41 ± 0.04 |
| Acetic anhydride | 2.3 ± 0.2 | 0.73 ± 0.09 |
| TNBS | 1.8 ± 0.3 | 0.64 ± 0.11 |

* The concentration of ouabain and of strophanthidin required to produce 50 per cent inhibition of the $(Na^+ + K^+)$-ATPase reaction, the $I_{50}$, was determined for untreated (control) enzyme preparations, and for those first reacted with 0.015% acetic anhydride or 0.03% TNBS, as described under Methods.

Table 2. Effect of strophanthidin on chemical modification of the enzyme*

| | | |
|---|---|---|
| None | 1.00 | 1.00 |
| Strophanthidin, 10 $\mu$M | 1.01 ± 0.03 | 0.92 ± 0.05 |
| Acetic anhydride, 0.015% | 0.26 ± 0.02 | 0.31 ± 0.04 |
| Strophanthidin plus acetic anhydride | 0.36 ± 0.02 | 0.42 ± 0.03 |
| TNBS, 0.03% | 0.53 ± 0.05 | |
| Strophanthidin plus TNBS | 0.80 ± 0.04 | |

* The enzyme preparation was first incubated in 30 mM imidazole (pH 7.8), plus the additions noted, for 10 min at 0° with acetic anhydride or for 15 min at 25° with TNBS, as described under Methods. The inactivation medium was then diluted with 10 vol. of 0.25 M sucrose and the enzyme preparation was collected by centrifugation and resuspended in 0.25 M sucrose. The residual $(Na^+ + K^+)$-ATPase and [$^3$H]ouabain binding (in the presence of $Mg^{2+}$ plus $Na^+$ plus ATP) were measured as described under Methods; data are presented relative to the control preparation (no additions to the inactivation medium).

vation medium protected against this loss of [$^3$H]-ouabain binding, in the same concentration range as it protected against the loss of enzymatic activity, and again $Na^+$ was ineffective (Fig. 1).

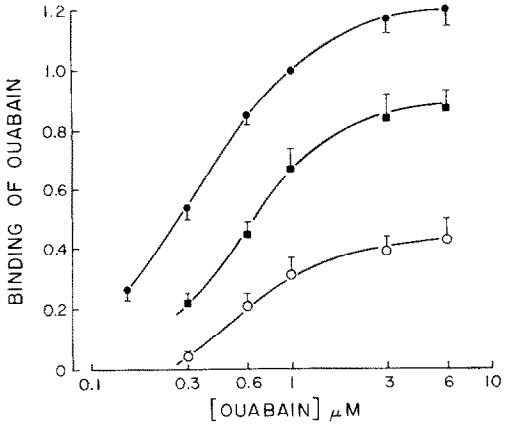


Fig. 5. Effects of acetic anhydride and TNBS on the binding of [$^3$H]ouabain. Binding of [$^3$H]ouabain to untreated enzyme (●) or to that first reacted with 0.015% acetic anhydride (○) or 0.03% TNBS (■) was measured as described under Methods: the enzyme preparations were incubated with the concentrations of [$^3$H]ouabain indicated, in the presence of 2 mM $MgCl_2$, 50 mM NaCl and 2 mM ATP, for 5 min at 37°. Data are presented relative to the binding of 1 $\mu$M ouabain to the untreated enzyme defined as 1.0.

Further evidence for the interrelationship between $K^+$- and ouabain-binding sites was provided by experiments testing whether the cardioactive steroids could, like $K^+$, protect against enzyme modification by acetic anhydride and TNBS. In these experiments strophanthidin was used because it is more easily removed from the enzyme than ouabain [23]. Inclusion of strophanthidin in the preincubation medium protected against the loss of both enzyme activity and of [$^3$H]ouabain binding (Table 2).

Finally, the observations by Pitts and Askari [12, 13] that cardioactive steroids could activate the phosphatase reaction in the absence of $K^+$ afforded another opportunity to test the relationship among the modifying reagents, the cardioactive steroids, and the $K^+$ at the $\alpha$-sites. With the purified enzyme preparation, phosphatase activity in the absence of KCl was less than 1 per cent of that with the optimal 10 mM KCl, whereas addition of 0.1 mM ouabain (Fig. 6) increased activity to nearly 20 per cent of that with 10 mM KCl. (These measurements were made with 0.2 mM umbelliferone phosphate as substrate, since the stimulation by ouabain is greater at non-saturating levels of substrate [12] and the fluorometric assay of umbelliferone is more sensitive than the colorimetric assay of nitrophenol formed from the more usual substrate nitrophenyl phosphate.) Treatment of the enzyme preparation with acetic anhydride reduced both the maximal level of stimulation by ouabain and the apparent affinity for ouabain, shifting the dose–response curve to the right (Fig. 6), just as it did in the experiments measur-

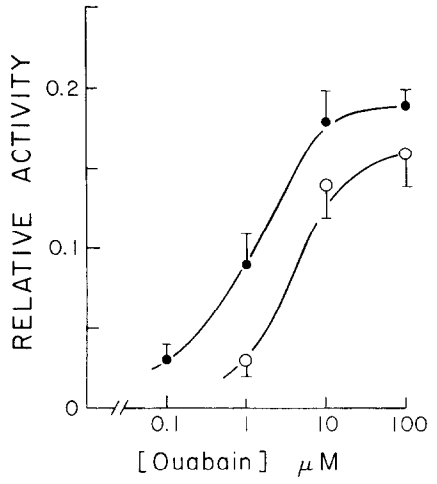


Fig. 6. Effect of ouabain on umbelliferone phosphatase activity in the absence of KCl. Umbelliferone phosphatase activity was measured, as described under Methods, with untreated control enzyme preparations (●) or with those first reacted with 0.015% acetic anhydride (○). Assay incubations were in the absence of KCl but in the presence of the ouabain concentrations indicated. Phosphatase activity is presented relative to that of untreated control enzyme preparations, incubated in the absence of ouabain and the presence of 10 mM KCl, defined as 1.0.

ing [$^3$H]ouabain binding (Fig. 5). The concentrations of ouabain to produce half-maximal stimulation correspond well with the $I_{50}$ values for ouabain (Table 1), also measured in kinetic experiments. The reduction in maximal activation by ouabain was less than that by $K^+$, although it corresponds to the reduction in maximal binding of [$^3$H]ouabain (Fig. 5).

## DISCUSSION

Previous studies [14] demonstrated that, after partial inactivation of the $(Na^+ + K^+)$-ATPase by either acetic anhydride or TNBS, the properties of the residual enzymatic activity were also modified. Thus, some of the enzyme molecules subjected to such treatment retained at least part of their catalytic activity, but their kinetic properties, and consequently the aggregate kinetic properties of the entire preparation, were irreversibly altered. Most notably, the $K_{0.5}$ for $K^+$ as activator of the phosphatase reaction was increased (Fig. 2), and the effect of $K^+$ on the $K_m$ for ATP was decreased [14]; both may be attributed to a diminished effect of $K^+$ at the $\alpha$-sites [15, 24]. Correspondingly, K+, with a concentration-dependence fitting its occupancy of the $\alpha$-sites, protected against inactivation (Fig. 1). By contrast, the $K_{0.5}$ for $K^+$ as activator of the $(Na^+ + K^+)$-ATPase reaction, an effect mediated through the $\beta$-sites [15], was not increased (Fig. 3), nor was the $K_{0.5}$ for $Na^+$ as activator of the ATPase (Fig. 4) or the $K_i$ for $Na^+$ as inhibitor of the phosphatase reaction [14]. The experiments with the purified enzyme preparation from kidney reported here confirm the earlier studies and extend such considerations to the relationships beteen the enzyme and the pharmacologically important cardioactive steroids.

The $(Na^+ + K^+)$-ATPase spans the plasma membrane *in vivo*, and has specific sites accessible from either extracellular or intracellular media. Ouabain binds only when added to the extracellular face [9] and $K^+$ activates the ATPase reaction at sites ($\beta$-sites) accessible from that face also [25]. In contrast to earlier reports [26, 27], recent studies [28] localize the $K^+$-sites that activate the phosphatase reaction ($\alpha$-sites) to the intracellular face. On this basis, early results [8] showing $K^+$ in the extracellular medium antagonizing inhibition by cardioactive steroids could not be attributable to $K^+$ at the $\alpha$-sites. More recent studies [11], however, indicate that $K^+$ at intracellular sites (or $Na^+$ at intracellular sites) is required for extracellular $K^+$ to exert its antagonism to cardioactive steroids.

The experiments described here support such formulations of interactions between cardioactive steroids at their (extracellular) binding sites and $K^+$ at its (probably intracellular) $\alpha$-sites. First, cardioactive steroids can, at their sites, substitute for $K^+$ at its $\alpha$-sites, as activator of the phosphatase reaction, as originally demonstrated by Pitts and Askari [12, 13], and the apparent affinities for both the cardioactive steroids and $K^+$ as activators are decreased by prior treatment of the enzyme with acetic anhydride or TNBS (Figs. 2 and 6). Second, binding of [$^3$H]ouabain to the enzyme and inhibition of the enzymatic reactions by cardioactive steroids are both modified by such treatment (Table 1; Fig. 5). Finally, both $K^+$ (at concentrations corresponding to its filling of the $\alpha$-sites) and cardioactive steroids protect against the modification of acetic anhydride or TNBS (Fig. 1; Table 2). The nature of the chemical modifications produced by acetic anhydride and TNBS that so affect enzyme activity and drug–receptor interaction is an intriguing question awaiting further study.

*Acknowledgements*—I wish to thank Grace Marin and Nancy Martin for their precise and thoughtful technical assistance.

## REFERENCES

1. J. L. Dahl and L. E. Hokin, *A. Rev. Biochem.* **43**, 327 (1974).
2. I. M. Glynn and S. J. D. Karlish, *A. Rev. Physiol.* **37**, 13 (1975).
3. T. Akera and T. M. Brody, *Pharmac. Rev.* **29**, 187 (1978).
4. E. T. Wallick, L. K. Lane and A. Schwartz, *A. Rev. Physiol.* **41**, 397 (1979).
5. J. D. Robinson and M. S. Flashner, *Biochim. biophys. Acta* **549**, 145 (1979).
6. A. Schwartz, H. Matsui and A. H. Laughter, *Science* **160**, 323 (1968).
7. R. W. Albers, G. J. Koval and G. J. Siegel, *Molec. Pharmac.* **4**, 324 (1968).
8. I. M. Glynn, *J. Physiol. Lond.* **136**, (1957).
9. J. F. Hoffman, *Am. J. Med.* **41**, 666 (1966).
10. T. Akera and T. M. Brody, *J. Pharmac. exp. Ther.* **176**, 545 (1971).
11. H. H. Bodemann and J. F. Hoffman, *J. gen. Physiol.* **67**, 497 (1976).
12. B. J. R. Pitts and A. Askari, *Biochim. biophys. Acta* **225**, 388 (1971).
13. B. J. R. Pitts and A. Askari, *Archs Biochem. Biophys.* **154**, 476 (1973).

14. J. D. Robinson and M. S. Flashner, *Archs Biochem. Biophys.* **196**, 350 (1979).
15. J. D. Robinson, *Biochim. biophys. Acta* **384**, 250 (1975).
16. S. M. Liang and C. G. Winter, *Biochim. biophys. Acta* **452**, 552 (1976).
17. P. L. Jørgensen, *Biochim. biophys. Acta* **356**, 36 (1974).
18. J. D. Robinson, *Biochemistry* **6**, 3250 (1967).
19. J. D. Robinson, *Biochemistry* **8**, 3348 (1969).
20. J. D. Robinson, *Biochim. biophys. Acta* **321**, 662 (1973).
21. W. B. Van Winkle, J. C. Allen and A. Schwartz, *Archs Biochem. Biophys.* **151**, 85 (1972).
22. J. D. Robinson, *Biochim. biophys. Acta* **482**, 427 (1977).
23. A. Yoda and L. E. Hokin, *Biochem. biophys. Res. Commun.* **40**, 880 (1970).
24. J. D. Robinson, *Biochim. biophys. Acta* **397**, 194 (1975).
25. I. M. Glynn, *J. Physiol. Lond.* **160**, 18P (1962).
26. A. F. Rega, M. I. Pouchan and P. J. Garrahan, *Science* **167**, 55 (1970).
27. A. F. Rega, P. J. Garrahan and M. I. Pouchan, *J. memb. Biol.* **3**, 14 (1970).
28. R. Blostein, H. A. Pershadsingh, P. Drapeau and L. Chu, in Na, K-*ATPase: Structure and Kinetics* (Eds. J. C. Skou and J. G. Norby), pp. 233–245. Academic Press, London (1979).