Biochemical Pharmacology, Vol. 21 ,pp. 1553–1560. Pergamon Press, 1972. Printed in Great Britain.

RATES OF DISSOCIATION OF ENZYME–OUABAIN COMPLEXES AND $K_{0.5}$ VALUES IN ($Na^+ + K^+$) ADENOSINE TRIPHOSPHATASE FROM DIFFERENT SPECIES

THOMAS TOBIN and THEODORE M. BRODY
Department of Pharmacology, Michigan State University, East Lansing, Mich. 48823, U.S.A.

(*Received* 2 *July* 1971; *accepted* 10 *December* 1971)

Abstract—The half-lives of rabbit kidney and dog brain ($Na^+ + K^+$) adenosine triphosphatase (ATPase)–ouabain complexes were 10 and 89 min respectively. The ratios between these half-lives and those previously reported for guinea-pig kidney and rat brain enzyme–ouabain are more than sufficient to account for the differences in the $K_{0.5}$ values for ouabain and those enzymes reported in the literature. The $K_{0.5}$ values for the ouabain–enzyme interaction could be reduced up to 12-fold by pre-incubating the enzyme with Na^+, Mg^{2+}, ATP and ouabain prior to the addition of K^+ to start the enzyme assay. These differences show that the $K_{0.5}$ values are not obtained under equilibrium conditions and suggest that the true equilibrium position is intermediate between the measured $K_{0.5}$ values. K_i values estimated from the dissociation rates of enzyme–ouabain fall between the measured $K_{0.5}$ values. It appears that the differences in the dissociation rates alone are sufficient to account for the observed differences in the $K_{0.5}$ values. The experiments also show a marked difference in glycoside sensitivity between rat brain and heart ($Na^+ + K^+$) ATPase.

RECENTLY, evidence has been accumulating in the literature which shows that the ($Na^+ + K^+$) ATPase (ATP phosphohydrolase; EC 3.6.1.3)–ouabain interaction is, at least under some conditions, a reversible interaction and that the half-life of the enzyme–glycoside complex (EOu) may differ considerably among enzyme preparations from different tissues and species.[1–6] These differences are interesting in view of the earlier suggestions of Allen and Schwartz[7], that species differences in the "stability" or rates of dissociation of the enzyme–glycoside complex may account for the observed species differences in sensitivity to the glycosides.[8] However, a preliminary examination of the published rates of dissociation of enzyme–ouabain[3,4] and those reported here showed that these differences were more than sufficient to account for the reported differences in glycoside concentrations required to half-maximally inhibit this enzyme ($K_{0.5}$ values) from different species.[8]

Recently, however, Akera[6] has shown that if the $K_{0.5}$ estimation is altered so that the glycoside is pre-incubated with the enzyme in the absence of K^+, before the ($Na^+ + K^+$) ATPase assay is started, a much lower $K_{0.5}$ value is obtained than if the assay is started with free enzyme and glycoside, the standard procedure. These observations suggest that the $K_{0.5}$ values previously obtained are underestimates of the true affinity of these enzymes for ouabain. In this communication we show that, when the $K_{0.5}$ determination is altered by the method of Akera,[6] the $K_{0.5}$ values of ouabain for these enzymes are reduced up to 12-fold. The $K_{0.5}$ differences are now greater than the differences in the dissociation rates. The data suggest that the true

K_i values for the ($Na^+ + K^+$) ATPase–glycoside interactions are between the $K_{0.5}$ values obtained by both assay methods and that the differences in dissociation rates can account for the differences in inhibitory potency of ouabain on the enzyme preparations investigated.

The experiments also show an approximately 2400-fold difference between the slowest half-life measured (dog brain) and fastest estimated (rat heart) rates of dissociation. An approximately 600-fold difference in sensitivity to ouabain between rat heart and brain preparations is demonstrated, which may explain the susceptibility of the rat to the neurotoxic effects of ouabain. Finally, the relative instability of the ouabain–enzyme complex at low temperatures in an enzyme preparation from a ouabain-insensitive tissue is demonstrated. A preliminary report has been communicated.[9]

MATERIALS AND METHODS

Determination of $K_{0.5}$ *for ouabain from inhibition of* ($Na^+ + K^+$) *ATPase activity.* Enzymes were prepared by the method of Akera *et al.*,[8] except for the guinea-pig kidney enzyme which was prepared by the method of Post and Sen.[10] Specific activities of the various enzyme preparations in micromoles of P_i per milligram of protein per hour were as follows: rat brain, 216–348; rabbit kidney, 180–252; dog brain, 138–179; guinea-pig kidney, 84–96; rat heart, 21·0; and dog heart, 10·2. The "normal" incubation medium contained 100 mM NaCl, 15 mM KCl, 5 mM MgATP and 16 μg enzyme protein in 50 mM tris–HCl buffer, pH 7·4. The incubation was for 10 min at 37°. Except where indicated, the reaction was started by the addition of ATP and terminated at 10 min by the addition of 1 ml of 15% trichloroacetic acid. Where "pre-incubation" is indicated, ATP was added to Na^+, Mg^{2+}, ouabain (Ou) and enzyme at 37°, 5–15 min before the addition of K^+, which started the ($Na^+ + K^+$) ATPase reaction. Since the rate of approach to equilibrium of an enzyme–inhibitor system at concentrations which half-maximally inhibit the system depends largely on the rate of dissociation of the enzyme–inhibitor complex,[11] the pre-incubation period was longer in the ouabain-sensitive tissues. This allowed fuller development of inhibition at the low ouabain concentrations which are effective in these preparations. The pre-incubation times were 5·0 and 12·0 min for the rabbit kidney and rat brain enzyme respectively. Because of the exceptionally long half-life of ouabain binding to dog brain preparations (Table 1) pre-incubation was for 15 min in this species. The P_i released and ($Na^+ + K^+$) ATPase activity were estimated as previously described.[8] Those concentrations of ouabain which produced 50 per cent inhibition of enzyme activity under the given conditions are referred to as the $K_{0.5}$ values for that particular enzyme and assay procedure.

Rate of release of [^3H]*ouabain.* Ouabain was bound to the enzyme by incubating 2·5 mg enzyme protein with 2×10^{-8} M [^{3}H]ouabain (specific activity, 3·7 c/m-mole), 80 mM NaCl, 2 mM MgATP in 50 mM tris–HCl buffer, pH 7·4; final volume 40 ml. The ouabain was allowed to bind at 37° for 20 min, after which the enzyme was centrifuged at 10^5 *g* for 30 min at 0°. The sediment was resuspended in 20 ml of 10 mM tris–HCl buffer, pH 7·4, and centrifuged as previously. The final sediment was resuspended in 3 ml of 10 mM tris–HCl buffer, pH 7·4, and the protein content assayed.[3]

To measure the rate of release, enzyme–ouabain complex containing 0·2 mg protein was incubated in a solution containing 0·1 mM unlabeled ouabain, 15 mM

KCl, and 50 mM tris–HCl buffer, pH 7·5; final volume 2·0 ml. The unlabeled ouabain was added to ensure that the actual rate of dissociation was being measured. KCl, 15 mM, was added because this is the concentration of KCl present in the $K_{0.5}$ determination, and it produces maximal slowing of enzyme–glycoside dissociation in all the tissues used in these experiments.[3,12] The reaction was stopped by cooling and centrifugation at 10^5 *g* for 30 min at 0° at time periods between 0 and 60 min, depending on the rate of dissociation being observed. Zero time determinations of radioactivity in the pellet were utilized as the 100 per cent value, and subsequent samples at each time period were calculated as a percentage of that value. The rate of loss of radioactivity was in all cases log linear, and half-lives were determined from the slopes of these dissociation curves. Because of the relative stability of the ouabain binding to some of these preparations, the measured half-lives in Table 1 are estimates from the initial rates of dissociation.

RESULTS

Rates of dissociation of the enzyme–ouabain complex prepared from various tissues. Recent experiments have shown that there is considerable variation in the rates of dissociation of the enzyme–ouabain complex in preparations of this enzyme from different species, and half-lives which vary from about 2·5 min in guinea-pig kidney preparations[4] to about 2 hr in dog kidney preparations have been reported.[12] Similarly Akera and Brody[3] have reported ouabain binding half-lives of about 40 min in preparations of this enzyme from the dog heart and rat brain in the presence of K^+.

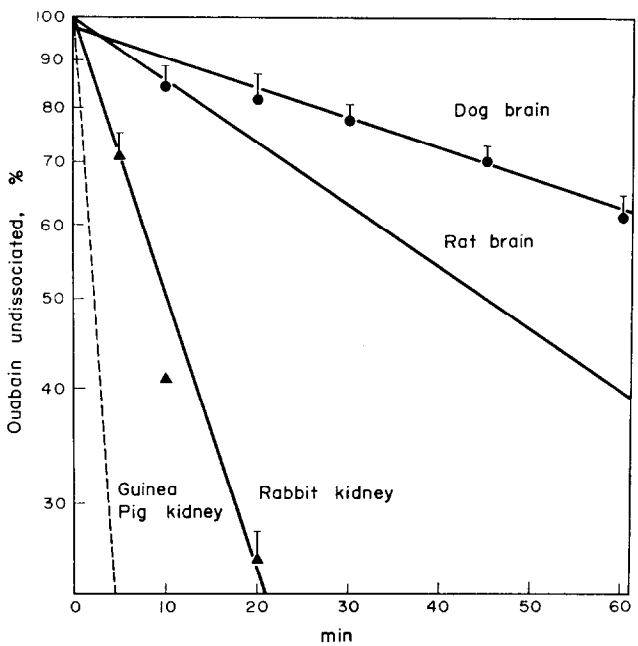

FIG. 1. Rates of dissociation of ouabin from rabbit kidney (n = 6) and dog brain (n = 4) preparations at 37°. [^{3}H]ouabain was bound to the enzymes as described in Methods, washed and then allowed to dissociate in a medium containing 15 mM KCl, $2{\cdot}5 \times 10^{-4}$ M unlabeled ouabain and 50 mM tris–HCl, pH 7·4. The vertical bars are the S.E.M. The solid and broken lines indicate the rates of dissociation of ouabain from rat brain and guinea-pig kidney enzymes.[3,4]

Figure 1 shows a similar study with dog brain and rabbit kidney preparations. The half-lives of the enzyme–ouabain complexes are about 10 and 90 min, respectively, putting the values within the range of estimated half-lives of this complex in preparations from different species.[3,4,12]

$K_{0.5}$ *values of ouabain for rat brain* ($Na^+ + K^+$) *ATPase.* As shown in Fig. 1, the half-life of the enzyme–ouabain complex in preparations from rat brain is approximately 40 min. This period (40 min) is much longer than the usual assay period for estimation of enzyme activity or glycoside $K_{0.5}$ values.[8,10] However, as shown by Akera and Brody,[3] both the binding to and dissociation of ouabain from these enzyme preparations can be remarkably slow. Figure 2 shows how the $K_{0.5}$ of ouabain for the

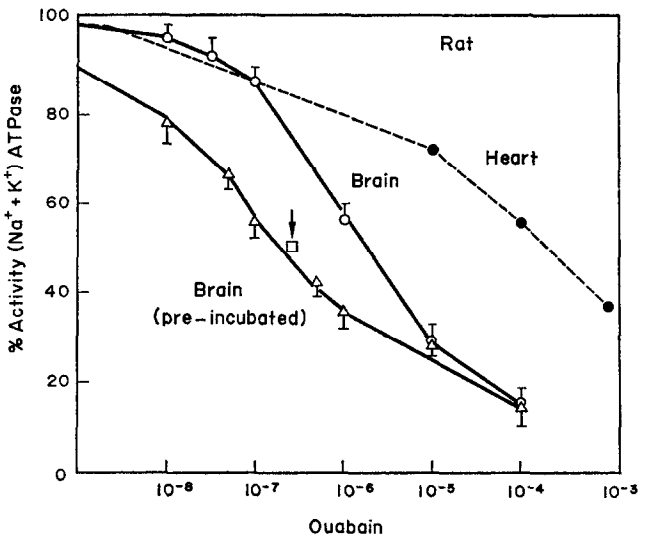

FIG. 2. $K_{0.5}$ values of rat brain enzyme for ouabain when measured by the "normal" (open circles) and "pre-incubated" (open triangles) methods. Each point is the mean of five individual estimations and the vertical bars indicate the S.E.M. The solid circles show the apparent affinity of rat heart enzyme for ouabain. The arrow indicates the "calculated $K_{0.5}$" value of ouabain for rat brain enzyme from Table 1.

rat brain enzyme preparation is affected by altering the sequence of addition in the assay system. The curve indicated by the open circles in Fig. 2 shows a "normal" assay for inhibitor $K_{0.5}$. Enzyme Na^+, K^+, Mg^{2+} and ouabain as indicated were allowed to temperature equilibrate for 12 min at 37°. Under these conditions, binding of ouabain to the enzyme does not occur.[4,13,14] After 12 min, the ATPase reaction was started by the addition of ATP and terminated after a 10-min incubation period. Under these conditions, the $K_{0.5}$ of ouabain for the rat brain enzyme is about $1{\cdot}6 \times 10^{-6}$ M. The left-hand curve (open triangles) shows the $K_{0.5}$ value obtained if the enzyme is allowed to complex with ouabain prior to starting the enzyme reaction. In this experiment, Na^+, Mg^{2+}, ATP and ouabain at the indicated concentrations were incubated together for 12 min at 37°.[6] Under these conditions, ouabain complexes rapidly with the enzyme and the relatively stable enzyme–ouabain complex is formed.[4,13,14] At 12 min, the ATPase reaction was started by the addition of 15 mM

K^+ and the reaction terminated at 10 min as previously. In this case, the $K_{0.5}$ of ouabain for the rat brain enzyme is about $1{\cdot}8 \times 10^{-7}$ M, an approximate 9-fold increase in the apparent affinity of the enzyme for ouabain. These observations show that under normal assay conditions the $K_{0.5}$ of ouabain for rat brain enzyme may be varied simply by altering the sequence of additions in the assay and allowing ouabain to complex with the enzyme prior to starting the reaction. Figure 2 also shows another characteristic of rat $(Na^+ + K^+)$ ATPase preparations, i.e. the much greater affinity of rat brain ATPase for ouabain than rat heart ATPase. Depending on the method of assay chosen for the brain enzyme, the difference in $K_{0.5}$ values between rat heart and brain $(Na^+ + K^+)$ ATPase varies between 100- and 1000-fold.

TABLE 1. RELATIONSHIP BETWEEN OBSERVED $K_{0.5}$ VALUES AND $K_{0.5}$ VALUES CALCULATED FROM THE ENZYME–OUABAIN STABILITY DATA*

Enzyme source	Treatment	Measured $K_{0.5}$	N	Measured $T_{\frac{1}{2}}$ (min)	N	Ratio of $T_{\frac{1}{2}}$	Calculated $K_{0.5}$
Dog brain	Pre-incub.	$6{\cdot}4 \times 10^{-8}$	4	87	4	1/35	$1{\cdot}1 \times 10^{-7}$
Dog brain	Normal	$7{\cdot}6 \times 10^{-7}$	4				
Dog heart	Normal	$7{\cdot}9 \times 10^{-7}$	4	45	4	1/18	$2{\cdot}2 \times 10^{-7}$
Rat brain	Pre-incub.	$1{\cdot}8 \times 10^{-7}$	5	45	4	1/18	$2{\cdot}2 \times 10^{-7}$
Rat brain	Normal	$1{\cdot}6 \times 10^{-6}$	5				
Rabbit kidney	Pre-incub.	$5{\cdot}0 \times 10^{-7}$	5	10	5	1/4	$1{\cdot}0 \times 10^{-6}$
Rabbit kidney	Normal	$1{\cdot}5 \times 10^{-6}$	5				
Guinea-pig kidney	Normal	$4{\cdot}0 \times 10^{-6}$		2·5		1	
Rat heart	Normal	$1{\cdot}8 \times 10^{-4}$	4	0·05†			

* The data on the dog and rat heart are from Akera, Larsen and Brody[8] and Akera and Brody.[3] Guinea-pig kidney $(Na^+ + K^+)$ ATPase was chosen as the standard, since the 2·5-min half-life of ouabain binding to this enzyme[4] is sufficiently fast to allow almost complete equilibration over the 20-min assay period used for this enzyme.[10] Calculated $K_{0.5}$ values were then extrapolated from the guinea-pig kidney $K_{0.5}$ in the ratios of the measured half-lives. The calculated $K_{0.5}$ values fall between the $K_{0.5}$ values obtained by the normal and pre-incubated assay methods. N = number of determinations.

† The half-life of ouabain binding to rat heart $(Na^+ + K^+)$ ATPase estimated from the $K_{0.5}$ data.

$K_{0.5}$ *values for ouabain of dog brain and rabbit kidney* $(Na^+ + K^+)$ *ATPases.* Similar experiments with rabbit kidney and dog brain $(Na^+ + K^+)$ ATPase preparations yielded qualitatively similar results which are presented in Table 1. In rabbit kidney preparations, pre-incubation with Na^+ and ATP for 5 min produced an approximate 3-fold shift in the $K_{0.5}$ value for ouabain (Table 1). In dog brain preparations, 15 min of pre-incubation produced a 12-fold decrease in the $K_{0.5}$ value (Table 1). Interestingly, however, there was little difference in the apparent $K_{0.5}$ values for the dog heart and brain enzymes, in agreement with the essentially similar dissociation rates for the enzyme–ouabain complexes in these preparations (Fig. 1 and reference 3).

Instability of enzyme–ouabain complex in ouabain-insensitive species. Ouabain

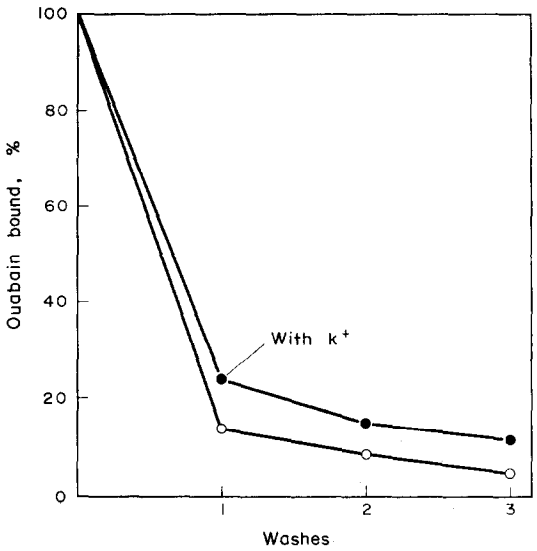

FIG. 3. Guinea-pig kidney enzyme was allowed to bind [^{3}H]ouabain, 4×10^{-8} M, in the presence of Na^+, Mg^{2+} and ATP. The enzyme was then washed as indicated in Methods by alternate centrifugation and resuspension. After each wash, ouabain binding to the enzyme as picomoles/per milligram of protein was estimated and plotted as a percentage of the binding prior to the first wash. Each point is the mean of three individual experiments.

administered *in vivo* to dogs remains bound to the heart and kidney ($Na^+ + K^+$) ATPases during relatively prolonged preparation procedures at 0°.[15,16] This does not seem true for preparations from glycoside-insensitive tissues. Figure 3 shows that ouabain is readily washed off guinea-pig kidney enzyme preparations even at 0°. Essentially similar effects have been observed with rabbit kidney preparations, though in this tissue the presence of alkali metal cations stabilized the binding to washing.

DISCUSSION

The ouabain–enzyme equilibrium has been successfully treated quantitatively in terms of a simple, reversible, 1:1 (one drug molecule inhibits one enzyme molecule) Michaelas–Menten type of interaction by two groups of workers.[1,2] Thus the ouabain–enzyme equilibrium is represented as

$$E - P + Ou \underset{k-1}{\overset{k+1}{\rightleftharpoons}} E - POu. \tag{1}$$

The affinity constant for the reaction is,

$$K_l = \frac{k-1}{k+1} = \text{(affinity of enzyme for ouabain under indicated conditions).} \tag{2}$$

The rate of association, $k + 1$, is expressed as litres, moles^{-1} sec^{-1} and $k-1$, the rate of dissociation is expressed as reciprocal time, sec^{-1}. Solving for K_m, the time dimension is eliminated leaving K_m as a molar concentration, the magnitude of which may vary directly with that of $k-1$, the rate of dissociation.

The problem with this simple approach is that the observed differences in the rates of dissociation are more than sufficient to account for the observed differences in $K_{0.5}$ values. This relationship is indicated in Table 1, which shows a 5-fold difference between the $K_{0.5}$ values for guinea-pig kidney and dog brain ($Na^+ + K^+$) ATPase, whereas there is a 35-fold difference between the half-lives of ouabain bound to these two enzymes.

A solution to this dilemma is presented by the work of Akera[6] and Akera and Brody.[3] These authors have shown that the rate of equilibration of the enzyme–glycoside interaction can be very slow at the low glycoside concentrations which are effective in the glycoside-sensitive species.[3] Thus, if the $K_{0.5}$ estimation is started from free enzyme and glycoside, the equilibrium point is not attained in 10 min[3] and the measured $K_{0.5}$ underestimates K_i.[2,3,6,8] Conversely, if the system is pre-incubated with ouabain in the absence of K^+, the $K_{0.5}$ estimation now starts from the pre-formed enzyme–inhibitor complex and the equilibrium point is approached from the left-hand side of equation 1. The $K_{0.5}$ values obtained by both methods thus might be expected to bracket the true K_i value. It turns out that if one calculates $K_{0.5}$ values from the guinea-pig $K_{0.5}$,[17] in the ratios of the half-lives of the enzyme–ouabain complexes, these values are intermediate between the measured values. It appears reasonable to suggest that the discrepancy between the ratio of the $K_{0.5}$ values obtained in "normal" assays and the ratio of the observed half-lives occurs because the $K_{0.5}$ values are underestimates of the true affinity of these glycosides for the ($Na^+ + K^+$) ATPase. Thus the differences between the true affinities, which are somewhere between the $K_{0.5}$ values obtained by both assay methods, are probably accounted for in terms of differences in the dissociation rates alone. This is in agreement with earlier results which showed little difference in the rate constants for formation of the enzyme–ouabain complex in a number of different tissues and species.[3,9] Since these enzymes are relatively unstable at 37°,[18] direct estimation of K_i at 37° does not appear feasible for the stable enzyme–ouabain complexes.

There appear to be a number of atypical effects in tissues from the less ouabain-sensitive species. The most marked of these is the large difference in apparent affinities of rat heart and brain enzymes for ouabain. These observations suggest that the susceptibility of the rat to the neurotoxic effects of ouabain is explainable in terms of ($Na^+ + K^+$) ATPase inhibition, further strengthening the hypothesis that this enzyme is the pharmacological receptor for the cardiac glycosides. Data of Friedman *et al.*[19] support this hypothesis. The latter investigators, studying the distribution of digoxin, observed no digoxin binding in dog brain, but observed a delayed entry of digoxin into rat brain at approximately one-tenth the level found in the heart. They attributed the delay to the blood–brain barrier. Further, the brain retained digoxin longer than any other tissue in the rat, which correlates well with the stable binding observed in the brain. Since dog brain ATPase is at least as sensitive to ouabain as cardiac ATPase (Table 1), it is presumably the blood–brain barrier which renders the dog less susceptible to the neurotoxic effects of ouabain.[20]

Lastly, it appears that, in the less glycoside-sensitive tissues, it is not possible to stabilize effectively the glycoside binding by lowering the temperature or by adding K^+. In the sensitive species, glycoside binding to ($Na^+ + K^+$) ATPase preparations is apparently stable for several hours at 0°.[15,16] However, the Na^+ATP-dependent binding to guinea-pig tissues is readily washed off at 0° in the presence or absence of

K^+. This lack of stability of the binding at 0° combined with its relatively high lability at 37° makes glycoside-insensitive tissues particularly unsuitable for the demonstration *in vitro* of binding or inhibition which has occurred *in vivo*.

Acknowledgements—This work was supported by grants from the Michigan Heart Association, the National Institute of Mental Health (M.H. 12783-05) and a General Research Support grant (NIH RR 05623-04) to the College of Veterinary Medicine, Michigan State University. The authors wish to thank Mr. Roxy So for excellent technical assistance.

REFERENCES

1. O. HANSEN, *Biochim. biophys. Acta* **223,** 122 (1971).
2. P. F. BAKER and J. S. WILLIS, *Nature, Lond.* **226,** 521 (1970).
3. T. AKERA and T. M. BRODY, *J. Pharmac. exp. Ther.* **176,** 17 (1971).
4. T. TOBIN and A. K. SEN, *Biochim. biophys. Acta* **198,** 120 (1970).
5. S. P. BANERJEE, V. K. KHANNA and A. K. SEN, *Biochem. Pharmac.* **20,** 1649 (1971).
6. T. AKERA, *Biochim. biophys. Acta,* **249,** 53 (1971).
7. J. C. ALLEN and A. SCHWARTZ, *J. Pharmac. exp. Ther.* **168,** 42 (1969).
8. T. AKERA, F. S. LARSEN and T. M. BRODY, *J. Pharmac. exp. Ther.* **170,** 17 (1969).
9. T. TOBIN, T. AKERA and T. M. BRODY, *Pharmacologist* **13,** 649 (1971).
10. R. L. POST and A. K. SEN, in *Methods in Enzymology* (Eds. S. P. COLOWICK and N. O. KAPLAN), Vol. 10, p. 762. Academic Press, New York (1967).
11. A. GOLDSTEIN, *Experientia* **8,** 442 (1952).
12. T. TOBIN and T. M. BRODY, *Fedn Proc.* **30,** 331 (1971).
13. A. K. SEN, T. TOBIN and R. L. POST, *J. biol. Chem.* **244,** 6956 (1969).
14. H. MATSUI and A. SCHWARTZ, *Biochim. biophys. Acta* **157,** 655 (1968).
15. J. B. HOOK, *Proc. Soc. exp. Biol. Med.* **131,** 731 (1969).
16. M. MARTINEZ-MALDONADO, J. C. ALLEN, G. EKNOYAN, W. SUKI and A. SCHWARTZ, *Science, N.Y.* **165,** 807 (1969).
17. H. BADER and A. K. SEN, *Biochim. biophys. Acta* **118,** 116 (1966).
18. J. C. SKOU and C. H. HILBERG, *Biochim. biophys. Acta* **110,** 359 (1965).
19. M. FRIEDMAN, S. ST. GEORGE, R. BINE, S. O. BYERS and A. B. BLAND, *Circulation* **6,** 367 (1962).
20. L. MEYER JONES, Ed., *Veterinary Pharmacology and Therapeutics*, 3rd edn, p. 606. Iowa State University Press, Ames, Iowa (1965).