[11] Goldstein, A., and B. J. Brown, *Biochim. Biophys. Acta*, **53**, 19 (1961).
[12] Ryter, A., and F. Jacob, *Ann. Inst. Pasteur*, **107**, 384 (1964).
[13] Ganesan, A. T., and J. Lederberg, *Biochem. Biophys. Res. Commun.*, **18**, 824 (1965).
[14] Lark, K. G., T. Repko, and E. J. Hoffman, *Biochim. Biophys. Acta*, **76**, 9 (1963).
[15] Pettijohn, D., and P. Hanawalt, *J. Mol. Biol.*, **9**, 395 (1964).

ALKYLATION OF A BRAIN TRANSPORT ADENOSINETRIPHOSPHATASE AT THE CARDIOTONIC STEROID SITE BY STROPHANTHIDIN-3-HALOACETATES*

By Lowell E. Hokin,† Michael Mokotoff,‡ and S. Morris Kupchan

DEPARTMENTS OF PHYSIOLOGICAL CHEMISTRY AND PHARMACEUTICAL CHEMISTRY, UNIVERSITY OF WISCONSIN, MADISON

Communicated by Henry Lardy, February 1, 1966

Our understanding of the molecular mechanisms involved in membrane transport has been hampered by our inability to isolate the macromolecules concerned. A promising development in the field of Na^+ and K^+ transport was the demonstration of an adenosinetriphosphatase (ATPase) activity closely related to Na^+ and K^+ transport (see review by Skou[1]). This enzyme is dependent on the simultaneous presence of Na^+ and K^+ and is inhibited by cardiotonic steroids, which rather specifically inhibit Na^+ and K^+ transport.[2, 3] The ATPase is usually isolated in a lipoprotein particulate fraction derived from cell membranes, and all attempts to purify it extensively have failed. One approach to the transport problem is to react the macromolecular transport components with radioactive irreversible inhibitors so as to tag the macromolecular components. Reagents such as diisopropylfluorophosphate (DFP) have been extensively used in labeling the active sites of numerous animal hydrolases (see, for example, Oosterbaan and Cohen[4]). In an earlier study we found that DFP irreversibly inhibited the ($Na^+ + K^+$) activated ATPase (hereafter referred to as transport ATPase) in a kidney membrane preparation;[5] the nontransport ATPase was unaffected. Although the inhibition by DFP and the labeling of the protein by DFP^{32} were reduced by ATP, in further studies with a brain transport ATPase it was not possible to establish conditions which demonstrated to our complete satisfaction a labeling of the transport ATPase distinct from the labeling of other proteins in the crude enzyme preparation;[6] this limited the use of DFP as a specific labeling agent for the transport ATPase.

In recent years the labeling of active sites of enzymes has been accomplished by incubating with substrates containing reactive functional groups which form covalent bonds at the substrate sites.[7–11] These reagents have been termed "active-site-directed irreversible inhibitors."[7] We thought that such a reagent would be more useful than DFP in labeling a specific site on the transport ATPase. Obviously, derivatives of the natural activators, i.e., Na^+ and K^+, could not be prepared. However, the high affinity of cardiotonic steroids for the transport ATPase offered an approach. The preparation of derivatives of strophanthidin was decided upon. This cardiotonic steroid offers several advantages for this kind of work. It is a specific inhibitor of the transport ATPase,[3] it inhibits reversibly,[5, 6]

and many derivatives can be prepared. We have prepared a large number of such derivatives, and a detailed study of their chemistry, reversible and irreversible inhibition of transport ATPase, cytotoxic action, and cardiotonic action will be presented elsewhere.[12] This paper reports on the behavior of two of these derivatives, strophanthidin-3-iodoacetate (SIA) and strophanthidin-3-bromoacetate (SBA).

The haloacetate ions, iodoacetate and bromoacetate, have been extensively used to react irreversibly with specific groups on proteins, particularly sulfhydryl groups;[13] they form stable carboxymethyl derivatives. Strophanthidin-3-acetate has been shown to inhibit the active transport of K^+ in erythrocytes.[14] In this paper it is shown that strophanthidin-3-acetate, SIA, and SBA inhibit the transport ATPase at low concentrations. The inhibition by strophanthidin-3-acetate was found to be reversible, but a large fraction of the inhibition by SIA and SBA was found to be irreversible. The studies presented here indicate that SIA and SBA alkylate the transport ATPase at the cardiotonic steroid site, and they offer the possibility of characterizing this site as well as isolating the transport ATPase protein.

Methods.—The preparation of the brain ATPase, the washing of the enzyme, and the ATPase assay have been previously described.[5, 15] The enzyme was treated with cardiotonic steroids as follows. Approximately 0.2 mg of enzyme protein in 2.0 ml of 0.1 *M* imidazole-HCl buffer (pH 7.0) was incubated at 37° with the cardiotonic steroid for the indicated period of time. The steroids were added in 10–40 μl of dimethylformamide. Controls contained an equivalent amount of dimethylformamide. If the sample was to be tested for irreversible inhibition, 10 ml of sucrose-EDTA was added after incubation, the enzyme preparation was sedimented by centrifugation, and the sediment was washed twice with sucrose-EDTA. The residue was finally suspended in 2.0 ml of 0.1 *M* imidazole-HCl buffer (pH 7.0). If irreversibility of the cardiotonic steroid was not to be tested, aliquots of the incubated sample were assayed directly for transport ATPase and non-transport ATPase activities.

Materials.—*Strophanthidin-3-iodoacetate (3β,5,14-trihydroxy-19-oxo-5β-card-20(22)-enolide-3-iodoacetate):* A solution of strophanthidin (0.730 gm, 1.80 mmole) in 30 ml of purified dioxane was treated with 2.0 ml of iodoacetyl chloride and allowed to stand at room temperature in the dark for 17 hr. The reaction was terminated by the addition of 140 ml of water, and the iodine color was discharged by the addition of a few crystals of $Na_2S_2O_3$. The colorless mixture was neutralized with 0.5 *N* Na_2CO_3 solution, and the dioxane was removed under reduced pressure. The resulting aqueous mixture was extracted with chloroform until a drop of the extract no longer gave a positive spot test for cardenolide with 3,5-dinitrobenzoic acid reagent.[16] The combined extracts were washed once with $Na_2S_2O_3$ solution, once with water, and dried over anhydrous Na_2SO_4. Removal of the chloroform under reduced pressure afforded a yellow oil which was crystallized from acetone-Skellysolve B, to yield yellow crystals (0.51 gm), mp 126–127° (dec). A second crop weighed 0.05 gm; total yield 0.56 gm (54%). Repeated crystallizations gave the analytical sample of strophanthidin-3-iodoacetate, mp 127–128°, $[\alpha]_D^{30}$ + 28° (c 0.69, dioxane).

Analysis for $C_{25}H_{33}IO_7$ (572.4): Calc.: C, 52.45; H, 5.82; I, 22.17. Found: C, 52.58; H, 5.74; I, 22.00.

Strophanthidin-3-bromoacetate (3β,5,14-trihydroxy-19-oxo-5β-card-20(22)-enolide-3-bromoacetate): A solution of strophanthidin (0.500 gm, 1.24 mmole) and 0.21 ml (2.6 mmole) of pyridine in 25 ml of purified dioxane was treated with 0.23 ml (2.6 mmole) of bromoacetyl bromide. The solution was stirred at room temperature for 1.5 hr, diluted with 75 ml of water, and the dioxane was removed under reduced pressure. A tan-colored precipitate remained, which was collected with water and dried in a vacuum desiccator over phosphorus pentoxide; yield, 0.385 gm. The analytical sample was obtained after repeated crystallizations from acetone-Skellysolve B, mp 191–194° (dec), $[\alpha]_D^{26}$ + 26° (c 0.31, dioxane).

Analysis for $C_{25}H_{33}BrO_7$ (525.4): Calc.: C, 57.14; H, 6.33; Br, 15.21. Found: C, 57.35; H, 6.54; Br, 15.29.

5,14-Dianhydrostrophanthidin-3-iodoacetate (3β-hydroxy-19-oxo-carda-5,14,20(22)-trienolide-3-

iodoacetate): A solution of 5,14-dianhydrostrophanthidin (0.100 gm, 0.27 mmole) prepared by the method of Jacobs and Collins[17] in 5.0 ml of purified dioxane was treated with 0.3 ml of iodoacetyl chloride. The mixture was allowed to stand at room temperature in the dark for 12 hr and was then added dropwise to cold Skellysolve B (75 ml). The precipitate which formed was collected, air-dried, and dissolved in chloroform. The chloroform solution was washed once with $Na_2S_2O_3$ solution, once with water, and dried over anhydrous Na_2SO_4. Evaporation of the solvent left an oil which crystallized upon treatment with a few drops of acetone. Thin-layer chromatography on silica gel G in 1% methanol in chloroform revealed one major spot with two minor spots of lower R_f. The component of highest R_f was obtained by thick-layer (1.0-mm thickness) chromatography on silica gel G, and recrystallization from acetone-water afforded 0.021 gm of colorless 5,14-dianhydrostrophanthidin-3-iodoacetate. Two more crystallizations gave an analytical sample, mp 153–155° (dec), $[\alpha]_D^{25}$ −165° (c 0.40, chloroform).

Analysis for $C_{25}H_{29}IO_5$ (536.4): Calc.: C, 55.97; H, 5.45; I, 23.66. Found: C, 56.16; H, 5.41; I, 23.62.

5,14-Dianhydrostrophanthidin-3-acetate (*3β-acetoxy-19-oxo-carda-5,14,20(22)-trienolide*): This compound was synthesized by the method of Jacobs and Collins;[17] mp 185–190°; $[\alpha]_D^{31}$ −231° (c 0.57, $CHCl_3$) (literature $[\alpha]_D^{22}$ −222°).

Strophanthidin-3-iodoacetate and strophanthidin-3-bromoacetate were reported to have been prepared by Welles *et al.*;[18] no data other than melting points were given. The melting points of their "strophanthidin-3-iodoacetate" and "strophanthidin-3-bromoacetate" were 186–188° and 220–222°, respectively. These melting points differ considerably from ours. A sample of their "strophanthidin-3-bromoacetate" was kindly provided by Dr. J. S. Welles and was shown to be 14-monoanhydrostrophanthidin-3-bromoacetate, by NMR spectroscopy and synthesis.[12] A sample of the "strophanthidin-3-iodoacetate" of Welles *et al.* was no longer available. However, the fact that our iodoacetate compound (mp 127–128°) was convertible to the same azidoacetate compound[12] as was obtained from our bromoacetate compound (mp 191–194°) indicated that our iodoacetate compound is 3β,5,14-trihydroxy-19-oxo-5β-card-20(22)-enolide-3-iodoacetate (strophanthidin-3-iodoacetate).

Results.—Reversible and irreversible inhibition of transport ATPase by cardenolides and sodium iodoacetate: The standard procedure for testing whether strophanthidin or a related cardenolide derivative was a reversible and also an irreversible inhibitor was to incubate two samples of the enzyme with the compounds, and then wash one samlpe twice with sucrose-EDTA.[5] As shown in Figure 1, the enzyme was almost completely inhibited by strophanthidin before washing. The washing procedure brought the enzyme activity back to that of the control which had been incubated without any inhibitor. Strophanthidin-3-acetate also inhibited the trans-

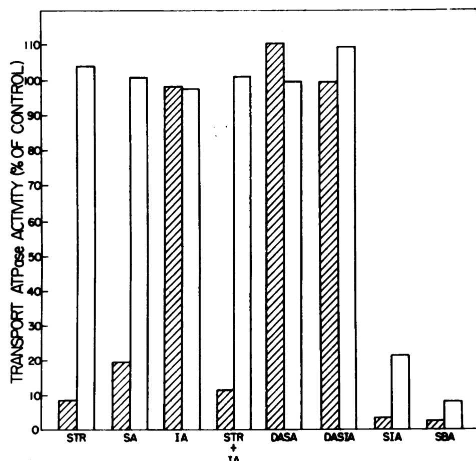

FIG. 1.—Reversible and irreversible inhibition of transport ATPase by cardenolides and iodoacetate.

The enzyme was incubated for 3 hr with the indicated compounds as described under *Methods*. Control enzymes were run without and with washings, and enzyme protein was determined on washed and unwashed samples.

Abbreviations:
STR, strophanthidin;
SA, strophanthidin-3-acetate;
IA, iodoacetate;
DASA, 5,14-dianhydrostrophanthidin-3-acetate;
DASIA, dianhydrostrophanthidin-3-iodoacetate;
SIA, strophanthidin-3-iodoacetate;
SBA, strophanthidin-3-bromoacetate.
All compounds were 0.1 mM.
Hatched bars, unwashed; *clear bars*, washed.

port ATPase before washing, and this inhibition was as fully reversible as was that of strophanthidin. SIA and SBA inhibited the enzyme as completely as strophanthidin, but the inhibition was only partly reversed on washing. Sodium iodoacetate, in a concentration ten times that of the strophanthidin haloacetates, showed no inhibition of the enzyme either before or after washing.

Two arguments could be raised against the idea that SIA and SBA were alkylating the cardiotonic steroid site. One is that strophanthidin or the strophanthidin haloacetates change the conformation of the transport ATPase so that it becomes more susceptible to inhibition by the iodoacetyl group. It is known, for example, that strophanthidin increases the susceptibility of transport ATPase to irreversible inhibition by DFP.[5, 6] If this explanation were correct, sodium iodoacetate would be expected to inhibit irreversibly the enzyme in the presence of strophanthidin. This was not found to be the case: the results with strophanthidin plus sodium iodoacetate were the same as with strophanthidin alone. Another argument is that any steroid related to strophanthidin might carry the iodoacetyl group into a hydrophobic region of the lipoprotein-enzyme where the iodoacetyl group would irreversibly react with a site on the enzyme which was not the cardiotonic steroid site. If this were the case, a cardenolide in which a minimum chemical change had been effected so as to render it inactive toward the ATPase should show irreversible inhibitory activity if it is esterified with iodoacetate on the 3 position. Dehydration of strophanthidin at positions 5 and 14, followed by esterification with acetic anhydride, afforded 5,14-dianhydrostrophanthidin-3-acetate, a steroid that was inactive toward the transport ATPase (Fig. 1). Also, 5,14-dianhydrostrophanthidin-3-iodoacetate did not inhibit the enzyme, either reversibly or irreversibly. Thus, the iodoacetyl group must be esterified to a cardenolide that is active toward the ATPase, and this supports the view that the alkylation takes place at the cardiotonic steroid site of the enzyme.

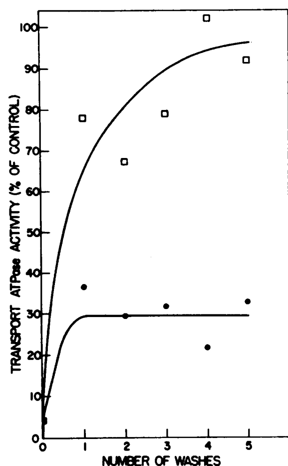

FIG. 2.—Effect of repeated washing on recovery of inhibition of transport ATPase by ouabain and SIA. The enzyme was incubated for 30 min and washed as described under *Methods*. Control samples without cardiotonic steroid were run for each point. □, Incubated with ouabain. ●, Incubated with strophanthidin-3-iodoacetate.

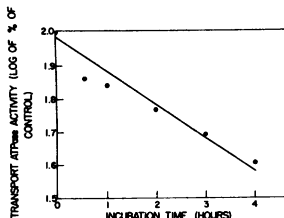

FIG. 3.—Rate of irreversible inhibition of transport ATPase by SIA. The enzyme was incubated for the indicated times and washed and assayed as described under *Methods*. Control samples without SIA were run for each point.

Effects of repeated washing on the inhibition of the enzyme by SIA and ouabain: Figure 2 shows that the inhibitory effect of ouabain, which has a higher affinity for the enzyme than does strophanthidin,[5] was gradually removed by repeated washing until full activity was restored after the fourth washing. SIA behaved quite differently. After one washing, 30 per cent of the activity of the enzyme was recovered. However, no further recovery of enzyme activity was observed after four additional washings. It would thus appear that at the end of the SIA treatment, but before washing,

70 per cent of the the total inhibition was due to irreversible binding at the cardiotonic steroid site, presumably through a covalent bond, while about 30 per cent of the inhibition was due to binding of this substance at the cardiotonic steroid site in a reversible manner. The restoration of the maximum recoverable activity with one wash suggests that the affinity of SIA for the cardiotonic steroid site before alkylation is not greater than that of strophanthidin or strophanthidin acetate.

Rate of inactivation by SIA, effect of SIA concentration, and effect of pH: The inactivation of transport ATPase by SIA followed first-order kinetics, as would be predicted for an irreversible inhibition of an enzyme by an inhibitor which is in great excess of the enzyme (Fig. 3). The inhibition increased with increasing concentrations of SIA from about 30 per cent at 0.01 mM SIA to about 90 per cent at 1.0 mM SIA (Fig. 4). The maximum irreversible inhibition lay between pH 7.0 and 8.5 (Fig. 5). Below pH 6.5 the inhibition was less. This pH dependence resembles that for

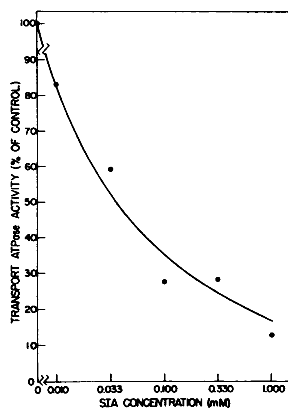

FIG. 4.—Effect of SIA concentration on irreversible inhibition of transport ATPase. The enzyme was incubated 3 hr, washed, and assayed as described under *Methods*.

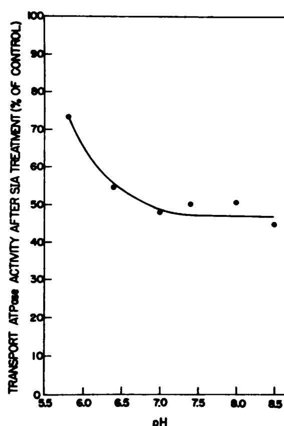

FIG. 5.—Effect of pH on irreversible inhibition of transport ATPase by SIA. The following buffers were used: pH 5.8, sodium maleate; pH 6.4–7.0, imidazole HCl; pH 7.4–8.5, Tris HCl. All buffers were 0.1 *M*. The enzyme was incubated 30 min, washed, and assayed as described under *Methods*.

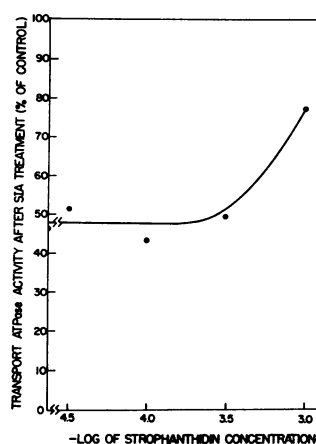

FIG. 6.—Effect of strophanthidin on the irreversible inhibition of transport ATPase by SIA. The enzyme was incubated with 0.1 mM SIA and the indicated concentrations of strophanthidin for 1 hr, washed four times, and assayed as usual.

reaction of haloacetates with thiol compounds,[19, 20] but further information is required before it can be concluded that the inactivation of the transport ATPase is by alkylation of a sulfhydryl group on the enzyme.

Effects of strophanthidin, K^+, ATP, and Mg^{++} on the irreversible inhibition of transport ATPase by SIA: Figure 6 shows that 1 mM strophanthidin partially counteracted the irreversible inhibition of the transport ATPase by 0.1 mM SIA. This is in keeping with the view that strophanthidin and SIA compete for the same site and that binding of strophanthidin to the site prevents binding of SIA and the resulting alkylation.

There are some indications that K^+ and cardiotonic steroids may both bind at

the site where K^+ activates the transport ATPase.[21, 22] In the present study there was only a slight antagonism by concentrations of K^+ up to 50 mM of the inhibition of the transport ATPase by partially inhibitory concentrations of strophanthidin or SIA (each at 0.01 mM) before washing and no K^+-antagonism of the irreversible inhibition by SIA.

Concentrations of ATP up to 10 mM had no effect on the irreversible inhibition by SIA, and concentrations from 10 mM to 20 mM enhanced the irreversible inhibition somewhat. Mg^{++} alone or added with ATP did not affect the inhibition by SIA. Since the ATP site and the cardiotonic steroid site are on opposite sides of the membrane,[23, 24] one would not necessarily expect a protection by ATP of the alkylation by SIA.

Effect of thiol compounds on the reversible and irreversible inhibition of transport ATPase by SIA: Sodium iodoacetate rapidly alkylates thiol compounds such as cysteine and glutathione.[25–27] Before washing, the inhibition of the enzyme was 90 per cent or more in the presence or absence of either cysteine, dithiothreotol,[28] or glutathione. However, the various thiol compounds reduced the irreversible inhibition considerably. These results can best be interpreted as follows. The thiol compounds rapidly react with SIA forming thioether derivatives of the strophanthidin ester. These are active reversible inhibitors of the transport ATPase, as would be anticipated from the fact that the inhibitor activity of strophanthidin is not lost by substitution on the 3 position. However, since the alkylating haloacetate group has been eliminated, the alkylation of the enzyme at the cardiotonic steroid site can no longer occur. This interpretation is confirmed by the experiment, also shown in Figure 7, in which SIA and cysteine were allowed to react for 1 hr and the reaction mixture was then added to the enzyme system. There was essentially complete inhibition before washing, but there was no irreversible inhibition as shown by the restoration to full activity after washing. The fact that the thiol compounds did not completely eliminate the irreversible component of the inhibition when added simultaneously with SIA to the enzyme system is presumably due to competition between the thiol compounds and the susceptible cardiotonic steroid site of the enzyme.

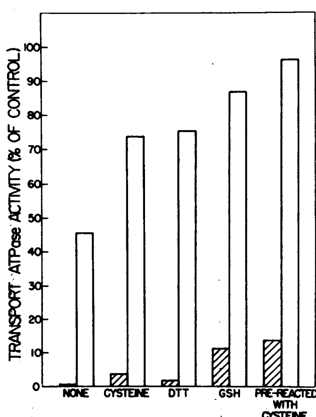

FIG. 7.—Effects of various thiol compounds on the reversible and irreversible inhibition of transport ATPase by SIA. The thiol compounds were all 1.0 mM. Cysteine HCl was neutralized with NaOH just before use. SIA (0.1 mM) was prereacted with 1 mM cysteine (pH 7.0) at room temperature for 1 hr before addition to the enzyme. Abbreviations: DTT, dithiothreotol; GSH, glutathione. Control samples without SIA were incubated with each thiol compound; there was no effect of thiol compounds alone on enzyme activity. *Hatched bars*, unwashed; *clear bars*, washed.

Effects of SIA and SBA on the nontransport ATPase: Table 1 shows that SIA

and SBA inhibited the nontransport ATPase about 50 per cent before washing and about 33 per cent after washing. This inhibition is probably not related to the cardenolide nucleus, but is due to the haloacetate functions alone, since sodium iodoacetate and 5,14-dianhydrostrophanthidin-3-iodoacetate—two compounds which are inactive toward the transport ATPase—also inhibited the nontransport ATPase. Others have reported that the nontransport ATPase is more sensitive to nonspecific sulfhydryl inhibitors than is the transport ATPase (see review by Skou[1]).

TABLE 1

REVERSIBLE AND IRREVERSIBLE INHIBITION OF NONTRANSPORT ATPASE BY HALOACETATE COMPOUNDS

		ATPase (μmoles/mg protein/hr)			
		Nontransport		Transport	
Expt. no.	Additions	Unwashed	Washed	Unwashed	Washed
1	Dimethylformamide	11.1	12.8	35.9	38.3
	Strophanthidin	12.9	12.8	3.9	39.8
	Strophanthidin acetate	9.4	13.3	13.4	32.1
	SIA	5.9	8.8	1.2	8.3
	SBA	4.9	8.3	0.9	3.1
	Water	13.2	15.4	35.1	40.2
	Sodium iodoacetate	9.5	11.6	38.3	40.5
2	Dimethylformamide	10.3	15.5	30.3	28.4
	5,14-Dianhydrostrophanthidin iodoacetate	8.6	9.0	30.2	31.5

Conditions were as in Fig. 1. The cardenolides were added in dimethylformamide as usual, and sodium iodoacetate was added in water. All compounds were 0.1 mM. The nontransport ATPase is the ATPase activity observed in the presence of 0.1 mM ouabain. The transport ATPase activity is the difference between the ATPase activities observed in the absence and presence of ouabain.

It should be noted that the ATPase activities in the washed control samples were somewhat greater than in the unwashed control samples. This enhancement due to washing was rather marked in some experiments; it may have been partly due to preferential removal of inert protein, since there was considerable loss of protein on washing.

Discussion.—The data presented in this paper strongly support the conclusion that the irreversible inhibition of transport ATPase by SIA and SBA is due to alkylation of the enzyme at the site where cardiotonic steroids bind. This is based on the following observations. (1) Sodium iodoacetate does not inhibit the enzyme in concentrations as high as 1 mM, while SIA shows some irreversible inhibition at 0.01 mM. (2) Strophanthidin does not increase the susceptibility of the enzyme to sodium iodoacetate. (3) Modification of the strophanthidin nucleus so as to form 5,14 dianhydrostrophanthidin-3-iodoacetate abolishes reversible and irreversible inhibition of transport ATPase. (4) Strophanthidin protects against irreversible inhibition by SIA. (5) Thiol compounds, which rapidly form thioethers with sodium iodoacetate, do not appreciably reduce the reversible inhibition of the transport ATPase by SIA but they do reduce the irreversible inhibition. Reaction of SIA with cysteine before addition to the enzyme abolishes the irreversible inhibition.

The demonstration of a "site-directed" irreversible inhibition of the transport ATPase by strophanthidin haloacetates affords an opportunity to label specifically the transport ATPase protein with an appropriate radioactive strophanthidin derivative. This approach may prove more useful in isolating and characterizing the transport ATPase than approaches utilizing relatively less specific irreversible inhibitors such as DFP and N-ethyl maleimide, although Fox and Kennedy[29] have

achieved some success with the latter compound in specifically labeling a protein involved in the permeation of β-galactosides into *E. coli*.

These studies may have considerable implications with respect to isolation of drug and hormone receptors generally. It should be possible chemically to modify a number of drugs and hormones so as to alkylate the binding sites of receptor molecules.

The authors are grateful to Mr. Radleigh Becker for technical assistance.

* Supported by grants from the National Institutes of Health (NB-01730 and CA 04500) and the American Cancer Society (T-275B).

† Research Career Awardee of the National Institutes of Health (5-K6-GM-1347).

‡ Fellow of the American Foundation for Pharmaceutical Education, 1963–1965.

[1] Skou, J. C., *Physiol. Rev.*, **45**, 596 (1965).

[2] Schatzmann, H. J., *Helv. Phys. Acta*, **11**, 346 (1953).

[3] Glynn, I. M., *Pharmacol. Rev.*, **16**, 381 (1964).

[4] Oosterbaan, R. A., and J. A. Cohen, in *Structure and Activity of Enzymes*, ed. T. W. Goodwin, J. I. Harris, and B. S. Hartley (New York: Academic Press, 1964), p. 87.

[5] Hokin, L. E., and A. Yoda, these PROCEEDINGS, **52**, 454 (1964).

[6] Hokin, L. E., A. Yoda, and R. Sandhu, submitted for publication.

[7] Baker, B. R., *J. Pharm. Sci.*, **53**, 347 (1964).

[8] Wolfsy, L., H. Metzger, and S. J. Singer, *Biochemistry*, **1**, 1031 (1962).

[9] Schoellmann, G., and E. Shaw, *Biochemistry*, **2**, 252 (1963).

[10] Lawson, W. B., and H. J. Schramm, *Biochemistry*, **4**, 377 (1965).

[11] Schaeffer, H. J., and E. Odin, *J. Pharm. Sci.*, **54**, 1223 (1965).

[12] Kupchan, S. M., M. Mokotoff, R. Sandhu, and L. E. Hokin, manuscript in preparation.

[13] Olcott, H. S., and H. Fraenkel-Conrat, *Chem. Rev.*, **41**, 151 (1947).

[14] Kahn, J. B., and G. H. Acheson, *J. Pharmacol. Exptl. Therap.*, **115**, 305 (1955).

[15] Hokin, L. E., P. S. Sastry, P. R. Galsworthy, and A. Yoda, these PROCEEDINGS, **54**, 177 (1965).

[16] Bush, I. E., and D. A. H. Taylor, *Biochem. J.*, **52**, 643 (1952).

[17] Jacobs, W. A., and A. M. Collins, *J. Biol. Chem.*, **59**, 713 (1924).

[18] Welles, J. S., R. C. Anderson, and K. K. Chen, *Proc. Soc. Exptl. Biol. Med.*, **65**, 218 (1947).

[19] Michaelis, L., and M. P. Schubert, *J. Biol. Chem.*, **106**, 331 (1934).

[20] Huggins, C., and E. U. Jensen, *J. Biol. Chem.*, **179**, 645 (1949).

[21] Dunham, E. T., and I. M. Glynn, *J. Physiol.*, **156**, 274 (1961).

[22] Hokin, L. E., and A. Yoda, *Biochim. Biophys. Acta*, **97**, 594 (1965).

[23] Caldwell, P. C., A. L. Hodgkin, R. D. Keynes, and T. I. Shaw, *J. Physiol.*, **152**, 561 (1960).

[24] Caldwell, P. C., and R. D. Keynes, *J. Physiol.*, **148**, 8P (1959).

[25] Dickens, F., *Biochem. J.*, **27**, 1141 (1933).

[26] Rapkine, L., *Compt. Rend. Soc. Biol.*, **112**, 790 (1933).

[27] Smythe, C. V., *J. Biol. Chem.*, **114**, 601 (1936).

[28] Cleland, W. W., *Biochemistry*, **3**, 480 (1964).

[29] Fox, F. C., and E. P. Kennedy, these PROCEEDINGS, **54**, 891 (1965).