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Studies on the Characterization of the Sodium-Potassium Transport Adenosine Triphosphatase

VIII. Effects of Ligands on Fluorescence Due to Interaction of the Enzyme with a Fluorescent Derivative of Hellebrigenin*

ATSUNOBU YODA AND LOWELL E. HOKIN

Department of Pharmacology, University of Wisconsin, Madison, Wisconsin 53706

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SUMMARY

The synthesis of the 1,4-sulfonaphthylhydrazone of hellebrigenin (HSNH) from hellebrigenin and 1,4-sulfonaphthylhydrazine is described. This compound is a specific inhibitor of the (Na^+ + K^+)-ATPase and serves as a hydrophobic probe of the enzyme. The effects of various ligands on the fluorescence intensity (I_f) due to the enzyme-HSNH system were examined, and many of the effects on I_f were considered to emanate from the cardiotonic steroid site on the enzyme. Among the monovalent cations only Na^+ enhanced I_f, while other monovalent cations (K^+, Rb^+, Li^+, Cs^+, Tl^+, and NH_4^+) suppressed it; the order of effectiveness of the suppressing ions paralleled the order of affinities for the K^+ site. ATP or Mg^{++} plus phosphate markedly diminished I_f. The effects of ligands were antagonized by hellibrigenin and were abolished or decreased if the enzyme was minimally denatured. The effects of the above ligands were specific for HSNH, since fluorescence due to 8-anilinonaphthalene-1-sulfonic acid or a biologically inactive analogue of HSNH, the 1,4-sulfonaphthylhydrazone of dianhydrostrophanthidin, was not affected by the cations and the effect of ATP was less.

INTRODUCTION

The mechanism whereby the (Na^+ + K^+)-activated ATPase effects the uphill movements of Na^+ and K^+ across animal cell membranes is not known. Recent models have invoked allosteric transitions coupled to cyclic phosphorylation and dephosphorylation of the enzyme to bring about translocation of the ions (1–3). The evidence supporting different conformational states of the enzyme is based on kinetics (4–6), changes in affinity for ouabain or digoxin (7, 8), and changes in the behavior of the enzyme on adding certain inhibitors and ligands (9–11). Because the enzyme has not been fully purified, direct physical evidence for different conformational states has not been obtained, although Schwartz and his collaborators claimed to have obtained some evidence for conformational changes in the membrane-bound enzyme using the nonspecific fluorescent probe ANS[1] (12) and circular dichroism (13).

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[1] The abbrevations used are: ANS, 8-anilinonaphthyl-1-sulfonic acid; HSNH, 1,4-sulfonaphthylhydrazone of hellebrigenin; DSSNH,

Fluorescence spectroscopy can give information about the polarity of the immediate environment of a fluorescent probe bound to a protein (14). Since the polarity of this environment can be influenced by the conformation of the protein, it is theoretically possible to obtain evidence for conformational changes in the protein from fluorescence spectroscopy. With some probes a shift to a more nonpolar environment increases the fluorescence intensity (I_f) emanating from the fluorescent probe and lowers the wavelength of maximum emission (15, 16).

Most studies have utilized nonspecific fluorescent probes such as ANS. Although such probes bind at only a few sites in many proteins, the binding appears to be rather nonspecific and extensive in membrane systems.

In the present study we have taken advantage of the highly specific binding of cardiotonic steroids to the $(Na^+ + K^+)$-ATPase to synthesize fluorescent derivatives which would act as probes at the cardiotonic steroid site. The formyl group at position 19 of strophanthidin and hellebrigenin offered an ideal functional group for attaching a naphthylaminesulfonic acid residue to the cardiotonic steroid via a hydrazone linkage.

Our laboratory has recently accomplished the large-scale preparation of beef brain $(Na^+ + K^+)$-ATPase of high specific activity and stability (17). The quantities of enzyme available made the concentration of cardiotonic steroid site in the preparation sufficient for measurement of fluorescence emanating from the cardiotonic steroid site.

This paper reports the synthesis of a biologically active sulfonaphthylhydrazone of hellebrigenin and describes the fluorescence elicited when this compound is added to the beef brain $(Na^+ + K^+)$-ATPase preparation in the presence of various ligands.

MATERIALS AND METHODS

Hellebrigenin and strophanthidin were prepared as described previously (18). The scheme for synthesis of the fluorescent steroids is shown in Fig. 1. Dianhydrostrophanthidin [3β-hydroxy-19-oxocarda-5, 14,20(22)-trienolide] was prepared by the method of Jacobs and Collins (19). The intermediate, 3β,19-oxidocarda-5,14,20(22)-trienolide 19-ethylal [m.p. 230–232°; recorded m.p. 249–251° (19), 229–235° (20)] and final product [m.p. 222–223°; recorded m.p. 233–236° (19), 220–223° (20)] showed only single spots on thin-layer chromatography on silica gel G (solvent, 25% acetonitrile in benzene). Infrared spectra of these compounds were coincident with the reference spectra (20). Dianhydrostrophanthidin did not inhibit the $(Na^+ + K^+)$-ATPase below 0.1 mM.

4-Sulfonaphthyl-1-hydrazine was prepared by reduction of the diazo compound derived from 1-naphthylamine-4-sulfonic acid (21, 22). The dicyclohexylammonium salt of the sulfonaphthylhydrazone of each steroid was prepared as follows: 0.5 mmole of sulfonaph-

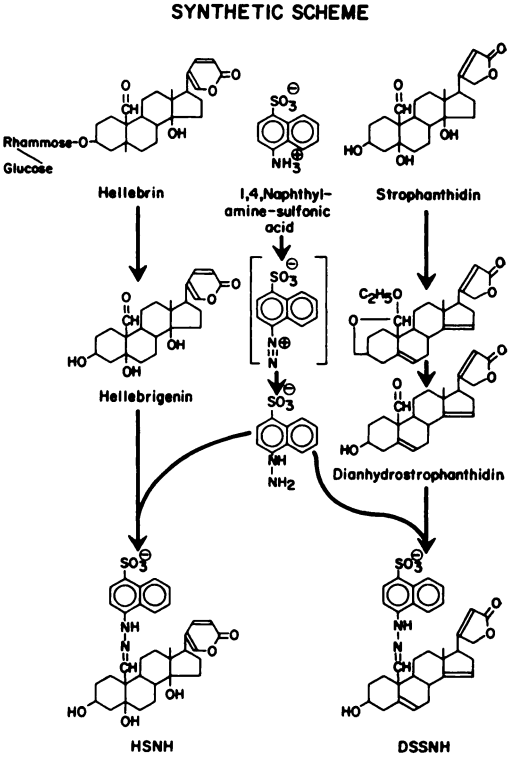

FIG. 1. *Schemes for synthesis of HSNH and DSSNH*

1,4-sulfonaphthylhydrazone of dianhydrostrophanthidin; SSNH, 1,4-sulfonaphthylhydrazone of strophanthidin; DFP, diisopropyl fluorophosphate.

thylhydrazine, 0.5 mmole of steroid (hellebrigenin or dianhydrostrophanthidin), 0.2 ml of dicyclohexylamine, 0.2 ml of acetic acid, and 10 ml of methanol were reflexed with stirring under nitrogen. After the insoluble hydrazine was converted to soluble hydrazone, the clear reaction mixture was dried under vacuum. From an ethanolic extract of this residue the dicyclohexylammonium salt of the hydrazone was precipitated with ether.

The dicyclohexylammonium salts of the 4-sulfonaphythyl-1-hydrazones of hellebrigenin and dianhydrostrophanthidin are soluble in methanol and ethanol, slightly soluble in water, and insoluble in ether. These products were tested for homogeneity by thin-layer chromatography on silica gel G [solvents, 1-butanol–acetic acid–water (4:1:2, v/v), chloroform–methanol–acetic acid (15:5:1), ethyl acetate–acetic acid–water (4:1:1), ethyl acetate–2-propanol–14 N ammonium hydroxide solution (7:9:4)]. In all solvent systems each product showed only one spot on visualization with an ultraviolet lamp (fluorescent spots) or the ceric sulfate reaction (detection of steroid). The spots detected by the above two methods coincided. They had different R_f values from the starting materials. Spectral and elemental analyses of these compounds were as follows.

HSNH: Yield, 53%. Ultraviolet spectrum: E_{max} 334 nm (ϵ 15,100), 237 nm (ϵ 19,100), 212 nm (ϵ 40,000). Infrared spectrum: 2400–3000 cm^{-1} ($=NH_2^+$), 1705 cm^{-1} (C=O of γ-pyrone), 1580 cm^{-1} (C=N of hydrazone), 1170 cm^{-1}, 1040 cm^{-1}, 680 cm^{-1} (S=O of sulfonic acid), 837 cm^{-1}, 760 cm^{-1} (naphthalene 1,4-derivative).

$C_{46}H_{63}N_3O_8S \cdot H_2O$

Calculated: C 66.07, H 7.83, N 5.02, S 3.96
Found: C 65.33, H 7.40, N 4.77, S 3.83

DSSNH: Yield, 45%. Ultraviolet spectrum: E_{max} 340 nm (ϵ 14,500), 245 nm (shoulder), 221 nm (ϵ 50,000). Infrared spectrum: 1780 cm^{-1}, 1740 cm^{-1} (C=O of unsaturated γ-lactone), 1620 cm^{-1} (C=H of α,β-unsaturated γ-lactone). Other peaks were the same as for HSNH, except the one at 1705 cm^{-1}.

$C_{45}H_{59}N_3O_6S \cdot H_2O$

Calculated: C 68.58, H 7.80, N 5.33, S 4.07
Found: C 68.24, H 7.67, N 5.11, S 4.19

The magnesium salt of HSNH was also synthesized. The synthesis and purification were the same as with the dicyclohexylammonium salt, except that 0.5 mmole of magnesium acetate was used instead of dicyclohexylamine and acetic acid. The yield was 63%. The magnesium salt of HSNH was a light pink powder which was soluble in water and methanol. The thin-layer chromatogram and spectral data were the same as with the dicyclohexylammonium salt.

$C_{34}H_{39}N_2O_8SMg_{0.5} \cdot 3H_2O$

Calculated: C 58.19, H 6.48, N 3.99, S 4.57
Found: C 57.77, H 6.03, N 3.70, S 4.95

The sulfonaphthylhydrazone of strophanthidin was synthesized as the dicyclohexylammonium salt and characterized by the same method as with HSNH. There were no qualitative differences between SSNH and HSNH in the experiments described below, although some quantitative differences were present. Only the results with HSNH are described.

All cations were added as the chlorides, and all anions were added as Tris salts. Tris salts were made by neutralization of the free acids with Tris base or by passage of sodium salts over a Dowex 50 column (Tris form).

The preparation of the beef brain "ammonium sulfate enzyme" is described elsewhere (17). Between 3 and 10 mg of the enzyme preparation (specific activity, 450–750 μmoles of P_i per hour per milligram of protein) were suspended in 10 ml of 0.1 M Tris-sulfate (pH 7.5) and sedimented by centrifugation at 105,000 $\times$ g for 60 min. After one washing, the pellet was homogenized with 10 mM imidazole HCl buffer, pH 7.5. The protein concentration in this suspension was adjusted to 2–5 mg/ml.

Fluorescence was measured with an Aminco-Bowman spectrophotofluorometer. Measurements were carried out at 23°.[2] The excitation and emission wavelengths were 330 nm and 410 nm, respectively, unless

[2] The temperature was maintained with ±0.5° by a cooling block (thermoelectric microcooler, Aminco).

otherwise indicated. Microcuvettes (3 mm square, inner diameter) were used, and sample solutions contained 10 mM imidazole HCl buffer, pH 7.5.

The fluorescence intensity (I_f) of 100 μl of the enzyme suspension was first measured; then 5 μl of 200 μM chromophore solution and 1 μl of each ligand solution were added sequentially with micropipettes. After each addition the contents of the cuvette were mixed with a polyethylene stirring rod and fluorescence was measured. The effects of ligands are expressed in terms of the enhancement of fluorescence intensity, I_e, which is defined as

$$I_e = \frac{(I_2 - I_1)}{I_0}$$

where $I_0 = I_f$ of the chromophore in buffer, $I_1 = I_f$ of the enzyme-chromophore system, and $I_2 = I_f$ of the ligand(s)-enzyme-chromophore system.

The I_f of the chromophore in buffer did not change more than 3% on adding ligands in the absence of the enzyme. I_0, I_1, and I_2 depend on the intensity of the exciting light and on the sensitivity of the instrument, which vary from day to day. In addition, I_1 varies with each batch of enzyme and is dependent on protein concentration, as shown in Fig. 2. I_e is far less dependent on protein concentration and is more reproducible among the batches of enzyme; the variation in this quantity was less than 3% within a given batch of enzyme or from batch to batch. Even so, in most instances, the same concentration of the same batch of enzyme was used in an experiment.

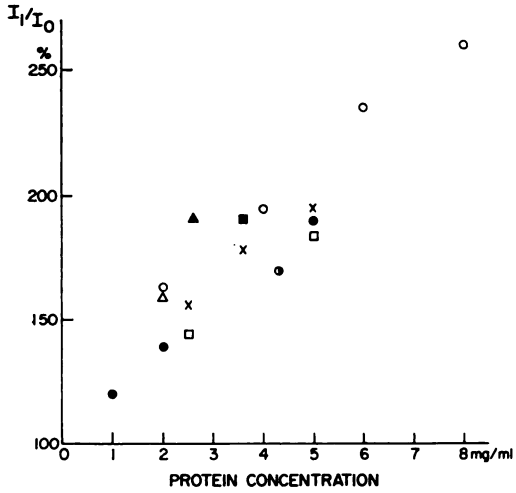

FIG. 2. *Dependence of fluorescence intensity on concentration of protein*

Different batches are represented by different symbols.

TABLE 1

Inhibition $(Na^+ + K^+)$-ATPase by HSNH and its congeners

The enzyme was assayed as reported previously (18), with the indicated concentrations of cardiotonic steroids added as aqueous solutions.

Inhibitor	Inhibition at various concentrations of steroid derivatives			
	2.5 μM	5.0 μM	10 μM	20 μM
	%	%	%	%
HSNH	40	54	66	71
SSNH	9	12	25	37
DSSNH	0	0	0	0

RESULTS

Inhibition of $(Na^+ + K^+)$-ATPase by fluorescent cardiotonic steroids. Table 1 shows the inhibition of $(Na^+ + K^+)$-ATPase by various fluorescent derivatives of cardiotonic steroids. The fluorescent derivatives of both hellebrigenin and strophanthidin (HSNH and SSNH) inhibited the enzyme. The affinity of HSNH was approximately 5 times that of SSNH; this parallels the relative affinities of the nonfluorescent parent compounds (18). The affinities of the sulfonaphthylhydrazone derivatives were two orders of magnitude less than those of the parent compounds. The sulfonaphthylhydrazone of the inactive steroid, DSSNH, did not inhibit up to 20 μM.

Effects of solvent and proteins on fluorescence spectra of sulfonaphthylhydrazones of cardiotonic steroids. The fluorescent spectra of the sulfonaphthylhydrazones of the cardiotonic steroids are shown in Fig. 3. They show properties similar to those of ANS (16): fluorescence was enhanced and the wavelength of maximum emission showed an appreciable blue shift with ethanol, albumin, or $(Na^+ + K^+)$-ATPase, and a smaller change with chymotrypsin or ribonuclease.[3] These data indicated that the sulfonaphthyl-

[3] The emission spectra of the chromophores were the same with either chymotrypsin or ribonuclease.

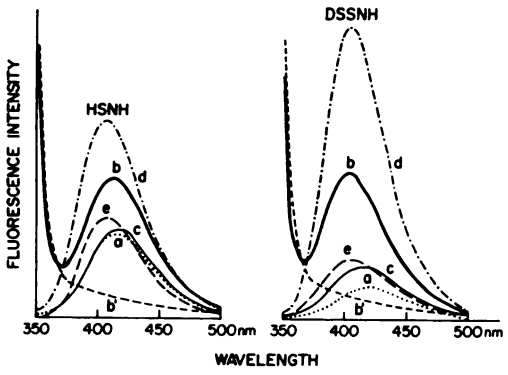

FIG. 3. *Emission spectra of HSNH and DSSNH*

The excitation wavelength was 330 nm. Curve *a*, 10 μM chromophore in 10 mM imidazole HCl buffer (pH 7.5); *b*, 10μM chromophore + 4.3 mg/ml of $(Na^+ + K^+)$-ATPase in the same buffer; *b'*, 4.3 mg/ml of $(Na^+ + K^+)$-ATPase in the same buffer without chromophore; *c*, 10 μM chromophore + 4.3 mg/ml of chymotrypsin in the same buffer; *d*, 10μM chromophore + 4.3 mg/ml of bovine serum albumin in the same buffer; *e*, 10 μM chromophore in 95% ethanol.

hydrazones should serve as hydrophobic probes.

Effects of various cations on fluorescence. Figure 4 depicts a typical continuous recording of fluorescence. The enzyme alone exhibited low fluorescence (not shown). The addition of 10 μM HSNH markedly enhanced I_f (curve *a*), and the addition of DSSNH to the enzyme enhanced I_f even more than with HSNH (curve *b*). ANS enhanced fluorescence many fold (curves *c* and *d*). The important point, however, was the characteristic change in I_f of the HSNH-enzyme system when various ligands were added, in contrast to the lack of a comparable change in the DSSNH-enzyme system. The addition of 10 mM Na^+, 2 mM K^+, 1 mM Mg^{++}, or 20 μM ATP to the HSNH-enzyme system produced significant changes in I_f: Na^+ and Mg^{++} enhanced I_f while ATP and K^+ reduced it. The same sequence of addition of ligands to the DSSNH-enzyme system was without effect on I_f except for the addition of Mg^{++}, which enhanced I_f slightly. The addition of ions to either HSNH or DSSNH in the absence of enzyme had no effect on I_f.

As shown in curves *c* and *d* (Fig. 4), Na^+ or Mg^{++} enhanced the I_f of the enzyme-ANS system, although neither ATP nor K^+ reduced it. Nagai *et al.* (12) found that ATP and K^+ reduced the I_f of a membrane-bound $(Na^+ + K^+)$-ATPase–ANS system. As shown in curve *d*, we found no effect of these ligands on our enzyme-ANS system with the same sequence of additions used by those authors. In a sarcoplasmic reticulum–ANS system, Vanderkooi and Martonosi (23) showed nonspecific enhancing effects of cations on I_f. These effects of cations appeared to be due primarily to the hydrophobic environment afforded by the phospholipids in the membrane. The effects of cations increased with charge. Similar ion effects were also reported in erythrocyte membranes (24). The effects observed here with ANS are presumably due to similar causes.

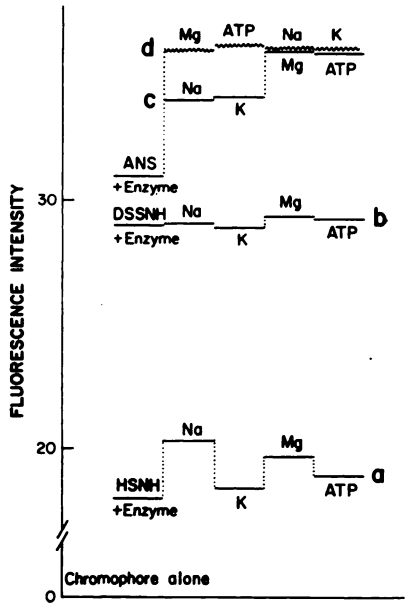

FIG. 4. *Effects of ligands on I_f of $(Na^+ + K^+)$-ATPase and chromophores*

The enzyme concentration was 3.6 mg/ml in each experiment. The chromophore (10 μM), NaCl (10 mM), KCl (2 mM), $MgCl_2$ (1 mM), or ATP (20 μM) was added sequentially as indicated. In curve *d* the sequence was different. With ANS (curves *c* and *d*), the excitation and emission wavelengths were 390 nm and 490 nm, respectively. With the other probes, the respective wavelengths were 330 nm and 410 nm. In each curve, the fluorescence intensity due to the chromophore alone is subtracted.

Effects of sodium, potassium, and potassium substitutes on I_f. Figure 5 shows the effects of Na^+ on I_f in the absence and presence of various monovalent cations. Among various monovalent cations, only Na^+ enhanced I_f. The concentration of

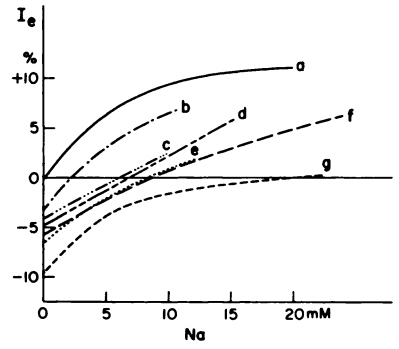

FIG. 5. *Effects of sodium on enhancement of I_f in HSNH-enzyme system in the absence and presence of other monovalent cations*

Curve *a*, no other cations added; *b*, with 1 mM CsCl; *c*, 5 mM LiCl; *d*, with 1 mM RbCl; *e*, with 1 mM NH_4Cl; *f*, with 1 mM KCl; *g*, with 1 mM TlCl. The enzyme concentration was 3.2–3.8 mg/ml. In this experiment, Na^+ was added after other monovalent cations.

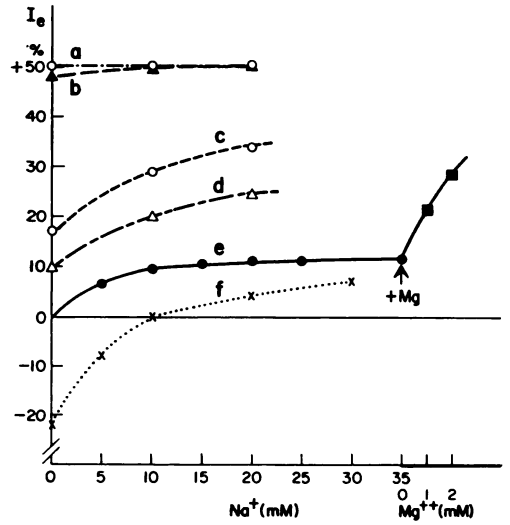

FIG. 6. *Effects of sodium on enhancement of I_f in HSNH-enzyme system in the presence of magnesium, calcium, or ATP*

Curve *a*, with 4 mM Ca^{++}; *b*, with 3 mM Mg^{++}; *c*, with 1 mM Ca^{++}; *d*, with 0.5 mM Mg^{++}; *e*, without any other ions, and magnesium effect after saturation with Na^+; *f*, with 30 μM ATP. The enzyme concentration was 2.0 mg/ml.

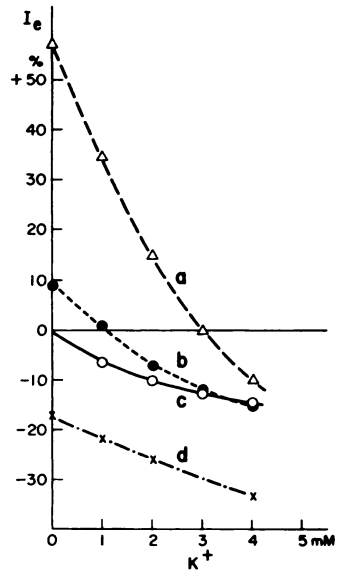

FIG. 7. *Effects of potassium on enhancement of I_f in HSNH-enzyme system in the presence of other ligands*

Curve *a*, with 3 mM Mg^{++} (the enzyme concentration was 2.0 mg/ml); *b*, with 10 mM Na^+ (the enzyme concentration was 3.2 mg/ml); *c*, without any other ligands (the enzyme concentration was 3.2 mg/ml); *d*, with 20 μM ATP (the enzyme concentration was 3.9 mg/ml).

Na^+ for half-maximal enhancement of I_f was 5 mM. This is close to the concentration for half-maximal activation of the enzyme. This Na^+ effect on I_f was obtained in the presence of other monovalent cations, low concentrations of Mg^{++} or Ca^{++}, or ATP (Figs. 5 and 6). However, Na^+ did not enhance I_f at high concentrations of divalent cations.

In contrast to Na^+, K^+ depressed I_f (Figs. 5 and 7 and Table 2). Monovalent cations other than Na^+ can substitute for K^+ in activating the (Na^+ + K^+)-ATPase. These cations also substituted for K^+ in depressing I_f, in the following order of effectiveness: $Tl^+ > K^+ \cong NH_4^+ > Rb^+ > Cs^+ > Li^+$. This order parallels that for activating the K^+ site on the enzyme (25–27). The concentrations of the cations effective in depressing I_f were less than 1 mM, with the exception of Li^+. The K^+ effect on I_f was not qualitatively affected by the other ligands (Fig. 7).

Effects of magnesium, calcium, and phos-

TABLE 2

Effects of monovalent cations on HSNH-enzyme system in the presence of sodium

The enzyme concentration was 3.2–3.8 mg/ml.

Additional ion	I_f produced by each ion added after 5 mM Na^+	Δ^a	I_f produced in absence of Na^{+b}
	%	%	%
None	+7 (±0.5)		
1 mM Li^+	+6.5	−0.5	<−1
5 mM Li^+	+4	−3	−4
1 mM Cs^+	+2	−5	−3
1 mM Rb^+	−3	−10	−5
1 mM K^+	−5	−12	−6
1 mM NH_4^+	−6	−13	−7
1 mM Tl^+	−9	−16	−10

[a] Increment in I_f above I_f with 5 mM Na^+ alone.
[b] Data taken from Fig. 5.

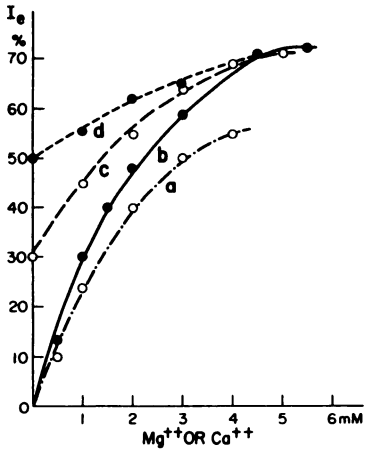

FIG. 8. *Effects of Mg^{++} and Ca^{++} on enhancement of I_f in HSNH-enzyme system*

Curve *a*, Ca^{++} alone; *b*, Mg^{++} alone; *c*, Ca^{++} effect with 1 mM Mg^{++}; *d*, Mg^{++} effect with 3 mM Ca^{++}. The enzyme concentration was 2.0 mg/ml.

phate on I_f. Either Mg^{++} or Ca^{++} markedly enhanced I_f (Fig. 8). These divalent cations were considerably more effective than Na^+. Ca^{++} was slightly less effective than Mg^{++}. The effects of these ions were partially additive. The Mg^{++} effect was not affected by Na^+ (Fig. 6, curve *e*), K^+, or ATP (Fig. 9), but P_i depressed the Mg^{++} effect considerably. Phosphate alone depressed I_f slightly (Fig. 10).

One explanation for the increased depression of I_f by P_i in the presence of Mg^{++} is complex formation between Mg^{++} and P_i. Calculations of the free Mg^{++} concentration from the apparent stability constant of the

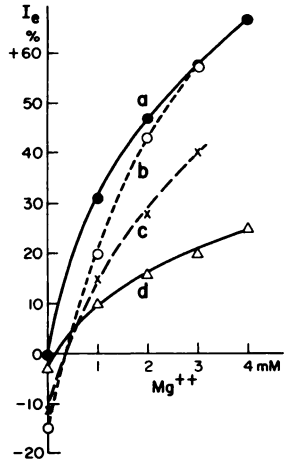

FIG. 9. *Effects of magnesium on enhancement of I_f in HSNH-enzyme system in the presence of other ligands*

Curve *a*, Mg^{++} alone (the enzyme concentration was 2.0 mg/ml); *b*, with 20 μM ATP (the enzyme concentration was 3.9 mg/ml); *c*, with 2 mM K^+ (the enzyme concentration was 2.0 mg/ml); *d*, with 5 mM phosphate (the enzyme concentration was 4.5 mg/ml).

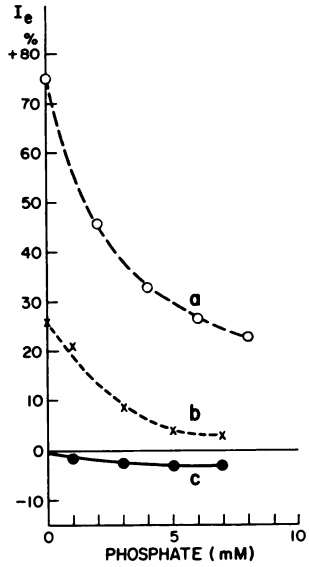

FIG. 10. *Effects of phosphate on enhancement of I_f in HSNH-enzyme system in the presence of Mg^{++}*

Curve *a*, with 7 mM Mg^{++}; *b*, with 1 mM Mg^{++}; *c*, with phosphate alone. The enzyme concentration was 4.5 mg/ml.

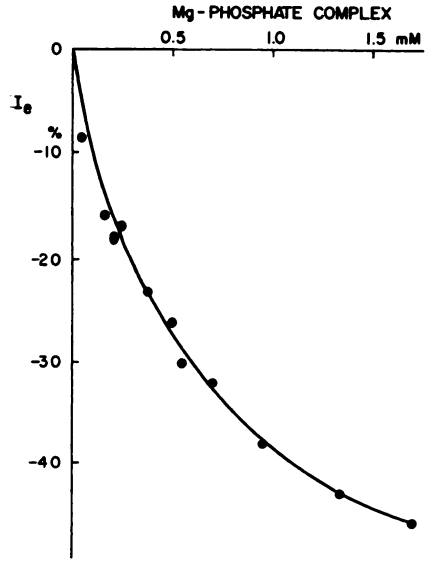

FIG. 11. *Effects of magnesium-phosphate complex ($MgHPO_4$) on enhancement of I_f in HSNH-enzyme system*

All points in this figure were calculated from the measured value shown in curve *d* of Fig. 9 and curves *a*, *b*, and *c* of Fig. 10. The concentration of the magnesium-phosphate complex was calculated from the stability constant (76 M^{-1}) (28) and the K_{a_2} of phosphate. I_e was also corrected for fluorescence changes due to free Mg^{++} and phosphate ions.

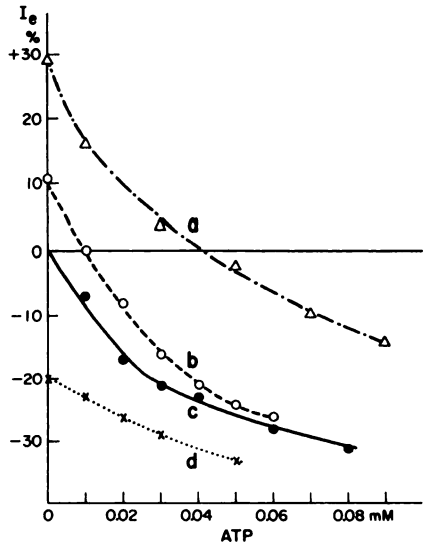

FIG. 12. *Effects of ATP on enhancement of I_f in HSNH-enzyme system in the presence of other ligands*

Curve *a*, with 1 mM Mg^{++}; *b*, with 10 mM Na^+; *c*, with ATP alone; *d*, with 2 mM K^+. The enzyme concentration was 3.7 mg/ml.

magnesium-phosphate complex (76 M^{-1}) (28) and the K_{a_2} of phosphate at various concentrations of P_i and Mg^{++} showed that the reductions in free Mg^{++} would be too slight to account for the fluorescence changes. On the other hand, as shown in Fig. 11, the depression of I_f correlated extremely well with the calculated concentration of the magnesium-phosphate complex after correction for fluorescence changes due to free Mg^{++} and P_i. The magnesium-phosphate complex is therefore probably the ligand responsible for the depression of fluorescence in the presence of Mg^{++} and P_i. This depression in fluorescence may be due to a conformational change in the $(Na^+ + K^+)$-ATPase affecting the cardiotonic steroid site. In this connection it has been shown recently that the magnesium-phosphate complex is the ligand responsible for binding of ouabain (measured by inhibition of the enzyme) to the $(Na^+ + K^+)$-ATPase in the presence of Mg^{++} and P_i.[4]

Effects of ATP on I_f. If ATP was added initially to the HSNH-enzyme system, there was a marked diminution in I_f (Fig. 12). This effect was achieved with concentrations of ATP as low as 10 μM. After the addition of ATP, Na^+ enhanced I_f in a manner parallel to that seen with Na^+ alone (see Fig. 6). If I_f was first enhanced with Na^+, ATP diminished it, and this effect paralleled the diminution in I_f produced by ATP alone. Similar results were obtained with Mg^{++} (see also Fig. 9, curve *b*). ATP still depressed I_f after K^+ addition (see also Fig. 7, curve *d*).

GTP, UTP, and ADP were only about one-fifth as effective as ATP in reducing I_f in the enzyme-HSNH system (Fig. 13). The weaker nucleotides did not differ among themselves in depressing I_f. AMP was only about 4% as effective as ATP in depressing I_f.

Effects of hellebrigenin on fluorescence. Hellebrigenin, which has roughly 100 times the affinity of HSNH for the enzyme, would be

[4] A. Yoda, unpublished observations.

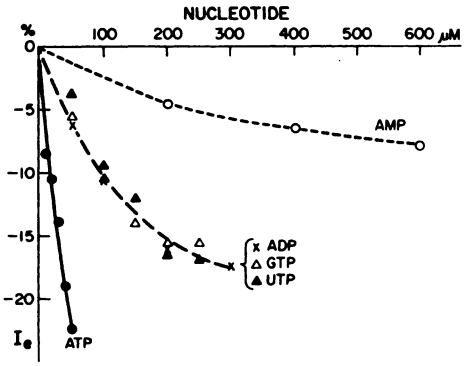

FIG. 13. *Effects of various nucleotides on enhancement of I_f in HSNH-enzyme system*

All measurements were carried out in the presence of 10 mM Na^+. The enzyme concentration was 2.0 mg/ml.

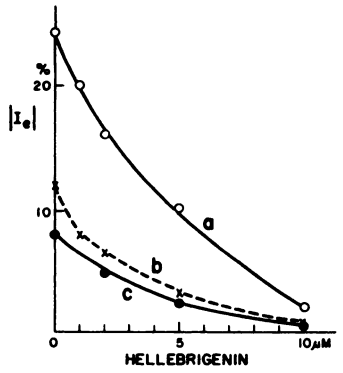

FIG. 14. *Antagonism of ion effects by hellebrigenin*

Curve *a*, enhancement with 10 mM Na^+ and 1 mM Mg^{++}; *b*, enhancement with 10 mM Na^+; *c*, depression with 1 mM K^+. Hellebrigenin was added in 20% dimethyl sulfoxide before the cation. The enzyme concentration was 3.5 mg/ml.

expected to antagonize the effects of the cations on fluorescence if the latter were related to fluorescence emanating from the specific cardiotonic site on the $(Na^+ + K^+)$-ATPase. As can be seen in Fig. 14, the addition of hellebrigenin prior to ions reduced the effects of the latter; at 10 μM hellebrigenin, all effects of ions on I_f were abolished. Hellebrigenin added after the ions also reversed their effect.

As shown in Fig. 15, the depressant effect of ATP on I_f was also counteracted by hellebrigenin. At 50 μM ATP, 5 μM hellebrigenin reduced the ATP effect by two-thirds.

Effects of ligands after incubation of enzyme with DFP or at low pH. It was previously shown (29) that DFP irreversibly inhibits the $(Na^+ + K^+)$-ATPase. Although the mechanism of this inhibition is not known [it does not appear to be due to liberated F^-, as has been suggested (30, 31), since organophosphorus compounds not containing F^- function analogously to DFP], it is unlikely to lead to extensive denaturation of the enzyme, since DFP generally is without effect on enzymes other than those containing an activated serine residue at the substrate site. As can be seen in Table 3, treatment of the enzyme with DFP under conditions similar to those reported previously (29) (with the present enzyme preparation, DFP treatment resulted in 99.9% inactivation) essentially abolished the effects of Na^+, K^+, and Mg^{++} on fluorescence. However, about half the ATP effect could still be observed. Nagai *et al.* (12) observed an effect of ATP or Mg^{++} on I_f in a heat-denatured $(Na^+ + K^+)$-ATPase–ANS system.

Treatment of the enzyme preparation at pH 4.5 and 25° is also likely to lead to minimal denaturation. Treatment of the enzyme under the conditions described in Table 2 led to 93% inactivation. Again, the effects of the cations on fluorescence were abolished, but about half the ATP effect remained.

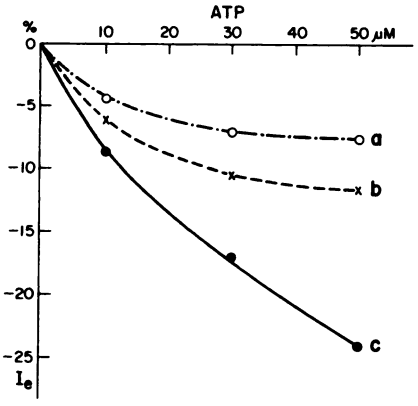

FIG. 15. *Antagonism of ATP effect by hellebrigenin*

Curve *a*, with 5 μM hellebrigenin; *b*, with 1 μM hellebrigenin; *c*, without hellebrigenin. All experiments were carried out in the presence of 10 mM Na^+. The concentration of enzyme was 2.4 mg/ml.

TABLE 3

Effects of enzyme inactivation on enhancement of fluorescence by various ligands

The enzyme concentration was 2.5–3.6 mg/ml.

Prior treatment	I_e				
	10 mM Na^+	2 mM K^+[a]	1 mM Mg^{++}[b]	20 μM ATP[a]	40 μM ATP[a]
	%	%	%	%	%
None[c]	+11 (±2)	−18 (±3)	+27 (±3)	−18 (±2)	−33 (±3)
Low pH[d]	0	−1	+2	−7.5	−16
DFP[d]	0	−3	+4	−9.5	−15

[a] In the presence of 10 mM Na^+.

[b] In the presence of 10 mM Na^+ and 2 mM K^+.

[c] The average value of six experiments.

[d] See the text.

DISCUSSION

Fluorescent probes (a) can provide information about the polarity of their immediate environments and (b) may also shed light on conformational changes in the protein that can affect the polarity of these environments. Both types of information would be of considerable interest with regard to the $(Na^+ + K^+)$-dependent ATPase. The former might help to elucidate the nature of the drug-receptor interaction, and the latter, the mechanism by which the enzyme effects translocation of Na^+ and K^+.

One drawback to fluorescence probes in general use, such as ANS, is the relatively nonspecific nature of their binding. Because of this we have synthesized a biologically active "site-directed fluorescent probe," HSNH, which has a sulfonaphthylhydrazone group at position 19 of hellebrigenin. It was felt that the very high affinity of hellebrigenin for the cardiotonic steroid site of the $(Na^+ + K^+)$-ATPase might minimize fluorescence due to nonspecific binding. DSSNH, a sulfonaphthylhydrazone of a structurally related but biologically inactive steroid, was also synthesized to serve as a control.

Several lines of evidence suggest that at least part of the fluorescence due to the $(Na^+ + K^+)$-ATPase–fluorescent probe system emanates from the cardiotonic steroid site. (a) All cations which bind at the K^+ site but not at the Na^+ site acted like K^+ in depressing fluorescence, and their effectiveness in this respect paralleled their effectiveness for activation of the enzyme. (b) Sodium, which binds at a different site, had an opposite effect on fluorescence; i.e., it enhanced it (c) Hellebrigenin, which has a higher affinity for the enzyme than HSNH by two orders of magnitude, and which would be expected to displace HSNH from its site, abolished all effects of ions on fluorescence of the enzyme-HSNH system. (d) The fluorescence due to interaction of a substance which is closely related structurally to HSNH (the sulfonaphthylhydrazone of dianhydrostrophanthidin), but which is biologically inactive, was unaffected by any cation. (e) ATP markedly diminished fluorescence. Other nucleotide triphosphates, ADP, and AMP were far less effective. Hellebrigenin counteracted up to two-thirds of the ATP effect. (f) In the presence of Mg^{++}, phosphate markedly reduced fluorescence; phosphate alone had virtually no effect on fluorescence. Mg^{++} and phosphate together are known to affect the conformation of the $(Na^+ + K^+)$-ATPase so as to increase cardiotonic steroid binding (7, 8). (g) Minimal denaturation of the enzyme by treatment with DFP or by incubation at 25° and pH 4.5 abolished the ion effects on fluorescence and roughly halved the ATP effect.

It seems likely that at least some of the effects on fluorescence summarized above are due to conformational changes in the enzyme induced by the various cations and nucleotides studied. These conformational changes might lead to an increased or decreased polarity in the immediate environment of the fluorescent probe. Nothing can

be stated at this time about the nature of these conformational changes.

It is difficult in all cases to distinguish between fluorescence intensity changes due to increased or decreased binding of the fluorescent probe and fluorescence intensity changes due to conformational changes in the (Na^+ + K^+)-ATPase. For example, part of the fluorescence enhancement due to Na^+ and Mg^{++}, on one hand, and the diminution in fluorescence due to K^+, on the other, could be attributed to their respective effects in enhancing or diminishing the binding of the cardiotonic steroid (see, for example, refs. 3, 7, 8, 10, 11, 18).

However, the fluorescence effects of ATP and phosphate (in the presence of Mg^{++}) cannot be explained in this manner, since these compounds markedly reduced fluorescence but are known to enhance binding of cardiac glycosides (1–3, 7, 8, 10). It should also be pointed out that Na^+, Mg^{++}, and ATP act more or less independently on fluorescence; yet they act cooperatively in enhancing the binding of cardiac glycosides (18).

There was a very high "blank" fluorescence due to interaction of the enzyme with all the fluorescent probes, including those which have no affinity for the cardiotonic steroid site. It is therefore likely that a high percentage of this fluorescence was due to nonspecific binding of the fluorescent steroids. This is not surprising in view of the high hydrophobicity of both the steroids and the enzyme [analysis of the roughly half-pure enzyme used here indicates 25% phospholipid (17)]; moreover, the unbound chromophores showed some fluorescence. Consequently, only the effects of ions and nucleotides on fluorescence can be regarded as meaningful. Although these effects were small in comparison with the blank fluorescence, they were highly reproducible (effects of ions and nucleotides on fluorescence agreed within 3%).

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