Biochimica et Biophysica Acta, 555 (1979) 299—306

BBA 78442

DIRECT PHOTOAFFINITY LABELING OF THE PRIMARY REGION OF THE OUABAIN BINDING SITE OF ($Na^+ + K^+$)-ATPase WITH [^{3}H]OUABAIN, [^{3}H]DIGITOXIN AND [^{3}H]DIGITOXIGENIN

BLISS FORBUSH III and JOSEPH F. HOFFMAN

Department of Physiology, Yale University School of Medicine, New Haven, CT 06510 (U.S.A.)

(Received December 28th, 1978)

Key words: Ouabain-binding site; ($Na^+ + K^+$)-ATPase; Photoaffinity labeling; Digitoxin; Digitoxigenin

Summary

The tritiated cardiotonic steroids, ouabain, digitoxin, and digitoxigenin are shown to photolabel the large polypeptide but not the glycoprotein or proteolipid component of the ($Na^+ + K^+$)-ATPase when they are bound to the inhibitory site and exposed to light of 220 or 254 nm. The extent of photolabeling is low, less than 1%, and is limited by photocross-linking of the enzyme. The mechanism of photoincorporation does not appear to be either photolysis of the lactone ring in ouabain or photolysis of tryptophan or tyrosine residues in the polypeptide.

Structure activity studies of cardiac glycoside binding to ($Na^+ + K^+$)-ATPase have led to the conclusion that the binding site has two functionally distinct regions [1]. A primary region recognizes the lactone ring and steroid portions of the inhibitor molecule, the principal determinants of inhibitory potency, and a secondary region binds the sugar groups that confer the property of slow reversibility on the cardiac glycosides. We have recently shown that a 2-nitro-5-azidobenzoyl derivative of ouabain (NAB-ouabain) photolabels both the large polypeptide subunit ($M_r \approx 95\,000$) and a small proteolipid component ($M_r \approx 12\,000$) of the ($Na^+ + K^+$)-ATPase with 15—20% efficiency, but does not appreciably label the glycoprotein subunit ($M_r \approx 35\,000$) [2]. Since the photoaffinity azido group is attached to the sugar in NAB-ouabain, the large polypeptide and proteolipid apparently comprise the secondary region of the cardiac glycoside binding site.

Abbreviations: NAB-ouabain, 2-nitro-5-azidobenzoyl derivative of ouabain; SDS, sodium dodecyl sulfate.

To answer the question as to the subunit location of the primary region of the binding site, we have pursued the observation of Ruoho and Kyte [3] that when a derivative of [^{14}C]cymarin reversibly bound to the ($Na^+ + K^+$)-ATPase was exposed to short-wave ultraviolet light, about 1% of the [^{14}C]glycoside became covalently incorporated into the large polypeptide chain of the enzyme. Due to lack of resolution of low molecular weight polypeptides, and to migration of the [^{14}C]cymarin derivatives to the bottom of the gel, it is not possible to tell if significant labeling of the proteolipid occurred. It was suggested to us by Dr. Boyd Haley that the mechanism of photolabeling by the cymarin derivatives could involve photolysis of the lactone ring: if so, other cardiotonic steroids should also behave as low-efficiency 'direct' photolabels. We report here that [^{3}H]ouabain, [^{3}H]digitoxin, and [^{3}H]digitoxigenin do indeed photolabel the large chain of the enzyme when they are bound to the inhibitory site and exposed to light of 220 or 254 nm. The proteolipid is not labeled, indicating that the primary region of the ouabain binding site is on the large polypeptide chain.

Purification of ($Na^+ + K^+$)-ATPase by the method of Jorgensen [5], ATPase assay, ouabain binding, and gel electrophoresis were performed as described previously [2]. The enzyme purified from pig kidney outer medulla had a specific activity of 700—900 μmol P_i/mg per h and bound 1.4—2.0 nmol ouabain/mg protein. [^{3}H]Ouabain and [^{3}H]digitoxin were obtained from NEN (New England Nuclear Co.); their specific activities were 10 and 11 Ci/mmol and their purities were 90% and 92%, respectively, as determined by the method of Hansen and Skou [6]. [^{3}H]Digitoxigenin was prepared by hydrolysis of 100 mCi of [^{3}H]digitoxin in 1 ml 90% ethanol containing 0.6 N HCl, for 12 h at 20°C [7]. Less than 1% of the tritium remained at the position of digitoxin on thin-layer chromatography (silica gel; ethylacetate saturated with H_2O), and 93% was in a spot corresponding to authentic digitoxigenin. Photolysis was performed with the short-wave (254 nm) ultraviolet bulb of a UVSL-25 lamp (Ultraviolet Products, San Gabriel, CA), without a filter. Samples were photolyzed 1—2 cm from the lamp: (a) from the side in quartz test tubes with magnetic stirrer; (b) from above in open polyethylene vial caps (1 cm × 1 cm), or (c) from below in flat-bottomed quartz tubes. A hydrogen lamp provided light of peak wavelength 220 nm (26 nm half-bandwidth) when filtered with 0.5 cm of 1.0% acetone, 3.3% $NiCl_2$, or light of 270—380 nm when filtered with a Corning UG-11 filter. Care was taken to keep the pathlength in the sample short or concentrations of absorbing species (ATP, ouabain, protein) low, so that light absorption by the sample did not exceed 40%.

In a typical direct photolabeling experiment 1.4 mg ($Na^+ + K^+$)-ATPase, 8.4 nmol [^{3}H]ouabain, 5 mM Mg^{2+}, 3 mM P_i, 2 mM EDTA, and 10 mM Tris (pH 7.5) in a volume of 5 ml were incubated for 45 min at 37°C. The protein was pelleted by centrifugation (120 000 × g, 2 h), washed once to remove unbound [^{3}H]ouabain, and resuspended in 2.1 ml of the binding medium without [^{3}H]ouabain. Control experiments have shown that ouabain bound under these conditions is bound specifically at the inhibitory site (cf. Ref. 2). The suspension was photolyzed in a nearly horizontal quartz test tube (maximum light pathlength in suspension = 2 mm) 2 cm from the 254 nm lamp at 0°C; 0.2 ml aliquots were removed at times from 0 to 30 min. The protein was collected by

filtration on 0.08 μm Nucleopore filters, solubilized in SDS, and submitted to SDS gel electrophoresis. Fig. 1 presents a scan of the Coomassie blue-stained gel of a sample that was photolyzed 2 min, and the tritium-labeling profile of the same gel. It is seen that the large polypeptide (marked 'a') but not the glycoprotein (marked 'b') is photolabeled; this is the same labeling pattern seen with [^{14}C]cymarin derivatives (see Ref. 3). In addition it is seen here that there are no significant counts at the position of the proteolipid (marked 'c') although the low molecular weight component is well resolved on gels of this composition [2]. The labeling profile was identical in unstained gels, except for a much higher background due to free [^{3}H]ouabain, and a large peak of free [^{3}H]ouabain that migrated variably to a position between the origin and the glycoprotein, depending on specific gel conditions.

Similar results to those in Fig. 1 were obtained when ouabain binding took place in the presence of 120 mM sodium, 3 mM MgATP. With either binding condition, covalent labeling did not occur if excess unlabeled ouabain (10^{-4} M) was included in the binding medium, or if photolysis was omitted. Furthermore, covalent labeling was directly proportional to the amount of [^{3}H]-

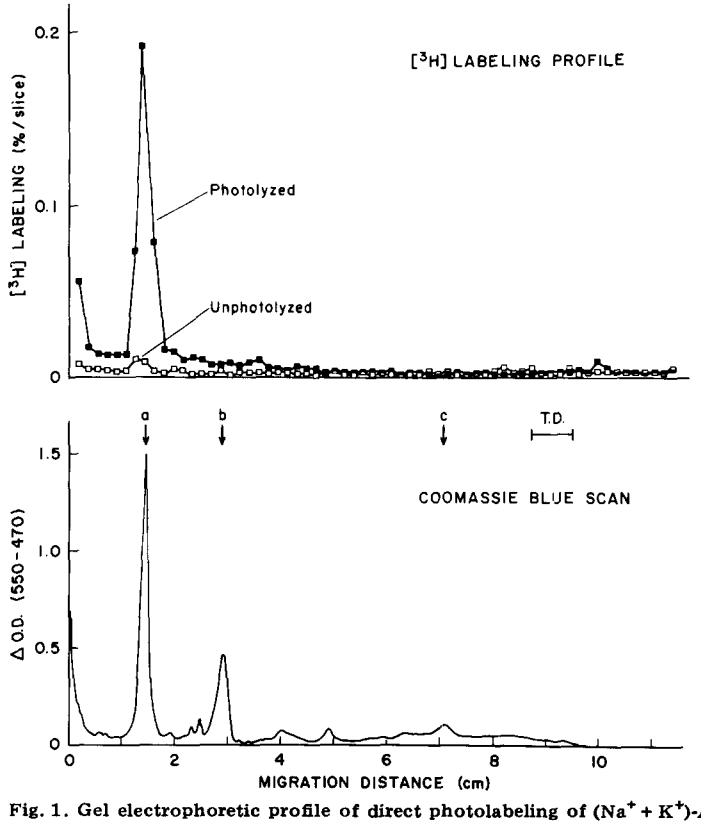

Fig. 1. Gel electrophoretic profile of direct photolabeling of ($Na^+ + K^+$)-ATPase by [^{3}H]ouabain. Experimental conditions are described in the text. Lower curve. Spectrophotometric scan of the gel stained with Coomassie blue and destained. Upper curve. ^{3}H-labeling profile in the same gel, sliced and digested in H_2O_2. Labeling in each slice is expressed as percent of the original reversibly bound [^{3}H]ouabain. $5 \cdot 10^5$ cpm were applied to the gel track.

ouabain bound to the $(Na^+ + K^+)$-ATPase as shown in the following experiment: $(Na^+ + K^+)$-ATPase (720 μg) was incubated with $1.28 \cdot 10^{-8}$ M [^{3}H]-ouabain in 10 ml of solution containing 5 mM Mg^{2+}, 3 mM P_i, 2 mM EDTA, 0.1% bovine serum albumin, 60 mM Tris, pH 7.2 at 37°C. At time intervals from 15 s to 30 min [^{3}H]ouabain binding was stopped by addition of 0.1 ml 10^{-3} M unlabeled ouabain to 1 ml aliquots of the binding medium; all samples were cooled, photolyzed for 6 min, filtered, solubilized in SDS, and submitted to gel electrophoresis. Fig. 2 shows that covalent labeling of the large polypeptide chain determined from sliced gel tracks increases with time in exact parallel to the increase in [^{3}H]ouabain binding determined from total counts on the nucleopore filters; this demonstrates that the [^{3}H]ouabain that is bound to the inhibitory site is responsible for photolabeling. The results also show that [^{3}H]ouabain does not leave the inhibitory site and equilibrate with ouabain in solution between the time of light absorption and the time of covalent attachment.

The extent of direct photolabeling of the large polypeptide is very low, about 0.3% in the sample of Fig. 1 and 0.2% in Fig. 2. Longer photolysis times gave increased incorporation, but concurrent photocross-linking of the $(Na^+ + K^+)$-ATPase resulted in disappearance of the large polypeptide and glycoprotein bands and an increase in the amount of protein that did not enter the gel. The severity of the problem, also mentioned by Ruoho and Kyte [3], is illustrated in Fig. 3; photoinactivation of $(Na^+ + K^+)$-ATPase activity, photolysis of the lactone ring of ouabain, disappearance of the large polypeptide band, and

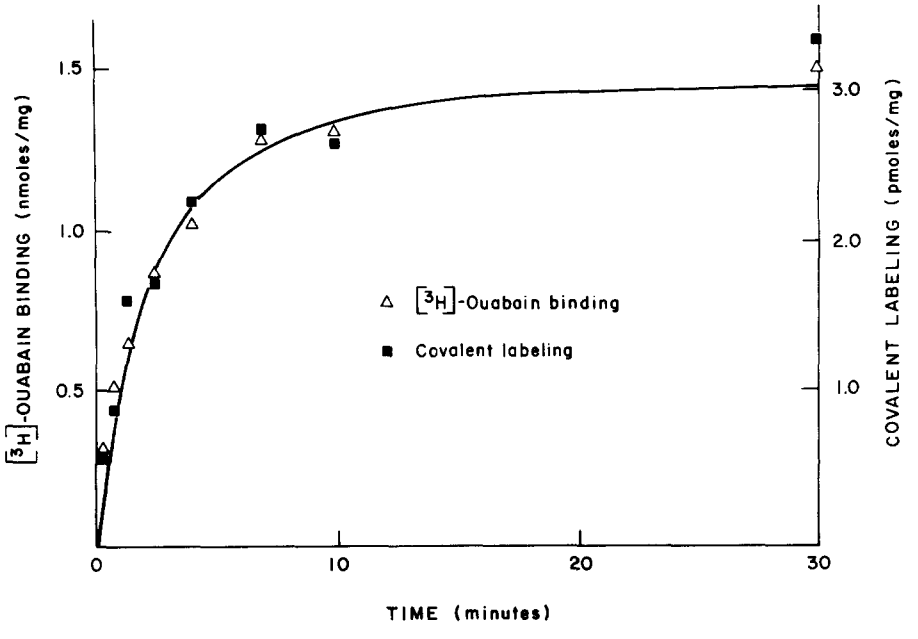

Fig. 2. [^{3}H]Ouabain binding and covalent labeling as a function of incubation time. The experimental protocol is described in the text. The line is a theoretical fit for a bimolecular reaction between ouabain and $(Na^+ + K^+)$-ATPase assuming total binding sites = 1.77 nmol/mg, $K_d = 5 \cdot 10^{-9}$ M, forward reaction rate constant = $5.33 \cdot 10^4$ $s^{-1} \cdot M^{-1}$.

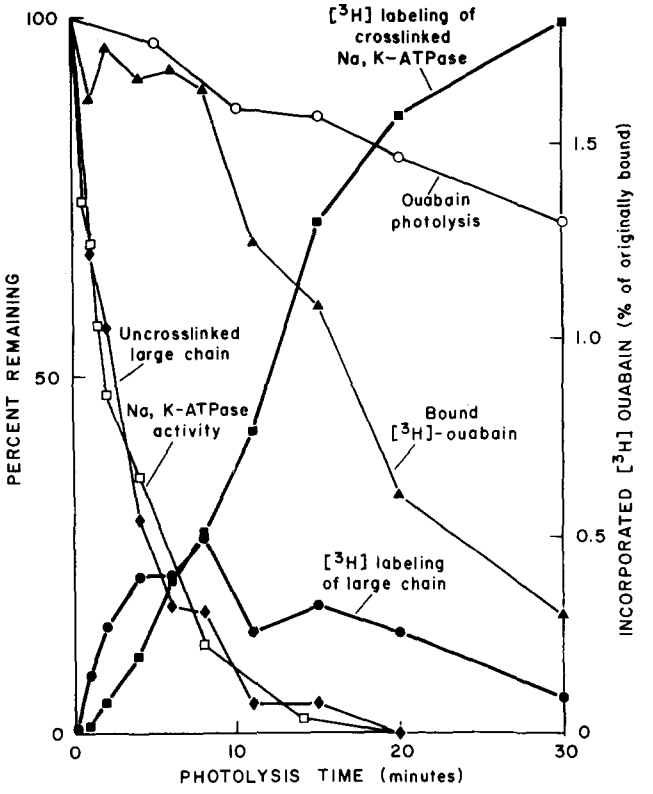

Fig. 3. Time course of effects of 254 nm light on ouabain, $(Na^+ + K^+)$-ATPase, and on [^{3}H]ouabain bound to $(Na^+ + K^+)$-ATPase. All samples were photolyzed in a quartz test tube (longest pathlength 1 cm) with magnetic stirrer, in an ice-filled quartz beaker 2 cm from the 254 nm lamp. ○———○, 10^{-4} M ouabain in H_2O was photolyzed and absorbance spectra were determined at intervals. The change in absorbance at 220 nm is plotted with 100% = 1.62 *A* and 0% = 0.62 *A*. (The absorbance of photoproducts extrapolated from long exposures.) □———□, $(Na^+ + K^+)$-ATPase (50 mg/ml) was photolyzed in 10 mM Tris, 1 mM EDTA (pH 7.5 at 20°C) at 0°C. $(Na^+ + K^+)$-ATPase activity, plotted as percent of control, was assayed at intervals. ▲,■,◆,●, [^{3}H]ouabain (0.84 nmol) was bound to $(Na^+ + K^+)$-ATPase (1.2 mg, approx. 1.7 nmol) in 0.8 ml of MgP_i binding medium for 15 min at 37°C, then diluted to 10 ml. 1 ml aliquots were photolyzed for indicated time intervals, filtered on 0.08 μm Nucleopore filteres, and washed with 1 ml 25 mM Tris (20°C). The amount of [^{3}H]ouabain remaining on the filtered protein is plotted (▲———▲). The protein was solubilized in SDS sample buffer, electrophoresed, stained, destained, scanned and sliced as previously described [2]. The amount of uncross-linked protein migrating to the position of the large polypeptide chain is determined as the area under the peak in the spectrophotometric scan of the Coomassie blue-stained gel and ploted as percent of the unphotolyzed control (◆———◆). The ^{3}H-label above background in the sliced gel at the origin (■———■) and at the position of the large polypeptide chain (●———●) is plotted as percent of original reversibly bound [^{3}H]ouabain.

covalent labeling are plotted as a function of time in the 254 nm light. Note that by the time photolabeling of the large polypeptide reaches a maximum (0.5%) at 8 min, only about 20% of the large subunit remains uncross-linked and the decrease in labeling beyond 10 min is clearly due to the loss of the labeled subunit. Also, although the $(Na^+ + K^+)$-ATPase is 90% photoinactivated within 10 min, [^{3}H]ouabain remains reversibly bound to the aggregated enzyme for much longer, with 35% left after 20 min. Thus the number of counts incorporated into the aggregate at the top of the gel continues to increase for 30 min.

In an attempt to improve the ratio of the rate of covalent labeling relative to the rate of photocross-linking, as well as to examine the mechanism of photolabeling, we tried photolysis with light of 220 and ⩾280 nm. The former is the wavelength of peak ouabain absorbance, while the latter is absorbed by tyrosine and tryptophan but not by ouabain. Table I presents the initial rate of change of parameters plotted in Fig. 2, for light of 220, 254, and ⩾280 nm. Comparison of absolute rates between one wavelength and another is not meaningful since the intensity of illumination was not determined; therefore, the ratio of the rate of [^{3}H]ouabain photoincorporation to the rate of ouabain photolysis is presented (V_l/V_0). At 220 nm the relative rate of photoincorporation is about ten-fold less than at 254 nm, indicating that photolysis of the lactone ring of ouabain is not directly responsible for photolabeling. A remaining possibility was that the photosensitive photolysis product of ouabain with an absorption maximum at 265 nm (unpublished observations) was the responsible species. This was ruled out in an experiment in which prephotolysis with 220 nm light of [^{3}H]ouabain bound to ($Na^+ + K^+$)-ATPase to produce the 265-nm absorbing species did not increase the rate of photolabeling in a subsequent exposure to 254 nm light (data not shown). The maximum attainable level of photoincorporation of [^{3}H]ouabain into the large polypeptide was near 0.6% in both 220 nm and 254 nm light.

An alternative hypothesis to photolysis of the lactone ring is that the responsible photoactive species is in the large polypeptide and that covalent labeling ('reverse photolabeling') [4] is thus analogous to photocross-linking. With ⩾280 nm light, the rate of photolabeling relative to the rate of photocross-linking (V_l/V_x, Table I) was 20-fold lower compared to the rate at 254 nm. Thus it appears unlikely that either tyrosine or tryptophane are the photoactive species. This contrasts to the recently published wavelength dependence of photoincorporation of cyclic [^{3}H]AMP into a receptor protein; in that

TABLE I

WAVELENGTH DEPENDENCE OF RATE OF PHOTOREACTIONS OF OUABAIN AND ($Na^+ + K^+$)-ATPase

The initial rates V_x, V_i, V_0, V_l were determined from Fig. 3, and similar plots of data at other wavelengths, using time points prior to 20% photoreaction. The rates are expressed as fractional change/h, and are analogous to first-order rate constants. The light intensity was the same for all measurements at a given wavelength, but was presumably different at the different wavelengths; it was not determined. Therefore, while absolute rates (e.g. V_l, V_0) at one wavelength cannot be directly compared to the corresponding rates at another wavelength, it is possible to compare relative rates (e.g. V_l/V_0, V_l/V_x) as presented at the bottom.

	Illumination wavelength		
	220 nm	254 nm	>280 nm
Duration of experiment (h)	0.33	0.5	6.0
Photocross-linking of ($Na^+ + K^+$)-ATPase (V_x, h^{-1})	6.8	20	0.16
Photoinactivation of ($Na^+ + K^+$)-ATPase (V_i, h^{-1})	9.7	20	0.21
Photolysis of ouabain (V_0, h^{-1})	2.4	0.58	<0.003
Photolabeling by [^{3}H]ouabain (V_l, h^{-1})	0.028	0.084	$<4 \cdot 10^{-5}$
V_l/V_0	0.012	0.15	—
V_l/V_x	0.004	0.004	<0.0002

case 280 nm light was found to be more effective than 254 nm light, and photolysis of aromatic amino acids was implicated. Phenylalanine and cystine (cf. Ref. 9) are among candidates for absorbing species that could be responsible for 'reverse photolabeling' with an optimum efficiency near 250 nm.

If 'reverse photolabeling' is the case, then attachment could be either to the steroid portion of the ouabain molecule from the primary region of the binding site, or to the ouabain rhamnose from the secondary region. To be certain that the primary site is photolabeled, we performed photolysis experiments with the aglycone [^{3}H]digitoxigenin, as well as with the parent glycoside [^{3}H]digitoxin. An excess of ($Na^+ + K^+$)-ATPase was used to keep the free [^{3}H]steroid concentration low; centrifugation could not be used since digitoxigenin dissociates rapidly. In 125 μl of MgP_i binding medium, 120 mg ($Na^+ + K^+$)-ATPase and 0.15 nmol [^{3}H]ouabain, [^{3}H]digitoxin, or [^{3}H]digitoxigenin were incubated with or without 4 nmol unlabeled ouabain for 15 min at 37°C and then photolyzed for 1.5 or 6 min at 4°C with the 254 nm lamp, then run on SDS-polyacrylamide gels which were stained, sliced, and counted. In three experiments the amount of photolabeling of the large polypeptide was the same with [^{3}H]-digitoxin as with [^{3}H]ouabain, and with [^{3}H]digitoxigenin it was 55% of the [^{3}H]ouabain level. In each case, photolabeling was prevented (less than 10%) by 10^{-4} M unlabeled ouabain. The two-fold lower level of incorporation with digitoxigenin could be due to: (a) a different conformation of the ($Na^+ + K^+$)-ATPase when the aglycone is bound; (b) more rapid dissociation of digitoxigenin when the enzyme is photoinactivated in the first minute, or (c) a contribution due to 'reverse photolabeling' of the cardiac glycosides by the secondary binding site. We have no evidence to decide among these alternatives. With [^{3}H]digitoxigenin and [^{3}H]digitoxin, as with [^{3}H]ouabain, there was no detectable photoincorporation of label into either glycoprotein or proteolipid. It should be noted that to assess labeling of the proteolipid it was necessary to first stain and destain the gels to remove free [^{3}H]digitoxin or [^{3}H]-digitoxigenin that migrated to the bottom of the gel.

Ruoho and Kyte [8] reported a failure to observe specific labeling of the ($Na^+ + K^+$)-ATPase from dog kidney, using [^{3}H]ouabain and 254 nm light. We have no explanation for this discrepancy, especially since the level of photoincorporation of their [^{14}C]cymarin derivatives was similar to the level we find with tritiated ouabain and digitoxin.

The above results confirm earlier findings that the large subunit of the ($Na^+ + K^+$)-ATPase forms at least part of the cardiac glycoside binding site [3,10], and they add three new pieces of information: (1) the direct photolabeling method used here and by Ruoho and Kyte [3] labels the primary region of the binding site. (2) The proteolipid is not labeled in this method. (3) The wavelength dependence of direct photolabeling does not appear to be consistent with either photolysis of the lactone ring of ouabain or of tryptophan or tyrosine groups on the protein as the mechanism. Thus it appears that the large polypeptide comprises the primary region of the cardiac glycoside binding site, while both the large polypeptide and the proteolipid participate at the secondary region [2].

Note added in proof (Received May 29th, 1979)

Rogers and Ladzunski recently reported [11] that a photoaffinity derivative of the aglycone strophantidin photolables only the large polypeptide chain of $(Na^+ + K^+)$-ATPase; that is the same pattern found here for direct photolabelling with [^{3}H]ouabain. They also confirmed our finding that derivatives of ouabain with a photoaffinity group on the rhamnose moiety covalently label both the large polypeptide and the proteolipid component.

Acknowledgments

We gratefully acknowledge the technical assistance of Ms. Bingh Loo and Ms. Gracie Jones, and the original suggestion of Dr. Boyd Haley to try direct photolabeling with [^{3}H]ouabain. This work was supported by USPHS grants HL-09906, AM-17433 and NIH Postdoctoral Fellowship 5-F22-AM-02262 to B.F.

References

1 Yoda, A. (1974) Ann. N.Y. Acad. Sci. 242, 598–616
2 Forbush, B., Kaplan, J.H. and Hoffman, J.F. (1978) Biochemistry 17, 3667–3676
3 Ruoho, A. and Kyte, J. (1974) Proc. Natl. Acad. Sci. U.S. 71, 2352–2356
4 Antonoff, R.S. and Ferguson, J.J., Jr. (1978) Photochem. Photobiol. 27, 499–501
5 Jorgensen, P.L. (1974) Biochim. Biophys. Acta 356, 36–52
6 Hansen, O. and Skou, J.C. (1973) Biochim. Biophys. Acta 311, 51–66
7 Cloetta, M. (1920) Arch. Exp. Pathol. Pharmacol. 88, 113–146
8 Ruoho, A. and Kyte, J. (1974) Methods Enzymol. 46, 523–531
9 Setlow, R. and Doyle, B. (1957) Biochim. Biophys. Acta 24, 27–41
10 Hegyvary, C. (1975) Mol. Pharmacol. 11, 588–594
11 Rogers, T.B. and Lazdunski, M. (1979) Febs. Lett. 98, 373–376