The catalytic subunit of sodium and potassium ion transport adenosine triphosphatase was isolated by sodium dodecyl sulfate-polyacrylamide electrophoresis and was subjected to isoelectric focussing on 3.5% acrylamide in 2% Triton X-100, 9 M urea, and 2% Bio-Lyte 3/10 from Bio-Rad Laboratories. At 20 °C this resolved 2 equal and closely spaced bands centered at pH 5.5 about 0.04 pH unit apart. The distribution of the polypeptide between the 2 bands came to a temperature-dependent equilibrium during focussing. At 15 °C predominantly the acidic band and at 25 °C predominantly the alkaline band appeared. Perhaps association of the nonionic detergent with the polypeptide resulted in its partitioning into bands corresponding to different physical states. A change of phase in a polypeptide-detergent complex might have altered its charge.

To test functional homogeneity of the subunit in the native enzyme, the active center for ATP binding was covalently labeled with fluorescein isothiocyanate, an acidic ligand. Isoelectric focussing of the derivatized subunit at 20 °C showed displacement of all of the alkaline band to the position of the acidic band, which was fluorescent. Isoelectric focussing at 25 °C showed displacement of almost half of the alkaline band to the position of the acidic band, and both bands were fluorescent. The results suggest that all of the subunit accepted the fluorescent label and that derivatization slightly raised the temperature at which the polypeptide equilibrated between the 2 states. A few experiments on the calcium-dependent ATPase of sarcoplasmic reticulum indicated that it responded similarly.

The (Na,K)-ATPase of plasma membranes has been highly purified from several tissues and contains at least 2 kinds of subunit (1-4). The larger a-subunit (M, about 85,000-120,000) carries the catalytic center for ATP hydrolysis and at least some of the receptor site for binding the cardioactive steroid inhibitor, ouabain. The smaller ß-subunit is a glycoprotein (M, about 37,000-56,000 with respect to protein and about 10,000 with respect to carbohydrate). Its principal function may be to brace the a-subunit, thereby permitting it to make more precise conformational changes (5). A small proteolipid (M, about 12,000), the ß-subunit, may also be a part of the enzyme since it is close to the receptor site for binding ouabain (1, 2), but it may not be required for activity (5).

In 1974 (with a correction in 1977) Jørgensen (6, 7) reported a highly purified preparation of (Na,K)-ATPase showing only the a- and ß-subunits by SDS-PAGE. The ratio of active sites (for binding ouabain, ATP, and the covalent intermediate phosphate group) was 1 per amount of protein in 2 a- and ß-subunits. These data supported speculation that the enzyme might have half-of-the-sites reactivity (4, 8, 9). In 1981 Moczylowski and Fortes (10) and Peters et al. (11, 12) concluded that the stoichiometry is 1 active site for each of these ligands per single aß pair. Both groups relied on a more accurate estimation of protein and an assignment of somewhat larger values to the molecular masses of the subunits. Estimates of the molecular mass of the functional enzyme by analytical centrifugation after solubilization by a nonionic detergent (1, 3) may be in error due to previously unsuspected heterogeneity of quaternary structure according to Craig (13).

In the purification of a soluble enzyme it is convenient that denatured molecules tend to aggregate and precipitate, allowing separation from the native enzyme. In contrast, the behavior of denatured intrinsic membrane proteins is not known. In the "negative" purification of Jørgensen (6) the enzyme remains embedded in membrane sheets after contaminating proteins have been stripped away by graded treatment with sodium dodecyl sulfate. We thought that this treatment with a strong detergent might leave denatured subunits of (Na,K)-ATPase embedded in the membrane along with the native enzyme (see also Freytag and Reynolds (14)). Denatured subunits would contribute protein in excess of active sites in a preparation apparently pure according to an SDS-PAGE gel. This could be interpreted mistakenly as evidence for half-of-the-sites reactivity.

To test the functional homogeneity of the (Na,K)-ATPase a-subunits we labeled them covalently in the active center for ATP binding with fluorescein isothiocyanate (15). We thus use acceptance of this label as a criterion for a functional subunit. In order to distinguish functional (derivatized) from nonfunctional (underivatized) a-subunits we subjected the isolated subunits to isoelectric focussing intending to identify the derivatized subunits through a decrease in their isoelectric pH. Furthermore, the fluorescence of the bands after isoelectric focussing would confirm successful labeling. This test of functional homogeneity is advantageous in that it avoids

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The abbreviations used are: (Na,K)-ATPase, sodium and potassium ion transport adenosinetriphosphatase (EC 3.6.1.3); SR Ca-ATPase, Ca2+- and Mg2+-dependent transport ATPase of sarcoplasmic reticulum (EC 3.6.1.3); AMPD, 3'-amino-2-hydroxypropyl,3',3'-dinitril0)tetraacetic acid; EGTA, ethylene glycol bis(ß-aminoethyl ether)-N,N,N',N'-tetraacetic acid; HEPES, N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid; PI, isoelectric point; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Composition of a solution given as (w/v) unless stated otherwise. Centrifugal force is the average value. Triton X-100 is polyoxyethylene p-t-octyl phenol with 9-10 ethylene groups.
reliance on protein analysis and estimation of molecular weight. It is expected isoelectric focussing of the native α-subunit displayed 2 bands in a temperature-sensitive equilibrium with each other. Labeling with fluorescein isothiocyanate did not produce a conspicuous shift of the isoelectric point, but it did shift the equilibrium between the bands.

**EXPERIMENTAL PROCEDURES**

**Materials**—The (Na,K)-ATPase was prepared from the outer membrane of fresh pig kidney according to the zonal gradient method of Jørgensen (6), except that 5 mM dithioerythritol was included in all the media used during and after the homogenization. Protease inhibitors, e.g. egg white trypsin inhibitor (0.1 mg/ml), leupeptin (50 μM), and pepstatin A (50 μM) were often included. The purified enzyme was stored at 0 °C in 30% (v/v) glycerol, 20 mM imidazole/HCl (pH 7.5), 0.7 mM EDTA, and 0.7 mM dithioerythritol; its ATPase activity was stable for more than 6 months.

The ATPase activity was 99.8% inhibitable by ouabain, and the specific activity ranged from 30-49 μmol P_i/min/mg at 37 °C in 30 mM imidazole glycylglycine (pH 7.4), 100 mM NaCl, 25 mM KC1, 4 mM Na_2ATP, 3.9 mM MgCl_2, 0.2 mM EDTA, and 0.2 mg/ml of fat-free bovine serum albumin.

The Ca-ATPase from sarcoplasmic reticulum of rabbit skeletal muscle was obtained from the laboratory of Dr. Sidney R. Ingwall (16). The specific activity was 3.6 μmol P_i/mg at 25 °C. In 1 ml of the assay mixture contained 0.1 mM KCl, 5 mM MgCl_2, 20 mM HEPES (pH 7.2), 40 μM EGTA, 5 mM Na_2ATP, and 5 μl/mg of A23187 (Ca_2+ ionophore) with or without 50 μM CaCl_2. One min after addition of the enzyme the reaction was stopped with 0.5 ml of 5% sodium dodecyl sulfate and P_i was estimated.

Fluorescin 5'-isothiocyanate was obtained from Molecular Probes, Inc., Plano, Texas. Bio-Lyte was from Bio-Rad Laboratories. Ultra-pure urea and sodium dodecyl sulfate were from Schwarz/Mann and BDH Chemical Corp.

**Isolation of Subunits**—Subunits were isolated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis essentially according to Laemmli (17). An enzyme pellet of 0.1-0.15 mg was solubilized in 200 μl of 5% sodium dodecyl sulfate in 50 mM histidine/HCl (pH 6.3). In order to block sulfhydryl groups the enzyme was incubated with 20 μl of 0.2 M N-ethylmaleimide for 20 min at room temperature (23 °C). The reaction was quenched by adding 2 μl of β-mercaptoethanol and 74 μl of 40% (v/v) sucrose. The samples were applied to tube gels (7.5% polyacrylamide + 0.2% bisacrylamide) containing 1% sodium dodecyl sulfate. After electrophoresis, polyamide bands were visualized by soaking the gel in ice-cold 0.5 M KCl, the desired bands were sliced out of the gel by using a razor blade. Each gel slice was homogenized in 1 ml of 30 mM imidazole/HCl (pH 7.4), 1 mM EDTA, 1 mM dithioerythritol, and 5% (v/v) Triton X-100. They were stored at −70 °C.

**Isoelectric Focussing of Subunits**—For isoelectric focussing within the pH range 3-10, 10-30 μg of subunit with its homogenized gel slice suspended in 1 ml were added to 1.5 ml of a solution such as the 0.25 ml of solution contained final concentrations of 12 mM imidazole/HCl (pH 7.4), 0.4 mM dithioerythritol, 0.4 mM EDTA, 2% (v/v) Triton X-100, plus 9 μl urea, 2% Bio-Lyte 3/10, 3.5% acrylamide, and 0.25% N,N,N'-methylenebisacrylamide. As a marker 5-10 μg of β-lactoglobulin B (isolectric pH of 5.23) were added. Then 12.5 μl of 10% ammonium persulfate followed by 1 μl of N,N,N',N'-tetramethylethlenediamine was added to start polymerization and the mixture was poured into the tube for isoelectric focussing, which was started 10 min later.

Isoelectric focussing over a narrow pH range Bio-Lyte 5/7 did not give clean bands when it simply replaced Bio-Lyte 5/10. However, it was satisfactory in a 2-layer compound system. Bio-Lyte 5/7 without protein replaced Bio-Lyte 3/10 in half of the gel mixture described above, which was polymerized separately in the bottom half of the tube. The other half of the mixture contained the Bio-Lyte 3/10 protein and a pI marker was polymerized in the upper half of the tube for only 10 min before electrofocussing. The Bio-Lyte 5/7 migrated into the upper half of the tube during the focussing and spread the protein bands apart giving clearer resolution. Otherwise the patterns were the same.

The isoelectric focussing apparatus was model GTS from Hoefer thermostated by a model 2209 Multiplate circulating bath from LKB. Some experiments were done without the circulating bath at room temperature, about 23 °C. Isoelectric focussing was started at 200 V for 30 min, continued at 400 V for 10 h at 20-25 °C or for 20 h at 15 °C, and terminated at 800 V for 1 h (a total of 4.9 kV hours). The cathodal solution (bottom) was 0.4% (v/v) ethanolamine and the anodal solution (top) was 0.2% (v/v) H_2SO_4. After isoelectric focussing the gels were immediately fixed with a solution containing 10% trichloroacetic acid, 3% sulfoisalicylic acid, and 26% (v/v) methanol for 2 h. They were stained for more than 6 h with a solution containing 0.04% Coomassie brilliant blue R-250, 0.05% Crocein scarlet, 0.5% CuSO_4, 27% (v/v) ethanol, and 10% (v/v) acetic acid. They were destained in a solution containing 12% (v/v) ethanol, 7% (v/v) acetic acid, and 0.5% CuSO_4. To prepare a sample for a repetition of isoelectric focussing and gel staining the fixed and stained gels were immersed in the trichloroacetic and acetic acids for 2 h, and then destained as above without the acetic acid for more than 6 h. The desired protein bands were cut from the gel with a razor blade and the slices were homogenized with 1 ml of 30 mM imidazole/HCl (pH 7.4), 1 mM dithioerythritol, and 1 mM EDTA. Immediately after homogenization the Triton, urea, and amylolide were added and the isoelectric focussing was performed as described above.

**Labeling with Fluorescein Isothiocyanate**—Labeling of (Na,K)-ATPase was done by a minor modification of the method of Karlish (15). The enzyme (at a final concentration of 0.4 or 0.5 mg/ml) was suspended in a buffer containing 30 mM NaCl, 0.2 mM Na_2ATP, and 0.3 mM Na_2EDTA at 23 °C. In experiments where labeling at the active site was to be prevented specifically by ATP, the buffer was changed to 30 mM CDTA/AMPD (pH 9.2) with 1 mM Na_2ATP/AMPD (pH 9.2). At zero time 0.16 mM fluorescein isothiocyanate freshly dissolved in 10% acetic acid was added to the final concentration of 1 μM. Immediately after homogenization the Triton, urea, and amylolide were added and the isoelectric focussing was performed as described above.

SR Ca-ATPase was labeled with fluorescein isothiocyanate essentially according to Pick (18). Membranes were suspended to a final concentration of 2 mg/ml in 0.2 M sucrose, 50 mM Tris/glycylglycine (pH 9.0), and 100 μM EGTA. Fluorescein isothiocyanate was added from a freshly made 1.2 mM solution in MeSO to a final concentration of 30 μM. After 1, 5, or 20 min at 25 °C the reaction was quenched by adding β-mercaptoethanol to 20 mM, and a 30-μl aliquot was taken for assay of ATPase activity. The remaining membranes were diluted with a solution of 50 mM sucrose, 17 mM KCl, 3.3 mM HEPES (pH 7.2), and spun down at 102,000 × g for 45 min. The pellet was solubilized in 5% sodium dodecyl sulfate in 50 mM histidine/HCl (pH 6.3) and treated with 20 mM N-ethylmaleimide as described above.

The protein concentration of the (Na,K)-ATPase was estimated by the method of Bradford (19) with bovine serum albumin as a standard. The values were multiplied by 1.144 to correct for the ratio of the color factors of the two proteins as estimated in this laboratory by amino acid analysis. Inorganic phosphate split from ATP in the ATPase assay was estimated on a Technicon autoanalyzer by the method of Hegvarry et al. (20).

**RESULTS**

**Isoelectric Focussing of α-, β-, and γ-Subunits of (Na,K)-ATPase**—When purified (Na,K)-ATPase was subjected to SDS/PAGE, bands were seen corresponding to the α- and β-subunits; a band of low molecular mass material near the position of the tracking dye (the “γ-band”) was also seen (Fig. 1A). When these bands were extracted from the gels and subjected to isoelectric focussing at 20 °C, the α-band resolved into 2 closely spaced bands (Fig. 1B). The β- and γ-bands resolved into more than 5 bands each (Fig. 1, C and D). Marshall and Hokin (21) reported microheterogeneity of the β-subunit after isoelectric focussing and attributed it to vari-
ation in the sialic acid content. Collins et al. (22) attributed heterogeneity in chromatography of the γ-band to aggregation.

In order to check for an artifact that might produce 2 bands during focussing, the procedure was modified in various ways. The α-band was applied to the isoelectric focussing gel at one end or the other; the results were the same. The duration of focussing was varied (4, 6, 8, or 10 kV hours) without effect. In a specific experiment the amount of protein applied to the gel was varied by a factor of 2 without affecting the relative distribution. Extraction of the α-subunit from heat-denatured or phenylmethylsulfonylfluoride-treated (protease inhibitor) (Na,K)-ATPase yielded the same 2 bands. Treatment of the isolated α-subunit with alkaline phosphatase also yielded the same 2 bands. When the 2 bands were extracted from the isoelectric focussing gel and each was subjected again to SDS/PAGE, the protein migrated with a single band corresponding to that of the temperature of the second half of the run. In one trial the polypeptide was run first at 20°C and then at 25 or 13°C and in both cases one band appeared. In another trial the polypeptide was run first at 25 or at 13°C and then at 20°C in both cases. In both cases 2 bands appeared (Fig. 2). In a repetition of this experiment the electrofocussing was done with addition of Bio-Lyte 5/7 as described under “Experimental Procedures” to separate the bands better. The results were the same as in the previous experiment (Fig. 3) except that the bands were less sharp than in Fig. 2. These data suggest a temperature-sensitive equilibrium between an acidic state and an alkaline state of the α-subunit.

In order to label the reactive α-subunits of the native enzyme we treated it with fluorescein isothiocyanate. We replaced the Tris buffer used by Karlish (15) with borate to

Fig. 1. Isoelectric focussing (IEF) at 20°C of selected bands from SDS/PAGE gel of purified (Na,K)-ATPase. Gel A, source gel (SDS/PAGE) showing from top downward, α-subunit (α), β-subunit (β), and “γ-band” (γ) running with the dye front; the negative electrode is at the top. Gels B, C, and D, isoelectric focussing gels of α-subunit, β-subunit, and γ-band, respectively, that were excised from a duplicate of gel A; the positive electrode is at the top. The isoelectric focussing medium contained 2% Bio-Rad ampholyte 3/10 in 9 M urea and 2% Triton X-100 in the full length of the gels, which were twice as long as gel A. Gel B shows 2 equal bands. Gel C shows 5 bands in the original gel (compare Reference 21).

Fig. 2. Isoelectric focussing gels of α-subunit of (Na,K)-ATPase and β-lactoglobulin B (isoelectric pH = 5.2) at various temperatures in a mixed Bio-Lyte 3/10 + 5/7 system. Gels A-D from left to right were run at 25, 22.5, 20, and 15°C, respectively. The α-subunit is α. The narrow band (LGB) is β-lactoglobulin B. The faint band above it is contaminating β-lactoglobulin A. The acidic and basic electrolytes are at the top and bottom, respectively.

Fig. 3. Effect of a temperature jump midway during isoelectric focussing of catalytic subunit. Isoelectric focussing was done as in Fig. 1 except for the temperature. Each period of focussing at a constant temperature was for 5 kV hours except for those at 13°C, which were for 9 kV hours. The jumps were: gel A, from 20 to 26°C; gel B, from 26 to 20°C; gel C, from 13 to 20°C; gel D, from 20 to 13°C.

In contrast, the appearance of β-lactoglobulins A and B and of the β- and γ-bands of (Na,K)-ATPase did not change in the temperature range from 20–25°C except that a section of the γ-band pattern appeared to condense a little (not shown). Deamidation or other covalent change did not seem to be involved since the changes were reversible. The difference between the isoelectric points of the 2 α-subunit bands was estimated to be 0.04 pH unit by multiplying the ratio of the distance between the 2 α-subunit bands to their average distance from the β-lactoglobulin B band (Fig. 2) by the separation of the isoelectric points of the α-subunit and β-lactoglobulin B, 0.3 pH unit (5.5–5.2). Addition of the following ligands of the native enzyme to the isoelectric focussing system did not change the distribution of polypeptide between the bands at 20°C: 1 mM ATP (Tris salt), 0.3 mM ouabain, 30 μg/ml of oligomycin, 1 mM RhCl, or a combination of 1 mM NH₄VO₃ + 1 mM RbCl + 0.2 mM MgCl₂.

Derivatization of α-Subunit with Fluorescein Isothiocyanate—Fluorescein isothiocyanate reacts covalently with the α-subunit of (Na,K)-ATPase at a specific location in the primary structure with a stoichiometry of 1 per ouabain-binding site (23). The reaction is inhibited by ATP, and the derivatized enzyme no longer reacts with nor responds to ATP nor ADP although it changes its fluorescence intensity in response to all other physiological ligands and ouabain (15).

In order to label the reactive α-subunits of the native enzyme we treated it with fluorescein isothiocyanate. We
reduce the concentration of amino groups that might react with the isothiocyanate. In this buffer the half-time for loss of inhibitory potency of fluorescein isothiocyanate was about 20 h. We stopped the reaction by adding β-mercaptoethanol, which was effective immediately (not shown). We measured disappearance of fluorescein isothiocyanate from the medium by the decrease in the amount of the β-mercaptoethanol derivative of the fluorescein isothiocyanate remaining in the supernatant after the enzyme was spun down. Loss of ATPase activity was proportional to the disappearance, but the ratio of activity to bound fluorescein isothiocyanate decreased with increasing fluorescein isothiocyanate concentration (Fig. 4). The time course of binding fitted the sum of a saturable component and a linear component. The linear component was about as large as the saturable component after 2 h in 8 μM fluorescein isothiocyanate. Binding to the saturable component was prevented and loss of (Na,K)-ATPase activity was slowed about 20-fold by addition of 0.5 mM ATP but the linear component was not affected (not shown). At a fluorescein isothiocyanate concentration of 8 μM, binding to the saturable component and loss of (Na,K)-ATPase activity were 3-fold faster in Na/borate than in K/borate buffer (not shown) (cf. Sen et al. (24)).

The fluorescein isothiocyanate-derivatized enzyme was solubilized in sodium dodecyl sulfate and run over a Sepharose CL-6B column in order to separate the enzyme was derivatized with 4 μM fluorescein isothiocyanate for 30 min at 24 °C in the presence or absence of 1 mM ATP. Inhibition of ATPase activity was 56% without ATP and 2% with it. The samples were subjected to SDS/PAGE, the gels were sliced, and fluorescent material was extracted and estimated as described in the legend to Fig. 5. The sample derivatized in the presence of ATP showed 3% as much fluorescence in the α-subunit as that derivatized in the absence of ATP (cf. Fig. 3 in Reference 15).

By extrapolation in Fig. 4 at 4 μM the amount of fluorescein isothiocyanate bound at complete inhibition of activity would be 5.2 nmol/mg. The ouabain binding capacity of one of our preparations with a specific activity of 44 μmol/min/mg was 5.3 nmol/mg. This value is close to the highest phosphorylation capacity (5.6 nmol/mg) reported by Peters et al. (11) and nucleotide (6.0 nmol/mg) and ouabain (5.2 nmol/mg) binding capacity reported by Moczydłowski and Fortes (10).

We conclude that fluorescein isothiocyanate rapidly made a covalent derivative of the α-subunit in the active center for ATP binding. It reacted more slowly elsewhere to inhibit (Na,K)-ATPase activity (and also p-nitrophenyl phosphatase activity according to Karlsh (15) and Sen et al. (24)). In subsequent experiments labeling with fluorescein isothiocyanate was done at 4 μM for up to 4 h at 23 °C to minimize nonspecific derivatization.

**Isoelectric Focussing of Derivatized α-Subunits—**Isoelectric focussing of the fluorescein isothiocyanate-derivatized α-subunit at 23 °C showed a relatively broad band of protein and fluorescence in the region of pH 5.5 (Fig. 5). Jørgensen (25) found minimum solubility for the isolated α-subunit at pH 4.5-5.5 and suggested that the pI is in this range.

In order to locate the fluorescent α-subunit after isoelectric focussing, the gels were taken out of the glass tubes and viewed under ultraviolet light in a dark room. Photography of the fluorescent bands was not satisfactory. Therefore, a black bristle was inserted into the gel where a fluorescent band was seen. Then the gels were stained for protein and the locations of the bristles and the stained bands of protein were compared.
There was good correspondence (Fig. 6). Faint fluorescent bands were also seen that did not correspond to the protein bands. They appeared in the absence of the α-subunit and their pattern varied with the source of the Triton X-100.

Effect of Derivatization on Isoelectric Focusing—The enzyme was treated with 4 μM fluorescein isothiocyanate for progressively longer times and the ATPase activity was progressively inhibited to completion. Isoelectric focusing of the isolated α-subunits at 25 °C showed corresponding progressive conversion of 1 band into 2 bands of equal staining intensity. Both bands were fluorescent (Fig. 7).

The presence of fluorescein in both bands might have been due to inhomogeneous derivatization, some of the fluorescein isothiocyanate being bound to the active center and some being bound elsewhere. In order to test the homogeneity of the labeling, the treatment was repeated with 4 μM fluorescein isothiocyanate in the presence of 1 mM ATP. In the presence and absence of ATP, 64% and 3% of the control (Na,K)-ATPase activity remained, respectively. The subunits treated in the absence of ATP showed 2 bands at 25 °C, both of which were fluorescent as in Fig. 7, but those treated in the presence of ATP appeared the same as the control (1 band) and no fluorescence was seen (not shown). The separation of the polypeptide into 2 bands at 25 °C thus depended upon derivatization at the active center. In another test the enzyme was treated with 4 or 8 μM fluorescein isothiocyanate for 4 h. Isoelectric focusing at 25 °C produced 2 stained bands with the same appearance in both cases. The bands were equally fluorescent after 4 μM fluorescein isothiocyanate, as before, but the acidic band was more fluorescent than the alkaline band after 8 μM fluorescein isothiocyanate (not shown).

The presence of fluorescence in both bands is consistent with an equilibrium between 2 states of a single α-subunit derivatized at the active center. If this is correct, derivatization shifted the equilibrium between the states in the same way that a reduction in temperature did in the experiment of Fig. 2. To test this possibility the experiment was repeated with isoelectric focusing at a lower temperature. At 20 °C isoelectric focusing of the untreated subunit produced 2 bands of almost equal intensity. As the enzyme was progressively inhibited and derivatized, the alkaline band shifted into the acidic band (Fig. 8). Only one fluorescent band was seen at complete loss of activity and this was in the position of the acidic band. Derivatization shifted all the α-subunits into the acidic band under conditions in which the native subunit appeared as 2 bands in equilibrium. If a significant amount of the subunit had not been derivatized, it would have remained at the position of the alkaline band. Apparently all of the subunit was derivatized.

Homogeneity of α-Subunits—In order to test the homogeneity of the material in the 2 bands from the native subunit, they were cut out of the isoelectric focusing gel and subjected to a repetition of isoelectric focusing at both 20 and 23.5 °C. At 20 °C the 2 excised bands ran differently; the acidic band reran almost entirely as 1 band but the alkaline band reran as 2 bands (Fig. 9). At 23.5 °C both excised bands reran like the alkaline band except that the acidic band showed a shadow on its acid side (Fig. 9). Since the material from the separated bands ran differently, presumably it was different. The α-subunit thus appeared to contain at least 2 components. The components appeared to differ in their distribution between the bands and, therefore, in their temperature for an equal distribution between the bands.

In order to test the homogeneity of the bands derivatized with fluorescein isothiocyanate, they were cut out of the isoelectric focusing gel and run again at 25 °C. As with the native subunit (Fig. 9) the material from each of the 2 bands did not give the same result. Each band gave 2 bands but the acidic band gave a more intense acidic band and the alkaline band gave a more intense alkaline band (Fig. 10). This difference is evidence for heterogeneity of the derivatized α-subunit. The marker band of β-lactoglobulin B was also cut out of the gel and rerun separately; a single band was obtained at about the same relative position as before (not shown).

Isoelectric Focusing of Polypeptide of Ca-ATPase of Sarcoplasmic Reticulum—The catalytic subunits of SR Ca-ATPase and of (Na,K)-ATPase share a similar molecular mass, active site, and reaction mechanism (2, 4). SR Ca-ATPase is derivatized with fluorescein isothiocyanate at a specific lysine residue (26). A preparation of sarcoplasmic reticulum was
was done at 25 °C. The experiment was done as in Fig. 7 with derivatization times of 5, 10, 60, or 240 min. The relative levels of activity retained were: gel A, 100% (control); gel B, 70% gel C, 60%; gel D, 8%; and gel E, 2%. In gels D and E only 1 fluorescent band was seen and marked with bristle (arrows). LGB, β-lactoglobulin B.

Gels alkaline, lower (L), and acidic upper (U) bands, respectively, of a-subunit from gel A were cut out separately and cast into a new gel. In gel B with addition of β-lactoglobulin B as a marker (LGB). Gel C, same as gel B with addition of catalytic subunit (a) (Na,K)-ATPase. Gel D, catalytic subunit of (Na,K)-ATPase (a) and β-lactoglobulin B (LGB).

Effect of progressive derivatization on isoelectric focussing at 20 °C of acidic, upper (L) and alkaline lower (L) bands respectively, of a-subunit (a) at 20 °C. Derivatization was done with 20 μM fluorescein isothiocyanate (18) for 1, 5, or 20 min at 23 °C. Gel A shows undervatized catalytic subunit (CAT) and β-lactoglobulin B (pI = 5.2) (LGB). Gels B, C, and D show enzyme with 32, 1, and 0.5% retained activity, respectively. Two fluorescent bands were seen in gels C and D. In gel C a bristle was placed between the bands. It was photographed end-on and shows as a black spot at the edge of the gel (arrow). In gel D one bristle was placed in each band (arrows).

subjected to isoelectric focussing at 20 °C. The catalytic subunit of SR Ca-ATPase appeared as a single broad band with a slightly more acidic isoelectric point (pI about 5.4) than the catalytic subunit of (Na,K)-ATPase (Fig. 11). At 25 °C isoelectric focussing of the isolated subunit produced a single rather broad polypeptide band that was not modified by derivatization with fluorescein isothiocyanate and showed only one fluorescent band (not shown). At 20 °C derivatization divided the single broad band into 2 bands, both of which were fluorescent (Fig. 12). These preliminary results indicate that the catalytic subunits of the two ion transport ATPases responded similarly to isoelectric focussing after derivatization with fluorescein isothiocyanate. Future work with sarcoplasmic reticulum may be improved by precautions to prevent spontaneous oxidation of sulfhydryl groups to sulfonic acid groups during preparation of this enzyme (27). Such precautions were not taken in our experiments.
Isoelectric Focussing of (Na,K)-ATPase

Discussion

The initial aim of these experiments was to test whether all or only half of the catalytic subunits are functional in the best available preparation of (Na,K)-ATPase. The test was designed to avoid estimation of the quantity of catalytic subunits in a sample since both the estimation of molecular weight of the catalytic subunit and the analysis for protein have given inconsistent results in the literature (1). The test aimed to derivatize each functional subunit with fluorescein isothiocyanate and then to separate the derivatized subunits by isoelectric focussing.

Equilibrium between 2 States—In actual fact shifts in isoelectric point after derivatization with fluorescein isothiocyanate were not estimated accurately because the pH gradient in the gel was not entirely consistent from one run to another. But unexpectedly the native subunit separated spontaneously into 2 bands in a slow temperature-sensitive equilibrium (Figs. 2 and 3). This equilibrium permitted an effect of derivatization to be detected. The bands appeared to correspond to acidic and alkaline states having a difference in isoelectric point of about 0.04 pH unit. The equilibrium temperature was 20 °C with a range of ±5 °C. The acidic state was stable at 15 °C and the alkaline state was stable at 25 °C (Figs. 2 and 3).

Model of Changes in Isoelectric Point of Native a-Subunit—In order to model a mechanism for the banding pattern of the a-subunit during isoelectric focussing, we simulated a pH titration curve of the a-subunit. We assumed that it contains 966 amino acids (1) in the proportions found by analysis (12) and assigned to each ionizable group a pK corresponding to that of the free amino acid. To obtain a pI of 5.51 we assumed that 83 out of 201 acidic residues are amidated. The resultant buffering capacity is 17.3 eq/mol/pH unit. Correspondingly a change of 1 eq of fixed charge on the model polypeptide changes its isoelectric point by 1/17.3 or 0.058 pH unit. Since histidine residues (pK = 6.1) are only partially charged, it would require the least energy to sequester a histidine residue. If a histidine residue were sequestered, sequestration would be in the acidic state.

Alternatively histidine residues might form salt bridges with acidic residues. Subtraction of 4 histidine and 4 acidic residues, glutamate or aspartate, from the model increased the isoelectric point by 0.05 unit. Accordingly the alkaline state could incorporate such salt bridges.

We also evaluated the effect of temperature. A 10 °C rise in temperature decreases the pK of free histidine (pK = 6.1) by 0.17 unit. This change decreases the pI of the model a-subunit by 0.07 unit. But the change is in the opposite direction to that observed experimentally where a rise in temperature shifted the equilibrium toward the alkaline state (Fig. 2). So whatever it is that separates the bands, it is opposed by the effect of temperature on the dissociation constant of their histidine residues.

Mechanism of Change in State—The mechanism of the change in state probably involves a complex between the a-subunit and the nonionic detergent, Triton X-100, used for solubilization. In isoelectric focussing of the γ-chain of human hemoglobin Saglio et al. (28) found that addition of a nonionic detergent separated the otherwise inseparable γγ and βγ polypeptides by 0.1 pH unit, even though these polypeptides differ only by replacement of a neutral glycine residue at position 136 by a neutral alanine residue. Their detergent, Nonidet P-40, separated the polypeptides also in cellulose acetate electrophoresis and in carboxymethylcellulose chromatography. The effect of the replacement of glycine by alanine was highly specific since replacement of the normal isoleucine residue at position 75 with a threonine residue in globin γγ had no effect. Nonidet P-40 is one of the components of Triton X-100.

In our system the proposed complex probably involves micelles since the concentration of Triton X-100 (2%) was about 100-fold higher than its critical micelle concentration. Triton X-100 micelles (Mγ ~ 90,000 at 20 °C (29)) are about as large as the a-subunit (Mγ ~ 106,000 (1)). Triton undergoes a phase change with elevation of temperature and separates in bulk from the aqueous phase (29). In the case of Triton X-114 (which is only 2 ethylene groups shorter than Triton X-100) intrinsic membrane proteins accumulate in the Triton phase while soluble proteins remain in the aqueous phase (30). (Na,K)-ATPase is an intrinsic membrane protein. The narrow temperature range of the change of state in our system, 10 °C, is consistent with a phase change of an amphiphatic molecule. On the basis of these considerations it seems likely in our isoelectric focussing system that Triton X-100 micelles formed one or more complexes with the a-subunit of (Na,K)-ATPase, these having different isoelectric points. With respect to the α-subunit itself a change in the state could reflect a change in tertiary or in quaternary structure, e.g. dimerization.

Heterogeneity—The a-subunit contained more than one component since each of its 2 bands, which were separated by isoelectric focussing at 20 °C, produced a different pattern after extraction and refocussing (Fig. 9). If the bands had been composed of homogeneous material, each would have yielded the same pattern. Therefore, the subunit contained at least 2 components. The components may have been distributed unevenly between the bands. Apparently the components differed slightly in their temperatures for an equal distribution between the acidic and alkaline states.

For the sake of discussion the putative components may be designated "warm" and "cool" corresponding to their relative temperature for an equal distribution between the 2 states. At 20 °C the warm component distributes more into the acidic state and the cool component distributes more into the alkaline state. (Actually the situation may be more complicated since the acidic band was not as sharp as the alkaline band; it tended to be broader and less sharply defined at its acidic edge.)

To account for the origin of the components one can imagine variant gene products, post-translational modifications, or artifacts arising during preparation of the enzyme. An SDS/PAGE study of heat-denatured (Na,K)-ATPase suggests heterogeneity of the a-subunit (31). The heterogeneity described here does not correspond to the two molecular forms of (Na,K)-ATPase found in brain (32). Reference 1 refers to other forms of heterogeneous ATPase.

Instead of accepting heterogeneity one might suppose that a homogeneous polypeptide did not reach equilibrium during the repetition of the isoelectric focussing. Or perhaps the staining after the first run stabilized the states during the second run. In the case of a homogeneous polypeptide in order for 2 states to separate on the gel, the time spent by a molecule in each state must be long relative to the time required for a molecule to move from the position on the gel corresponding to one state to the position corresponding to the other. This implies a slow equilibrium.

A rapid equilibrium between the states would be possible with a heterogeneous polypeptide consisting of 2 components if the components themselves were relatively stable. For instance, at 15 °C both the warm and the cool components of the a-subunit would be at the acidic position. If the preparation were warmed from 15 to 20 °C, the cool component would
migrate as a single band from the acidic position to the alkaline position. At 20 °C the cool component would be in the alkaline position and the warm component would remain at the acidic position. Thus there would be 2 bands. Then with further warming the warm component would migrate as a single band to the alkaline position giving again one band for both components at 25 °C. An analogous mechanism is shown by plasma albumin in which relatively stable permu-
tations and thus microheterogeneity of disulfide pairing per-
mit electrophoretic separation of rapidly equilibrating "normal" and "fast" forms. (Consult this reference for earlier references.) In the α-subunit Esmann (34) did not find any disulfide bonds.

Derivation of the α-Subunit with Fluorescein Isothio-
cyanate—Fluorescein isothiocyanate derivatized the enzyme easily in the active center for ATP binding and possibly to some extent elsewhere (Fig. 4 and text). Sen et al. (24) observed different patterns of inhibition by fluorescein isothio-
cyanate at different pH values. In our experiments derivati-
zation of the active center of the α-subunit appeared to shift the distribution of the α-subunit from the alkaline state to the acidic state and so to elevate the temperature required for an equal distribution between the 2 states from 20 to 25 °C (Figs. 7 and 8 and text). This change is consistent with the presence of a carboxyl group on fluorescein.

Heterogeneity of Derivatization—We will discuss these data from 2 points of view.

The first point of view regards heterogeneity as insignificant or at least unrelated to derivatization. It considers that all the α-subunits were derivatized in the active center. It claims support from the experiment of Fig. 7 at 25 °C in which derivatization shifted half of the material that was in the alkaline band to the position of the acidic band and both bands became fluorescent. It relies on evidence for an equilib-
rium between the bands of the native subunit in Fig. 3 to claim an equilibrium for the derivatized bands in the experi-
ment of Fig. 7. It interprets the experiment of Fig. 8 at 20 °C as indicating that both bands were derivatized but the acidic band did not change its pl because it was already in the acidic state and addition of a fluorescent group could not make it more so. The conclusion from this point of view is that all subunits were functional in the native enzyme.

The second point of view considers that the "cool," more alkaline, component was derivatized in the active center for ATP binding and that the "warm," more acidic, component was derivatized elsewhere. The derivatization elsewhere did not shift the pl. In Fig. 8 at 20 °C the cool component was in the alkaline state and the warm component was in the acidic state. Derivatization of the cool component in the active center shifted it to the acidic state and derivatization of the warm component elsewhere did not shift its pl. In Fig. 7 at 25 °C both components were in the alkaline state. Derivati-
zation of the cool component shifted it to the acidic state and derivatization of the warm component made it fluorescent but left it in the alkaline state. Consequently this point of view predicts that while derivatization did not change the equilib-
rium temperature of the warm component, it elevated that of the cool component from a level below that of the warm component to a level above it. A shift of about 10 °C would be required from say, 17.5±2.7 °C, the warm component having an equilibrium temperature of 22.5 °C.

We prefer the first point of view.

Relationship of Isoelectric Focussing States to Conforma-
tions of Native Enzyme—Our most interesting speculation relates the isoelectric focussing states to the well established conformations E1 and E2 of the native enzyme (2–4). Against any such relationship is our failure to find an effect of ligands of the native enzyme on the isoelectric focussing equilibrium. In favor is the finding of a similar equilibrium for the catalytic subunit of the SR Ca-ATPase (Fig. 11). Skou and Esmann (35) have evidence for a shift from E1 to E2 upon acidi-
fication of (Na,K)-ATPase. Pick and Karlish (36) have evidence for a similar effect in SR Ca-ATPase. Furthermore, they show that an increase in temperature favors a shift from E1 to E2. The effect of temperature on the E1 ↔ E2 equilibrium in (Na,K)-
ATPase is not well defined. Toda and Post have an experiment indicating by an indirect method (37) a 9-fold reduction in the affinity for Na⁺ as the temperature was raised from 4 to 23 °C. Such changes are consistent with the isoelectric focussing data if the alkaline band corresponds to E2 and the acidic band corresponds to E1. E1 is the stronger acid and conversion of E1 to E2 is endothermic. In contradiction to this Swann and Albers (38) show activation of Li⁺-dependent p-
itrinophosphatase activity with a reduction in tempera-
ture as if E1 were more stable in the cold. If these speculations have any validity, they suggest that the conformational changes in the "cool," more alkaline, component to a level above it.

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