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Active Calcium Transport Via Coupling Between the Enzymatic and the Ionophoric Sites of Ca^{2+} + Mg^{2+}-ATPase

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The 20K dalton fragment of Ca^{2+} + Mg^{2+}-ATPase obtained from the tryptically digested sarcoplasmic reticulum has been further purified using Bio-Gel P-100. This removed low-molecular-weight UV-absorbing and positive Lowry-reacting contaminants. The ionophoric activity of the 20K fragment in both oxidized cholesterol and phosphatidylcholine:cholesterol membranes is unaltered by this further purification. The 20K selectivity sequence in phosphatidylcholine:cholesterol membranes is $Ba^{2+} > Ca^{2+} > Sr^{2+} > Mn^{2+}$ Mg^{2+}.

Digestion of intact sarcoplasmic reticulum vesicles with trypsin, which results in the dissection of the hydrolytic site (30K) from the ionophoric site (20K), is shown to disrupt energy transduction between ATP hydrolysis and calcium transport. This further implicates the 20K dalton fragment as a calcium transport site.

These data and previous evidence are discussed in terms of a proposed model for the ATPase molecular structure and the mechanism of cation transport in sarcoplasmic reticulum.

Key words: Ca^{2+} + Mg^{2+} ATPase, transport, inophore, energy coupling

The nature of the mechanism for directing the energy released by ATP hydrolysis to the vectorial work of ion transport is a fundamental problem of biological research. The customary approach in dealing with this problem has been to study the activation and kinetics of ATP hydrolyzing membrane-bound enzymes. More recently reconstitution of membrane proteins into vesicles has allowed a direct measure of transport.

Our approach has been to identify the ion-transporting site (ionophoric site) as an entity separate from the enzymatic site of the transport ATPases. This seems feasible for a number of reasons. The high-molecular-weight Na^+ + K^+-ATPase from excitable tissue and the Ca^{2+} + Mg^{2+}-ATPase from muscle sarcoplasmic reticulum have been shown to contain the entire mechanism necessary for active transport (1–4). The ion translocating site is probably contained within a small part of the much larger membrane-bound enzyme. Under conditions of assay, the isolated ion-bearing site must have an inherent affinity for the transported ion. Separation of this ion transporting or ionophoric site from the parent molecule may result in the isolation of a valinomycin-type ionophore (5, 6) with a hydro-

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phobic exterior but in any case must have a specific affinity for the transported ion. This approach has led to our identification of several ionophores isolated as part of membrane-bound transport proteins (7–12).

In our definition, ionophoric activity is the ability of a substance to increase black lipid membrane conductance. Criteria which link this ionophoric activity to transport have recently been reviewed by us (5, 6). Briefly, these are: the ability of the ionophore to move the ion transported by the parent protein across a black lipid membrane; this phenomenon must show dependence and/or selectivity to that ion; evidence showing that this activity is isolated from definite parts of the parent protein and inhibitor work demonstrating that the transport is affected in both the native enzyme system and an ionophore-doped black lipid membrane (BLM).

Rabbit white skeletal muscle sarcoplasmic reticulum has been shown to contain an ATP-dependent pump for Ca^{2+} (13). Vesicles assembled from this sarcoplasmic reticulum purified $Ca^{2+} + Mg^{2+}$-ATPase are able to pump Ca^{2+}. The pure $Ca^{2+} + Mg^{2+}$-ATPase has also been shown to exhibit Ca^{2+}-dependent and -selective ionophoric activity in the BLM (8). The 100K dalton $Ca^{2+} + Mg^{2+}$-ATPase as part of the sarcoplasmic reticulum vesicles is cleaved into peptides of 45K and 55K daltons. The enzymatic and ionophoric activities are retained on the 55K dalton fragment.

Subsequent cleavage of the 55K dalton fragment to 30K and 20K dalton fragments separates the enzymatic and ionophoric sites. The 30K dalton fragment contains the site of ATP hydrolysis and the 20K dalton fragment the ionophoric site (10–12). Ruthenium red and mercuric chloride, inhibitors of transport in the intact system, inhibit the ionophoric activity of the 55K and 20K dalton fragments. Methylmercury, an inhibitor of the hydrolytic site of the enzyme, does not inhibit the ionophoric activity (14).

This report presents evidence indicating that the 20K dalton fragment purified on Bio-Gel A-1.5m contains smaller protein pieces not detectable using sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis. The contaminating proteins were removable by SDS exclusion chromatography on Bio-Gel P-100. Evidence is presented that cleavage of the 55K dalton fragment to 30K and 20K dalton fragments in sarcoplasmic reticulum vesicles causes an interruption of Ca^{2+} transport but not ATPase activity. Data is also presented on the ionophoric properties of the 20K dalton fragment in phosphatidylcholine: cholesterol (5:1 mg/mg) membranes.

MATERIALS AND METHODS

Preparation

Sarcoplasmic reticulum and the $Ca^{2+} + Mg^{2+}$-ATPase were prepared from rabbit white skeletal muscle by the method of MacLennan (15). The tryptic fragments of the ATPase of 55K, 45K, 30K, and 20K daltons were prepared and purified by the method of Stewart et al. (11). At times a Sephacryl S-200 column was substituted for Bio-Gel A-1.5m.

Purification of the 20K Dalton Fragment on Bio-Gel P-100 Column

A Bio-Gel P-100 column (180 × 2.5 cm) was equilibrated with 0.5% SDS, 50 mM Tris-HCl, pH 7.0, 1 mM dithiothreitol, 0.02% NaN_3. The column was eluted with the same column buffer and 2-ml fractions were collected every 20 min.

Thin Layer Chromatography of 20K

Fractions presumed to be the 20K dalton fragment from Bio-Gel A-1.5m were con-

centrated with the aid of an Amicon concentrator. Then 1–2 μl of 1 mg/ml was spotted on a silica gel G glass plate. The plate was subjected to ascending solvent of 4:1:1.67 (butyl alcohol:acetic acid:H_2O). Fluorescamine staining was performed according to the method of Udenfriend et al. (16). Plates were also stained in an iodine tank.

Analytical Methods

Protein was determined by the method of Lowry (17). SDS-polyacrylamide gel electrophoresis was carried out according to the methods of Weber and Osborn (18); Laemmli (19); and Swank and Munkres (20).

Ca^{2+} + Mg^{2+}-ATPase activity was assayed in 25 mM Tris-Cl, pH 7.50, 100 mM KCl, 5 mM $MgCl_2$, 5 mM [^{32}P] ATP, and 10 μM added $CaCl_2$ and $^{32}P_i$ determined by the method of Martin and Doty (21). Calcium uptake was measured in 25 mM HEPES pH 7.0, 120 mM KCl, 5 mM $MgCl_2$, 5 mM ATP, and 50 μM free ^{45}Ca by Millipore filtration.

Conductance Measurement

The lipid bilayer was formed from egg phosphatidylcholine:cholesterol (5:1 mg/mg in decane). Conductance, capacitance, and ionic selectivities were all measured according to published methods (5, 12).

RESULTS

Purification of 20K Dalton Fragment of Ca^{2+} + Mg^{2+}-ATPase

The tryptically digested sarcoplasmic reticulum is treated with potassium deoxycholate (KDOC, pH 8.0) to remove calsequestrin and high affinity calcium binding protein as described previously (11, 12). The digested sarcoplasmic reticulum is solubilized with 10% SDS and passed through an SDS-equilibrated Bio-Gel A-1.5m column. The column is eluted with the previously described buffer (12). Figure 1 represents the elution pattern of the column. Fractions 167–200 are UV-absorbing material which show no protein bands on SDS-gel electrophoresis. Fractions 220–241 contain the 20K dalton fragment as tested on SDS-gel electrophoresis. Fractions 220–232 show the 20K dalton fragment on SDS-gel electrophoresis and contain no impurities as tested on the thin-layer chromatography (TLC) plate (Fig. 2). Fractions 232–243 contain 2 extra fluorescamine stained spots. An 8 M urea SDS-gel electrophoresis of fractions 220–241 shows only one protein band. The 8 M urea gel is able to separate proteins of molecular weight as low as 1,000 daltons (e.g., bacitracin). Therefore, the fluorescamine sensitive spots on silica gel may represent small peptidic fragments below 1,000 daltons. The small fragments may be the result of the tryptic digestion of sarcoplasmic reticulum.

In controlled thin-layer chromatography experiments, we have shown that the fluorescamine sensitive spot with R_f = 0.26 on the silica gel is due to Tris-HCl. However, fractions 6–9 show I_2-sensitive staining indicative of other contaminants. The spot at the origin represents the 20K dalton fragment. The spot with R_f = 0.6 is only slightly I_2 sensitive, and may represent slight lipid contaminants. The spots with R_f values of 0.36 and 0.47 are fluorescamine sensitive and may represent the small peptidic fragments.

In order to purify the 20K dalton fragment eluted from Bio-Gel A-1.5m (fractions 220–241) further, the fractions were concentrated with an Amicon P-10 membrane and passed through an SDS-equilibrated Bio-Gel P-100 column. Figure 3 represents the elution pattern of this column. Fractions 40–56 represent purified 20K dalton fragment as shown by both SDS-gel electrophoresis (Fig. 4) and silica gel TLC patterns as in spots numbered

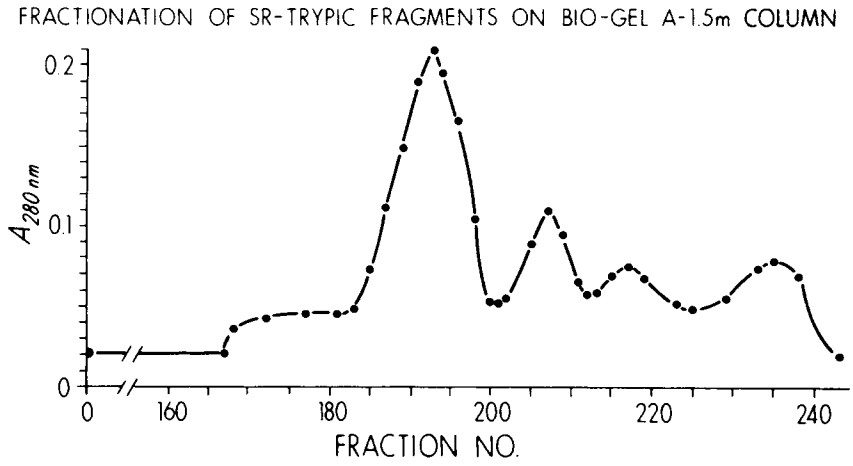

Fig. 1. Fractions of sarcoplasmic reticulum tryptic fragments from a Bio-Gel A-1.5m column (180 × 5 cm) equilibrated with 0.5% SDS, 50 mM Tris-HCl, pH 7.0, 1 mM DTT, and 0.02% NaN_3. The tryptic digest of sarcoplasmic reticulum was run through the column similar to the published method, Stewart et al. (11).

1–5 in Fig. 2. Fractions 60–80 represent the contaminants described earlier. The 20K dalton fragment purified with Bio-Gel P-100 is considered our standard purified 20K dalton fragment for subsequent work described in this paper.

Ionophoric Activity Associated With the 20K Dalton Fragment

The purified 20K dalton fragment just described was dialyzed against 8 M urea for 5 days followed by water for several days as described previously (12). In certain experiments, in order to insure complete removal of SDS, the 20K dalton fragment was treated further by solubilization in 1% potassium cholate and dialysis against the same for several days followed by dialysis against water. The ionophoric data presented here will be those of the cholate-treated 20K dalton fragment. We found no noticeable changes in the ionophoric properties of the 20K dalton fragment after cholate treatment.

Measurement of the diffusion potential in the presence of the 20K dalton fragment and a gradient of calcium chloride yields a permeability ratio $P_{Ca}{}^{2+}:P_{Cl}{}^{-}$ of 2.3:1, consistent with previous data in oxidized cholesterol bilayers (10, 12).

Table I presents the biionic potential in mV in the presence of calcium ion on one side of a phosphatidylcholine:cholesterol (5:1 mg/mg) BLM and another divalent cation on the other side. The permeability ratios $P_{Ca}{}^{2+}/P_X$, x denoting the other cation, were calculated as detailed in our review (5). The selectivity ratios seen here are consistent with the published selectivity sequence of the 20K dalton fragment in oxidized cholesterol membranes (12) and those of the intact enzyme (8, 9).

The fluorescamine-sensitive contaminant of the 20K dalton fragment when tested alone on oxidized cholesterol or phosphatidylcholine:cholesterol BLMs, shows non-ion-dependent, nonselective ionophoric activity. A collection of the small contaminants from fractions further away from the 20K dalton fragment peak has no ionophoric activity.

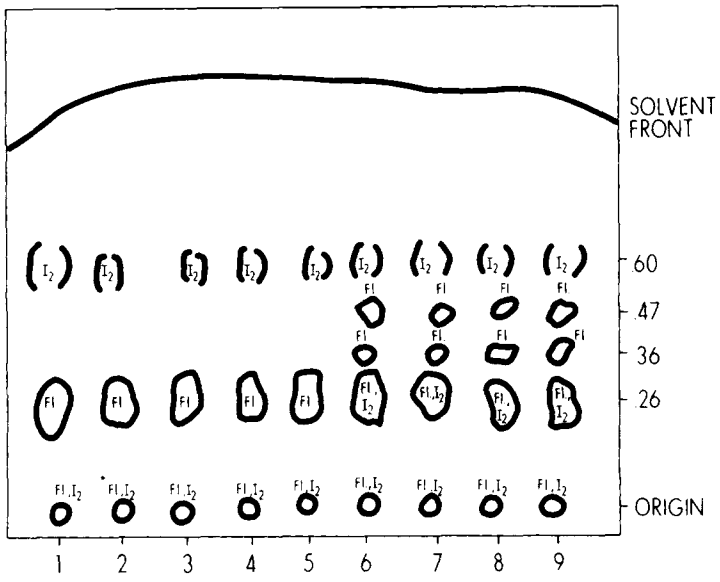

Fig. 2. Silica gel G thin-layer chromatography of 20K dalton fractions from Bio-Gel A-1.5m column. Solvent 4:1:1.67 (butyl alcohol: acetic acid:H_2O). Spots 1–9 represents fractions taken in order of elution from the Bio-Gel A-1.5m column. Fl. and I_2 represent fluorescamine and iodine-stained spots, respectively.

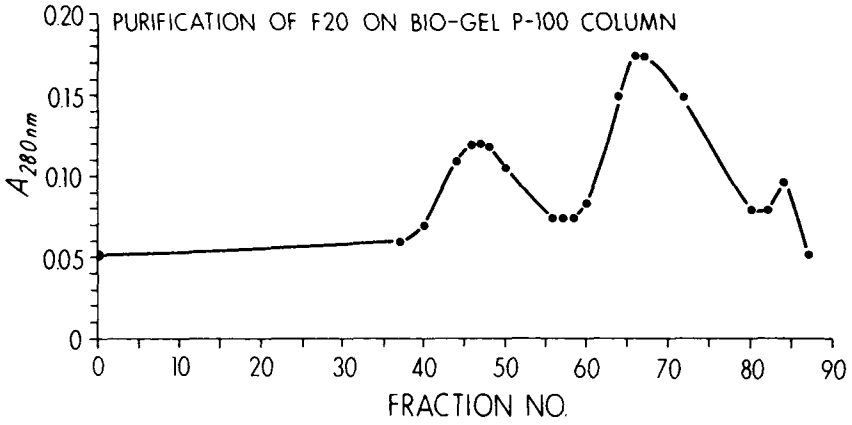

Fig. 3. Purification of 20K dalton fragment. The 20K dalton peak fractions from Bio-Gel A-1.5m were concentrated with an Amicon P-10 membrane and passed through a Bio-Gel P-100 column (180 X 5 cm) equilibrated with 0.5% SDS, 50 mM Tris-HCl, pH 7.0, 1 mM DTT, and 0.2% NaN_3.

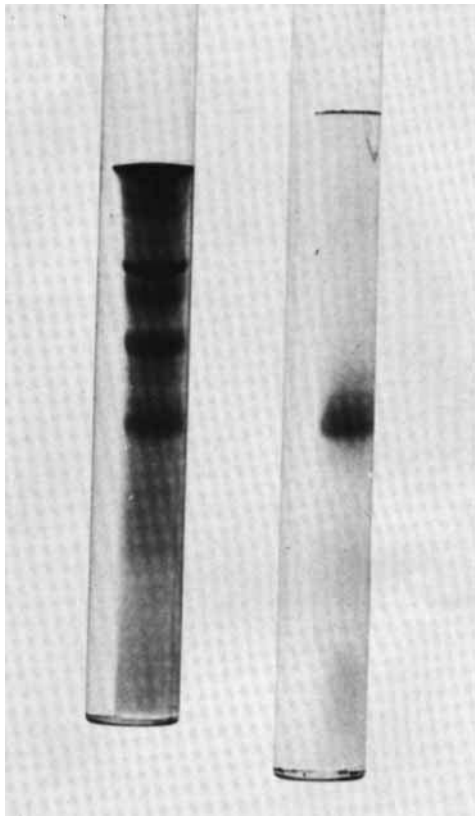

Fig. 4. 10% SDS-polyacrylamide gels of DOC-treated tryptically digested sarcoplasmic reticulum (left) and the 20K dalton fragment after further purification on Bio-Gel P-100 (right).

Uncoupling of Hydrolysis and Transport by Tryptic Digestion of Sarcoplasmic Reticulum Vesicles

Sarcoplasmic reticulum vesicles were digested as described in Methods but with a range of trypsin:protein ratios from 1:5 to 1:1,400. The control was treated with trypsin which had been previously inhibited with trypsin inhibitor. ATPase activity is substantially unaffected by tryptic digestion throughout, but while calcium uptake is unimpaired by cleavage of the 100K dalton molecule to 55K and 45K dalton fragments, it is abolished upon digestion of the 55K dalton fragment to 30K and 20K dalton fragments. Thus, the cleavage of an essential bond between the 30K and 20K dalton fragments uncouples ATP hydrolysis and calcium transport (22).

DISCUSSION

The extreme sensitivity of the bilayer membrane assay requires the highest purity of the material to be tested in order to provide meaningful results. In view of the discrepancies in the amino acid analyses of the 20K dalton fragment prepared in the laboratories of MacLennan (11) and Green (23) by similar digestion procedures, we have further purified

TABLE I. Selectivity of 20K Dalton Fragment in PC:Cholesterol (5:1) Membranes

	Ba^{2+} >	Ca^{2+} >	Sr^{2+} >	Mn^{2+} >	Mg^{2+}
Biionic potential (mV)	−2.1 >	0.0 >	1.4 >	5.8 >	8.8
$P_{Ca^{2+}}/P_X$	0.82 >	1.00 >	1.14 >	1.80 >	2.53

Biionic potential in mV in the presence of the 20K dalton fragment and 5 mM $CaCl_2$ on one side of a phosphatidylcholine:cholesterol (5:1, mg:mg) bilayer and 5 mM of the other divalent cation on the opposite side.

TABLE II. Uncoupling of Transport From Hydrolysis by Tryptic Digestion of Sarcoplasmic Reticulum

Activity (% control)		Protein (% maximum)	
Ca-dependent ATPase	Ca-uptake	100K dalton fragment	55K dalton fragment
100	100	100	0
106	103	39	95
85	3	0	5
102	0	0	0

Calcium-dependent ATP hydrolysis and ATP-dependent calcium uptake assayed as described in Methods, presented as percent of control activities. Amount of fragments determined by measurement of the area under the peaks in gel scans, expressed as percent of the maximum amounts attained before or during tryptic digestion.

the 20K dalton ionophoric polypeptide whose properties are described here. The 20K dalton fragment purified by the standard procedure contained 2 fluorescamine-sensitive spots in addition to the 20K dalton fragment. This material did not appear on SDS gels which are sensitive down to 1,000 daltons. The isolated contaminants removed by further purification of the 20K dalton fragment with SDS-gel chromatography showed non-specific ionophoric activity or no activity, while the properties of the further purified 20K dalton fragment were the same as before.

Phosphatidylcholine:cholesterol bilayers provide a lipid environment which is more consistent with the composition of the sarcoplasmic reticulum membrane than that of oxidized cholesterol bilayers used previously. In bilayers of either composition, the ionophoric activity of the 20K dalton fragment possesses the same ion dependence and the same selectivity for divalent cations. This is further evidence that the ionophoric activity of the 20K dalton fragment is not the result of a nonspecific protein-lipid interaction. The 20K dalton fragment requires Ca^{2+} ions for the expression of its ionophoric properties and has a selectivity sequence for divalent cations which is consistent with the selectivity of transport in intact sarcoplasmic reticulum. The ionophoric activity of the intact ATPase (100K daltons), and the 55K and the 20K dalton fragments exhibit the same selectivity sequence for divalent cations (25). Further, specific inhibition of the ionophoric function by mercuric chloride, ruthenium red, etc., has been shown to be the same for the intact ATPase, the 55K dalton cleavage product and the 20K dalton fragment (14). This is con-

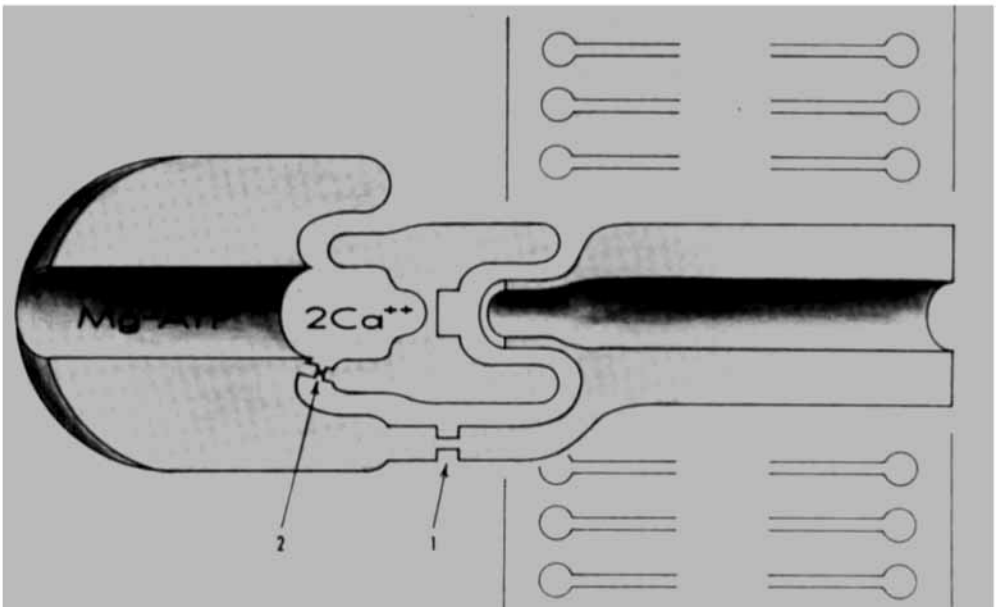

Fig. 5. Model for active transport of calcium in sarcoplasmic reticulum.

sistent with inhibition of transport in intact sarcoplasmic reticulum.

The disruption of calcium transport upon the cleavage of a bond connecting the 30K dalton fragment phosphorylation site to the 20K dalton fragment in intact sarcoplasmic reticulum vesicles is consistent with the identification of the 20K dalton polypeptide as a Ca^{2+}-ionophoric site of the ATPase molecule and is further evidence for localization of the hydrolytic and ionophoric functions in separate portions of the ATPase molecule. ATP hydrolytic activity is unimpaired while Ca transport is uncoupled from hydrolysis by the disruption of this essential bond.

These studies and our previously published work (5–12, 14, 22) lead then to a schematic model of the ATPase molecular structure (Fig. 5) which is consistent with all of the evidence for coupling between hydrolysis and transport in sarcoplasmic reticulum. The 100K dalton ATPase has a hydrophobic portion which may be a channel spanning the membrane, this being the 45K dalton fragment produced by trypsin digestion (9, 24, 25). The 55K dalton portion exists on the exterior, cytoplasmic face and contains both the 30K dalton phosphorylation site and the 20K dalton ionophoric site which is a gate partially buried in the hydrophobic interior at the mouth of the 45K dalton fragment channel. The 20K dalton fragment contains the site of specific Ca^{2+} interaction as demonstrated by the ionophoric properties in this study and previous work. Thus, while both ATP hydrolysis and Ca^{2+} transport remain coupled despite tryptic cleavage of the bond denoted 1, disruption of energy transduction ensues with the breaking of the bond denoted 2, hydrolysis being still functional while transport is abolished since the gate can no longer interact with the hydrolytic site.

ACKNOWLEDGMENTS

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