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# ATP-dependent proton transport by isolated brain clathrin-coated vesicles. Role of clathrin and other determinants of acidification

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**We have systematically investigated certain characteristics of the ATP-dependent proton transport mechanism of bovine brain clathrin-coated vesicles. $H^+$ transport specific activity was shown by column chromatography to co-purify with coated vesicles, however, the clathrin coat is not required for vesicle acidification as $H^+$ transport was not altered by prior removal of the clathrin coat. Acidification of the vesicle interior, measured by fluorescence quenching of acridine orange, displayed considerable anion selectively ($Cl^- > Br^- \gg NO_3^- \gg$ gluconate, $SO_4^{2-}$, $HPO_4^{2-}$, mannitol; $K_m$ for $Cl^- \simeq 15$ mM), but was relatively insensitive to cation replacement as long as $Cl^-$ was present. Acidification was unaffected by ouabain or vanadate but was inhibited by *N*-ethylmaleimide ($IC_{50} < 10$ μM), dicyclohexylcarbodiimide (DCCD) ($IC_{50} \simeq 10$ μM), chlorpromazine ($IC_{50} \simeq 15$ μM), and oligomycin ($IC_{50} \simeq 3$ μM). In contrast to *N*-ethylmaleimide, chlorpromazine rapidly dissipated preformed pH gradients. Valinomycin stimulated $H^+$ transport in the presence of potassium salts (gluconate $\gg NO_3^- > Cl^-$), and the membrane-potential-sensitive dye Oxonol V demonstrated an ATP-dependent interior-positive vesicle membrane potential which was greater in the absence of permeant anions (mannitol > potassium gluconate > KCl) and was abolished by *N*-ethylmaleimide, protonophores or detergent. Total vesicle-associated ouabain-insensitive ATPase activity was inhibited 64% by 1 mM *N*-ethylmaleimide, and correlated poorly with $H^+$ transport, however *N*-ethylmaleimide-sensitive ATPase activity correlated well with proton transport ($r = 0.95$) in the presence of various $Cl^-$ salts and $KNO_3$. Finally, vesicles prepared from bovine brain synaptic membranes exhibited $H^+$ transport activity similar to that of the coated vesicles. Collectively these findings indicate that: (1) the $H^+$ transport mechanism of bovine brain clathrin-coated vesicles is not dependent upon the clathrin coat, and closely resembles that of rat liver clathrin-coated vesicles, (2) the $H^+$ transport mechanism is intrinsically electrogenic and pH and electrical gradients established by the proton transport mechanism vary inversely in the presence of permeable or impermeable anions, (3) at concentrations above 3 μM, oligomycin inhibits vesicle acidification, (4) chlorpromazine inhibits coated vesicle acidification, at least in part, by increasing proton conductance, and (5) other smooth membranes from bovine brain, including synaptic membranes, exhibit a similar $H^+$ transport mechanism.**

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Abbreviations: Mes, 4-morpholineethanesulfonic acid; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; DCCD, dicyclohexylcarbodiimide; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; Oxonol V, bis(3-phenyl-5-oxoisoxazol-4-yl)pentamethine oxonol, EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid.

## Introduction

Proton transport may play an important role in many biological processes including intracellular pH regulation [1], transepithelial bicarbonate or proton transport [2–4], and both uptake and secretion of macromolecules [5,6]. Reports from a number of laboratories, including our own, have described ATP-dependent proton transport mechanism(s) ($H^+$ pumps) in a variety of subcellular organelles derived from mammalian tissues, including clathrin-coated vesicles [7–10], endosomes [11,12], lysosomes [13–15], chromaffin granules [16], and Golgi [5,17] as well as in plasma membrane vesicles prepared from turtle bladder [2,3] and synaptosomes from rat brain [18]. To provide additional insights into the physiologic role and regulation of these pumps, we have characterized in detail the effects of ions and a variety of inhibitors, including oligomycin and chlorpromazine, on the proton transport mechanism of bovine brain clathrin-coated vesiscles. In addition, we have examined the electrogenicity of proton transport and the relationship between vesicle acidification and ATPase activity. Finally, we have studied two aspects of coated vesicle $H^+$ transport which have important implications for endocytosis, exocytosis, and membrane recycling: (1) the effect of removing the clathrin coat on ATP-dependent proton transport by these vesicles and (2) evidence for a similar $H^+$ transport mechanism in vesicles prepared from brain synaptic plasma membranes, which are a site of formation of neuronal coated pits and coated vesicles [19].

## Materials and Methods

*Materials.* Hepes, DCCD, oligomycin, ouabain, *N*-ethylmaleimide, vanadate, valinomycin, CCCP, monensin, polyoxyethylene 20 acetyl ether (Brij 58), NADH, phospho*enol*pyruvate, lactate dehydrogenase and pyruvate kinase were purchased from Sigma. ATP was obtained from Boehringer Mannheim; Mes from Calbiochem, Behring; $^2H_2O$ from Aldrich Chemical Co.; and ultrapure sucrose from Bethesda Research Labs. Oxonol V was purchased from Molecular Probes, Inc. (Junction City, OR), chlorpromazine hydrochloride was a gift of Dr. C Kaiser (Smith, Kline and French Laboratories), and chlorpromazine sulfoxide was a gift of Dr. Stephen Kennedy (NIMH). DCCD, oligomycin, valinomycin, CCCP, Oxonol V, and monensin were prepared as stock solutions in absolute ethanol. Fresh stocks of *N*-ethylmaleimide and chlorpromazine were prepared as aqueous solutions each day and vanadate was prepared at least one week prior to use.

*Isolation of coated vesicles.* Coated vesicles were isolated by a slight modification [20] of the procedure of Nandi et al. [21]. In a typical preparation, nine bovine brains were homogenized in Buffer A containing 100 mM Mes (pH 6.5), 1 mM EGTA, 0.5 mM $MgCl_2$, and 0.02% (w/v) sodium azide. All procedures were carried out at 4°C and involved a series of low speed (approx. $10\,000 \times g_{av}$), and high speed (approx. $100\,000 \times g_{av}$) centrifugations [20]. The enriched coated vesicle suspension was layered onto an 8% sucrose/$^2H_2O$ single-step gradient (maintained at pH 6.5 with Buffer A salts) and centrifuged at $130\,000 \times g_{av}$ for 2 h at 18°C. The coated-vesicle pellets were washed several times with the appropriate final suspension buffer. For most studies, the brain coated vesicle pellets were resuspended in suspension buffer using a type A loose-fitting Dounce homogenizer, centrifuged at $17\,000 \times g_{av}$ for 10 min to remove the bulk of smooth membrane contaminants, and were stored at a final concentration of 1–2 mg/ml The suspension buffer contained 200 mM mannitol, 3 mM $MgSO_4$, and 10 mM Hepes (pH 7.0 with KOH); however, for certain experiments 100 mM KCl or potassium gluconate was substituted for mannitol.

For the purposes of some studies unwashed coated vesicles suspended in buffer A were purified further by Sephacryl S-1000 gel filtration column chromatography (Pharmacia Fine Chemicals, Piscataway, NJ, lot No. G1-23033). Approximately 8 ml of sample containing 5–8 mg/ml of coated-vesicle protein was layered onto a Sephacryl S-1000 superfine (2 × 85 cm) column preequilibrated in buffer A. The material was eluted in similar buffer at approx. 45 ml/h and collected in 2.0-ml fractions. The purified coated vesicles and the contaminating structures eluted in two well defined peaks as determined by absorbance at 280 nm as previously described [22]. Fractions

from the first peak were pooled and termed column contaminants. Fractions from the second peak were pooled and termed column-purified coated vesicles. The pooled fractions were centrifuged at $100\,000 \times g_{av}$ for 60 min and resuspended overnight in a small volume (approx. 10 ml) of Buffer A or the mannitol suspension buffer. All vesicle preparations were kept at 4°C and were assayed for $H^+$ transport activity within 36–48 h of preparation although $H^+$ transport activity was well preserved after storage for several weeks at −70°C.

*Preparation of uncoated vesicles.* Uncoated vesicles were prepared from either brain coated vesicles or from the Sephacryl S-1000 column-purified coated vesicles by overnight dialysis against buffer containing 10 mM Tris-HCl, pH 8.2 at 4°C. Separation of the uncoated vesicles from the dissociated clathrin coats was achieved by the centrifugation at $100\,000 \times g_{av}$ for 45 min. The supernatants were analyzed by SDS-polyacrylamide gel electrophoresis, Lowry protein determination [23] and spectroscopy to determine the extent of vesicle uncoating. An extinction coefficient of 10.0 (at $A_{280nm}$) was used to estimate the concentration of clathrin coat protein in the supernatant [24].

*Analytical gel electrophoresis.* Samples were solubilized in buffer containing (final concentrations) 2% SDS, 5% β-mercaptoethanol, 60 mM Tris-HCl (pH 6.8), 10% glycerol and 0.001% bromophenol blue and were boiled for 5 min. Aliquots of 120 μl were layered onto a 4% stacking gel and electrophoresed at 15 mA through a 9% separating gel according to the procedure of Laemmli [25]. Gels were stained with 0.15% Coomassie blue R-250 in a mixture of 10% acetic acid and 50% methanol and destained in 10% glacial acetic acid and 5% methanol or were silver stained using the procedure of Merril et al. [26] (Bio-Rad Silver Stain Kit).

*Isolation of synaptic plasma membrane.*

Synaptic plasma membranes from bovine brain were isolated according to the procedure of Jones and Matus [27]. The plasma membrane band at the 28.5–34% (w/w) sucrose interface was collected, diluted 2-fold with cold distilled water and centrifuged at $87\,000 \times g_{av}$ for 2 h. The slightly darker center of the pellet was gently aspirated and the remaining white pellet of synaptic plasma membrane was resuspended in buffer (100 mM mannitol, 10 mM Hepes, 3 mM $MgSO_4$, pH 7.0) as described for the coated vesicles.

*$H^+$ transport.* $H^+$ transport was assayed using the fluorescence quenching of acridine orange (excitation = 493 nm, emission = 530 nm) as described previously [7,28,29] in a Perkin-Elmer 44B spectrofluorimeter. Clathrin-coated vesicles (0.27–0.61 mg protein) or equivalent amounts of the other preparations were added to acrylic cuvettes (Sarstedt) with incubation medium containing acridine orange (6 μM) in a final volume of 2 ml and preincubated for 60 min at 4°C and then warmed to 23°C for 10 min prior to measuring $H^+$ transport. Details of the composition of all assay solutions are given in the table legends. $H^+$ transport was initiated by the addition of 3 mM $Na_2ATP$. The final pH of all solutions was 7.0. The rate of $H^+$ transport was measured as the initial rate of decrease of fluorescence determined from the slope of a tangent to the trace immediately after addition of ATP [7,29]. Preliminary studies indicated that the $H^+$ transport rate measured in this fashion for a given batch of coated vesicles was linearly related ($r = 0.99$) to vesicle protein concentration over the range used in these studied and was independent of both acridine orange concentration (over the range of 0.5 to 6 μM) and buffer concentration (over the range 3–50 mM Hepes). $H^+$ transport rate per mg vesicle protein varied between batches of clathrin-coated vesicles, but was constant within a given batch. Therefore, for most studies, the rate of $H^+$ transport in the presence of ions or inhibitors was expressed as a percentage of the rate measured in concurrent control studies performed with coated vesicles from the same batch.

In all assays, fluorescence quenching achieved a steady-state level within 5 min which was assumed to reflect the steady-state pH gradient across the vesicle membrane. In some studies, chlorpromazine or *N*-ethylmaleimide was added to vesicles after an ATP-dependent steady-state pH gradient had developed in order to examine the effects of these agents on preformed gradients.

*Membrane potential studies.* Changes in the coated vesicle membrane potential were assessed directly using the fluorescent anionic dye Oxonol V [30]. Brain coated vesicles (0.25 mg protein, in

mannitol suspension buffer) were added to cuvettes containing 200 mM mannitol or 100 mM salt, 10 mM Hepes (pH 7.0) and 3 mM $MgSO_4$ in a total volume of 2 ml and were preincubated for 60 min at 4°C. Oxonol V (1.3 $\mu$M) was added to the cuvettes which were then warmed to 23°C for 10 min. Fluorescence was measured at an excitation wavelength of 570 nm (slit width 5 nm) and emission wavelength of 640 nm (slit width 10 nm). Each assay was initiated by the addition of 3 mM $Na_2ATP$. Preliminary studies showed that, in the absence of vesicles, *N*-ethylmaleimide had no effect on the fluorescence intensity or emission spectrum of Oxonol V. ATP, monensin, and CCCP changed the fluorescence by +0.8%, −3.3%, and −0.3%, respectively; and 0.2% Brij 58 increased fluorescence about 25% without changing the shape of the emission spectrum.

*Electron microscopy.* The various coated vesicle preparations were fixed for 1 h at 4°C in 1.5% glutaraldehyde in 0.1 M sodium cacodylate buffer and then pelleted by centrifugation at $100\,000 \times g_{av}$ for 60 min. Pellets were rinsed with normal saline, osmicated in 2% osmium tetroxide containing 1.5% KCN for 90 min, rinsed in distilled water, dehydrated and embedded in LX-112. Sections were stained with lead citrate and uranyl acetate and examined in a Phillips 380 electron microscope [31].

*ATPase activity.* $Mg^{2+}$-ATPase activity was measured at 23°C by a recording spectrophotometric assay [32] using an Aminco DW-3 spectrophotometer. Each 1-ml incubation contained Hepes (10 mM, pH 7.0), $MgSO_4$ (3 mM), ouabain (2 mM), NADH (0.5 mM), phospho*enol*pyruvate (2.5 mM) with salts (100 mM) and with or without inhibitor. Coated vesicles (stored in mannitol suspension buffer) were preincubated on ice for 60 min in the ATPase incubation medium and prewarmed for 10 min in a room-temperature bath. Lactate dehydrogenase (10 units) and pyruvate kinase (10 units) were added to each cuvette and the assay was initiated by the addition of 3 mM $Na_2ATP$. Each assay was performed in duplicate and *N*-ethylmaleimide-sensitive ATPase activity was calculated as the difference between activities in incubations with and without 1 mM *N*-ethylmaleimide.

*Other analytical techniques.* Protein concentrations were determined according to the method of Lowry et al. [23]. Total vesicle-associated lipids were extracted by ultrasonification in chloroform/methanol (2:1, v/v), were rectified according to the procedure of Folch et al. [33], and were dried under nitrogen. Total lipid content was determined gravimetrically on triplicate samples using a Mettler UM-3 electrobalance.

## Results

*Characterization of the brain coated-vesicle preparation*

The coated-vesicle preparation consisted of a population of vesicles approximately 50–150 nm in diameter, most of which were surrounded by a rod-like lattice, characteristic of clathrin [34] (Fig. 1A), as well as some smooth membrane vesicles, most of which were larger than the coated vesicles. SDS-polyacrylamide gel electrophoresis (SDS-PAGE) (Fig. 2) of this preparation demonstrated the predominant and characteristic 180 kDa clathrin band.

Passage of the isolated coated vesicles over a Sephacryl S-1000 column produced two distinct peaks (as assessed by absorbance at 280 nm). The first peak which was termed column contaminants consisted predominantly of large smooth-surfaced membrane vesicles, as well as some coated vesicles (Fig. 1D). SDS-PAGE revealed a striking loss of the 180 kDa band in this peak (Fig. 2). The second peak, which contained almost exclusively coated vesicles as determined by thin section electronmicroscopy (Fig. 1B), also demonstrated the 180 kDa clathrin band (Fig. 2) on SDS-PAGE.

Thin section electronmicroscopy of uncoated vesicles, prepared from column-purified coated vesicles, revealed a population of small, smooth-membrane structures (Fig. 1C), and on SDS-PAGE there was a marked loss of the 180 kDa clathrin band as well as the 33-kDa and 36-kDa clathrin-associated protein bands (Fig. 2). SDS-PAGE of the supernatants from the uncoating process demmonstrated that the solubilized protein consisted almost exclusively of clathrin and clathrin-associated proteins (data not shown). These proteins account for approximately 58% of all brain coated vesicle protein [34], and, as supernatant proteins accounted for about 50% of total coated vesicle

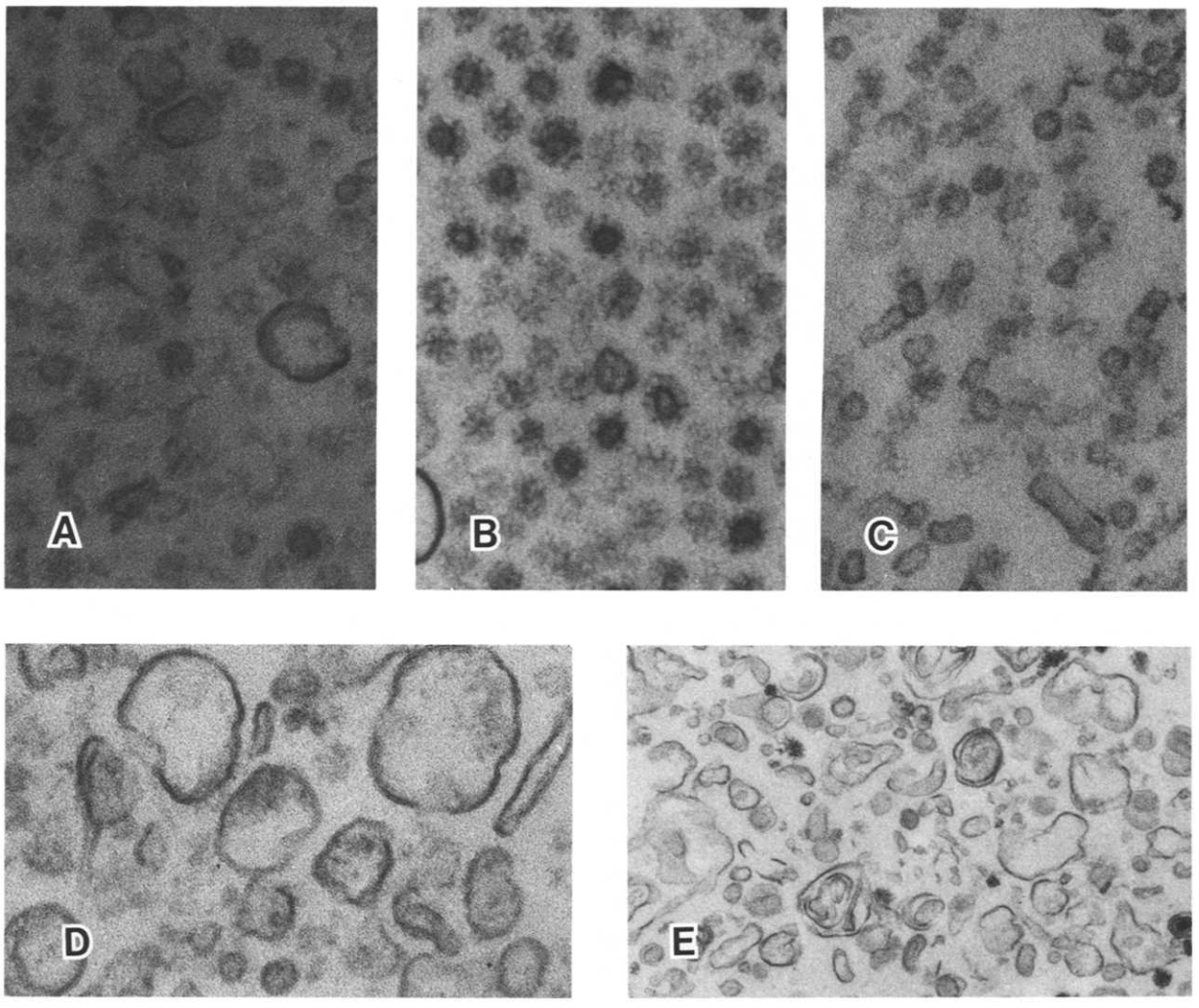


Fig 1 Thin section electron micrographs of (A) enriched unwashed coated vesicles from the 8% sucrose/$^2H_2O$ step gradient; (B) Sephacryl S-1000 column purified coated vesicles; (C) uncoated vesicles prepared from (B); (D) Sephacryl S-1000 column contaminants; and (E) synaptic plasma membrane vesicles. Magnification: A–D, 90000×; E, 60000×

protein (as measured by Lowry's method and spectroscopically), it can be estimated that ≥ 86% of the clathrin coat was removed from these vesicles.

The synaptic membrane preparation contained large smooth membrane vesicles, many of which were larger than the coated vesicles, as well as sheets of membrane (Fig. 1E). The 180 kDa clathrin band was not seen in this preparation (Fig. 2).

Although silver-staining of the SDS-polyacrylamide gels revealed many more protein bands than did Coomassie blue staining, the relative patterns for the various preparations did not differ from that seen with the conventional Coomassie blue stain.

*$H^+$ transport*

Acridine orange, a fluorescent weak base, is concentrated within acidic compartments, and concomitant with development of a pH gradient (acidic vesicle interior), acridine orange fluorescence is quenched [28,29]. Fluorescence quenching

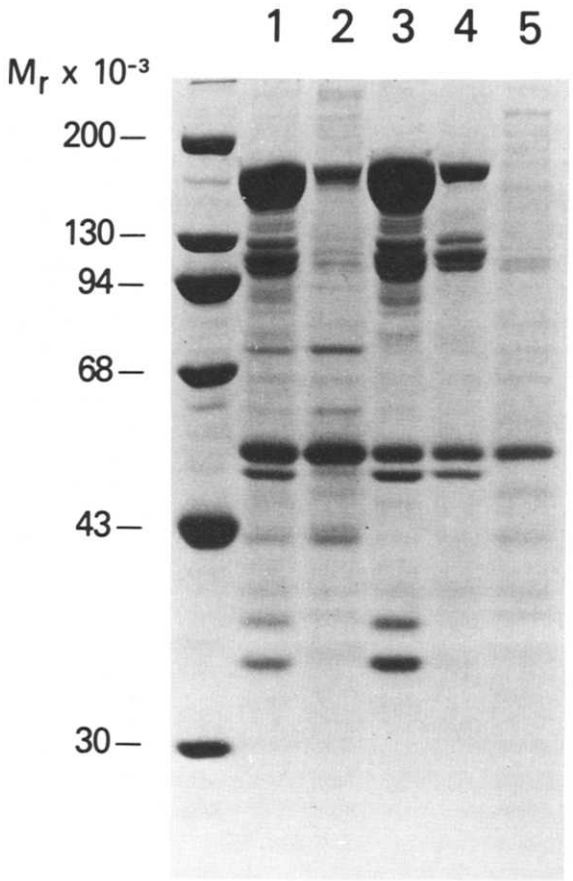


Fig 2 SDS-polyacrylamide gel electrophoresis of samples from preparations shown in Fig 1 Separation was carried out as described in Materials and Methods through a 9% separating gel The gels were stained with 0 15% Coomassie blue R-250 Gels were calibrated with standard molecular weight markers (unlabeled lane) myosin, 200000, β-galactosidase, 130000; phosphorylase B, 94000; bovine serum albumin, 68000; ovalbumin, 43000; carbonic anhydrase, 30000 The gel lanes contained (1) brain coated vesicles, 80 μg, (2) column contaminant, 85 μg, (3) column-purified brain coated vesicles, 75 μg, (4) column-purified brain uncoated vesicles, 40 μg; (5) synaptic plasma membrane vesicles, 120 μg

due to proton transport is rapidly reversed by agents such as proton ionophores (CCCP), proton/cation-exchanging ionophores (monensin, nigericin), and detergents (Brij 58) which abolish the proton gradient. Indeed, in these studies acridine orange fluorescence was quenched upon addition of ATP to solutions containing isolated brain coated vesicles but not after addition of ATP to solutions containing either no coated vesicles or coated vesicles previously exposed to CCCP (5 μM) or the detergent Brij 58 (0.2%). Fluorescence quenching was rapidly reversed upon addition of CCCP (5 μM) or monensin (5 μM). Ethanol alone (0.25% in the final incubation medium) had no effect on acridine orange fluorescence.

*Effects of inhibitors on $H^+$ transport*

The effects of a number of agents thought to inhibit proton transport were studied. *N*-Ethylmaleimide virtually abolished $H^+$ transport by coated vesicles at all concentrations greater than 10 μM, and was equally effective whether it was present in the pre-incubation medium or added just prior to the addition of ATP. In contrast, when it was added to vesicles which had already established an ATP-dependent pH gradient, *N*-ethylmaleimide (1 mM) caused a slow, but progressive, loss of the pH gradient and return of acridine orange fluorescence toward baseline (data not shown). DCCD inhibited $H^+$ transport with an $IC_{50} \simeq 10$ μM. Micromolar concentrations of oligomycin also inhibited acidification of these vesicles with an $IC_{50} \simeq 3$ μM. Ouabain and vanadate, at concentrations known to inhibit other ATPases such as $(Na^+ + K^+)$-ATPase and $H^+$ transport by yeast membranes [35], had no effect on $H^+$ transport (Table I).

Chlorpromazine, a drug which alters many membrane transport processes, abolished vesicle acidification (Table II) with an $IC_{50} \simeq 15$ μM. This effect of chlorpromazine did not require a pre-incubation period, i.e. as with *N*-ethylmaleimide, chlorpromazine was equally effective when added simultaneously with ATP or when added to the pre-incubation medium. In contrast to the effects of *N*-ethylmaleimide, chlorpromazine, when added to vesicles which had already established an ATP-dependent pH gradient, caused rapid reversal of the steady-state fluorescence quenching of acridine orange. The extent to which chlorpromazine reversed steady-state fluorescence quenching was concentration-dependent (Table II). Chlorpromazine sulfoxide, at concentrations up to 200 μM, had virtually no effect on vesicle acidification (data not shown).

*Effect of ions on $H^+$ transport*

$H^+$ transport by the coated vesicles was maximal in the presence of 100 mM KCl but exhibited

TABLE I

EFFECTS OF INHIBITORS ON ATP-DEPENDENT PROTON TRANSPORT OF BRAIN COATED VESICLES

All assays were performed in 2 ml of medium containing 100 mM KCl, 10 mM Hepes (pH 7.0), 3 mM $MgSO_4$ and 6 $\mu$M acridine orange with ATP as a substrate, and results are expressed as a percentage of concurrent controls. Coated vesicles were preincubated with inhibitor (or solvent) in assay medium for 60 min at 4°C and warmed to 23°C for 10 min prior to initiation of the assay by addition of ATP. All values, expressed as a percentage of concurrent controls, represent the mean ($\pm$S.E. for $n \geq 3$) of determinations from 1–8 different preparations of coated vesicles.

| Inhibitor | Concn (M) | $H^+$ transport initial rate (% control) |
|---|---|---|
| *N*-Ethylmaleimide | $2 \cdot 10^{-3}$ | 0.56 |
| | $1 \cdot 10^{-3}$ | 3.61 ± 2.20 |
| | $5\ 10^{-4}$ | 0.88 ± 0.88 |
| | $2.5\ 10^{-4}$ | 0.57 ± 0.41 |
| | $1 \cdot 10^{-4}$ | 4.7 ± 4.4 |
| | $1\ 10^{-5}$ | 12.6 |
| Dicyclohexyl-carbodiimide | $5 \cdot 10^{-4}$ | 0.23 |
| | $2 \cdot 10^{-4}$ | 3.1 ± 1.8 |
| | $2\ 10^{-5}$ | 6.3 ± 2.5 |
| | $1 \cdot 10^{-5}$ | 43.8 ± 13.8 |
| | $2.5 \cdot 10^{-6}$ | 68.8 ± 14.1 |
| | $1 \cdot 10^{-6}$ | 80.9 ± 6.2 |
| | $1\ 10^{-7}$ | 88.5 ± 15.4 |
| Oligomycin | $1 \cdot 10^{-4}$ | 5.1 |
| | $2.6 \cdot 10^{-5}$ | 10.5 ± 6.9 |
| | $2.6 \cdot 10^{-6}$ | 62.0 ± 8.7 |
| | $5.3\ 10^{-7}$ | 94.8 ± 19.0 |
| | $2.6 \cdot 10^{-7}$ | 84.6 ± 13.0 |
| | $2.6 \cdot 10^{-8}$ | 79.2 ± 5.3 |
| | $2.6\ 10^{-9}$ | 103.8 ± 5.2 |
| | $2.6 \cdot 10^{-11}$ | 108.6 |
| | $2.6 \cdot 10^{-13}$ | 101.7 |
| Ouabain | $2 \cdot 10^{-3}$ | 109 |
| Vanadate | $1 \cdot 10^{-4}$ | 100 |

TABLE II

EFFECTS OF CHLORPROMAZINE ON $H^+$ TRANSPORT BY BRAIN COATED VESICLES

All assays were performed in medium containing 100 mM KCl, 10 mM Hepes (pH 7.0), 3 mM $MgSO_4$, and 6 $\mu$M acridine orange with ATP as substrate. To assess the effects of chlorpromazine on the initial rate of $H^+$ transport and the steady-state pH gradient, coated vesicles were preincubated in assay medium with chlorpromazine for 60 min at 4°C and warmed to 23°C for 10 min prior to initiation of the assay by the addition of ATP. Results are expressed as a percentage of concurrent controls without chlorpromazine. The effect of chlorpromazine on pre-established pH gradients was measured by adding small aliquots of chlorpromazine (10 mM stock in water) to coated vesicles, previously incubated with acridine orange, 100 mM KCl and ATP, which had already established a stable steady-state pH gradient. The recovery of the total fluorescence after addition of chlorpromazine is expressed as a percentage of the total fluorescence recovery seen after addition of monensin. All values represent the mean of determinations performed on 1–4 different preparations of coated vesicles.

| Concn ($\mu$M) | $H^+$ transport initial rate (% control) | Steady-state pH gradient achieved in the presence of chlorpromazine (% control) | Relaxation of established pH gradient after addition of chlorpromazine (% gradient relaxed) |
|---|---|---|---|
| 200 | 1.76 | 0.0 | – |
| 150 | 0.54 | 0.0 | – |
| 100 | 1.52 | 0.0 | – |
| 75 | 0.0 | 2.2 | – |
| 50 | 10.5 | 2.6 | 100 |
| 25 | 25.1 | 24.9 | 76 |
| 10 | 65.4 | 73.1 | 29 |
| 5 | 80.9 | 85.0 | 17 |
| 1 | 89.7 | 96.7 | – |
| 0.1 | 93.8 | 100.8 | – |

no absolute dependence upon any ion (Table III). In $Cl^-$-containing buffer, $H^+$ transport was similar in the presence of $K^+$, $Na^+$, and $Li^+$ but was decreased somewhat in the presence of choline. In contrast, although $H^+$ transport was supported by substitution of $Br^-$ for $Cl^-$, it was markedly diminished when relatively permeant ($NO_3^-$) or impermeant (gluconate, $SO_4^{2-}$ or $HPO_4^{2-}$) anions were substituted for $Cl^-$ or when mannitol was used in place of salts.

Further studies were performed to examine the relationship between $H^+$ transport and buffer chloride concentration. The initial rate of fluorescence quenching of these vesicles varied nonlinearly with increasing chloride concentration, with an apparent $K_m$ of about 15 mM.

*Effects of column purification and of removal of the clathrin coat on $H^+$ transport by coated vesicles*

Purification of brain coated vesicles using a Sephacryl S-1000 column removed virtually all of

TABLE III

EFFECTS OF ION SUBSTITUTION ON ATP-DEPENDENT PROTON TRANSPORT

All $H^+$ transport studies were performed in buffer containing salt, 10 mM Hepes (pH 7.0), 3 mM $MgSO_4$ and 6 $\mu$M acridine orange after a 1-h preincubation at 4°C, and the results are expressed as a percentage of $H^+$ transport activity in the presence of KCl with ATP (3 mM) as substrate All salts were present at a concentration of 100 mM, except for $K_2SO_4$ and $K_2HPO_4$ which were present at a concentration of 50 mM, with mannitol used to maintain isosmolarity KCl and NaCl were present at a concentration of 50 mM each in the KCl/NaCl incubation Mannitol alone (200 mM) was present in the no salt incubation Each value represents the mean ($\pm$S E for $n \geq 3$) of determinations on 1–8 different preparations of coated vesicles, and the mean of determinations on two preparations of uncoated vesicles

| Salt | $H^+$ transport initial rate (% of control) | |
|---|---|---|
| | Brain coated vesicles | Brain uncoated vesicles |
| KCl | 100 | 100 |
| NaCl | 98 5 $\pm$ 5 1 | 98 0 |
| KCl/NaCl | 93 8 $\pm$ 9 2 | – |
| LiCl | 75.5 $\pm$ 10 9 | – |
| Choline chloride | 59 6 $\pm$ 11 0 | 85 8 |
| KBr | 85 2 $\pm$ 13.9 | 112 0 |
| $KNO_3$ | 40.9 $\pm$ 5 4 | 62 8 |
| Potassium gluconate | 4.5 $\pm$ 2 1 | – |
| $K_2SO_4$ | 3 5 $\pm$ 0 6 | 4 1 |
| $K_2HPO_4$ | 4 8 $\pm$ 2.1 | – |
| No salt | 0 24 | – |

the smooth membrane vesicle contaminants, although a number of coated vesicles remained in the contaminant fraction. $H^+$ transport specific activity calculated per mg protein was reduced greater than 50% by the purificaton procedure in both the contaminant fraction and in the column-purified coated vesicle fraction (Table IV). However, $H^+$ transport specific activity calculated per mg lipid was increased by 44% in column-purified coated vesicles compared with the contaminant fraction, indicating that $H^+$ transport activity co-purified with the coated vesicles. $H^+$ transport in the purified coated vesicles exhibited the same ion and inhibitor sensitivities as did the parent coated vesicles; it was completely inhibited by 1 mM *N*-ethylmaleimide, inhibited 56% by 10 $\mu$M DCCD,

TABLE IV

SPECIFIC ACTIVITY OF PROTON TRANSPORT

All assays were performed in medium containing 100 mM KCl after a 70-min pre-incubation as described in the legend to Table I Results are expressed as a percentage of values obtained using brain coated vesicles from the same batch Values are given as the mean($\pm$S E for $n \geq 3$) of determinations on 1 to 3 different preparations

| Preparation | $H^+$ transport initial rate, specific activity (% control) | |
|---|---|---|
| | per mg protein | per mg lipid |
| Brain coated vesicles | 100 | 100 |
| Column-purified brain coated vesicles | 45 5 $\pm$ 3 5 | 47 0 |
| Column contaminants | 48 6 $\pm$ 19 7 | 47 0 |
| Brain uncoated vesicles | 123 1 $\pm$ 42 4 | 81 3 |
| Synaptic membrane vesicles | 39 3 | – |

and unaltered by 0.53 $\mu$M oligomycin. $H^+$ transport by the smooth vesicle contaminants was inhibited 69% by 1 mM *N*-ethylmaleimide and 24% by 0.53 $\mu$M oligomycin.

The clathrin coat was removed from aliquots of four different preparations of column-purified coated vesicles. The specific activity of $H^+$ transport in these smooth vesicles was decreased by 19% compared to coated vesicles when calculated on a mg lipid basis, but was increased by 23% when calculated per mg protein. Since approximately 50% of vesicle-associated protein was lost during the uncoating process, these figures indicate that about 80% of $H^+$ transport activity remained after more than 86% of the clathrin coat had been removed. Uncoated vesicles exhibited ion (Table III) and inhibitor sensitivities very similar to those of the parent coated vesicles; it was inhibited 96% by 1 mM *N*-ethylmaleimide and 76% by 10 $\mu$M DCCD, and was unaffected by 0.53 $\mu$M oligomycin.

Synaptic plasma membranes were also prepared from bovine brains and assayed for $H^+$ transport. By thin-section electron microscopy, this preparation consisted of smooth membrane vesicles of varying sizes as well as sheets of membrane (Fig. 1E). These membrane vesicles exhibited ATP-dependent $H^+$ transport that was sensitive to *N*-ethylmaleimide (95.3% inhibition by 1 mM) and DCCD (55% inhibition by 10 $\mu$M) and had a

specific activity (per mg protein) 39% that of concurrently prepared brain coated vesicles (Table IV).

*Electrogenicity of $H^+$ transport*

The results of experiments which examined the effects of valinomycin and potassium on $H^+$ transport by isolated brain coated vesicles are shown in Table V. Although the absolute rate of $H^+$ transport differed in the presence of various potassium salts, valinomycin (0.1 $\mu$M) consistently increased the initial rate of acidification by these vesicles and the percentage increase was greater in the presence of an impermeant anion (gluconate) than a permeant anion ($Cl^-$). Furthermore, the effect of valinomycin in the presence of potassium gluconate was enhanced when vesicles were suspended in salt for 36 h, suggesting that these vesicles are relatively impermeable to potassium gluconate and require prolonged exposure to achieve equilibration of this salt across the vesicle membrane.

*Membrane potential measurements*

The membrane potential-sensitive anionic fluorescent dye Oxonol V was used to assess directly the ATP-dependent membrane potential of coated vesicles. The fluorescence of Oxonol V is quenched with development of an interior positive membrane potential and this fluorescence quenching is thought to be due, at least in part, to increased binding of dye to the vesicle membrane [30]. Addition of 3 mM $Na_2ATP$ to the coated vesicles caused an abrupt decrease in Oxonol V fluorescence (Fig. 3), which was greatest for vesicles in mannitol buffer and least for vesicles in KCl buffer. Much of this decrease in fluorescence (75–80% in mannitol, 43–57% in KCl) was abolished by pretreatment of vesicles with the protonophore CCCP (5 $\mu$M), *N*-ethylmaleimide (1 mM), or the detergent Brij 58 (0.2%), treatments which consistently abolished the development of a proton gradient as assessed by acridine orange fluorescence quenching. After addition of ATP, Oxonol V fluorescence was further quenched by the addition of monensin, an electroneutral $Na^+$ or $K^+/H^+$ ionophore. The effect of monensin was abolished in vesicles pretreated with CCCP, *N*-ethylmaleimide or Brij. Finally, ATP-dependent fluorescence quenching of Oxonol V was partially reversed by the addition of the electrogenic protonophore CCCP.

It should be noted, however, that some of the

TABLE V

EFFECTS OF VALINOMYCIN ON $H^+$ TRANSPORT BY BRAIN COATED VESICLES

Coated vesicles (0.34–0.41 mg protein) were added to buffer containing 100 mM salt, 10 mM Hepes (pH 7.0), 3 mM $MgSO_4$, and 6 $\mu$M acridine orange with or without 0.1 $\mu$M valinomycin (assays without valinomycin contained an equal volume of the solvent ethanol [0.25%]) in a total volume of 2 ml. Following preincubation at 4°C for 60 min, mixtures were warmed to 23°C for 10 min. Assays were initiated by the addition of 3 mM ATP. All studies were performed using vesicles from the same batch of coated vesicles. $H^+$ transport is expressed as the absolute rate of fluorescence quenching.

| Salt | H transport initial rate (% fluorescence quenching/min) | | % Increase with valinomycin |
|---|---|---|---|
| | No valinomycin | 0.1 $\mu$M valinomycin | |
| KCl [a] | 46.0 ± 3.6 | 54.1 ± 1.5 | 17.6 |
| Potassium gluconate [a] | 1.13 ± 0.42 | 9.50 ± 2.36 | 741.1 |
| KCl [b] | 59.8 | 76.4 | 27.8 |
| $KNO_3$ [b] | 25.6 | 35.4 | 38.3 |
| Potassium gluconate [b] | 3.73 | 11.54 | 209.4 |

[a] Vesicles were suspended in medium containing 100 mM KCl or potassium gluconate, 10 mM Hepes, 3 mM $MgSO_4$ during preparation and were stored in this buffer for 36 h at 4°C prior to assay. Values are given as mean ± S.E. of triplicate determinations.

[b] Vesicles were suspended in medium containing 200 mM mannitol, 10 mM Hepes, 3 mM $MgSO_4$ during preparation and were stored in this buffer for 36 h at 4°C prior to assay. Consequently, vesicles were exposed to the respective salts only for the 70-min preincubation period as outlined above.

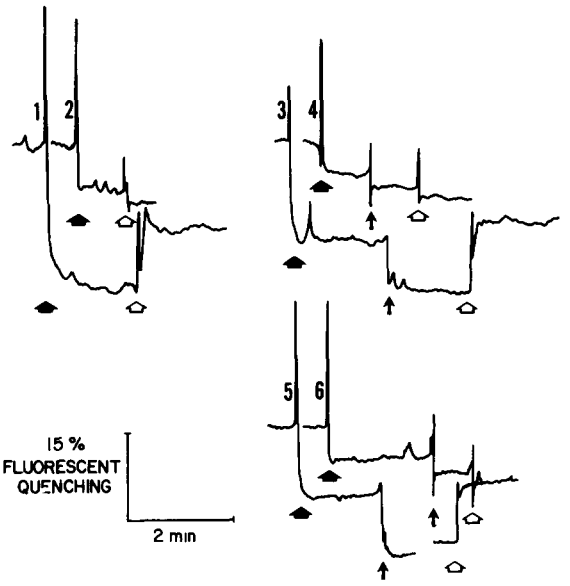


Fig 3. ATP-dependent fluorescence quenching of Oxonol V by brain coated vesicles. Coated vesicles (0.25 mg protein) were added to cuvettes containing, in a total volume of 2 ml, 1 3 μM Oxonol V, 10 mM Hepes (pH 7.0), 3 mM $MgSO_4$ and (1) 200 mM mannitol, (2) 200 mM mannitol plus 1 mM *N*-ethylmaleimide, (3) 100 mM potassium gluconate, (4) 100 mM potassium gluconate plus 0.2% Brij 58, (5) 100 mM KCl, (6) 100 mM KCl plus 1 mM *N*-ethylmaleimide Assays were initiated by the addition of 3 mM ATP (broad closed arrow) and terminated by the addition of 5 μM CCCP (broad open arrow) or 10 μM monensin (narrow closed arrow).

effects of ATP on Oxonol V fluorescence appeared to be non-specific. As shown in Fig. 3, a modest portion of ATP-dependent fluorescence quenching was not abolished by *N*-ethylmaleimide, CCCP or Brij and is probably unrelated to changes in membrane potential or pH.

*Relationship between $H^+$ transport and ouabain-insensitive ATPase activity*

Total ouabain-insensitive ATPase activity in the coated vesicle preparation averaged $1.57 \pm 0.18$ μmol/mg protein per h (mean ± S.E.) when measured in six separate preparations of coated vesicles. Total ouabain-insensitive ATPase activity was inhibited by $63.9 \pm 1.4\%$ ($n = 3$) in the presence of 1 mM *N*-ethylmaleimide, by $57.9 \pm 9.4\%$ ($n = 3$) in the presence of 200 μM DCCD and by 8% in the presence of 100 μM chlorpromazine. Linear regression analysis was used to examine the relationship between $H^+$ transport (fluorescent units/mg per min) and ATPase activity. When coated vesicles were suspended in a variety of salts or exposed to various inhibitors, the correlation between $H^+$ transport and ouabain-insensitive ATPase activity was poor ($r = 0.59$; $n = 36$). Therefore, in two preparations we examined the relationship between $H^+$ transport and *N*-ethylmaleimide-sensitive, ouabain-insensitive ATPase activity, which constituted 65.1% of total ATPase activity. $H^+$ transport correlated poorly with *N*-ethylmaleimide-sensitive ATPase activity ($r = 0.57$; $n = 12$) when both permeant and impermeant salts were used (KCl, NaCl, LiCl, $KNO_3$, potassium gluconate, $K_2SO_4$, $K_2HPO_4$). Thus, although the impermeant anions gluconate, $SO_4^{2-}$ and $HPO_4^{-2}$ virtually abolished $H^+$ transport (> 93% inhibition), they had much less effect on *N*-ethylmaleimide-sensitive ATPase activity (15–51% inhibition). However, $H^+$ transport and *N*-ethylmaleimide-sensitive ATPase activity were well correlated when vesicles suspended in media containing the more permeant $Cl^-$ salts or $KNO_3$ were evaluated ($r = 0.95$; $n = 6$), and for these studies the slope of the linear regression line approximated 1.

## Discussion

Recent work in a number of areas of cell biology has led to an increasing appreciation of the important role that proton transport, mediated by ATP-dependent mechanisms ($H^+$ pumps), may play in many biologic processes, including intracellular pH regulation [1], transcellular $H^+$ (or $HCO_3^-$) transport [2–4], receptor-mediated endocytosis of proteins and hormones [34,36,37], protein secretion [5,17,38], and lysosomal degradation processes [13–15]. Additional information on the biochemical and physiologic properties of these $H^+$ pumps would be expected to provide further insight into the physiologic role and regulatory mechanisms of proton transport. Previous reports from this as well as other laboratories have identified an ATP-dependent $H^+$ transport mechanism in clathrin-coated vesicles and have provided some information regarding ion dependency, inhibitor sensitivity, and electrogenicity of transport [7–10]. Therefore, we chose to investigate in detail a num-

ber of specific characteristics of the $H^+$ transport mechanism found in clathrin-coated vesicles derived from bovine brain.

In the present study, brain coated vesicles were prepared by differential centrifugation in a manner similar to that described by others [8–10], and ATP-dependent proton transport was readily demonstrable in this preparation using the technique of acridine orange fluorescence quenching. Proton transport was independent of $Na^+/H^+$ exchange and $(Na^+ + K^+)$-ATPase, as it occurred readily in the absence of $Na^+$ or cation gradients and was not inhibited by ouabain. Therefore, it is likely due to a primary $H^+$-transporting ion pump.

Because $H^+$ pump activity has been demonstrated in a number of subcellular organelles, we undertook an additional purification step designed to remove any large smooth-membrane vesicle contaminants from our preparation of coated vesicles. The purified coated vesicles were enriched in $H^+$ pump activity compared to the smooth-membrane vesicle contaminants (Table IV), indicating that $H^+$ pump activity was indeed associated with coated vesicles. The $H^+$ transport activity detected in the contaminant fraction may represent activity associated with the coated vesicles contained in this fraction as well as with membrane vesicles derived from other subcellular organelles such as endosomes, lysosomes, Golgi, and plasma membranes that may contain a similar $H^+$ transport mechanism. Our findings differ somewhat from those of Forgac et al. [9] who were unable to identify ATP-dependent proton transport in their pooled contaminant fraction. We cannot explain these disparate results although they may reflect differences in the isolation method used to prepared coated vesicles or in the techniques used to assess proton transport (acridine orange fluorescence quenching versus [$^{14}$C]methylamine uptake).

We also sought to determine the role of the clathrin coat in vesicle acidification. The presence of the clathrin coat per se appears to play little role in acidificaton of isolated coated vesicles, as, when over 86% of clathrin and clathrin-associated protein was removed from these vesicles, less then 20% of the specific activity (calculated per mg lipid) of $H^+$ transport was lost. These calculations depend upon the assumption that vesicle-associated lipid predominantly derives from the membrane bilayer and should be unaffected by simple removal of clathrin.

Detailed studies of coated vesicle acidification were performed with a number of agents thought to inhibit $H^+$ pumps. *N*-Ethylmaleimide, a sulfhydryl agent, was extremely effective in inhibiting the $H^+$ pump of coated vesicles ($IC_{50} < 10$ $\mu$M), observations which agree with the earlier findings of ourselves and others that *N*-ethylmaleimide effectively inhibits $H^+$ transport by clathrin-coated vesicles derived from rat liver (70 $\mu$M–1mM) [7] and bovine brain (0.1, 1 mM) [10]. *N*-Ethylmaleimide (100 $\mu$M or 1 mM has also been used to inhibit $H^+$ transport by endosomes and lysosomes [12,14,15], Golgi [17], as well as plasma membrane vesicles prepared from turtle bladder [2] and yeast [39]. This is in contrast to $H^+$ transport by submitochondrial particles which is unaffected by similar concentrations of *N*-ethylmaleimide [7,10]. These observarions suggests that sulfhydryl groups are critical to the normal functioning of the nonmitochondrial $H^+$ pumps found in eukaryotic cells.

Detailed concentration/inhibition curves for the inhibitors DCCD and oligomycin, which have not been previously available, were determined in this study. DCCD is known to inhibit a wide variety of $H^+$ pumps [2,7,9,10,35,40], and it effectively inhibited $H^+$ transport by coated vesicles with a higher $IC_{50}$ (10 $\mu$M) than we previously reported for submitochondrial particles ($IC_{50} = 1$ $\mu$M) [7]. Oligomycin, which is thought to be relatively selective for the mitochondrial $H^+$-ATPase, inhibited $H^+$ transport in coated vesicles at concentrations ($IC_{50} \simeq 3$ $\mu$M) approximately 10-fold higher than those which inhibit $H^+$ transport by submitochondrial particles ($IC_{50} \simeq 0.3$ $\mu$M) [7]. These results differ somewhat from those of others [8–10], even though all these groups used oligomycin obtained from the same source (Sigma). However, Xie et al. [8] and Stone et al. [10] used only a single concentration (0.62 $\mu$M) and did show [10] a slight decrease in acidification in the presence of oligomycin, findings compatible with those presented in this study. In contrast, Forgac et al. [9] showed no difference in [$^{14}$C] methylamine uptake by coated vesicles in the presence of 23 $\mu$M oligomycin. The discrepancy between their results and ours is difficult to explain; however, it may

result from differences in coated vesicle preparation or handling. Indeed, Lorenson and Jacobs [41] have demonstrated that the oligomycin sensitivity of the $Mg^{2+}$-ATPase of prolactin secretory granules can be markedly altered when membranes are prepared from intact granules.

We also chose to examine the effects of chlorpromazine on acidification of these coated vesicles, as chlorpromazine is thought to alter neurotransmitter availability in mammalian brain, to act as a mitochondrial uncoupler [42] and to abolish the membrane potential of chromaffin granules [43]. At concentrations greater than 10 $\mu$M, chlorpromazine virtually abolished acidification of isolated brain coated vesicles; however, the metabolite chlorpromazine sulfoxide had no significant effect on $H^+$ transport. The observations that total vesicle-associated ATPase activity was decreased only 8% by 100 $\mu$M chlorpromazine and that the ATP-dependent pH gradient of coated vesicles was rapidly reversed by addition of chlorpromazine (as compared to *N*-ethymaleimide), suggest that chlorpromazine may increase the rate at which protons leak out of coated vesicles. Our findings imply therefore that some of the known effects of chlorpromazine may be due to altered intracellular proton gradients.

We also undertook a detailed characterization of the ion dependency of $H^+$ transport by brain coated vesicles. Unlike the ($H^+/K^+$)-ATPase of gastric parietal cells [4], the $H^+$ pump of coated vesicles has no specific requirement for a particular cation as long as $Cl^-$ was present and $H^+$ transport exhibits a saturable relationship to $Cl^-$ concentration. These findings are consistent with the recent observations that $H^+$ transport by brain coated vesicles is well supported by $Cl^-$ and $Br^-$ but poorly supported by $F^-$, $SO_4^{2-}$, $HPO_4^{2-}$ and gluconate [8]. Of interest, others have reported that $H^+$ transport by lysosomes and Golgi is well supported by $Cl^-$or $Br^-$ but not by other anions [14,17]. Thus $H^+$ transport in all of these organelles exhibits anion selectivity.

Mechanisms which might explain the apparent anion selectivity of coated vesicle $H^+$ transport include: (1) an electroneutral $H^+$ pump which transports both $H^+$ and $Cl^-$ or $Br^-$, or (2) an electrogenic pump which transports $H^+$ and is associated with a selective $Cl^-$ (halide) conductance pathway. We utilized valinomycin and the potential-sensitive dye Oxonol V to distinguish these two mechanisms.

In the presence of valinomycin, an electrogenic potassium ionophore, and equal internal and external $K^+$ concentrations, $K^+$ would be expected to move rapidly across vesicle membranes to diminish any membrane potential generated by an electrogenic ion pump, thereby enhancing the rate of development of the chemical component, i.e., the pH gradient. In the studies reported here, $H^+$ transport was consistently increased by valinomycin and $K^+$, and the relative increase was greatest in the presence of gluconate, particularly after prolonged incubation in this salt. These results are similar to the report of Xie et al. [8] in which valinomycin was shown to increase $H^+$ transport in brain clathrin-coated vesicles in the presence of various potassium salts Our findings are consistent with an electrogenic $H^+$ transport mechanism and constitute further evidence that (1) $Cl^-$ acts as a permeable anion rather than as a specific cosubstrate for $H^+$ transport, and (2) brain coated vesicles are relatively impermeant to gluconate.

Fluorescent lipophilic ions have been used by a number of investigators to monitor rapid changes in membrane potential in vesicles and cells. We chose to use Oxonol V, an anion, to look for evidence of an ATP-dependent positive interior potential. The evidence that fluorescence quenching of Oxonol V in our studies was due to changes in vesicle potential included the following: (1) fluorescence quenching was abolished by exposure to agents (*N*-ethylmaleimide , CCCP, and Brij 58) which inhibit the $H^+$ pump or prevent development of a $H^+$ gradient; (2) fluorescence quenching was reversed by the electrogenic protonophore CCCP, but was increased by the electroneutral ionophore monensin; and (3) virtually no change in fluorescence was observed when these agents were used in the absence of vesicles.

The results depicted in Fig. 3 are consistent with an electrogenic $H^+$ transport mechanism which generates and, in turn, is limited by electrical and pH gradients across the vesicle membrane. These observations confirm and extend the findings of Xie et al. [8] that uptake of the potential-sensitive anion $SCN^-$ by brain coated vesicles is ATP-dependent and is inversely related to $Cl^-$

concentration. Taken together with our observations regarding the ion selectivity of vesicle acidification, these findings suggest that the regulation of $Cl^-$ conductance or availability of $Cl^-$ may determine the relative magnitudes of the electrical and chemical gradients attributable to the $H^+$ pump.

Previous reports have shown that brain clathrin-coated vesicles exhibit ouabain-insensitive ATPase activity [9] or $^{32}P_i$-ATP exchange [8,10]. We have extended these studies to examine the relationship between $H^+$ transport and ouabain-insensitive ATPase activity when the former was varied by the use of different salts. *N*-ethylmaleimide-sensitive ouabain-insensitive ATPase activity was linearly correlated with $H^+$ transport in the presence of $Cl^-$ salts and $KNO_3$, but not in the presence of the impermeant anions $SO_4^{2-}$, $HPO_4^{2-}$, and gluconate. In conjunction with the observation of Xie et al. [8] that $^{32}P_i$-ATP exchange in coated vesicles was not altered when potassium gluconate was substituted for KCl, these findings suggest that in the presence of impermeant anions, ATP-dependent proton transport leads to development of a large electrical gradient and a small chemical (pH) gradient, and thus to a disparity between vesicle acidification and *N*-ethylmaleimide-sensitive ATPase activity. Other factors which may contribute to this disparity include the following: (1) *N*-ethylmaleimide may not be completely specific for the $H^+$ pump; (2) *N*-ethylmaleimide-sensitive ATPase activity may be uncoupled from $H^+$ transport in the presence of impermeant anions; or (3) the various anions may have additional allosteric or non-specific effects on ATPase activity.

Finally, we have shown that synaptic membrane vesicles prepared from bovine brain also exhibit ATP-dependent proton transport, indicating that the $H^+$ transport mechanism may also be present in the plasma membranes of neurons. A similar proton mechanism was also recently described in rat brain synaptosomes by Cidon et al. [18]. These results are particularly intriguing in light of recent observations that coated vesicles derive from neuronal plasma membranes [19], and they suggest that a $H^+$ pump may be present in and cycle between these two organelles.

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