We have synthesized the radiolabeled "loop" diuretics [3H]bumetanide and [3H]benzmetanide (3-benzylaminoo-4-phenoxy-5-sulfamoylbenzoic acid) and have tested their potential as reversible labels of the Na,K,Cl co-transport system. These compounds bind with high affinity (Kd ≤ 30 nM, under optimal conditions) to membranes isolated from dog kidney; we found ~2 pmol/mg of sites in crude membraees from the outer medulla, and ≤0.5 pmol/mg in a similar preparation from kidney cortex. On sucrose gradient centrifugation, a peak of [3H]bumetanide binding activity (30 pmol/mg) is obtained at 37% (w/v) sucrose, distinct from the basolateral membranes in outer medulla and from brush borders in proximal tubule; our hypothesis is that this peak contains luminal membranes from the thick ascending limb of the loop of Henle. [3H]Bumetanide is displaced from its binding sites by various unlabeled loop diuretics at concentrations that have previously been shown to inhibit co-transport. Na+, K+, and Cl- (Kd = 2, 1, and 1 mM, respectively) are required for [3H]bumetanide binding, and Cl- inhibits at higher concentrations. We interpret these data to demonstrate that the Na,K,Cl co-transport system is the site involved in [3H]bumetanide binding in kidney membranes.

The coupled transmembrane movement of Na+, K+, and Cl- is mediated in many animal cells by a plasma membrane transport system that is inhibited by furosemide, bumetanide, and other loop diuretics (1-4). Recently, it has been shown that furosemide-sensitive active Cl uptake in the TALH in mammalian kidney is Na+ and K+ dependent (5, 6) and probably involves a Na,K,Cl co-transport system localized in the luminal membranes of the tubule cells. In view of the extraordinarily high rate of transport across the luminal membrane, approximately 2 nmol/cm².s (5), this tissue should be a rich source of the transport system.

In order to study the molecular properties of the Na+, K+, Cl- co-transport process, it will be necessary to identify and purify the transport protein; for this purpose, a specific marker is needed with which the transporter can be followed through steps of membrane isolation, and protein solubilization and purification. Here we report the synthesis of [3H]bumetanide and show that it binds with high affinity to membranes isolated from dog kidney outer medulla; the ion requirements for binding and the pharmacological specificity are convincing evidence that the Na,K,Cl cotransport system is involved. A preliminary report has been presented (7).

**EXPERIMENTAL PROCEDURES**

Synthesis of [3H]Bumetanide and [3H]Benzmetanide—These were prepared by reduction with NaB3H4 of the Schiff base adduct between 3-amino-4-phenoxy-5-sulfamoylbenzoic acid (Ref. 8; gift of P. Feit, Leo Pharmaceuticals, Ballerup, Denmark) and butyraldehyde or benzaldehyde. The synthesis of [3H]bumetanide is described; [3H]benzmetanide was synthesized in a parallel reaction (half-scale) with identical procedures. Butyraldehyde (35 amol, 3 μl) was added to 75 μmol of 3-amino-4-phenoxy-5-sulfamoylbenzoic acid in 75 μl of dimethyl sulfoxide, and after 30 min, 7 μmol of NaB3H4 (40 μCi, New England Nuclear; in 120 μl of 0.1 N NaOH) was added. After 1 h, 3 M acetic acid (1.2 ml) and H2O (5 ml) were added and product was extracted into methylene chloride (four times, 3 ml each); the pooled organic phase was evaporated, and the residue taken up in 1 ml of MeOH (2.5 μCi yield at this stage). [3H]Bumetanide was purified on a reverse phase analytical high performance liquid chromatography column (Whatman Partisil ODS III, 10 μm) with 30 MeOH, 20 CH3CN, 0.2 CH3COOH, 50 H2O as solvent (2 ml/min). The peak fractions were pooled, evaporated, and taken up in 1 ml 10% dimethyl sulfoxide (yield, 0.7 μCi; for benzmetanide, 0.5 μCi from 20-μCi reaction). From the absorption spectrum (using E280 = 9200 and E400 = 4000), the specific activity of the product was found to be 1.25 Ci/mmol. Purity of the radioactive material, which co-migrated with the authentic compound, was greater than 90% as determined by scintillation counting of samples from a TLC plate (Silica Gel 60 F run with 80:CHCl3, 25 MeOH, 10 CH3COOH, 1 cyclohexane; or Whatman KC-18 run with 60 EtOH, 4 H2O, 2 CH3COOH; visualization by fluorescence in 254 nm light). We have determined the ether/H2O partition coefficient of [3H]bumetanide to be 8.8, 8.7, 8.0, 3.9, 0.88, 0.25, 0.04, 0.02, and 0.02 at pH 2, 3, 4, 5, 6, 7, 8, 9, and 10, respectively, indicating that the relevant pK is near 5.

**Isolation of Plasma Membranes**—The dark red outer medulla was dissected from dog kidneys and homogenized in 0.25 M sucrose, 30 mM histidine, pH 7.2, as previously described (9). The homogenate was centrifuged successively at 500 × g for 10 min ("nuclear" pellet), 7,500 × g for 15 min ("mitochondrial" pellet), and 48,000 × g for 90 min ("plasma membrane" pellet). We have noted large differences in the amount of [3H]bumetanide-binding activity in preparations from different individual dogs, even when the membranes are prepared in parallel; for example, plasma membranes from renal outer medulla of three dogs killed at the same time exhibited 1.6, 24, and 4.7 pmol/mg of specific [3H]bumetanide binding. Membranes from renal cortex were prepared by a modification (10) of the detergent precipitation method of Booth and Kenney (11) to give a preparation that is predominantly brush borders from the proximal tubule (gift of S.
**Results**

**Equilibrium Binding of \[^{3}H\]Bumetanide to Kidney Membranes**—When we incubated plasma membranes from outer medulla of dog kidney with 10^{-6} \text{M} \[^{3}H\]bumetanide in a medium containing Na, K, and Cl, we found that a fraction of the drug remained bound to the membranes when they were filtered on a cellulose ester filter and washed briefly at 0°C; as shown in Fig. 1A, a component of the binding showed saturation behavior with increasing concentrations of \[^{3}H\]bumetanide. As will be shown below, the 20-min incubation was sufficient to attain at least 90% of the equilibrium binding level, at concentrations above 0.1 \text{mM} \[^{3}H\]bumetanide. From the Scatchard analysis in Fig. 1B, the apparent number of binding sites was found to be 4.7 pmol/mg, and the affinity of the sites for \[^{3}H\]bumetanide, under these ionic conditions, was 45 mM. \[^{3}H\]Benzetanide also exhibited saturable binding and in a given membrane preparation the amount of specific binding was the same (within ±20%) as that with \[^{3}H\]bumetanide, consistent with the hypothesis that the two compounds bind to the same membrane sites. The affinity \((K_d = 22 \text{ nM under the same conditions})\), as expected from its greater inhibitory potency and diuretic efficacy in other preparations (15).

**Partial Purification of Membranes that Bind \[^{3}H\]Bumetanide**—We examined different membrane fractions in a conventional differential centrifugation procedure (see "Experimental Procedures") and found that while the "plasma membrane" fraction (48,000 x g pellet) always had the highest specific binding activity, 1-5 pmol of \[^{3}H\]bumetanide/mg of protein compared to ≤ 1 pmol/mg in the 7,500 x g pellet and 500 x g pellet, the total amount of binding was usually greater in the low speed pellets, which contained most of the protein. It appears that different size fragments of the same membranes are responsible for the wide dispersion in differential centrifugation, since when the three fractions were further purified...
Fractions were assayed for saturable [3H]bumetanide binding. (○), (Na,K)-ATPase activity (O), alkaline phosphatase activity (□), and protein (■; by dye binding) as described under "Experimental Procedures."
other diuretics, and ionic requirements for bumetanide binding.

**Time Course of Bumetanide Binding**—Time courses of [3H]bumetanide interaction with the binding site are shown in Fig. 3, for the same incubation conditions used in Fig. 1. To determine the [3H]bumetanide association rate (Fig. 3A), 20-μl samples of 0.3 μM [3H]bumetanide were mixed with equal volumes of 2.6 mg/ml membrane protein, and after an appropriate time, 8-μl aliquots were diluted into 5 ml of cold stop medium and filtered (see "Experimental Procedures"). As shown in Fig. 3A, the data describing the association of [3H]bumetanide with the binding site are adequately fit by a theoretical curve for the second order association of binding site and ligand, with a rate constant of Kt = 2.5 × 10^7 M⁻¹ s⁻¹, using the dissociation rate constant from Fig. 3B (Kd = 1.23 × 10⁻⁴ s⁻¹). The equilibrium binding constant Kt = 49 nM that is obtained from the ratio of Kt and kᵣ is in excellent agreement with the value obtained by analysis of the data shown in Fig. 1.

The dissociation rate was determined (Fig. 3B) after preincubation of 3.9 mg/ml membrane protein with 0.2 μM [3H]bumetanide for 20 min, by dilution of 40-μl aliquots into 4.6 ml of the "binding medium" containing 100 μM unlabeled bumetanide; after appropriate times, 200-μl aliquots were further diluted into 5 ml of cold stop medium and filtered. In other experiments, not shown, it was found that at this dilution the rate of dissociation of [3H]bumetanide was the same with or without the addition of unlabeled bumetanide. At 0°C, dissociation of [3H]bumetanide is very slow (kᵣ = 8 × 10⁻⁹ s⁻¹; upper curve, Fig. 3B) confirming that dissociation of label from this population of sites is negligible during the 10-20-s dilution-filtration/wash procedure. [3H]Bumetanide dissociation at 22°C is fit by an exponential decay curve with a rate constant of kᵣ = 1.23 × 10⁻³ s⁻¹ (Fig. 3B, middle curve). Although it is readily seen that a multieponential curve would yield a more accurate fit, we have not investigated possible reasons for this complexity. The rate of dissociation was found to be unaffected by the ionic composition of the medium, in that the rate in the absence of Na⁺, K⁺, and Cl⁻ was identical with the rate in the presence of 100 mM Na⁺, 100 mM K⁺, and 200 mM Cl⁻ (not shown; the same as the rate of Fig. 3). Thus, the ionic effects on the equilibrium binding constant (Kᵣ) and the dissociation rate constant are not significant.

**Competition by Other Loop Diuretics**—To find if saturable [3H]bumetanide binding is due to binding to the inhibitory site on the Na⁺,K⁺,Cl⁻ co-transporter, we have examined the ability of related compounds to compete with [3H]bumetanide for the binding sites in a displacement assay; the results obtained with five compounds are presented in Fig. 4. The authenticity of the radioactive compounds [3H]bumetanide and [3H]benzmetanide is confirmed by the finding that the affinity for the unlabeled drugs is the same as that previously determined for the radiolabeled compounds under the same conditions (not shown). On comparing the concentrations at which these analogs prevent half of the [3H]bumetanide binding in kidney membranes (Kᵣ values) with the concentrations at which they inhibit Na⁺,K⁺,Cl⁻ co-transport in isolated kidney tubules (18) and in avian red cells (15), an excellent correlation is seen (top, Fig. 4). Note, for instance, that benzmetanide is the most potent compound in each case, whereas 4'-methoxybenzmetanide is ineffective as an inhibitor and (as a diuretic; Ref. 5) and is 300-fold less effective than benzmetanide in competition with [3H]bumetanide for binding to kidney membranes. We have also obtained an excellent correlation between the affinity of these drugs for membranes from the dogfish shark rectal gland and their corresponding inhibitory potencies in that organ, although the affinities are about 10-fold lower than in kidney (7). We feel that these data are very strong evidence that we are measuring [3H]bumetanide binding to the Na⁺,K⁺,Cl⁻ co-transport system in epithelial membranes.

**Ion Requirements for Bumetanide Binding**—Since earlier studies had indicated that both Na⁺ and K⁺ decrease the Kᵣ for bumetanide inhibition of co-transport in avian red cells (15, 19), and our preliminary data showed that [3H]bumetanide did not bind to kidney membranes in the absence of salts, we examined the appropriate ligands of the Na⁺,K⁺,Cl⁻ co-transport system as to their effect on saturable [3H]bumetanide binding. Fig. 5 shows the results of an experiment in which either Na⁺, K⁺, or Cl⁻ in the incubation medium was replaced with choline or SO₄²⁻. It is seen that all three ions are required for high affinity [3H]bumetanide binding, since with any of the ions omitted, only a low level of saturable binding is obtained. We have found that optimal binding is obtained with 10 mM Na⁺, 30 mM K⁺, 4 mM Cl⁻ (18 mM SO₄²⁻) in 12 mM histidine, pH 7.2 (Fig. 4, and from other experiments with lower total salt concentrations). Under these conditions, the Kᵣ values for [3H]bumetanide and [3H]benzmetanide are 30 and ≤15 nM, respectively (by Scatchard analysis, not shown). The Kᵣ values for stimulation of binding by Na⁺ is ~2 mM, and by K⁺ ~1 mM (Fig. 5); these correlate well with the Kᵣ values of the transport system in the TALH of rabbit kidney: 3.6 and <3 mM, respectively (1, 6). Although there is no direct evidence that the sites stimulating binding are the same as the transport sites, we could speculate that diuretics bind to the conformation of the transport protein in which Na⁺ and

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*Assuming competitive binding to a single population of sites, the affinities of the binding site for the unlabeled drugs (Kᵣ values) are related to the Kᵣ values determined in a displacement binding assay (Fig. 4) by a proportionality factor that is dependent on the concentration of the labeled compound in relation to the affinity for the labeled compound (cf. Ref. 18); this factor is approx. 0.67 for the concentration of [3H]bumetanide used in the experiment of Fig. 4.

* B. Forbush, III, and H. C. Palfrey, unpublished results.
FIG. 5. Effect of Na\textsuperscript{+}, K\textsuperscript{+} and Cl\textsuperscript{−} concentrations on saturable binding of [\textsuperscript{3}H]bumetanide. Kidney membranes (2 mg/ml) were incubated with [\textsuperscript{3}H]bumetanide (0.23 μM) in the presence of various salts, and [\textsuperscript{3}H]bumetanide binding was determined after 30 min. Unless otherwise stated, the ion concentrations were 128 mM Na\textsuperscript{+}, 64 mM K\textsuperscript{+}, 128 mM Cl\textsuperscript{−}, 32 mM SO\textsubscript{4}\textsuperscript{2−}. In each experiment, the concentration of one ion was varied, as plotted on the abscissa. K\textsuperscript{+} concentration was varied by substitution with choline\textsuperscript{+}. Na\textsuperscript{+} concentration was varied by substitution with choline\textsuperscript{+}. Chloride concentration was varied by substitution with SO\textsubscript{4}\textsuperscript{2−}. Each curve is a separate experiment and has been normalized to the maximal binding in that experiment. Chloride substitution; data from a separate experiment in which membranes were isolated in Cl\textsuperscript{−} free media. Na\textsuperscript{+} replacement in an experiment with 10 mM K\textsuperscript{+}, 15 mM Cl\textsuperscript{−}.

DISCUSSION

We have shown that [\textsuperscript{3}H]bumetanide binds with high affinity to a population of membranes from dog kidney outer medulla. [\textsuperscript{3}H]Bumetanide binding requires the presence of all three transported ions, Na\textsuperscript{+}, K\textsuperscript{+}, and Cl\textsuperscript{−}, and binding is competed for by various other "loop" diuretics at concentrations similar to those needed for inhibition of transport. We feel that this is very strong evidence that the [\textsuperscript{3}H]bumetanide binding site is the inhibitory site on the Na,K,Cl co-transport protein.

Our finding that cations are required for [\textsuperscript{3}H]bumetanide binding is in agreement with earlier reports that increasing concentrations of extracellular Na and K promote bumetanide inhibition of co-transport in avian red cells (15, 19). Recently, Haas (21) has extended the red cell studies in showing that extra cellular Cl increases the IC\textsubscript{50} for bumetanide inhibition of cotransport. Since in Fig. 5 it is shown that Cl\textsuperscript{−} is required at low concentrations to give optimal [\textsuperscript{3}H]bumetanide binding, we suggest that the diuretic-binding site may only involve one of the two anion-binding sites, and that the stable inhibited conformation is that of the fully loaded carrier (Na\textsuperscript{+} + K\textsuperscript{+} + Cl\textsuperscript{−} + bumetanide). However, this is clearly speculative in view of the possibility that allosteric interactions could yield similar behavior in models with separate sites and in view of the possibility that there are modifying sites for the various ions.

Since it is known that NO\textsubscript{3}\textsuperscript{−} does not substitute for Cl\textsuperscript{−} in the co-transport process (1−4), it would be expected that it would not affect [\textsuperscript{3}H]bumetanide binding. Instead we found that it behaved exactly as did Cl\textsuperscript{−}, with somewhat lower affinities. Thus, it appears that either 1) the anion sites involved in stimulation and inhibition of [\textsuperscript{3}H]bumetanide binding are not transport sites, or 2) NO\textsubscript{3}\textsuperscript{−} can bind to the transport sites but cannot be transported. The latter possibility could be tested in a future experiment by looking for competitive inhibition of Na,K,Cl co-transport by NO\textsubscript{3}\textsuperscript{−}.

The amount of specific [\textsuperscript{3}H]bumetanide binding per weight of protein is rather low even in the best preparations from outer medulla, considering the very high rate of transport across the cells of the TALH (5). However, it is clear from the overlap with (Na,K)-ATPase activity (cf. Fig. 2) that the sucrose gradient peak is not comprised of a homogeneous population of membranes and, thus, that the specific binding activity of pure luminal membranes would be somewhat higher than attained so far. Furthermore, it is possible that the transport system is hormonally modulated (4), and is "turned off" in the membrane preparation, or that binding sites are inaccessible on inside-out vesicles; it is also possible that proteolytic degradation takes place on membrane purification. These possibilities will be investigated in future studies.

Although we have no information as to the "tightness" and sidedness of membrane vesicles that most probably comprise our membrane preparation, it seems unlikely that these are important considerations with regard to the results reported here. Given the high lipid solubility of [\textsuperscript{3}H]bumetanide (ether water partition coefficient = 0.2; see "Experimental Procedures"), the lipid bilayer will not be a significant barrier to
diffusion of bumetanide into vesicles, and we can estimate that a membrane vesicle (≤1 μm in diameter) will equilibrate within milliseconds. It might be expected that entry of the ions required for bumetanide binding would be rate limiting, in vesicles of one orientation or the other. However, given the very high co-transport rate reported by Greger (5) for the intact cell membrane, we have calculated that the Na, K, and Cl would equilibrate within seconds at 37 °C (flux = 2 nmol/cm²/s; diameter = 0.5 μm, 40 mM salts; t₀ = 0.5 s). Thus, even at 22 °C, we anticipate that ion concentrations will equilibrate rapidly on the time scale of the binding experiments (cf. Fig. 3).

The radiolabeled compounds [³H]bumetanide and [³H]benzmetanide should be of great use in the identification, purification, and further characterization of the membrane protein responsible for Na,K,Cl co-transport. Because the binding assay is very simple and involves few assumptions, and because ion-promoted [³H]bumetanide binding is very specific, this method appears preferable to the possible alternative assay for the co-transport system, detection of diuretic-specific, this method appears preferable to the possible alternative assay for the co-transport system, detection of diuretic-sensitive co-transport after reconstitution of membrane protein into phospholipid vesicles. The high affinity of the binding site for the diuretic molecule (bumetanide Kᵦ = 30 nM, under optimal conditions) and the number of modifications that can be made to the drug molecule with partial retention of potency (22) argue the feasibility of photoaffinity labeling as an approach to identification of the transport protein. In addition, because of the high binding affinity, it is also hoped that these compounds will be of use in localization and characterization of the Na,K,Cl co-transporting system in tissues in which the density of transport sites is much lower than in the renal medulla. Finally, since we have shown the binding of [³H]bumetanide to be affected by the concentrations of Na⁺, K⁺, and Cl⁻, most probably through interactions of the ions at the binding site, we expect that further characterization of bumetanide binding will assist in gaining an understanding of the co-transport process.

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