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Fluorescent Indicators for Cytosolic Sodium*

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Fluorescent indicators sensitive to cytosolic concentrations of free Na^+ have been synthesized and characterized. They consist of a crown ether, 1,7-diaza-4,10,13-trioxacyclopentadecane, linked via its nitrogens to fluorophores bearing additional liganding centers. In the currently preferred dye, SBFI (short for sodium-binding benzofuran isophthalate), the fluorophores are benzofurans linked to isophthalate groups. Selectivities for Na^+ over K^+ of about 20 are observed, resulting in effective dissociation constants for Na^+ of about 20 mM against a background of 120 mM K^+. Increasing $[Na^+]$ increases the ratio of excitation efficiency at 330–345 nm to that at 370–390 nm with emission collected at 450–550 nm, so that ratio fluorometry and imaging can be performed with the same wavelengths as used with the well known Ca^{2+} indicator fura-2. If the macrocyclic ring is increased in size to a 1,10-diaza-4,7,13,16-tetraoxacyclooctadecane, the chelators become selective for K^+ over Na^+.

Nearly all animal cells maintain a large difference in sodium concentrations between their interiors (typically 10–40 mM) and the extracellular milieu (120–450 mM). This gradient is used to power nutrient uptake, epithelial transport, regulation of other intracellular ions, and transmission of electrical impulses, functions so important that organisms devote a major part of their metabolic energy to maintaining the sodium gradient (Skou *et al.*, 1988; Guernsey and Edelman, 1983). Usually, extracellular Na^+ is easily measurable or controllable, so that intracellular Na^+ is the major unknown of interest. Current techniques for measuring Na^+ fall into three categories: 1) assays that measure total cell Na^+ destroy the tissue, *e.g.* flame photometry, atomic absorption, neutron activation, counting of ^{22}Na at isotopic equilibrium, or electron microprobe analysis (Somlyo, 1986). The destructive nature of these techniques is obviously a drawback when time courses are desired. Except for the electron microscopic methods, these techniques lack spatial resolution and demand careful removal of extracellular fluid, which usually has a much higher concentration of Na^+ than the cells. The most general problem (Tsien, 1983; Horowitz and Paine, 1979; Slack *et al.*, 1973) is that the total intracellular $[Na^+]$ usually considerably exceeds the intracellular concentration of free sodium ($[Na^+]_i$),[1] and it is the latter that affects binding equilibria, transmembrane electrochemical gradients, and cell function. Free and total $[Na^+]$ are known to be able to vary independently (Slack *et al.*, 1973). 2) NMR techniques using dysprosium shift reagents can quantify the amount of intracellular Na^+ that is readily exchangeable on the NMR time scale (Springer, 1987; Liebling and Gupta, 1987). This probably includes weakly bound Na^+ as well as free. Although nondestructive, this technique requires relatively large amounts of tissue packed at high density in a magnet cavity, an environment awkward for other manipulations. 3) Techniques relying on well defined physicochemical equilibria measure free $[Na^+]$ (or Na^+ activity) nondestructively (Tsien, 1983). Examples include ^{19}F NMR of Na^+-sensitive chelators (Smith *et al.*, 1986) and Na^+-selective microelectrodes (Slack *et al.*, 1973), methods that have found limited applicability so far. A fluorescent indicator for Na^+ would be a very valuable addition to this group of techniques, since such dyes have the advantages of excellent spatial and unsurpassed temporal resolution, compatibility with cell types too small or fragile to impale with ion-selective and voltage reference barrels, and applicability to single cells as well as to populations (Tsien, 1986), as long as the tissue is optically clear enough.

An ideal $[Na^+]_i$ indicator would have the following properties.

1) Na^+ should bind with a dissociation constant (K_d) of 5–50 mM at pH 7, obviously in aqueous solution with no organic cosolvents permitted. Such a K_d would approximately match the expected range for $[Na^+]_i$ and maximize sensitivity to small changes in $[Na^+]_i$. Excessive Na^+ affinity would be undesirable, since the indicator would then either be Na^+-saturated and unresponsive, or if applied in excess would depress $[Na^+]_i$.

2) The indicator should have enough discrimination against K^+ (at least 20-fold or a $K_d > 150$ mM), H^+ (highest $pK_a < 6.5$), Mg^{2+} ($K_d > 10$ mM), and Ca^{2+} ($K_d > 10$ μM) so that physiological variations in those ions have little effect.

3) It would show reasonably strong fluorescence, characterizable by a product of extinction coefficient and fluorescence quantum yield exceeding 10^3 M^{-1} cm^{-1}.

4) Its excitation wavelengths should exceed 340 nm, because shorter wavelengths demand expensive quartz rather than glass microscope optics and are absorbed strongly by nucleic acids and aromatic amino acids.

5) Emission wavelengths should exceed 500 nm to reduce overlap with tissue autofluorescence from reduced pyridine nucleotides peaking near 460 nm.

6) Either the excitation or emission spectrum or both should

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[1] The abbreviations used are: $[Na^+]_i$ intracellular free sodium concentration; EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid; MOPS, 3-(*N*-morpholino)propanesulfonic acid; SBFP, sodium-binding benzofuran phthalate; SBFO, sodium-binding benzofuran oxazole; SBFI, sodium-binding benzofuran isophthalate; PBFP, potassium-binding benzofuran phthalate.

undergo a large wavelength shift upon binding Na^+, so that ratioing of signals at two excitation or two emission wavelengths can cancel out the local path length, dye concentration, and wavelength-independent variations in illumination intensity and detection efficiency.

7) The indicator should have enough polar groups such as carboxylates to render it highly water-soluble and impermeant through membranes, so that it does not rapidly leak out of cells.

8) The polar groups just mentioned should be maskable by nonpolar protecting groups hydrolyzable by cytoplasm, so that large populations of cells can be loaded with the indicator by incubating them with the membrane-permeant nonpolar derivative rather than requiring microinjection or other techniques of membrane disruption. The most obvious protecting groups are acetoxymethyl esters, which have proven to be useful with a wide variety of cation indicators (Tsien, 1981, 1989).

No such compound has yet been demonstrated to work in living cells, despite nearly two decades' chemical development of ligands for alkali metal cations. Indicator dyes with visible absorbance and moderate preference for Na^+ over K^+ have been reported (Dix and Vögtle, 1980; Löhr and Vögtle, 1985), but their operation is limited to nonaqueous solvents like acetonitrile, and no quantitative data are available on their cation binding constants. Higher affinity and selectivity for Na^+ over K^+ in water can be obtained with macrobicyclic chelators, for example the cryptand (Lehn and Sauvage, 1975) "[2.2.1]" (Fig. 1). Recently, fluorine-substituted cryptands have been introduced for measurement of $[Na^+]_i$ by ^{19}F NMR (Smith *et al.*, 1986). A promising fluorescent version was also described (Smith *et al.*, 1988), but its excitation and emission spectra peaked at rather short wavelengths, 320 and 395 nm, respectively, and no demonstration of intracellular use was given. The highest selectivities for Na^+ over K^+ are obtained in very large inflexible chelators called "spherands;" so far these require organic solvents for solubility and are so rigid that hours to days are required for equilibration with Na^+ (Lein and Cram, 1982; Cram, 1983; Cram *et al.*, 1988). The main mechanism by which they give optical shifts upon metal binding has been the displacement of a proton from the binding cavity, but this equilibrium must inherently be pH-sensitive, an unwanted feature. We chose to explore crown ethers rather than the more elaborate cryptands and spherands both for ease of synthesis and because of a concern that the conformational rigidity and preorganization of cryptands and spherands would tend to reduce the spectroscopic shift upon metal binding.

We now wish to report the design and synthesis of a different series of macrocyclic ligands (**1A–2P**), which ultimately attain the basic goals described above for a fluorescent sodium indicator. The strategy was modeled on the development of fluorescent Ca^{2+} indicators (Tsien, 1980; Grynkiewicz *et al.*, 1985) and consisted of three phases: (*a*) invention of a binding site with adequate affinity and selectivity for aqueous Na^+, in which Na^+ binding causes significant spectral change; (*b*) extension of the chromophore to render it fluorescent with sufficiently long wavelengths of excitation and emission; (*c*) adjustment of the polar groups to improve intracellular trapping and retention by acetoxymethyl ester permeation and hydrolysis. Tests in lymphocytes, hepatocytes, fibroblasts (Harootunian *et al.*, 1989), smooth muscle cells (Moore *et al.*, 1988), and gastric glands (Negulescu *et al.*, 1988) show the biological utility of such molecules for nondestructive observation of $[Na^+]_i$ in individual cells viewed by fluorescence microscopy.

EXPERIMENTAL PROCEDURES

The structures of the chelators are schematized in Fig. 1 and their syntheses described in detail in the Miniprint Supplement.[2]

UV absorbance spectra were recorded initially on a Cary 210 and later on a Perkin-Elmer Lambda Array 3840 spectrophotometer. Fluorescence excitation and emission spectra and quantum efficiencies were measured on a Spex Fluorolog 111 as described previously (Grynkiewicz *et al.*, 1985).

Proton dissociation constants (pK_a values) of the chelators were measured by spectrophotometry or spectrofluorometry of buffered solutions, containing either 100 mM tetramethylammonium chloride as inert supporting electrolyte or 121.5 mM K^+, 13.5 mM Na^+, and 1 mM Mg^{2+} to simulate the cation environment of vertebrate cytoplasm (Tsien *et al.*, 1982). Traces of UV-absorbing impurities in the tetramethylammonium chloride (Alfa Inorganics, Danvers, MA) were removed by filtration through acid-washed activated charcoal. The concentration of the tetramethylammonium chloride was then measured by chloride titration, and the absence of significant Na^+ contamination was verified with a sodium-selective glass electrode (Microelectrodes Inc., Londonderry, NH). When the chelators contained two protonatable nitrogens, the curve of absorbance or fluorescence *versus* pH was analyzed by computerized least squares fitting to the equations for two arbitrary proton equilibria (Rossotti, 1978), with the added assumption that the two protonations each caused the same change in extinction coefficient or fluorescence. This assumption was based on the presence of two identical chromophores in each chelator and produced good fits to the experimental data.

Dissociation constants (K_d) for metal cations were similarly measured by recording the absorbance or fluorescence spectra at constant indicator concentration while the cation concentration was increased stepwise by either of two procedures. The simplest titration method started with the dye in 100 mM of tetramethylammonium chloride, usually with a few millimolar tris(hydroxymethyl)aminomethane base to hold the pH high enough (pH 8–9) to insure that protonation was negligible. After recording the spectrum, successive aliquots of metal chloride were added either as solid or from concentrated stock solutions, taking care of minimize dilution and correct for it by scaling the spectra. A convenient way to mass-produce premeasured micromole to millimole aliquots of solid NaCl or KCl was to pipette saline solutions into polypropylene micro test tubes and evaporate the water in an oven. This convenient titration procedure had the slight disadvantage that the ionic strength was not constant, especially when large quantities of a salt had to be added due to weak affinities. When constant ionic strength was desired, a different procedure was adopted, in which the chelator was made up at identical concentrations in matched solutions of NaCl, KCl, or tetramethylammonium chloride, then these stocks were mixed in the desired proportions. All measurements were made at room temperature (22 ± 2 °C).

RESULTS

Design and Organic Syntheses—This process was longer and more contorted than the basic design of tetracarboxylate Ca^{2+} indicators, because at the outset no suitable model molecules were known with good Na^+ to K^+ selectivity at pH 7 in water, whereas the Ca^{2+} indicators could be based (Tsien, 1980) on the established chelator EGTA. Our starting point was structure **1A**, chosen to combine the bare minimum of molecular parts, a macrocyclic ring of ligand groups with at least theoretically (Vögtle and Weber, 1980) the right size to favor Na^+ over K^+ binding, connected via an sp^3-hybridized nitrogen to a rudimentary chromophore. The use of such aniline-type nitrogens to link chelating groups to chromophores has proved highly successful in the rational design of calcium indicators (Tsien, 1980, 1983) and gives far larger spectral shifts than reliance on aryl ether oxygens (Wun *et al.*, 1977). Structure **1A** was synthesized by Dix and Vögtle (1980) but not characterized for cation binding properties; we found its dissociation constants for Na^+ and K^+ on the order

[2] The syntheses of the chelators are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are included in the microfilm edition of the Journal that is available from Waverly Press.

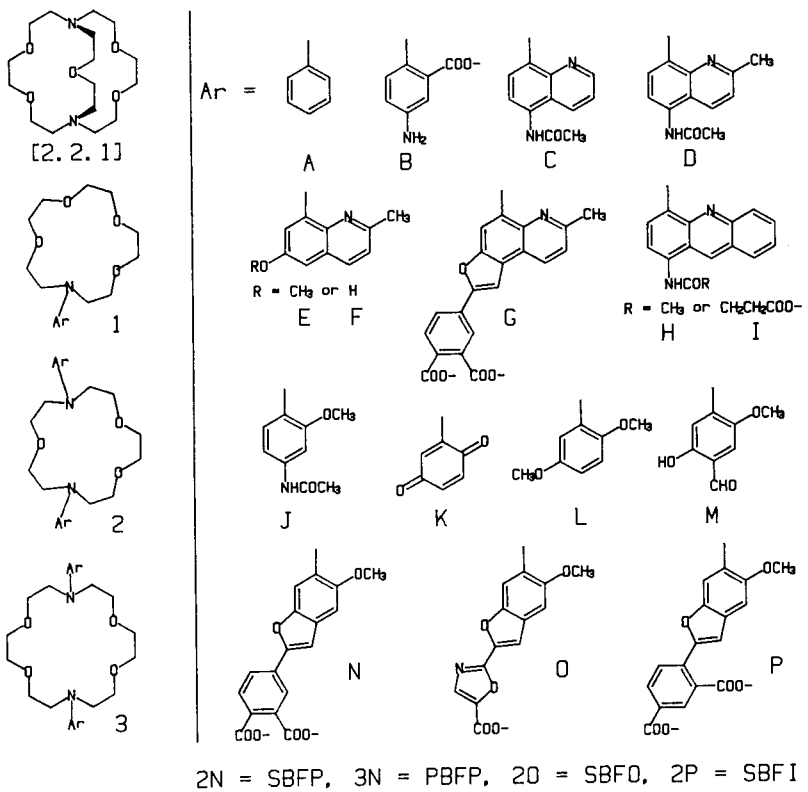

FIG. 1. **Structures of the compounds prepared in this study.** For comparison, Lehn's cryptand [*2.2.1.*] is shown in the *upper left-hand* corner. Below it are the three generic types of crown ethers prepared. To the *right* of the *vertical* dividing *line* are the aromatic and heterocyclic substituents attached to the nitrogens of the crown ethers. Compound SBFI is **2P**; SBFO is **2O**; PBFP is **3N**; PBFI is **3P**.

of 2 and 5 M in water, too high to be characterized accurately. Obviously, more donor groups were needed, especially out of the plane approximately defined by the macrocyclic ring. Synthetic convenience suggested **1B**, made by reaction of 1-aza-4,7,10,13-tetraoxacyclopentadecane with 2-chloro-5-nitrobenzoic acid, followed by catalytic reduction of the nitro group to counteract its extreme electron-withdrawing power. Compound **1B** proved indeed to have considerably higher Na^+ affinity, $K_d \approx 71$ mM, and Na^+:K^+ selectivity (13:1) than **1A** had shown, but its pK_a was too high, 9.21. Comparably high pK_a values in other *N,N*-dialkylanthranilates are attributed to internal hydrogen bonding between zwitterionic amino and carboxylate groups (Tramer, 1969). To eliminate such chelation of protons, the carboxylate was abandoned in favor of an sp^2-hybridized nitrogen in **1C**, in which the two nitrogens are too far apart to engage the same proton at once. Chelator **1C** was prepared by reaction of 1-aza-4,7,10,13-tetraoxacyclopentadecane with 8-tosyloxy-5-nitroquinoline followed by reduction of the nitro group. The resulting primary amino group was acetylated to block a tendency to autoxidize. Despite the replacement of the carboxylate of **1B** by an uncharged ligand group in **1C**, the K_d for Na^+, 67 mM, was practically unchanged. As desired, the pK_a was lowered below 7. Since addition of one additional donor group out of the main ring plane increased the Na^+ affinity by more than an order of magnitude, we tried adding a second donor group, as shown in structure **2C**. Molecular models suggested that this complex could neatly fold up around a sodium cation with the macrocyclic ring forming an equatorial belt and the two additional donor groups capping the north and south poles. Chelator **2C** was prepared from the commercially available 1,7-diaza-4,10,13-trioxacyclopentadecane with 8-tosyloxy-5-nitroquinoline, followed by reduction and acetylation. Compound **2C** indeed bound Na^+ much more strongly, $K_d \approx 5.3$ mM at 0.1 M ionic strength. Fortunately the K^+ affinity did not increase to anything like the same extent. Since the spectral change associated with K^+ binding was only half that caused by Na^+ binding, K^+ probably could interact with only one of the two quinoline rings, presumably because K^+ was too big to fit fully inside the macrocyclic ring but rather had to stay on one side of it. However, **2C** was found to have a major drawback in its excessive affinity for Mg^{2+}, K_d only 78 μM, which would give overwhelming interference from the typical value of 1 mM free intracellular Mg^{2+}. This Mg^{2+} binding was unusual in its kinetic sluggishness, with association and dissociation rate constants of only 1.48 M^{-1} s^{-1} and 1.28×10^{-4} s^{-1} at 25 °C, easily observable in a spectrophotometer without rapid mixing equipment. A reasonable explanation for the high affinity of Mg^{2+} for **2C** is that the binding site can readily collapse compactly to fit the small Mg^{2+} ion. In confirmation of this hypothesis, **2C** proved to have an affinity for Li^+ comparable to that for Na^+. To prevent such compaction of the binding site, we added methyl groups to the quinoline 2-positions to act as buttresses to prevent the quinoline nitrogens from approaching too closely the plane of the macrocyclic ring. The resulting chelator, **2D**, showed a $>10^4$ increase in the K_d for Mg^{2+} and a $>10^2$ increase in the K_d for Li^+ compared to **2C**, yet its K_d for Na^+ increased by less than 2-fold. Since the two methyl substituents did not affect the Na^+:K^+ selectivity, they clearly made a major improvement overall. Nevertheless, although **2D** had highly satisfactory ionic selectivities for Na^+ over the other alkali and alkaline earth metals, the pK_a for its first protonation was too high (7.55), and its extinction coefficient ($\approx$5000 M^{-1} cm^{-1}) and fluorescence quantum yield (0.01) were too low.

In the hope of increasing the fluorescence quantum efficiency, several analogues of **2D** were synthesized with 6-alkoxy substituents on the quinaldines instead of the 5-acetamido groups. The rationale was that 6-methoxyquinolinium fluorophores are responsible for the strong fluorescence of quinine. Also, addition of a 6-methoxy substituent did turn a weakly fluorescing Ca^{2+} indicator, quin-1, into a

considerably stronger fluorescent dye, quin-2 (Tsien, 1980). The vacancy of the 5-position would permit attachment of more extensive conjugation to extend the wavelengths of excitation and emission. However, an entirely different synthetic strategy was needed since the 5-position could not readily bear the nitro group needed to activate aromatic nucleophilic substitution. Now the crown macrocycle was synthesized from scratch by reaction of 6-methoxy-8-aminoquinaldine (Wan *et al.*, 1974) with 3,6-dioxaoctanedioyl chloride (Dietrich *et al.*, 1973), reduction of the diamide with diborane, re-acylation of the diamine with diglycolic acid chloride, then aluminum hydride (Yoon and Brown, 1968) reduction of the amide. Aluminum hydride was found to give better results than diborane, since the latter gave products from which it was difficult to remove boron fully. The re-acylation with diglycolic acid chloride was run under high dilution conditions to favor macrocycle closure over polymer formation. Conventionally (Dietrich *et al.*, 1973), triethylamine is added to neutralize the HCl generated during acylation, but this base itself slowly destroyed the acid chloride, presumably through ketene intermediates. When the amine to be acylated is reactive, this side reaction is not serious, but with a bulky and less nucleophilic aromatic diamine such as 1,8-bis(2-methyl-6-methoxy-8-quinolinylamino)-3,6-dioxaoctane, a weaker tertiary base such as *N,N*-dimethylaniline was preferable to triethylamine.

First trials of 6-substituted quinolines were conducted on the 6-hydroxyquinaldine **2F** obtained by demethylation of the precursor **2E** bearing methoxyls. However, **2F** proved to be yet another disappointment in its quantum efficiency, about 0.01 and 0.005 with and without Na^+. Therefore **2E** was formylated at the 5-position, demethylated, and coupled with dimethyl 4-bromomethyl phthalate (Anzalone and Hirsch, 1985) to form the quinolinofuran **2G**. The Na^+ affinity of **2G** was even higher than that of **2D**, perhaps aided by some long range electrostatic attraction of the cation to the four negative charges. Unfortunately, the pK_a for the first protonation rose to 7.9. Since the quantum efficiencies were the same low values as those of **2F**, this approach had to be abandoned.

We hoped to increase both the wavelength and quantum efficiency of fluorescence by shifting from quinoline nuclei to acridines as in **2H** and **2I**. These compounds were prepared from 4-methoxy-9-acridone by reduction to the acridine (Irving *et al.*, 1949), nitration at the 1-position, replacement of the methoxy group by hydroxy, tosylation, reaction with 1,7-diaza-4,10,13-trioxacyclopentadecane, finishing with reductive acylation. The acetamido derivative **2H** analogous to **2C** and **2D** proved insufficiently soluble in water for spectrophotometry, so the more hydrophilic hemisuccinamide **2I** was also prepared. This had the highest Na^+ affinity ($K_d \approx 0.32$ mM at 0.1 M ionic strength) and Na^+:K^+ selectivity (>500) yet obtained in our work. Sadly the pK_a, 8.19, was also near a record. Although the absorbance band was indeed shifted into the visible, the fluorescence quantum yield remained very poor, so that the acridine nucleus was only accentuating the undesirable features of **2D**.

The excessively high pK_a values of the quinaldines and acridines seemed attributable to protonation on the heterocyclic rather than the amino nitrogens, because protonation gave rise to bathochromic shifts whereas metal cations were hyposochromic. One obvious tactic to eliminate the high pK_a would be to replace the heterocyclic nitrogens by less basic donor atoms such as ether oxygens. Our first attempt at such a molecule was **2J**, prepared by reaction of the diaza crown with 2-fluoro-5-nitroanisole (Ruyle *et al.*, 1977) then reduction and acetylation as usual. **2J** indeed had its highest pK_a at 6.31, much lower than the pK_a values for the quinaldines and acridines. Also protonation of **2J** gave a hyposochromic shift very similar to metal cation binding, confirming that the site of protonation had shifted to an amino nitrogen. Despite the decreased donor strength of oxygens compared to nitrogens, the K_d for Na^+ was still respectable, 12 mM. However, **2J** absorbed only in the deep UV, as expected from the small size of its chromophore. A useful fluorescent indicator would require a chromophore with a much longer conjugation path. The normal site of attachment of such conjugation would be *para* to the dialkylamino group, but that position is occupied by a nitrogen. All common substituents that extend conjugation through a –N= are significantly electron-withdrawing, so they would depress the Na^+ affinity strongly.

To escape this quandary, we needed an electrophile other than a nitrohalobenzene that would react with amines and then be reducible to an electron donor-substituted aromatic ring. An attractive electrophile was *p*-benzoquinone. By reaction (Hikosaka, 1970; Ulrich and Richter, 1977) of a large excess of this cheap reagent with the diaza crown, it was easy to produce **2K** in which each quinone bears only one amino substituent. Reduction and alkylation gave aminoquinol ether **2L**. Vilsmeier formylation of **2L** followed by regioselective demethylation (Dean *et al.*, 1966) of the phenol ortho to the formyl gave salicylaldehyde **2M**. From this intermediate, various benzofurans can be prepared, all representing styryl fluorophores with cis-trans isomerism prevented by heterocyclic ring formation. Thus **2M** reacted with 2 mol of dimethyl 4-bromomethylphthalate to form the tetramethyl ester of the benzofuran phthalate **2N**. Alternatively, **2M** with ethyl 2-chloromethyloxazole 5-carboxylate gives the ester of benzofuran oxazole **2O**, a close relative of the successful fluorescent Ca^{2+} indicator fura-2. **2N** and **2O** were promising enough to justify biological trials, but their acetoxymethyl esters were found not to load cells properly (Harootunian *et al.*, 1989). Therefore yet another analog, **2P**, was synthesized from **2M** plus 2 mol of dimethyl 4-bromomethylisophthalate. For convenience we refer to **2N**, **2O**, and **2P** as SBFP, SBFO, and SBFI, respectively, short for sodium-binding benzofuran phthalate, oxazole, or isophthalate. Ultimately SBFI proved satisfactory enough to allow useful biological studies (Harootunian *et al.*, 1989), so the following description focuses on it.

Spectral and Cation-binding Properties—SBFI has extinction coefficients of 42,000–47,000 M^{-1} cm^{-1}, as expected for a molecule containing two styryl chromophores. Its fluorescence quantum efficiency is respectable, 0.08 and 0.045 with and without Na^+. Both the Na^+ and K^+ dissociation constants, 7.4 and 166 mM, are a little lower than those of the model compound **2J**. Some or all of this increased affinity may just be the electrostatic attraction of the four remote carboxylates for cations. However, the highest pK_a, 6.1, is still low enough so that most physiological pH variations will have little effect on the dye spectra or effective Na^+ affinity. To enable physicochemical comparison with earlier chelators, the above affinities were measured against truly inert background cations such as tetramethylammonium or Cs^+, at a pH high enough for protonation to be completely negligible. Biologically more relevant values are obtained in Na^+-K^+ mixtures. When the sum of the two cations is held constant at 135 mM, with 1 mM Mg^{2+} present at pH 7.05 as would be reasonable for vertebrate cytoplasm, the apparent dissociation constant for Na^+ is 17–19 mM, as may be seen in Fig. 2. Na^+ binding shifts both the excitation and emission spectra to shorter wavelengths. Although Na^+ shifts the excitation peak wavelength only 8 nm from 344 to 336 nm, it also makes the long wavelength side of the excitation spectra roll off much more

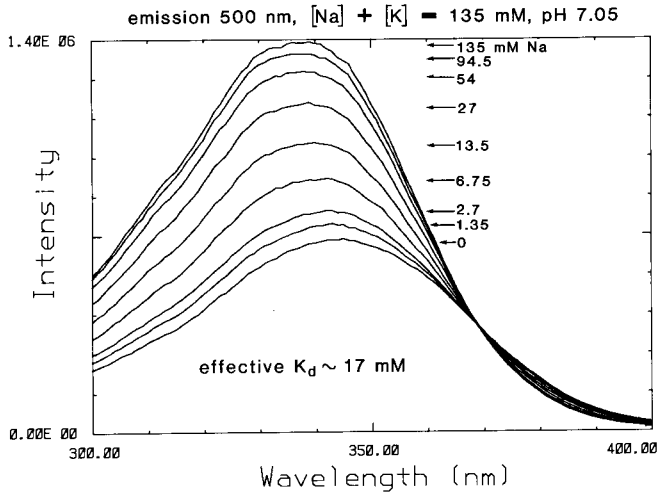

FIG. 2. **Fluorescence excitation spectra of SBFI as a function of increasing [Na^+] and decreasing [K^+].** The *lowest curve* ([Na^+] = 0) was obtained with 5 μM SBFI in 130 mM KCl, 10 mM MOPS, KOH to pH 7.05, approximately 135 mM total K^+. The *highest curve* ([Na^+] = 135 mM) was recorded analogously from 5 μM SBFI in 130 mM NaCl, 10 mM MOPS to pH 7.05. The *intermediate curves* from 1.35 to 94.5 mM Na^+ were obtained by iteratively replacing 1/100, 1/99, 3/98, 1/19, 1/9, 1/4, and 1/2 of the K^+-rich SBFI solution by the SBFI in sodium medium. The excitation bandwidth was 1.85 nm; emission was collected at 500 nm with 9.3 nm bandwidth. The temperature was 22 ± 2 °C.

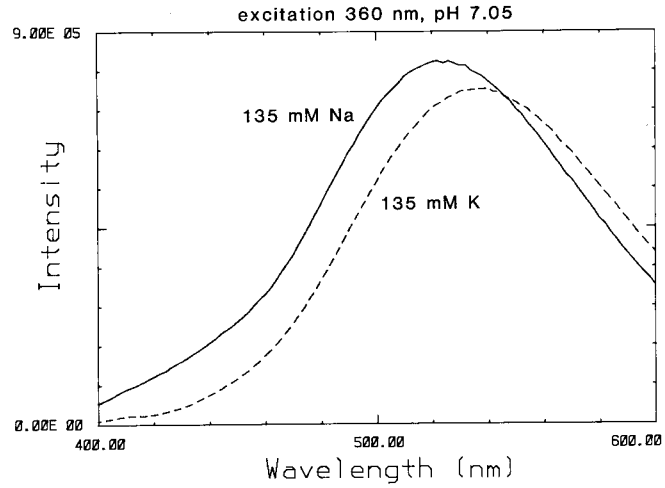

FIG. 3. **Fluorescence emission spectra of SBFI at 135 mM K^+ and 135 mM Na^+, recorded from the same solutions as used in Fig. 2 for 0 and 135 mM Na^+.** Excitation was at 360 nm; bandwidths were the same as in Fig. 2.

steeply. The ratios of excitation efficiencies at 335–340 to that at 375–380 nm therefore undergo a 2.8-fold increase with binding. This sort of spectral shift is roughly similar to the way fura-2 responds to Ca^{2+}, except that SBFP and SBFI are excited at slightly shorter wavelengths and do not change their ratio quite as much as fura-2 does (Grynkiewicz *et al.*, 1985). The emission spectra of SBFP and SBFI (Fig. 3) shift very little as Na^+ replaces K^+, so that these dyes are like fura-2 in being most sensitive in excitation ratioing rather than emission ratioing (Grynkiewicz *et al.*, 1985).

Selectivities of SBFP and SBFI against other cations are also adequate (Table I). In the presence of 13.5 mM Na^+ and 121.5 mM K^+, the highest apparent pK_a of SBFI is 6.09. Moreover, acidification depresses the 335–340 nm and the 375–380 nm excitation amplitudes about equally (Fig. 4), so that the ratio does not change significantly. Ratioing therefore not only normalizes for amount of dye in the optical path but also improves the discrimination against pH changes. Li^+ binding causes a greater shift of the SBFI excitation peak to shorter wavelengths but a lesser increase in amplitude than Na^+ binding does; the K_d for Li^+, 67 mM, is also weaker than the Na^+ affinity. Dissociation constants for Mg^{2+} and Ca^{2+} are high enough (about 60 and 38 mM, respectively) for cytosolic levels of those ions to have insignificant effect. Curiously, Ca^{2+} is unique in causing a large hypsochromic shift of the emission peak to 432 nm. All the above-mentioned salts except tetramethylammonium seem to cause a slight nonspecific quenching of SBFI at very high concentrations. For example, CsCl causes no spectral shift at all, but 100, 200, and 500 mM CsCl depress the fluorescence by 9, 14, and 21% from the metal-free level. This effect is not a heavy atom effect of cesium, since large excesses of Li^+, Na^+, and K^+ also slightly quench their SBFI complexes. It may represent weak quenching by Cl^-, since acetate and fluoride gave much less of the effect.

Compared with SBFP and SBFI, SBFO has even higher quantum efficiencies of fluorescence, 0.44 and 0.14 with and without Na^+. Because the oxazole group in SBFO is more electron withdrawing than the phthalate in SBFP, SBFO has somewhat longer wavelengths of excitation and emission as well as higher K_d for Na^+, 50 mM against a background of 100 mM tetramethylammonium. This rises to 95 mM when measured against a K^+ background with [Na^+] + [K^+] = 135 mM (Fig. 5). Again, Na^+ binding causes a large change in the ratio of excitation efficiencies at 340–350 nm to 380–390 nm, rather like the effect of Ca^{2+} on the related fura-2. Competition from protons is also reduced, with a highest pK_a of only 5.34.

The effect of ionic strength on the affinities for Na^+ and K^+ was checked in a few instances (Table I, see entries for **2C, 2D, 2I**, and **2O**). In general, increasing the ionic strength from about 0.1 to 3.0 M strengthened the binding slightly. Both the sign and the magnitude of the ionic strength effect differ from that found for Ca^{2+} complexation to tetracarboxylate chelators and indicators, where increasing ionic strength markedly weakens the binding (Harrison and Bers, 1987; Tsien, 1989). The difference in behaviors is probably because the crown ethers present an uncharged binding site to a monovalent cation whereas the tetracarboxylates offer four negative charges to a divalent cation, so that electrostatic effects are much more important in the latter case. The slight decrease in the apparent K_d values of the crown ethers at 3 M ionic strength may be due to the variations in monovalent cation activity coefficients at very high concentrations of the somewhat hydrophobic tetramethylammonium ion.

Potassium Indicators—To check the basis for the Na^+ selectivity of the above indicators, we synthesized **3J–3P**, analogs of **2J–2P** but with six heteroatoms in an 18-membered ring instead of the usual five heteroatoms in a 15-membered ring. As expected, the increased cavity size made K^+ the preferred cation, although by only a small margin over Na^+ (Table I). **3N** is named PBFP for potassium-binding benzofuran phthlate. Its excitation spectra are shown in Fig. 6 under the usual conditions of [Na^+] + [K^+] = 135 mM. Now it is increasing K^+ or decreasing Na^+ that enhances the fluorescence at 340 nm excitation. The apparent K_d for K^+ is 70 mM. Curiously, K^+ increases the intensity without much change in wavelength, so that PBFP works best at a single wavelength rather than in dual-wavelength ratio mode. Though its K^+:Na^+ selectivity is modest, it may find some use for intracellular measurements because $[K^+]_i$ usually far exceeds $[Na^+]_i$.

TABLE I

Cation-binding and spectroscopic properties of chelators prepared in this study

Structure[a]	Dissociation constants (K_d)[b]					pK_a[c]	K_d for Na^+ in K^+, pH 7.05[d]	pK_a in 14 mM Na^+, 126 mM K^+, 1 mM Mg^{2+}[e]	Absorbance maxima[f]		Emission maxima[g]		Quantum efficiency[g]	
	Na^+	K^+	Mg^{2+}	Ca^{2+}	Li^+				−Na^+	+Na^+	−Na^+	+Na^+	−Na^+	+Na^+
	mM													
1A									252 (14.2)	249 (8.7)				
1B	71	~900				9.21			295 (shoulder, 1.1)	296 (1.1)				
1C	67	~1000	12	10	63	6.32			349 (2.4) 252 (9.2)	304 (2.6) 229 (13)				
2C	5.3, 2.4[h]	225	0.078[i]	0.075	2.8	6.85, 5.43			346 (4.7) 250 (19)	304 (5.0) 230 (23)				
2D	9.4, 4.4[h]	470	>500	200–250	500	7.55, 5.62			344 (4.9) 256 (22)	306 (6.0) 237 (24)	525	510	0.01	0.01
2F	8.9								363 (2.4)	362 (2.3)	497	466	0.0035	0.0040
2G (SQFP)	4.7					7.9, 6.8	~300		373 (24) 308 (22)	362 (31) 307 (28)	485	525	0.005	0.01
2I	0.32, 0.4[h]	178, 110[h]	–[j]	–[j]	11.5	8.19, 6.86			414 (4.2) 359 (4.1) 244 (48)	360 (6.1) 251 (48)	480	625	<0.001	<0.001
2J	12	290	>100	>100	72	6.31, 5.0	30		261 (10.8)	280 (5.4) 249 (10.7)				
2L	10–20								295 (4) 213 (13)	291 (4)				
2N (SBFP)	8.5	161	18	40		6.3, 5.5	19	6.4, 5.6	342 (46)	334 (52)	528	505	0.036	0.091
2O (SBFO)	50, 22[h]	170[h]	63		347	5.34, 4.15	95	4.82, 4.03	355 (25)	343 (27)	515	500	0.14	0.44
2P (SBFI)	7.4	166	60	38	68	5.89, 5.19	17–18[k]	6.09, 5.51	346 (42)	334 (47)	551	525	0.045	0.083
3J	20	10							258 (3.5)	281 (2.0)[l] 249 (3.4)[l]				
3N (PBFP)	260	83					70[m]	6.68	344 (23)	337 (25)[l]	518	494[l]	0.0075	0.12[l]
3P (PBFI)	21	8	40	16	380		100[m]	5.78, 5.17	350 (42)	344 (42)[l]	546	504[l]	0.024	0.072[l]

[a] For molecular structures, see Fig. 1.

[b] Except where otherwise noted, these dissociation constants were measured with the chelator dissolved in 0.1 M tetramethylammonium chloride, at a pH held high enough with 1–5 mM tris(hydroxymethyl)aminomethane for protonation to be insignificant. The absorbance spectrum, or the fluorescence excitation spectrum for those compounds (**2G, 2N, 2O, 2P, 3N, 3P**) with acronyms, was measured as a function of the concentration of cation added as a solid or concentrated aqueous solution of the halide salt. No correction has been made for the changing ionic strength of the solution, which started from 0.1 M and increased, but the absorbances and fluorescence amplitudes were corrected for dilution of the dye and any slight quenching due to high concentrations of Cl^-.

[c] These protonation constants were determined in 0.1 M tetramethylammonium chloride, 10 mM tris(hydroxymethyl)aminomethane, titrated with 5 M H_3PO_4 to successively lower pH values measured with a Radiometer PHM84 meter. Other details resembled the metal titrations above.

[d] These dissociation constants are effective values for Na^+ against a K^+ background such that $[Na^+]+[K^+]$ = 135 mM, at pH 7.05 buffered with 10 mM *N*-(morpholino)propanesulfonic acid. They were measured by spectrofluorometry (or spectrophotometry for **2J**) as shown in Figs. 2 and 5 and are in units of millimolar.

[e] These protonation constants are for dye in 126 mM KCl, 14 mM NaCl, 1 mM $MgCl_2$, 4 mM tris(hydroxymethyl)aminomethane, titrated with 5 M H_3PO_4 as in Fig. 4.

[f] Absorbance maxima refer to the main peaks, measured in 100 mM tetramethylammonium chloride plus 1–5 mM tris(hydroxymethyl)aminomethane (−Na^+), or with enough NaCl added (0.2–1 M) to saturate the Na^+ binding (+Na^+). The first number is the wavelength in nanometers, followed in parentheses by 10^{-3} times the corresponding extinction coefficient, M^{-1} cm^{-1}. Extinction coefficients for most of the compounds are lower limits because most of the chelators were obtained by chromatographic purification as gums or oils, which revealed no other significant absorbing species but may have included traces of chromatographic solvents or other nonabsorbing impurities.

[g] Emission maxima in nanometers and quantum efficiencies were measured in the same solutions as used for absorbance ±Na. Emission maxima are not corrected for the spectral sensitivity of the emission detection. Quantum efficiencies were measured (Grynkiewicz *et al.*, 1985) by comparing the integral of the corrected emission spectrum with the corresponding integral for a solution of quinine bisulfate in 1 N H_2SO_4 of matched absorbance at the excitation wavelength. Quinine was assumed to have a quantum efficiency of 0.55.

[h] These dissociation constants were measured at 3.0 M ionic strength by mixing solutions of dye in 3.0 M NaCl with the same dye concentration in 3.0 M tetramethylammonium chloride.

[i] Free $[Mg^{2+}]$ was controlled by Mg^{2+}-nitrilotriacetate buffers at pH 8.5.

[j] At several mM concentrations of Mg^{2+} or Ca^{2+}, precipitate began to form, so dissociation constants could not be quantified.

[k] An effective K_d of 28 mM was determined additionally for SBFI-Na^+ when $[Na^+]+[K^+]$ = 280 mM as might be more appropriate for the cytoplasm of marine organisms.

[l] These values for the K^+ selective chelators **3J, 3N,** and **3P** refer to spectra in saturating K^+ rather than Na^+.

[m] Effective K_d in millimolar for K^+ against a Na^+ background, where $[Na^+]+[K^+]$ = 135 mM, as measured in Fig. 6.

DISCUSSION

Basis for Na^+ Affinity and Selectivity—The Na^+ indicators presented here are derivatives of 1,7-diaza-4,10,13-trioxacyclopentadecane in which both nitrogens bear aryl or heterocyclic substituents (Fig. 1), which in turn include additional liganding atoms such as –N= in substituents **C–I**, or –OMe in substituents **J–P**. The parent crown ether (also trivially known as "diaza[15]-crown-5" or "Kryptofix 21") proved to

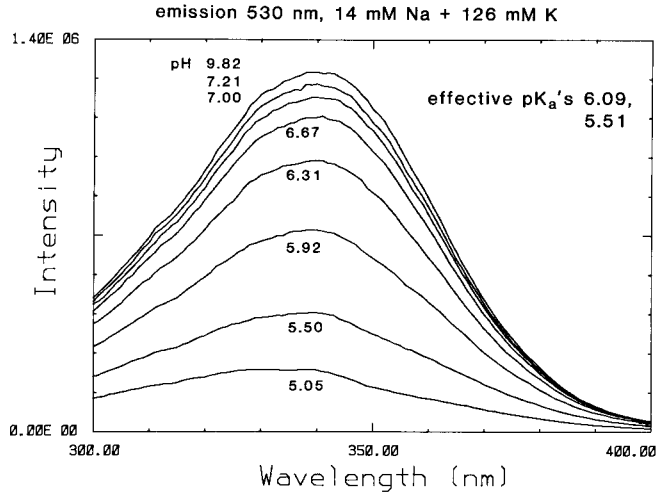

FIG. 4. **Fluorescence excitation spectra of 5 μM SBFI in 14 mM NaCl, 126 mM KCl, 1 mM $MgCl_2$, 4 mM Tris, titrated to the indicated pH values by small additions of 5 M H_3PO_4.** Emission was collected at 530 nm. Bandwidths and temperature were as in Fig. 2.

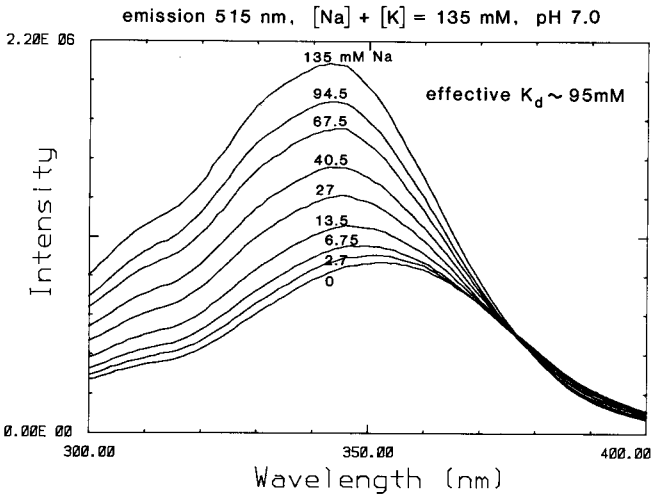

FIG. 5. **Fluorescence excitation spectra of 6 μM SBFO as a function of increasing [Na^+] and decreasing [K^+] in solutions similar to those in Fig. 2.** The intermediate Na^+ concentrations from 2.7 to 94.5 mM were obtained by the iterative replacement of 1/50, 3/98, 1/19, 1/9, 1/8, 2/7, and 2/5 of the high K^+ medium by the 135 mM Na^+ mixture. Excitation bandwidth was 1.85 nm; emission was collected at 515 and 4.7 nm bandwidth.

have several advantages: it was commercially available, it could be attached as a preformed unit to a variety of aryl chromophores, enabling a considerable number of the latter to be tested without too much effort, and it formed a belt of about the right size to fit equatorially around a Na^+ cation (Vögtle and Weber, 1980). However, the unsubstituted diaza crowns actually have very poor cation affinities and selectivities (Gramain and Frère, 1979) and of course lack any optical properties. Several workers have previously tried adding one liganding substituent above the plane formed by the main crown ring (Nakatsuji *et al.*, 1988; Takagi and Ueno, 1984; Shiga *et al.*, 1983). Such structures, dubbed "lariats" for their shape, can have modestly improved Na^+ affinities. Some lariats can extract alkali and alkaline-earth cations into 1,2-dichloroethane from aqueous medium at high pH (Takagi and Ueno, 1984; Shiga *et al.*, 1983). However, selectivities for Na^+ over K^+ are poor, <1 log unit, and none has been shown to respond optically to Na^+ in a purely aqueous medium. Our

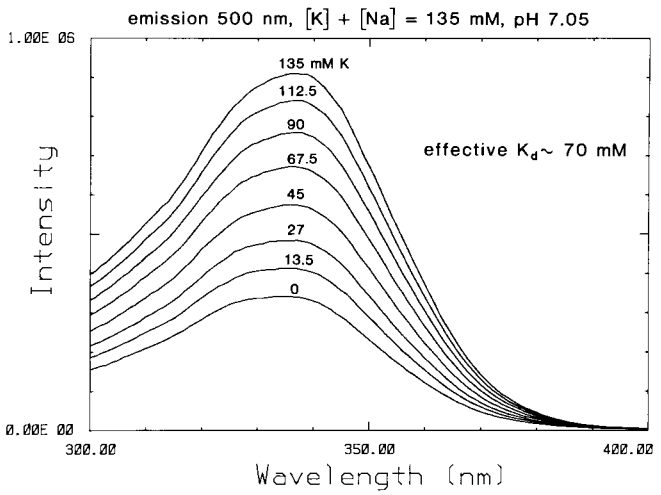

FIG. 6. **Fluorescence excitation spectra of 10 μM PBFP as a function of increasing [K^+] and decreasing [Na^+] in solutions similar to those of Fig. 2.** The *lowest curve* was obtained in 130 mM NaCl, 10 mM MOPS, NaOH to pH 7.05. The *highest curve* was in 130 mM KCl, 10 mM MOPS, KOH to pH 7.05. The *intermediate curves* with 13.5–112.5 mM K^+ were obtained by iteratively replacing 1/10, 1/9, 1/6, 1/4, 1/3, and 1/2 of the low K^+ medium by the 135 mM KCl solution of PBFP.

initial trials of crowns with one axial substituent (**1A–1C**) confirmed their inadequate Na^+ affinity and selectivity. However, when we increased the number of axial substituents to two aromatic ether or sp^2 nitrogen ligand groups, adequate to very good selectivity and affinity for Na^+ resulted. The highest Na^+ affinities and selectivities over K^+ were obtained with quinoline or acridine ring nitrogens as the axial donors, but despite many permutations, all such chelators suffered from inadequate proton rejection and low fluorescence quantum efficiencies. The latter properties were greatly improved by changing the axial donors to aromatic ether oxygens.

In retrospect, the Na^+:K^+ selectivities (20 to 500) and Na^+ to divalent cation selectivities (>1) of **2C–2P** are unexpectedly good compared to reports of other derivatives of diaza[15]crown-5 (Tazaki *et al.*, 1982; Chang and Ochaya, 1986; Gandour *et al.*, 1986; Gatto *et al.*, 1986). Addition of carboxymethyl groups to both nitrogens gives a chelator dianion (Tazaki *et al.*, 1982; Chang and Ochaya, 1986) with a strong preference for divalent and trivalent metals over Na^+. Gokel and coworkers (Gandour *et al.*, 1986; Gatto *et al.*, 1986) have synthesized several "bibracchial lariat ether" derivatives of diaza[15]crown-5. These "BiBLEs" have liganding side arms on each nitrogen, linked through flexible aliphatic linkages rather than rigid aromatic rings. They show Na^+:K^+ selectivities of only 1.0 to 4.9 even though they were measured (Gatto *et al.*, 1986) in anhydrous methanol, a solvent known to foster much higher absolute affinities and selectivities (Vögtle and Weber, 1980) than the aqueous salt solutions in which biologists must work. The improved selectivities of the present compounds may at least partly arise from the rigidity of their pendant aromatic groups, which force the crown nitrogen and the pendant ligand –OMe or –N= into a *cis* conformation ready to make the desired five-membered chelate ring with the Na^+ ion. The bulk of the two aromatic groups may also play a role in forcing them to sit on opposite sides rather than the same side of the macrocyclic plane. Indeed, the bulkier and more rigid the heteroaromatic ligand, the higher the observed Na^+:K^+ selectivity (compare **2J**, **2D**, and **2I**).

If the size of the crown ring really controls the preference between alkali metal ions, one should be able to change Na^+

indicators into Li^+ or K^+ indicators by using the next smaller or next larger crown ethers, 1,7-diaza-4,10-dioxacyclododecane or 1,10-diaza-4,7,13,16-tetraoxacyclooctadecane, respectively. The former has not been tested, but the latter did indeed confer K^+ selectivity, as shown by compounds **3J**, **3N**, and **3P**. Expansion of the macrocycle not only increases the K^+ affinity but decreases Na^+ affinity. Therefore, the attractively simple idea that ring size correlates with the preferred cation (Vögtle and Weber, 1980) does explain our findings so far, although it has been shown to fail with simpler crowns with small or no substituents (Gokel *et al.* 1983; Gandour *et al.*, 1986).

Spectral Properties—The spectral similarity between these new Na^+ indicators and the established Ca^{2+} indicator fura-2 (Grynkiewicz *et al.*, 1985), means that much the same equipment can be used for both. Likewise, the shift of the excitation peak to shorter wavelengths upon binding of Na^+ is analogous to the spectral effect of protonating the aromatic amino groups or of Ca^{2+} binding to tetracarboxylate indicators like fura-2. Such shifts are consistent with a mechanism in which cation binding causes a major loss of conjugation between the amino groups and the rest of the chromophore, at least partly by twisting the >N–Ar bond (Grynkiewicz *et al.*, 1985; Tsien, 1980, 1983). Unfortunately, the change in shape of the excitation spectrum due to Na^+ binding is not as drastic as the effect of Ca^{2+} on fura-2. Thus the 340/380 nm excitation ratio of SBFI changes by only a factor of 2.8 between 0 Na^+ (135 mM K^+) and 135 mM Na^+ (0 K^+) (Fig. 2). Therefore any photometric equipment used with SBFI must be able to resolve fairly small changes in ratio.

Suitability for Biological Use—Although the Na^+:K^+ selectivity of SBFI barely meets the criterion given in the Introduction, and is considerably less than achieved by some of the other chelators in this study, SBFI manages to be useful inside cells for three reasons. First, Na^+ binding affects its spectra more strongly than does K^+ binding, so that replacement of a bound K^+ by a Na^+ is spectroscopically visible. Second, under most circumstances cellular Na^+ and K^+ are not free to vary independently but are constrained by osmotic balance and electroneutrality to have a constant sum which is approximately known (Horowitz and Paine, 1979; Thomas and Cohen, 1981). Third, ionophores are available that can clamp $[Na^+]_i$ and $[K^+]_i$ to define values inside cells so that the spectral responses can be calibrated *in situ*. With their aid, $[Na^+]_i$ can be calibrated even when it is <10% of $[K^+]_i$, and even though the properties of the dye are significantly altered by cytoplasm (Harootunian *et al.*, 1989). Better Na^+:K^+ selectivity and greater sensitivity of excitation ratio to $[Na^+]_i$ would be valuable improvements, but SBFI is already good enough for many interesting biological applications.

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SUPPLEMENTARY MATERIAL to
"Fluorescent Indicators for Cytosolic Sodium"
by Akwasi Minta and Roger Y. Tsien

Proton NMR spectra were recorded on a Varian Instruments EM-390 at 90 MHz and on a 200 MHz Fourier-transform instrument (UCB-200) constructed in the Dept. of Chemistry, University of California, Berkeley. Peaks are reported below in the following format: NMR (solvent, operating frequency): chemical shift δ in ppm downfield from tetramethylsilane, multiplicity (s = singlet, d = doublet, dd = doublet of doublets, t = triplet, q = quartet, m = multiplet, br = broad), spin-spin coupling constant if appropriate, integrated number of protons; sometimes several adjacent peaks are too close for their integrals to be separated, in which case only the total integral for a cluster is stated. Column chromatography, reverse-phase thin layer chromatography, and preparative layer chromatography were done on EM Sciences (Cherry Hill, NJ) types 9385, 15687, and 5717 media respectively; centrifugal chromatography was performed on 1 mm layers cast from EM Sciences 7749 silica mounted in a model 7924T Chromatotron (Harrison Research, Palo Alto, CA).

N-(4-amino-2-carboxyphenyl)-aza[15]-crown-5 (1B)

2-Chloro-5-nitrobenzoic acid (Aldrich) (50.5 mg, 0.25 mmole) and 1-aza-4,7,10,13-tetraoxacyclopentadecane (Maeda et al, 1983) (220 mg, 1 mmole) were heated together under reflux in pyridine (1 ml) overnight. The reaction mixture was evaporated to dryness *in vacuo* and purified by preparative thin layer chromatography (silica gel) to give N-(4-nitro-5-carboxyphenyl)-aza[15]-crown-5 as a yellowish brown gum (100 mg, 26% yield). NMR (CD_3OD, 90 MHz) δ 8.25, d, 3 Hz, 1H; 8.10, dd, 7 Hz, 3Hz, 1H; 7.55, d, 7 Hz, 1H; 3.65, s + m, 16H; 3.20, t, 4H. The nitro-compound (60 mg) was dissolved in ethanol (2 ml) and hydrogenated at room temperature and atmospheric pressure with 15 mg palladium (5% on charcoal) catalyst. After full hydrogen uptake the mixture was filtered and the solvent evaporated *in vacuo* to give an off-white solid (50 mg, 94% yield) of 1B. NMR (CD_3OD, 90 MHz) δ 6.93, d, 7 Hz, 3 Hz, 1H; 3.50, s + m, 16H; 3.10, t, 4H.

N-(5-acetamido-8-quinolinyl)-aza[15]-crown-5 (1C)

8-Tosyloxy-5-nitroquinoline (80 mg, 0.25 mmole) [prepared by tosylation of commercial 8-hydroxy-5-nitroquinoline in pyridine] and 1-aza-4,7,10,13-tetraoxacyclopentadecane (220 mg, 1 mmole) were heated together under reflux in pyridine (2 ml) overnight. The reaction mixture was evaporated to dryness *in vacuo* and purified by preparative thin layer chromatography (silica gel) to give N-(5-nitro-8-quinolinyl)-aza[15]-crown-5 as a brown gum (105 mg, 25% yield). NMR ($CDCl_3$, 90 MHz) δ 8.90, dd, 9 Hz, 2 Hz, 1H; 8.45, d, 3 Hz, 1H; 8.09, d, 9 Hz, 1H; 7.25, dd, 9 Hz, 3 Hz, 1H; 6.72, d, 9 Hz, 1H; 3.72, m, 8H; 3.30, s + m, 12H. The nitro compound (50 mg) was dissolved in acetic anhydride and hydrogenated at room temperature and atmospheric pressure with 10 mg palladium (5% in charcoal) catalyst. After full hydrogen uptake the mixture was filtered and the solvent evaporated to give 1C as a brown solid (40 mg, 80%). NMR (CD_3OD, 90 MHz) δ 2.30, s, 3H; 3.40, m, 16H; 3.60, m, 4H; 7.20, m, 2H; 7.60, d, 9 Hz, 1H; 8.50, dd, 9 Hz, 2 Hz, 1H; 8.40, m, 1H.

N,N-bis-(5-acetamido-8-quinolinyl)-diaza[15]-crown-5 (2C)

8-Tosyloxy-5-nitroquinoline (0.85 g, 2.5 mmole) and commercial 1,7-diaza-4,10,13-trioxacyclopentadecane (Kryptofix 21, EM Sciences) (0.19 g, 0.8 mmole) were heated together under reflux in pyridine (5 ml) overnight. The reaction mixture was evaporated to dryness *in vacuo* and purified by preparative thin layer chromatography (silica gel) to give N,N-bis-(5-nitro-8-quinolinyl)-diaza[15]-crown-5 as a brown gum (180 mg, 32%). NMR ($CDCl_3$, 90 MHz) δ 9.35, dd, 2 Hz, 8 Hz, 1H; 8.70, dd, 2 Hz, 3 Hz, 1H; 8.40, d, 9 Hz, 1H; 7.5, dd, 8 Hz, 3 Hz, 1H; 6.89, d, 9 Hz, 1H; 4.10, m, 16H; 3.70, s, 4H. The nitro compound (60 mg) was dissolved in acetic anhydride (2.5 ml) and hydrogenated at room temperature and atmospheric pressure with 20 mg palladium (5% on charcoal) catalyst. After full hydrogen uptake, the mixture was filtered and the solvent evaporated to give 2C as a light brown solid (50 mg, 83%). NMR (CD_3OD, 90 MHz) δ 8.50, dd, 2 Hz, 8 Hz, 2H; 8.20, m, 2H; 7.85, d, 5 Hz, 2H; 7.40, m, 4H; 3.78, s, 4H; 3.48, m, 16H; 3.30, s, 6H.

N,N-bis-(5-acetamido-2-methyl-8-quinolinyl)-diaza[15]-crown-5 (2D)

8-Tosyloxy-5-nitro-2-methylquinoline (0.8 g, 2 mmole), prepared by tosylation of 5-nitro-2-methyl-8-quinolinol (Perez-Cistue, 1956), was heated under reflux with 1,7-diaza-4,10,13-trioxacyclopentadecane (0.110 g, 0.5 mmole) in pyridine (5 ml) overnight. The reaction mixture was evaporated to dryness *in vacuo* and purified by preparative thin layer chromatography (silica gel) to give N,N-bis-(5-nitro-2-methyl-8-quinolinyl)-diaza[15]-crown-5 as a reddish brown gum (85 mg, 29%). NMR ($CDCl_3$, 90 MHz) δ 9.25, d, 9 Hz, 2H; 8.45, d, 9 Hz, 2H; 7.35, dd, 9 Hz, 2 Hz, 2H; 6.85, dd, 9 Hz, 2 Hz, 2H; 4.00, m, 16H; 3.60, t, 4H; 2.62, s, 3H. The nitro compound (60 mg) was dissolved in acetic anhydride (5 ml) and hydrogenated at room temperature under atmospheric pressure with 0.02 g palladium (5% on charcoal) catalyst. After full hydrogen uptake the mixture was filtered and the solvent evaporated *in vacuo* to give 2D as a brown solid (0.04 g, 78%). Mass spec. (FAB) m/e = 615 ($M^+ + 1$).

1,8-bis-(6-methoxy-2-methylquinolinyl-8-amino)-3,6-dioxaoctane

6-Methoxy-2-methyl-8-aminoquinoline [prepared by the method of Wan et al (1974)] (3 g, 16 mmole) was dissolved in chloroform (25 ml) and triethylamine (8 ml) was added. Then 3,6-dioxaoctanedioyl chloride (Dietrich et al, 1973) (2.6 g, 12 mmole) in chloroform (5 ml) was added slowly with stirring under nitrogen. After 30 minutes the reaction mixture was diluted with more chloroform and washed with sodium bicarbonate solution and then brine. The chloroform solution was then passed through a plug of alumina and then evaporated *in vacuo* to obtain N,N-bis-(6-methoxy-2-methyl-8-quinolinyl)-3,6-dioxaoctane-1,8-diamide as a white solid (2.4 g, 60%). M.p. 191-193°. NMR ($CDCl_3$, 90 MHz) δ 8.2, d, 3 Hz, 2H; 7.58, d, 7 Hz, 2H; 6.95, d, 7 Hz, 2H; 6.45, d, 3 Hz, 2H; 4.20, s, 4H; 3.92, s, 4H; 3.72, s, 6H; 2.50, s, 6H.

The amide (3 g, 6 mmole) was dissolved in dry tetrahydrofuran (50 ml) and aluminum hydride (Yoon & Brown, 1968) solution (0.6M) in tetrahydrofuran (70 ml) was added slowly, and stirred over a period of two hours. Tetrahydrofuran-H_2O (1:1, 100 ml) was added followed by ether (200 ml). Stirring was continued for 30 more minutes and sodium hydroxide solution (20%) was added. The total reaction mixture was extracted with ether and the combined organic extracts were evaporated to dryness. The residue obtained was purified by column chromatography (silica gel, ethyl acetate:hexane:triethylamine, 75:25:1 v/v) to give the amine as a white solid (1.6 g, 57%). M.p. 89-91°. NMR ($CDCl_3$, 90 MHz) δ 7.85, d, 7 Hz, 2H; 7.20, d, 7 Hz, 2H; 6.35, s, 2H; 3.90, s, 6H; 3.80, t, 4H; 3.50, t, 4H; 2.70, s, 6H.

N,N-bis-(6-methoxy-2-methyl-8-quinolinyl)-diaza[15]-crown-5 (2E)

The above amine (2.25 g, 4.5 mmole) was dissolved in chloroform (200 ml) containing dimethylaniline (2.5 ml) and acylated under high dilution conditions (5 drops per second) with diglycolic acid chloride (855 mg, 5 mmole) in chloroform (200 ml). The reaction mixture was evaporated *in vacuo* and the residue purified by column chromatography (silica gel) to give the amide as a white solid (1.8 g, 70%). M.p. 231-233°. NMR ($CDCl_3$, 90 MHz) δ 8.15, d, 9 Hz, 2H; 8.00, m, 2H; 7.40, d, 9 Hz, 2H; 7.30, d, 3 Hz, 2H; 4.25, s, 6H; 4.00, s + m, 20H; 2.85, s, 6H.

The macrocyclic amide (1.2 g, 2 mmole) was dissolved in dry THF (120 ml) and aluminum hydride (0.6M) in THF (25 ml) was added slowly and stirred over a period of two hours. THF-H_2O (1:1, 100 ml) was added, followed by ether (200 ml). Stirring was continued for 30 more minutes and sodium hydroxide solution 20% was added. The total reaction mixture was extracted with ether and the combined ether extracts were evaporated to dryness. The residue obtained was purified by column chromatography (silica gel) to give 2E as a whitish foam (340 mg, 30%). NMR ($CDCl_3$, 90 MHz) δ 7.75, d, 9 Hz, 2H; 7.05, d, 9 Hz, 2H; 6.70, d, 3 Hz, 2H; 6.48, d, 3 Hz, 2H; 3.70, s + m, 26H; 2.50, s, 6H.

N,N-bis-(6-hydroxy-2-methyl-8-quinolinyl)-diaza[15]-crown-5 (2F)

N,N-bis-(6-methoxy-2-methyl-8-quinolinyl)-diaza[15]-crown-5 (50 mg, 0.9 mmole) was dissolved in dry tetrahydrofuran (1 ml) and added to a solution (2 ml) of diphenylphosphine 0.5 ml in 1.5 ml of dry THF and 0.35 ml of 9.5M n-butyllithium in hexane. The mixture was stirred for three hours and water was added. It was then extracted three times with chloroform-methanol 9:1 and two times with ethyl acetate. The combined organic extracts were evaporated to dryness and triturated with hexane. The residue of 2F was purified by centrifugal chromatography with chloroform-methanol (4:1 v/v).

N,N-bis(2-(3,4-dicarboxyphenyl)-7-methylfuro[3,2-f]quinolin-5-yl)-diaza[15]-crown-5 (2G methyl ester) (= SQFP/Me)

The above hydroxyquinoline was used directly for formylation by dissolving it in dimethylformamide (300 µl) and adding 0.5 ml of a 1:4 (v/v) mixture of $POCl_3$ and dimethylformamide. After stirring for two hours, water was added to quench the reaction mixture followed by saturated potassium carbonate to basify the solution. The reaction mixture was then extracted three times with chloroform and the combined chloroform extracts were back-washed with water and evaporated to give the salicylaldehyde as a yellow gum. The gum was purified by centrifugal chromatography with ethyl acetate to give the salicylaldehyde derivate (20 mg, 38%). NMR ($CDCl_3$, 90 MHz) δ 10.30, s, 1H (aldehyde); 8.30, d, 9 Hz, 2H; 7.15, d, 9 Hz, 2H; 6.45, s, 2H; 3.90, m, 16H; 3.60, s + m, 4H; 2.50, s, 6H.

The salicylaldehyde (10 mg, 0.017 mmole), potassium carbonate (40 mg), dimethyl 4-bromomethylphthalate (Anzalone & Hirsch, 1985) (14 mg, 0.05 mmole) and dimethylformamide (500 µl) were heated together at 140° (bath temperature) for four hours. The mixture was allowed to cool and chloroform with 10% methanol was added. The entire mixture was washed with water and evaporated *in vacuo*. The residue was purified by centrifugal chromatography with chloroform-methanol (9:1 v/v) to give SQFP (2G) methyl ester as a light brown gum (5.5 g, 33%). NMR ($CDCl_3$, 200 MHz) δ 2.75, s, 6H; 3.80, s + m, 32H; 8.10, d, 2H; 7.50, d, 2H; 7.60-7.80, s + m, 8H.

N,N-bis-(1-acetamidoacridin-4-yl)-diaza[15]-crown-5 (2H) and N,N-bis-(1-succinamidoacridin-4-yl)-diaza[15]-crown-5 (2I)

4-Methoxyacridine (1.4 g, 6.67 mmole) was dissolved in acetic anhydride (5 ml) and cooled to 0°. Concentrated nitric acid (1 eq, 0.7 ml) was added, followed by concentrated sulphuric acid (1 eq, 0.7 ml). After stirring for 1 hr, the product was filtered and the residue was extracted into methylene chloride and washed with sodium bicarbonate solution. It was then dried and evaporated *in vacuo* to give 1.2 g (70%) of 4-methoxy-1-nitroacridine. NMR ($CDCl_3$, 90 MHz) δ = 4.20, s, 3H; 6.95, d, 9 Hz, 1H; 6.70-8.00, m, 3H; 8.34, d, 9 Hz, 1H; 8.55, d, 9 Hz, 1H; 9.82, s, 1H. The methoxy compound (500 mg) was converted to the 4-hydroxy derivative by heating with KOH (2 ml of an 11 M aqueous solution) in DMSO (20 ml). This was tosylated with pyridine and toluenesulfonyl chloride to give the 4-tosyloxy derivative (260 mg, 34%). NMR ($CDCl_3$, 90 MHz) δ 2.2, s, 3H; 7.00, m, 2H, 6.30-6.80, m, 7H; 8.20, d, 9 Hz, 1H; 9.45, s, 1H. The tosyloxy compound (600 mg, 1.5 mmole) was dissolved in acetonitrile (4 ml) and 1,7-diaza-4,10,13-trioxacyclopentadecane (110 mg, 0.5 mmole) were added, followed by 1,8-bis(dimethylamino)naphthalene (290 mg, 2 mmole) and the reaction mixture heated under reflux overnight. The reaction mixture was evaporated *in vacuo* and the residue taken into chloroform and purified by column chromatography (silica gel, ethyl acetate, hexane 1:1) to give the N,N-bis-(1-nitro-4-acridinyl)-diaza[15]-crown-5 as a yellow gum (180 mg, 27%). NMR ($CDCl_3$, 90 MHz) δ 3.40, s, 4H; 3.82, m, 16H; 6.50, d, 9 Hz, 2H; 6.95, d, 9 Hz, 2H; 7.30, t, 9 Hz, 4H; 7.65, d, 9 Hz, 2H; 8.20, d, 9 Hz, 2H; 9.68, s, 2H.

The nitro-crown compound (70 mg, 0.1 mmole) was added to stannous chloride (200 mg in 2 ml ethanol), concentrated hydrochloric acid (3 ml), and ethanol (3 ml) and stirred overnight at room temperature. The reaction mixture was neutralized with sodium bicarbonate and extracted into chloroform. The chloroform layer was treated with acetic anhydride to give 2H, which was purified by column chromatography (silica gel, chloroform: methanol 85:15) to a soft yellow solid (30 mg, 41.6%). NMR ($CDCl_3$, 90 MHz) δ 2.45, s, 6H; 3.55, m, 20H; 6.30, t, 4H; 6.80, t, 2H; 7.30, d, 9 Hz, 2H; 7.50, d, 9 Hz, 2H; 7.82, d, 9 Hz, 2H; 9.15, s, 2H.

The succinamide derivative (2I) was obtained by reduction of the nitro compound (45 mg, 0.064 mmole) with stannous chloride (150 mg) and conc. HCl (3 ml) in ethanol. The resulting amine hydrochloride was washed repeatedly with ethanol, dissolved in pyridine (1.5 ml), and treated with succinic anhydride with stirring. The reaction was complete in three hours and the product (2I) was purified by column chromatography (silica gel, 10% methanol in chloroform) (15 mg, reddish yellow soft solid, 28%). Mass spec. (FAB) m/e = 802 (M^+).

N,N-bis-(4-acetamido-2-methoxyphenyl)-diaza[15]-crown-5 (2J)

2-Fluoro-5-nitroanisole (Ruyle et al, 1977) (250 mg, 1.5 mmole) was heated under reflux with 1,7-diaza-4,10,13-trioxacyclopentadecane (108 mg, 0.5 mmole) in pyridine (5 ml) overnight. The reaction mixture was evaporated *in vacuo* and the residue purified by preparative thin layer chromatography (silica gel) to obtain N,N-bis-(4-nitro-2-methoxyphenyl)-diaza[15]-crown-5 as a light yellow oil (120 mg, 23%). NMR ($CDCl_3$, 90 MHz) δ 7.80, dd, 8 Hz, 3 Hz, 2H; 7.70, d, 3 Hz, 2H; 6.89, d, 8 Hz, 2H; 3.87, s, 6H; 3.68, s + m, 20H. The nitro compound (0.05 g) was dissolved in acetic anhydride and hydrogenated at room temperature and atmospheric pressure with 0.02 g palladium (5% on charcoal) catalyst. After full hydrogen uptake the mixture was filtered and the solvent evaporated *in vacuo* to give 2J as a whitish semi-solid (43 mg, 82%). Mass spec.: m/e = 544 (M^+); m/e = 513 (M^+ - OMe).

N,N-bis-(3,6-dioxocyclohexa-1,4-dienyl)-diaza[15]-crown-5 (2K)

p-Benzoquinone (2.0 g, 18.5 mmole) and 1,7-diaza-4,10,13-trioxacyclopentadecane (400 mg, 1.8 mmole) were dissolved in a 1:1 mixture of chloroform and methanol (13 ml) and heated under reflux overnight. The reaction mixture was evaporated off *in vacuo* and the residue dissolved in a large volume of chloroform and filtered through a column of silica gel packed in ethyl acetate. The excess benzoquinone was removed with more ethyl acetate and the product (2K) was obtained by eluting with 4% methanol in ethyl acetate. Evaporation of the solvent gave 2K as a deep red foam (600 mg, 76%). NMR ($CDCl_3$, 90 MHz) δ 3.75, s, 4H; 3.88, s, 16H; 5.68, d, 1.5 Hz, 2H; 6.48, dd, 1.5 Hz, 4H.

N,N-bis-(2,5-dimethoxyphenyl)-diaza[15]-crown-5 (2L)

The bis-quinone (2K) (210 mg, 0.49 mmole) was dissolved in 2 ml methanol and hydrogenated with 33 mg of palladium (5% on charcoal) catalyst at atmospheric pressure and room temperature. When H_2 uptake ceased after 1.5 hrs, the solution had changed to a dull yellowish brown color. The reaction flask was stirred under a slight positive pressure of H_2 while approximately 1 mmole of tetramethylammonium hydroxide pentahydrate was injected as a 4 M solution in methanol through a gas-tight rubber septum into the mixture. Then 1 mmole of neat dimethyl sulfate was similarly injected using a separate syringe. The alternate injection of tetramethylammonium hydroxide then dimethyl sulfate was repeated nine more times over the next 2.5 hrs, for a total of 10 mmoles each of base and alkylating reagent. This cyclical procedure of partial deprotonation and methylation was found to give better results than adding all the base and all the alkylating reagent simultaneously, in which case the latter two mainly destroy each other. Also by conducting the alkylation under H_2, the strong tendency of the phenoxide anion to re-oxidize was suppressed. Once the alkylation was complete, the product was reasonably stable to air and could be worked up by evaporating the methanol under vacuo, dissolving the residue in water, neutralizing the alkalinity with acetic acid, and extracting with chloroform. Evaporation of the chloroform gave 252 mg crude 2L (105% of the stoichiometrically expected weight), which could be purified by centrifugal chromatography. NMR (CF_3COOH, 90 MHz) δ 3.95, m + s, 32H; 7.15, m, 6H.

N,N-bis-(2-methoxy-5-hydroxy-4-formylphenyl)-diaza[15]-crown-5 (2M)

The dimethoxy compound (2L) (30 mg, 0.06 mmole) was dissolved in dimethylformamide (200 µl) and kept at 0°. 0.5 ml of a 1:4 (v/v) mixture of $POCl_3$ and dimethylformamide was added and the reaction mixture stirred for 1 hour. Water (2 ml) was added to quench the reaction mixture followed by saturated potassium carbonate to basify the solution. The reaction mixture was then extracted 3 times with chloroform. The combined chloroform extracts were back-washed with water and evaporated *in vacuo* to give the dimethoxyaldehyde as a yellow gum (25 mg, 75%). NMR ($CDCl_3$, 90 MHz) δ 3.80, m, 20H; 3.98, s, 6H; 4.20, s, 6H; 6.70, s, 2H; 7.48, s, 2H; 10.68, s, 2H.

The dimethoxyaldehyde (15 mg, 0.027 mmole) was dissolved in nitromethane (2 ml). Saturated zinc chloride in nitromethane solution (1 ml) was added, followed by 2 ml of 1.0 M BCl_3 solution in dichloromethane. The reaction mixture was stirred for 1.5 hours and a 1:1 mixture of water and methanol (2 ml) was added. Stirring was continued for 30 min and potassium carbonate-EDTA solution was added. Stirring was continued for 30 more minutes and the mixture was extracted with chloroform (3 times), followed by ethyl acetate (1 time). The combined organic extracts were washed with water and purified by centrifugal chromatography on ethyl acetate to give 2M as a yellow soft solid (11 mg, 78%). NMR ($CDCl_3$, 90 MHz) δ 3.75, m, 20H; 3.85, s, 6H; 6.50, s, 2H; 6.88, s, 2H; 9.84, s, 2H (aldehyde); 11.50, s, 1H, OH hydrogen bonded.

N,N-bis-(2-(3,4-dicarboxyphenyl)-5-methoxybenzofuran-6-yl)-diaza[15]-crown-5 (2N Me ester) (= SBFP/Me)

The salicylaldehyde (2M) (9 mg, 0.017 mmole), potassium carbonate (40 mg), dimethyl 4-bromomethylphthalate (11 mg, 0.04 mmole), and dimethylformamide (0.5 ml) were heated together at 140° (bath temp.) for 4 hours. The mixture was allowed to cool. Chloroform with 10% methanol (3 ml) was added. The entire mixture was washed with water and evaporated *in vacuo*. The residue was dissolved in 5% MeOH in chloroform and purified by centrifugal chromatography with chloroform-methanol (24:1 v/v) to give SBFP/Me as a light brown gum (5 mg, 33%). NMR (CD_3OD, $CDCl_3$ 1:9, 200 MHz) δ 3.70, m, 20H; 3.97, 2s, 6H; 3.99, 2s, 6H; 4.10, 2s, 6H; 7.04, m, 2H; 7.10, s, 2H; 7.85, d, 2H; 7.95, d, 2H; 8.13, s, 2H.

N,N-bis-(2-(5-carboxyoxazol-2-yl)-5-methoxybenzofuran-6-yl)-diaza[15]-crown-5 (2O ethyl ester) (= SBFO/Et)

The salicylaldehyde (2M) (6 mg, 0.012 mmole), potassium carbonate (40 mg), ethyl 2-chloromethyloxazole-5-carboxylate (Grynkiewicz et al, 1985) (12 mg, 0.068 mmole), and dimethylformamide (300 µl) were heated together at 100° for 1 hour. The reaction mixture was allowed to cool and chloroform (3 ml) was added and the entire mixture washed with water to get rid of the solid potassium carbonate. The organic layer was evaporated *in vacuo* and taken into 5% methanol-chloroform for purification by centrifugal chromatography using chloroform-methanol (24:1 v/v) into 5% product, SBFO/Et, was obtained as a yellow gum (5 mg, 57%). NMR (CD_3OD, $CDCl_3$ 1:9, 200 MHz) δ 1.21, t, 6H; 3.60-3.80, m, 16H; 3.98, s, 4H; 4.45, q, 4H; 4.80, d, 2H; 7.18, s, 2H; 7.55, s, 2H; 7.75, s, 2H; 7.92, s, 2H.

N,N-bis-(2-(2,4-dicarboxyphenyl)-5-methoxybenzofuran-6-yl)-diaza[15]-crown-5 (2P methyl ester) (= SBFI/Me)

Dimethyl 4-bromomethylisophthalate was obtained by the method of Anzalone & Hirsch (1985) using methanol instead of ethanol in the esterification procedure. M.p. 80-82°. NMR ($CDCl_3$, 90 MHz) δ 3.90, s, 3H; 3.92, s, 3H; 4.90, s, 2H; 7.45, d, 7 Hz, 1H; 8.05, dd, 2 Hz, 7 Hz, 1H; 8.50, d, 2 Hz, 1H.

The above isophthalate ester (150 mg, 0.52 mmole), the salicylaldehyde 2M (40 mg, 77 µmole), K_2CO_3 (250 mg, 1.8 mmole), and dimethylformamide (2 ml) were heated together at 150° for 2.5 hrs. The mixture was diluted with chloroform and filtered. The filtrate was washed with water and evaporated in vacuo. The gummy residue was purified by centrifugal chromatography to give SBFI/Me (28 mg, 41%). NMR ($CDCl_3$, 200 MHz) δ 3.60-3.80, m, 20H; 3.90, s, 12H; 3.95, s, 6H; 7.05, s, 2H; 7.20, s, 2H; 7.30, s, 2H; 7.80, d, 2 Hz, 2H; 8.20, dd, 7 Hz, 2 Hz, 2H; 8.35, d, 2 Hz, 2H.

N,N-bis(2-(3,4-dicarboxyphenyl)-5-methoxybenzofuran-6-yl)-diaza[18]-crown-5 (3N Me ester) (=PBFP/Me)

The preparation of PBFP/Me was similar to that of SBFP/Me (2N) except that 1,10-diaza-4,7,13,16-tetraoxacyclooctadecane (Kryptofix 22, EM Sciences) was used as the crown instead of 1,7-diaza-4,10,13-trioxacyclopentadecane. The properties of the various intermediates and PBFP are as follows:

N,N-bis-(3,6-dioxocyclohexa-1,4-dienyl)-diaza[18]-crown-6 (3K) was obtained in 70% yield as reddish-brown needles, M.p. 153-155°. NMR ($CDCl_3$, 90 MHz) δ 3.60-3.80, s + m, 24H; 5.60, d, 1.5 Hz, 2H; 6.48, dd, 1.5 Hz, 4H.

N,N-bis-(2,5-dimethoxyphenyl)-diaza[18]-crown-6 (3L) was obtained in 55% yield as a gum. NMR (CF_3COOH, 90 MHz) δ 3.40-3.80, s + m, 36H; 6.80, m, 6H.

N,N-bis-(1,4-dimethoxy-5-formyl-2-phenyl)-diaza[18]-crown-6 was obtained as an off-white soft solid. M.p. 131-133°. NMR ($CDCl_3$, 90 MHz) δ 3.50-3.70, s + m, 24H; 3.75, s, 6H; 3.85, s, 6H; 6.50, s, 2H; 7.20, s, 2H; 10.25, s, 2H (formyl).

N,N-bis-(1-methoxy-4-hydroxy-5-formyl-2-phenyl)-diaza[18]-crown-6 (3M) was obtained as an orange solid. M.p. 134-136°. NMR ($CDCl_3$, 90 MHz) δ 3.50-3.70, s + m, 24H; 3.75, s, 6H; 6.40, s, 2H; 6.90, s, 2H; 9.65, s, 2H (formyl); 11.40, s, 2H (hydrogen-bonded OH).

PBFP/Me (3N methyl ester) was obtained as a light yellow gum. NMR ($CDCl_3$, 200 MHz) δ 3.60, m, 8H; 3.67, m, 16H; 3.90, s, 12H; 3.93, s, 6H; 6.90, s, 2H; 7.10, s, 2H; 7.48, s, 2H; 7.68, d, 2 Hz, 2H; 7.80, dd, 2 Hz, 1 Hz, 2H; 7.98, d, 1 Hz, 2H.

Saponification of methyl or ethyl esters; preparation of acetoxymethyl esters

The esters of polycarboxylic acids 2N, 2O, 2P, and 3N were hydrolyzed by dissolving them in methanol or dioxane or a mixture of the two solvents, then adding excess base, usually tetramethylammonium hydroxide or cesium hydroxide so that the cation would show negligible tendency to bind to the chelator. Acetoxymethyl (AM) esters were prepared by the standard procedure of realkylation of the polycarboxylate anions using acetoxymethyl bromide (Grynkiewicz et al, 1985). A typical procedure is given below for 2P (=SBFI) and its AM ester:

SBFI/Me (6 mg, 6.7 µmole) was dissolved in 200 µl methanol and 200 µl dioxane. 1M TMA^+OH^- (200 µl) was added and the reaction left overnight. When hydrolysis was complete as judged by reverse-phase thin layer chromatography, the mixture was evaporated to dryness. The residue was dissolved in dimethylformamide (2 ml) and ethyldiisopropylamine (200 µl) and acetoxymethyl bromide (300 µl) were added. The suspension was stirred overnight. Chloroform was added and the alkylammonium bromide salts filtered off. The filtrate was evaporated *in vacuo* and the residue purified by centrifugal chromatography (silica) to give the product as a hard gum (4 mg, 53%). NMR ($CDCl_3$, 200 MHz) δ 2.10, s, 6H; 2.18, s, 6H; 3.50-3.80, m, 20H; 3.88, s, 6H; 5.95, s, 4H; 6.05, s, 4H; 6.98, s, 2H; 7.05, s, 2H; 7.20, s, 2H; 7.87, d, 7 Hz, 2H; 8.20, dd, 7 Hz, 2 Hz, 2H; 8.35, d, 2 Hz, 2H.

PBFI and its AM ester

The synthesis of PBFI and its AM ester was similar to those for PBFP except that dimethyl 4-bromomethylisophthalate was used instead of dimethyl 4-bromomethylphthalate. The salicylaldehyde (3 M) [1.6 g, 2.8 mmoles] was suspended with potassium carbonate, 2-5 g, dimethyl 4-bromomethylisophthalate (2 g, 6.9 mmoles) in acetonitrile (25 ml) and heated under reflux for 2 hours. The orange solid which formed on cooling was dissolved in chloroform and filtered to remove the potassium carbonate. The filtrate was washed with water and evaporated *in vacuo*. The solid residue was purified by column chromatography (silica gel, ethyl acetate followed by 3% methanol in chloroform) to give orange solid 2-5 g, 90% yield. NMR ($CDCl_3$, 300 MHz) δ 10.80, s, 2H formyl; 8.70, d, 2 Hz, 2H; 8.20, dd, 7 Hz, 2H; 7.80, d, 7 Hz, 2H; 7.30, s, 2H; 6.50, s, 2H; 5.60, s, 4H; 3.95, s, 3H; 3.90, s, 3H; 3.80, s, 3H; 3.60-3.75, m, 24H.

The ether (500 mg, 0.51 mmole) was dissolved in dry dimethylformamide (8 ml). Potassium carbonate (600 mg) was added and the suspension heated at 145° for 3 hours. Thin layer chromatography showed a mixture of free acid and ester. The mixture was filtered and washed with 10% methanol in chloroform. The total filtrate was evaporated *in vacuo* to dryness. The brown gummy residue was dissolved in methanol and 2 M KOH (5 ml) was added. The solution was stirred overnight and evaporated *in vacuo* to an aqueous solution. Water (10 ml) was added followed by 2 M HCl (5 ml) to give the free acid as brownish-yellow solid. This was dried over P_2O_5 to give 400 mg of product (88% yield). This free acid (50 mg, 0.06 mmoles) was converted to the AM by the usual procedure to give 30 mg of product as yellowish-brown gum (45% yield). NMR ($CDCl_3$, 300 MHz) δ 8.40, s, 2H; 8.20, d, 7 Hz, 2H; 7.80, d, 7 Hz, 2H; 7.40, s, 2H; 7.30, s, 2H; 7.15, s, 2H; 6.10, s, 2H; 5.90, s, 2H; 3.80, s, 3H; 3.30-3.70, m, 24H; 2.10, s, 3H; 2.20, s, 3H.