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Design and properties of a fluorescent indicator of intracellular free Na^+ concentration

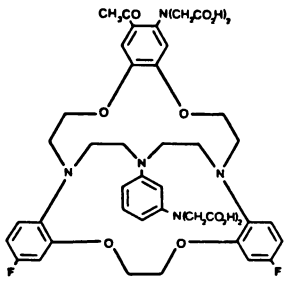
Gerry A. SMITH, T. Robin HESKETH and James C. METCALFE*
Department of Biochemistry, University of Cambridge, Tennis Court Road, Cambridge CB2 1QW, U.K.

We have recently described a cryptand structure, FCryp-1, with appropriate properties for an indicator of intracellular free Na^+ concentration using the ^{19}F-n.m.r. chemical shift of the incorporated 5FBAPTA [1,2-bis-(2-amino-5-fluorophenoxy)ethane-*NNN'N'*-tetra-acetic acid] reporter group to measure the free cytosolic Na^+ concentration ($[Na^+]_i$) [Smith, Morris, Hesketh and Metcalfe (1986) Biochim. Biophys. Acta **889**, 82–83]. FCryp-1 carries four carboxylate groups to confer aqueous solubility and the indicator is membrane-permeant when the carboxyls are esterified with acetoxymethyl ester groups. Here we describe the synthesis of FCryp-2 to provide a fluorescent indicator of $[Na^+]_i$. FCryp-2 retains the parent tribenzo (2:2:1) cryptand structure of FCryp-1, in which the benzenoid ring at C-21 in FCryp-1 is replaced by an indole derivative which acts as the fluorophor in FCryp-2. With excitation at 340 nm, FCryp-2 gives an emission maximum at 460 nm in the absence of Na^+ which shifts to 395 nm when FCryp-2 is saturated with Na^+, with an isosbestic point at 455 nm. The apparent dissociation constant of FCryp-2 in a buffer solution of 100 mM-KCl/20 mM-KH_2PO_4/K_2HPO_4, pH 7.0, at 37 °C is 6.0 mM and the free Na^+ concentration can be measured either from the calibrated fluorescence intensity at 395 nm, which increases 25-fold when Na^+ is bound to FCryp-2, or from the ratio of fluorescence intensities at 395 nm and 455 nm. The measurement of free $[Na^+]$ by either method is unaffected by K^+, Ca^{2+} or Mg^{2+} in the normal intracellular concentration ranges. Free $[Na^+]$ measurements by the ratio method are unaffected by pH from 6.6 to 7.6.

INTRODUCTION

The development of specific indicators incorporating fluorescent or ^{19}F-n.m.r. reporter groups has enabled measurement of the free cytosolic concentrations of Ca^{2+} and H^+ ($[Ca^{2+}]_i$ and pH_i) to be made in a wide variety of systems from single cells to isolated perfused organs [1–6]. One example of a field in which the use of the indicators has been of particular interest is the stimulation of quiescent somatic cells by mitogens to re-enter the cell cycle. Many growth factors and mitogens generate a rapid increase in $[Ca^{2+}]_i$ and pH_i in quiescent cells [7], although the role of these ionic signals in the subsequent mitogenic pathway remains largely undefined. In one fibroblast cell line it has been shown that the increase in pH_i in response to mitogenic stimulation is required both for the activation of protein synthesis in the quiescent cells and for subsequent progression to DNA synthesis [8,9]. From studies using inhibitors of the pH_i response, however, it appears unlikely that the response is generally obligatory for mitogenic stimulation in other types of fibroblast or in cells of different phenotype [10]. The increase in pH_i has been attributed to activation of an amiloride-sensitive Na^+/H^+ antiporter in the plasma membrane. The response is dependent on extracellular Na^+, is coupled to an increased influx of $^{22}Na^+$ [11], and in some cells (e.g. pig lymphocytes) there is a small increase in total cell Na^+ concentration after mitogenic stimulation [12]. Evaluation of whether the stimulation of Na^+ influx is accompanied by a change in $[Na^+]_i$, and any functional significance such a change might have, has been hindered by lack of suitable indicators of $[Na^+]_i$. The potential applications of such indicators are extensive. One example is from a recent study of the mitogenic stimulation of mouse thymocytes by the Ca^{2+} ionophore A23187, in which the activation of the c-*fos* and c-*myc* proto-oncogenes by A23187 was blocked by removal of Na^+ from the medium [13]. Indirect evidence suggested that this could not be attributed to the block of the pH_i response to the ionophore when external Na^+ was removed, but might depend instead on a decrease in $[Na^+]_i$ which we were unable to measure. More generally, indicators of $[Na^+]_i$ would be useful, for example, in studies of Na^+ exchange in heart [14] and smooth muscle [15], in stimulation–secretion coupling in exocytosis [16] and in the coupling of G protein(s) to adenylate cyclase [17].

We have therefore developed a fluorescent $[Na^+]_i$ indicator, FCryp-2. The starting point was the structure of FCryp-1 which we described recently as a ^{19}F-n.m.r. indicator of $[Na^+]_i$ [18]:



FCryp-1

Abbreviations used: 5FBAPTA, 1,2-bis-(2-amino-5-fluorophenoxy)ethane-*NNN'N'*-tetra-acetic acid; $[Ca^{2+}]_i$, $[Na^+]_i$, pH_i, cytosolic free concentrations of Ca^{2+}, Na^+ and H^+; e.b., electron bombardment; f.a.b., fast atom bombardment; NOBA, *o*-nitrobenzyl alcohol.

* To whom correspondence and reprint requests should be addressed.

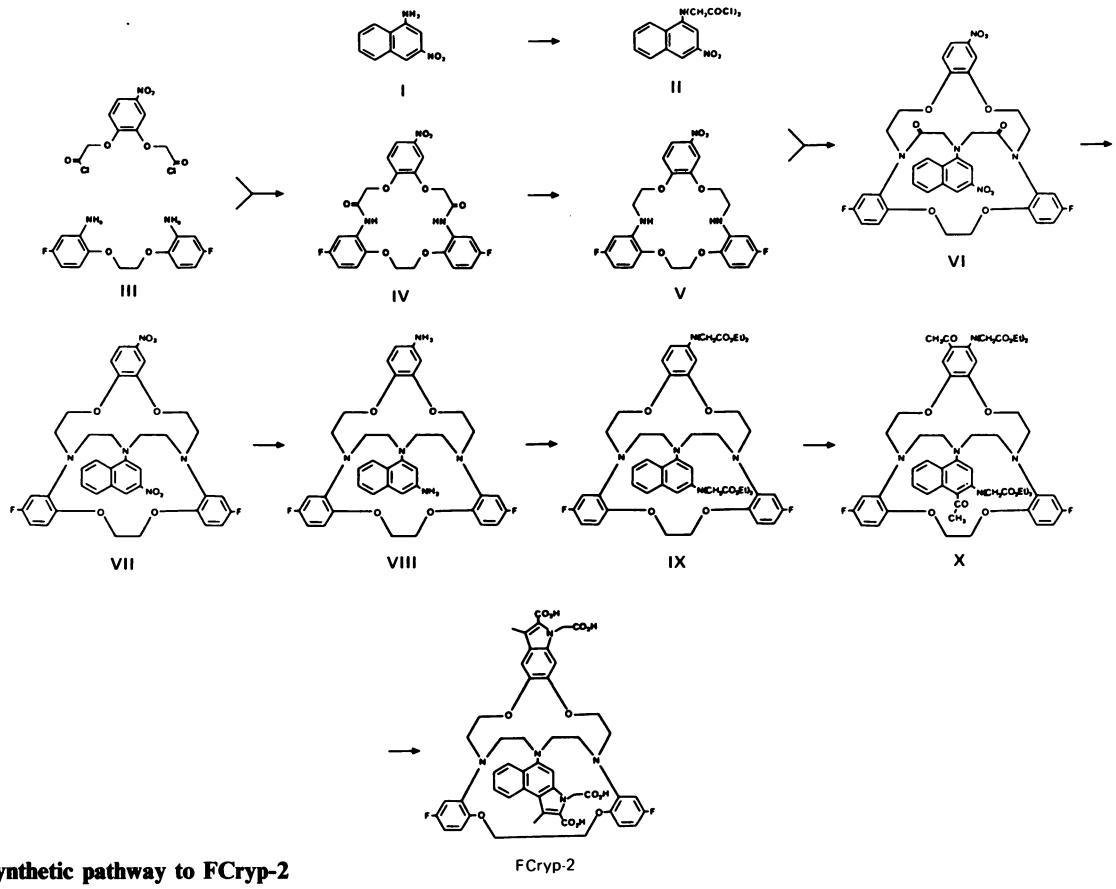


Fig. 1. The synthetic pathway to FCryp-2

FCryp-1 incorporated the 5FBAPTA reporter group, first used as an indicator of $[Ca^{2+}]_i$ [5], into a (2:2:1) cryptand with appropriate Na^+ affinity, Na^+/K^+ selectivity and insensitivity to pH. The chemical shift of the ^{19}F resonance provided a direct readout of the free Na^+ concentration. The synthesis of the FCryp-2 structure and its properties are described.

MATERIALS AND METHODS

The synthetic pathway to FCryp-2 is summarized in Fig. 1.

3-Nitro-1-naphthylamine (I)

1,3-Dinitronaphthalene (25 g) in ethanol (1 litre) was heated under reflux with rapid stirring and sufficient sodium hydrosulphide (approx. 40 g) was added in portions to complete monoreduction (t.l.c: silica; 15% ethyl acetate in toluene). The cooled reaction mixture was diluted with 1 litre of 1 M-NaOH solution, stirred, and the title compound recovered by filtration and recrystallized from aqueous ethanol (10 g, 46%); m.p. 128–129 °C.

3-Nitro-1-naphthylamine-*N*-diacetoyl chloride (II)

This compound was prepared from **I** as for 3-nitroaniline diacetic acid [18] using a large excess of ethyl bromoacetate. Ethyl ester (80%); m.p. 99–100 °C; n.m.r. (C^2HCl_3, 60 MHz), δ 1.5 (t, 7 Hz, 6H), 4.1 (q, 7 Hz, 4H), 4.2 (s, 4H), 7.65 (m, 2H), 7.95 (dd, 3 and 10 Hz, 1H), 8.05 (d, 3 Hz, 1H), 8.3 (dd, 3 and 10 Hz, 1H), 8.5 (d, 3 Hz, 1H); *M* 360 (e.b.). Free acid (75%); m.p. 197–198 °C (d). The acid chloride was prepared by dissolution of the free acid in excess thionyl chloride containing 0.2% dimethyl formamide for 12 h at room temperature before evaporation to dryness *in vacuo*; *M* 340, 343, 345 (e.b.).

1,2-Bis-(2-amino-4-fluorophenoxy)ethane (III)

This was prepared from 2-nitro-4-fluorophenol according to the reaction described previously for the 5-fluoro isomer [5,18]. Dinitro intermediate, m.p. 134–135 °C; *M* 340 (e.b.). Diamine (**III**), m.p. 117–118 °C; *M* 280 (e.b.).

2,3:9,8-Bis-[1-fluoro-3,4-benzo]-14,15-[1-nitro-3,4-benzo]-1,10-diaza-4,7,13,16-tetraoxacyclo-octadecane-11,18-dione (IV)

The diamine (**III**, 32.8 g), and pyridine (20 ml) in dichloromethane (1 litre), and a solution of the acid chloride [18] made from 4-nitro-*OO*-diacetic acid (31.75 g) in dichloromethane (1 litre) were added simultaneously to well-stirred dichloromethane (1 litre) over 2 h and left overnight. The resulting solution was stirred with HCl (2 M, 500 ml), filtered through Hyflo Supercel, separated and dried with Na_2CO_3. The product was absorbed onto a column of silica gel (500 g); on attempted elution with 1% ethyl acetate in chloroform the product crystallized on the column. The column was extruded, the top portion discarded and the remainder extracted by boiling with 2% ethyl acetate in chloroform (3 × 2 litres). Evaporation and filtration from ethyl acetate gave the title compound (44.8 g, 74%); m.p. 265–266 °C; *M* + 1, 516 (f.a.b., NOBA).

2,3:9,8-Bis-[1-fluoro-3,4-benzo]-14,15-[1-nitro-3,4-benzo]-1,10-diaza-4,7,13,16-tetraoxacyclo-octadecane (V)

The diamide (**IV**, 40 g) was dissolved in borane in tetrahydrofuran (1 M, 500 ml) under reflux, and water (12 ml) in tetrahydrofuran (100 ml) was added dropwise over 1 h. Note that borane reductions did not proceed rapidly or smoothly without the slow addition of water. The solution was treated with conc. HCl (50 ml), poured with fast stirring into excess NaOH solution (1 M) and light petroleum (b.p. 40–60 °C). The product was collected by filtration and dried (35.5 g, 95%); m.p. 243–244 °C; $M+1$, 520 (f.d.). The extreme insolubility of the crown ether **V** gave two problems: it was not possible to obtain a f.a.b. mass spectrum or n.m.r. spectrum, and it was not possible to add the compound normally in infinite dilution reactions.

2,3:9,8-Bis-[1-fluoro-3,4-benzo]-14,15-[1-nitro-3,4-benzo]-21-(3-nitro-1-naphthyl)-4,7,13,16-tetraoxa-1,10,21-triazabicyclo[8.8.5]tricosane-19,23-dione (VI)

The diaza crown ether (**V**, 10.8 g) was prepared in a finely divided state by dissolution in boiling ethanol (100 ml) with the addition of conc. HCl (50 ml), before the hot solution was poured into well-stirred ammonia (*d* 0.88, 1 litre). The fine suspension was collected by filtration, dried *in vacuo* first at 80 °C overnight and then over phosphorus pentoxide at 150 °C for 5 h. The dry solid was suspended in dichloromethane (1.5 litre) and stirred well. 3-Nitro-1-naphthylamine-*N*-diacetyl chloride (**II**; 8.3 g, 10% excess) in dichloromethane (1 litre) was added over 4 h and after a 30 min delay a solution of pyridine (3.4 g) in dichloromethane was also added concurrently. When additions were complete (4–5 h), more pyridine (4 g) was added and the reaction was stirred for 1 h. The resulting solution was washed with dilute HCl (2 M, 500 ml), dried with Na_2CO_3, filtered through Hyflo Supercel and absorbed onto a column of silica (200 g). Traces of remaining diamine were eluted with dichloromethane (1 litre). The product was eluted with chloroform (1 litre) as a coloured band, seeded, left to crystallize overnight and collected by filtration (12.5 g, 75%); decomp. 200 °C, non-melting < 330 °C; $M+1$, 756 (f.a.b., NOBA).

2,3:9,8-Bis-[1-fluoro-3,4-benzo]-14,15-[1-nitro-3,4-benzo]-21-(3-nitro-1-naphthyl)-4,7,13,16-tetraoxa-1,10,21-triazabicyclo[8.8.5]tricosane (VII)

The diamide (**VI**, 12.5 g) was dissolved in borane in tetrahydrofuran (1 M, 600 ml) under reflux, water (5.4 ml) in tetrahydrofuran (150 ml) was added dropwise over 1 h, and further water (25 ml) was added after 45 min. The solution was evaporated to dryness *in vacuo* and triturated with water (500 ml). The product was collected by filtration, washed with water and dried (10.2 g, 85%); sintered 150 °C, decomp. 200 °C; $M+1$, 728 (f.a.b., NOBA).

2,3:9,8-Bis-[1-fluoro-3,4-benzo]-14,15-[1-amino-3,4-benzo]-21-(3-amino-1-naphthyl)-4,7,13,16-tetraoxa-1,10,21-triazabicyclo[8.8.5]tricosane (VIII)

The dinitro cryptand (**VII**, 10.2 g) in tetrahydrofuran (200 ml) was treated with stannous chloride dihydrate (50 g) in conc. HCl (100 ml) for 2 h. The reaction solution was poured into KOH (500 g) in water (1 litre) and extracted with dichloromethane until all visible solids were dissolved. The combined extracts were washed with KOH solution (5 M), dried over $MgSO_4$, evaporated with toluene (approx. 50 ml), allowed to crystallize at 0 °C and collected by filtration (7.3 g, 79%); m.p. 155–160 °C; $M+1$, 668 (f.a.b., NOBA). (N.B. All solutions were deoxygenated with nitrogen.)

2,3:9,8-Bis-[1-fluoro-3,4-benzo]-14,15-[1-di(ethoxycarbonylmethyl)amino-3,4-benzo]-21-[3-di(ethoxycarbonylmethyl)amino-1-naphthyl]-4,7,13,16-tetraoxa-1,10,21-triazabicyclo[8.8.5]tricosane (IX)

The amine (**VIII**, 7.3 g), proton sponge (5 g), ethyl bromoacetate (15 g), sodium iodide (15 g) and acetonitrile (50 ml) were heated under reflux in N_2 for 24 h. The reaction mixture was diluted with toluene and ammonium phosphate solution (1 M, pH 4), filtered through Hyflo Supercel, separated, the toluene phase was washed with ammonium phosphate (3×), then with potassium bicarbonate (1 M), dried with $MgSO_4$, evaporated and purified by chromatography in ethyl acetate/toluene over silica gel (9 g, 78%); $M+1$, 1012 (f.a.b., NOBA).

Acylation reagent

Zinc chloride (14 g) was dried by fusion over a bunsen flame, and sodium chloride (6 g) was added to the melt and mixed well over gentle heat to give a clear solution. On cooling the solid was dissolved in acetic anhydride (140 ml). Note that the zinc chloride acylation reaction will not proceed past the first stage without sufficient sodium present.

2,3:9,8-Bis-[1-fluoro-3,4-benzo]-14,15-[1-acetyl-2-di(ethoxycarbonylmethyl)amino-4,5-benzo]-21-[3-di(ethoxycarbonylmethyl)amino-4-acetyl-1-naphthyl]-4,7,13,16-tetraoxa-1,10,21-triazabicyclo[8.8.5]tricosane (X)

The cryptand ethyl ester (**IX**, 1.5 g) was dissolved in the acylation reagent (30 ml) and stirred in an oil bath at 100 °C for 30 min. Acetic anhydride (10 ml) was added, taken off by vacuum distillation (1 h) to remove any acetic acid side-product, and the reaction heated for a further 1 h. The yellow solution was cooled, poured onto a mixture of water and toluene with potassium bicarbonate (50 g) and stirred well for 1 h. After filtration with Hyflo Supercel, the phases were separated and the organic phase washed with potassium chloride solution dried with $MgSO_4$, and evaporated (t.l.c: activated silica; 30% ethyl acetate in toluene or 5% propan-2-ol in dichloromethane). The oil was chromatographed on dry silica (100 g) in 15% ethyl acetate in toluene, the first band eluted was collected, to ensure that the slower running impurity was not included as this distorted the second stage of chromatography. The product was rechromatographed on dry silica (150 g), pre-equilibrated with 3% propan-2-ol in dichloromethane (2 litres), and eluted with the same solvent to give the pure ethyl ester of **X** as a dry froth (0.7 g, 43%); $M+1$, 1096 (f.a.b., NOBA); i.r. 1750 cm^{-1} (vs), 1680 cm^{-1} (s), 1600 cm^{-1} (s); n.m.r. (C^2HCl_3, 400 MHz) δ 1.139 (t, 7.1 Hz, 6H), 1.173 (t, 7.0 Hz, 6H), 2.685 (s, 3H), 2.690 (s, 3H), 2.941 (m, 2H), 3.21 (m, 2H), 3.44 (m, 2H), 3.64 (m, 2H), 3.65 (m, 2H), 3.91 (m, 4H), 4.07 (m, 4H), 3.950 (s, 4H), 3.956 (q, 7.1 Hz, 4H), 3.970 (s, 4H), 4.055 (q, 7.0 Hz, 2H), 4.060 (q, 7.0 Hz, 2H), 4.45 (m, 2H), 6.630 (dt, 3 and 9 Hz, 1H), 6.636 (s, 1H), 6.653 (dt, 3 and 9 Hz, 1H), 6.760 (dd,

3 and 10 Hz, 1H), 6.785 (dd, 3 and 10 Hz, 1H), 6.872 (dd, 5 and 9 Hz, 1H), 6.881 (dd, 5 and 9 Hz, 1H), 6.912 (s, 1H), 7.316 (ddd, 1.5, 7.0 and 8.5 Hz, 1H), 7.364 (ddd, 1.5, 7.0 and 8.5 Hz, 1H), 7.504 (s, 1H), 7.642 (dd, 1.5 and 8.5 Hz, 1H), 8.246 (dd, 1.5 and 8.5 Hz, 1H).

2,3:9,8-Bis-[1-fluoro-3,4-benzo]-14,15-[1-carboxymethyl-2-carboxy-3-methyl-5,6-indolo]-21-(1-carboxymethyl-2-carboxy-3-methyl-8-benz[*e*]indolyl)-4,7,13,16-tetraoxa-1,10,21-triazabicyclo[8.8.5]tricosane (FCryp-2)

The diacylated cryptand (**X**, 50 mg) was dissolved in absolute ethanol (2 ml) containing diethyl malonate (320 mg), treated with sodium ethoxide in ethanol (1 M, 1 ml) and heated under reflux for 3 h. The reaction mixture was cooled and poured into well-stirred Tris/phosphate buffer (20 ml, 1 M, pH 7.5) and the product recovered by filtration, washed with water and dried. Crystallization from dichloromethane/ethanol yielded the title compound as its ethyl ester (40 mg, 80%); m.p. 226–228 °C; $M+1$, 1060 (f.a.b., NOBA); i.r. 1690 cm^{-1} (vs) 1750 cm^{-1} (s) 1605 cm^{-1} (vw); n.m.r. (C^2HCl_3, 400 MHz) δ 0.98 (t, 7.1 Hz, 3H), 1.23 (t, 7.1 Hz, 3H), 1.36 (t, 7.1 Hz, 3H), 1.39 (t, 7.1 Hz, 3H), 2.48 (s, 3H), 2.79 [t(b), 12 Hz, 2H], 2.97 (s, 3H), 3.13 (t, 12 Hz, 1H), 3.15 (t, 12 Hz, 1H), 3.44 (d, 12 Hz, 1H), 3.47 (d, 12 Hz, 1H), 3.76 (m, 12 Hz, 6H), 3.95 (d, 11 Hz, 1H), 3.98 (m, 4H), 4.0 (q, 7.1 Hz, 2H), 4.17 (q, 7.1 Hz, 1H), 4.18 (q, 7.1 Hz, 1H), 4.30 (q, 7.1 Hz, 2H), 4.34 (q, 7.1 Hz, 2H), 4.82 [m(b), 2H], 5.07 (s, 2H), 5.21 (s, 2H), 6.40 (s, 1H), 6.66 (m, 2H), 6.77 (s, 1H), 6.80 (m, 2H), 6.88 (m, 2H), 7.42 (dt, 1.3 and 8 Hz, 1H), 7.52 (dt, 1.3 and 8 Hz, 1H), 7.85 (s, 1H), 8.37 (dd, 1.3 and 8 Hz, 1H), 8.48 (dd, 1.3 and 8 Hz, 1H); ^{19}F-n.m.r. (C^2HCl_3, 376 MHz) shifts in p.p.m. upfield from 4-nitrofluorobenzene at 25 °C: 14.11 p.p.m. (0.05 rel. signal), 14.27 (0.05), 16.25 (0.05), 16.39 (0.05), 17.01 (0.9), 17.21 (0.9); at 45 °C: 17.07 (1.0), 17.15 (1.0); at 52 °C: 17.11 (broad singlet 2.0).

Hydrolysis of the ethyl esters

The indole cryptand (FCryp-2 tetraethyl ester, 10 mg) was dissolved in dichloromethane (0.2 ml) and ethanol (0.5 ml) and treated with KOH (5 M, 0.1 ml). The solution was heated at 50 °C for 2 h, the organic solvents removed under a stream of N_2, replaced with water (0.3 ml) and the heating continued for 24 h. The solution was diluted with ethanol (5 ml) and the solid collected by centrifugation, washed once by resuspension in ethanol, collected and dried to give the potassium salt of FCryp-2 (8 mg); m.p. 210–220 °C (d); $M+1$, 948 (f.a.b., glycerol/HCl).

Fluorescence titrations

The ionic titrations of FCryp-2 fluorescence were performed on solutions containing 10 μM of FCryp-2, 100 mM-KCl, 20 mM-KH_2PO_4/K_2HPO_4, pH adjusted to between 7.0 and 7.2 at 37 °C as stated, using a Perkin–Elmer 44E spectrofluorimeter in the ratio mode with excitation and emission bandwidths set at 2 nm at the wavelengths indicated.

RESULTS AND DISCUSSION

The reaction pathway from compound X to FCryp-2

The reaction sequence producing the tetraethyl ester of FCryp-2 from **X** is complex (Fig. 2), and generates several intermediates. In the reaction enolate anions are formed most readily from the acetyl substituents on **X**,

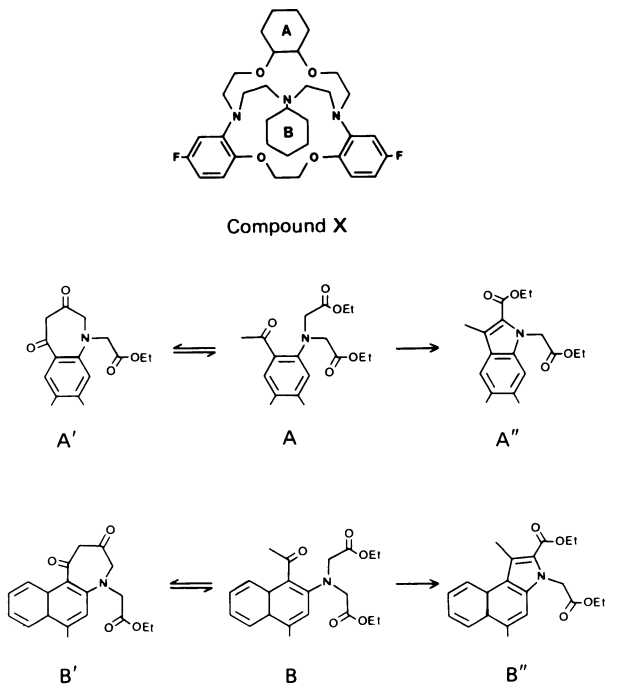


Fig. 2. The reaction pathway from compound X (structure A,B) to the tetraethyl ester of FCryp-2 (A″,B″)

The reactions A to A′ and B to B′ occur rapidly and reversibly. The parallel reactions A to A″ and B to B″ are much slower, but B to B″ is faster than A to A″.

which displace ethoxide from the adjacent ethyl ester groups to form the seven-membered cyclic diketones, A′ and B′ (Fig. 2) when buffered with malonate. These initial reactions are reversible (to A and B) and slower, parallel reactions of cyclization with dehydration of the alternative carboxyl enolates give the two indole moieties, A″ and B″. Given the complexity of the reaction and the need to confirm the structure of the end-product (the tetraethyl ester of FCryp-2), two intermediates between the starting material **X** (structure A,B) and the end product (A″,B″) were isolated and shown by n.m.r. and mass spectroscopy to have the structures A′B′ (formed first) and A′B″. The initial cyclizations to generate the first isolated intermediate, A′B′, were too rapid to permit detection of the intermediates with the structures AB′ and A′B. However the subsequent much slower formations of the two indole structures differed sufficiently in rate to allow the isolation of A′B″. Thus the analysis of the FCryp-2 structure was fully supported by the structural determinations of the intermediates.

An interesting feature of the ^{19}F-n.m.r. spectra of FCryp-2 tetraethyl ester in chloroform was the reversible changes which occurred on raising the temperature. At 25 °C the spectrum consisted of six peaks (four minor components of equal intensity and two major peaks of equal intensity) which converged to a partially resolved doublet at 45 °C and merged to a broad singlet at 52 °C. The spectra at 25 °C therefore represent conformers of the FCryp-2 tetraethyl ester with exchange rates in chloroform in the range 10^2–10^3 s^{-1} at 52 °C.

Properties of FCryp-2

FCryp-2 is the first of the many cryptands synthesized in the evolution of a prototype fluorescent Na^+ indicator

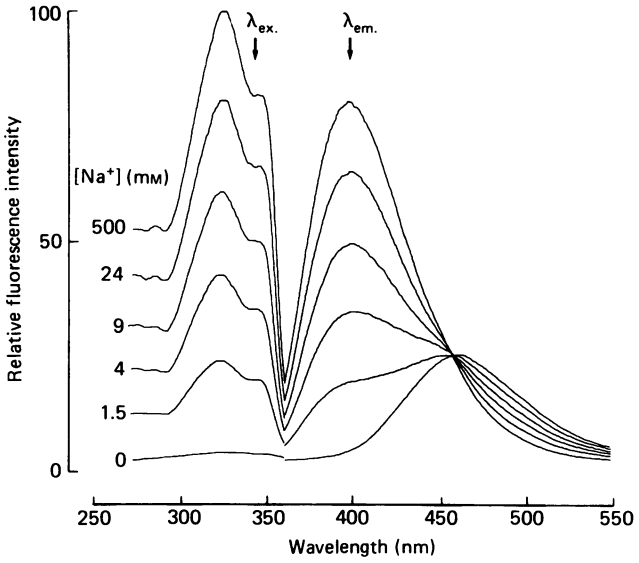


Fig. 3. Na^+ titrations of the fluorescence excitation and emission spectra of FCryp-2 (10 μM) in 100 mM-KCl/20 mM-KH_2PO_4/K_2HPO_4, pH 7.0 at 37 °C

The excitation and emission wavelengths were 340 nm and 395 nm respectively.

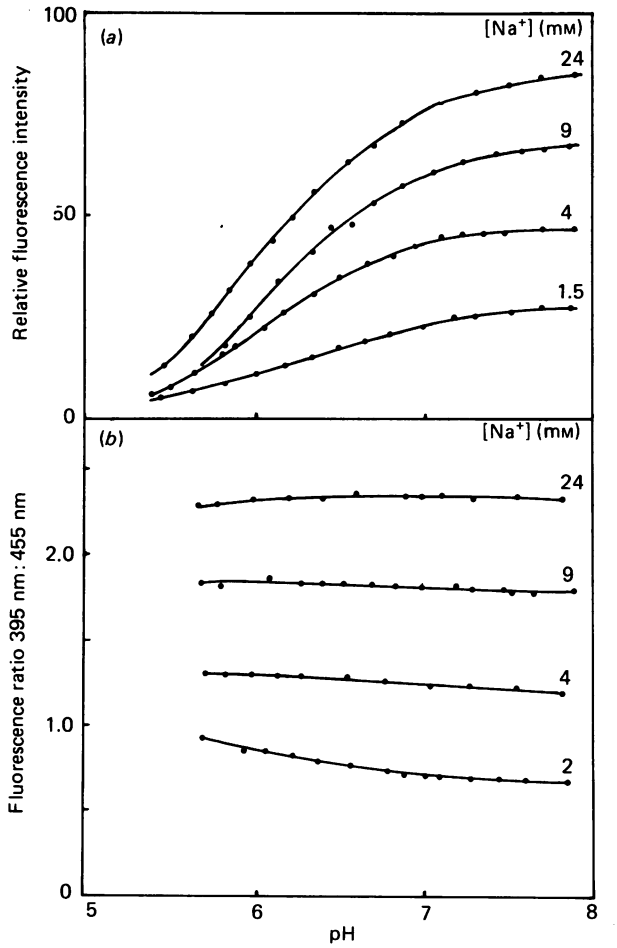


Fig. 4. pH titrations of FCryp-2 fluorescence at 37 °C with excitation at 340 nm

FCryp-2 (20 μM) in 100 mM-KCl/20 mM-KH_2PO_4/K_2HPO_4 with added NaCl as indicated. The pH was adjusted with 12 M-HCl and 5 M-KOH. (*a*) Fluorescence intensity at 395 nm; (*b*) ratio of fluorescence intensities at 395 nm to 455 nm.

that has been crystallized. The pure compound is stable in aqueous solutions and soluble to more than 10 mM.

The fluorescence excitation and emission spectra of FCryp-2 are shown in Fig. 3. With excitation at the inflexion at 340 nm, the FCryp-2 emission maximum shifted from 460 nm to 395 nm with an isosbestic point at 455 nm as the Na^+ concentration was increased from zero to 500 mM. The absorption coefficient of FCryp-2 at 340 nm was 5.0×10^3 $M^{-1} \cdot cm^{-1}$ in the absence of Na^+ and 5.7×10^3 $M^{-1} \cdot cm^{-1}$ with 200 mM-Na^+ in the medium with a quantum yield of > 0.7. The fluorescence intensity of FCryp-2 at 395 nm increased 25-fold on binding Na^+. With excitation of FCryp-2 or quin 2 at 340 nm the uncorrected fluorescence intensity at 395 nm of FCryp-2 saturated with Na^+ was at least 20-fold greater than the fluorescence of quin 2 saturated with Ca^{2+} at the 492 nm emission maximum [1]. FCryp-2 therefore provides adequate fluorescence intensity, comparable with the Ca^{2+} indicator fura [19], for studies on cell populations in a spectrofluorimeter or by microscopic photometry of single cells [20] which do not autofluoresce strongly at 395–450 nm (e.g. lymphocytes and fibroblasts).

The dissociation constant of FCryp-2 for Na^+ was estimated as 6.0 ± 0.1 mM from a plot of log(bound [FCryp-2]/free [FCryp-2]) against log(free [Na^+]) which had a slope of 1.02 ± 0.06, indicating a single homogeneous binding site for Na^+ per FCryp-2 molecule. The affinity of FCryp-2 for K^+ was too low to be determined accurately by fluorescence titration and the dissociation constant for K^+ was estimated to be greater than 10 M. K^+ will not therefore interfere with measurement of $[Na^+]_i$. The fluorescence intensity of FCryp-2 was unaffected by Ca^{2+} or Mg^{2+} concentrations up to 1 mM and neither of these ions will affect the use of FCryp-2 at their normal intracellular concentrations. The fluorescence intensity of FCryp-2 at 395 nm with 9 mM-Na^+ in the medium increased by 2.0% between pH 7.0 and 7.1 (Fig. 4*a*). However the ratio of fluorescence intensities at 395 nm and 355 nm was insensitive to pH over the normal intracellular range from 6.8 to 7.4 (Fig. 4*b*). It should be noted that both the apparent dissociation constant for Na^+ and the pH titrations of FCryp-2 were affected by the major cation in the medium. The apparent dissociation constant for Na^+ was increased to 10.1 mM in 100 mM-tetramethylammonium/20 mM-KH_2PO_4/K_2HPO_4, pH 7.0 at 37 °C. Changes in K^+ concentration from 100 to 200 mM or in the anion did not affect the fluorescence properties of FCryp-2 significantly. The data presented were therefore obtained in a K^+ medium appropriate for measurement of $[Na^+]_i$.

The above data imply that the FCryp-2 indicator can be used to measure $[Na^+]_i$ by calibrating the fluorescence intensity of the indicator at 395 nm by lysis of the cells at the end of the experiment [21] and making a small correction for any change in intracellular pH_i during the experiment. Alternatively, measurement of the ratio of fluorescence intensities of the indicator at 395 nm and 450 nm provides direct measurement of $[Na^+]_i$ which is insensitive to pH_i changes and does not require calibration at the end of the experiment.

The overall properties of FCryp-2 represent a promising prototype for fluorescent $[Na^+]_i$ indicators. The compound did not permeate into mouse thymocytes or 3T3 fibroblasts over several hours and is therefore very likely to remain trapped in the cytosol when introduced into cells. The structure of FCryp-2 facilitates adjustment of the dissociation constant for Na^+ without affecting the selectivity for Na^+ over K^+. For example, moving the fluorine atoms from the 1 position in FCryp-2 to the 6 position on each ring (as in the n.m.r. indicator FCryp-1 [18]) decreased the dissociation constant for Na^+ to 3.0 mM without any detectable effect on K^+ binding and caused only small changes in the fluorescence spectra compared with FCryp-2. Analogous changes in Ca^{2+} binding to the *n*FBAPTA series were found in a previous study [5] in which moving the two ^{19}F atoms from the position *para* to the oxygen to *para* to the nitrogen (i.e. from 4 to 5FBAPTA) resulted in a 4-fold increase in binding affinity for Ca^{2+}. Based on studies of model compounds, it is very likely that adjustment of the dissociation constant to higher values, closer to the $[Na^+]_i$ value of 13.8 mM determined for pig lymphocytes by FCryp-1, can be achieved by further manipulation of the F atom substitution pattern in the rings. Synthesis of a range of naphthyl analogues of compound **X** has established that the fluorophor at ring **B** (Fig. 2) can be varied without adverse effect on the tailored properties of the parent FCryp structure. We therefore expect to be able to design other new $[Na^+]_i$ indicators operating at longer wavelengths.

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