Antipyrylazo III, a "Middle Range" Ca^{2+} Metallochromic Indicator†

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ABSTRACT: Antipyrylazo III [bis(4-antipyrylazo)-4,5-dihydroxy-2,7-naphthalenedisulfonic acid] is a metallochromic indicator which can be used for selective and sensitive kinetic measurements of ionized Ca^{2+} in biological systems. The presence of Ca^{2+} induces a marked increase in absorbance of antipyrylazo III in the spectral area between 660 and 800 nm. At the same wavelengths, Mg^{2+} and Mn^{2+} are without effect. This allows for selection of wavelength pairs (720–790, 675–690 nm) where Ca^{2+} transients can be measured in cells or cell fractions without interference from Mg^{2+}. In a buffered reaction mixture at pH 7 containing 100 mM KCl, the antipyrylazo III–calcium complex has a $\Delta_\epsilon = \sim 7 \times 10^3$ (cm mol)$^{-1}$, a K_D of 160 μM, and a relaxation time, τ, of 180 μs. Antipyrylazo III, even at large concentrations, did not bind to cells or subcellular organelles. Antipyrylazo III was tested for and found to be without side effects on a variety of cellular functions. The affinity of antipyrylazo III toward Ca^{2+}, and therefore the range of Ca^{2+} concentrations at which antipyrylazo III is most effective, is between that of arsenazo III and murexide, the two most often used metallochromic Ca^{2+} indicators. Kinetic measurements of Ca^{2+} transport by isolated mitochondria, sarcoplasmic reticulum, and chromaffin vesicles obtained by recording the absorbance changes of antipyrylazo III by dual-wavelength spectrophotometry are shown. Measurements of ionized Ca^{2+} in the cytosol of a single cell by detection of the differential absorbance changes of antipyrylazo III in situ are also illustrated. If properly used, antipyrylazo III is a "middle range" Ca^{2+} indicator which permits measurements of Ca^{2+} binding and transport across cells or subcellular organelles in Ca^{2+} concentration ranges where other known metallochromic indicators are less effective. In addition, antipyrylazo III presents some interesting features with respect to selectivity, response time, and spectral area of absorption, so as to indicate a wider applicability to Ca^{2+} measurements in biological systems.

Sensitive kinetic measurements of Ca^{2+} binding and transport in cells and cell fractions are central not only to the elucidation of basic mechanisms of Ca^{2+} transport in various systems but also to the understanding of the control of cellular events such as contraction, excitation, secretion, fusion, vision, and hormone interaction.

Ca^{2+} distribution and atomic absorption spectrophotometry are the most routinely used techniques for measuring Ca^{2+} binding and transport in the presence of biological material. Both techniques, however, measure total—and not ionized Ca^{2+} and require the separation of the biological material by centrifugation or filtration, a time-consuming process which does not allow for fast kinetic measurements of Ca^{2+} binding and transport.

Quantitative kinetics of Ca^{2+} transients in cells and cell fractions have recently been measured with Ca^{2+} electrodes, photoluminescent indicators, and metallochromic indicators. All of these techniques, however, have intrinsic advantages or disadvantages with respect to selectivity, sensitivity, time resolution, interference, and applicability. These are discussed elsewhere (Scarpa, 1978).

Metallochromic indicators for Ca^{2+} have become increasingly popular during the last 5 years and are now a common tool in several laboratories for measuring Ca^{2+} binding and transport in cells and subcellular organelles. More recently, Ca^{2+} metallochromic indicators have been successfully used for measuring ionized Ca^{2+} concentrations in single cells and the kinetic changes during various metabolic, electric, and contractile events.

The two most commonly used indicators of Ca^{2+} transport in biological systems are ammonium purpurate (murexide) and 2,2′-(1,8-dihydroxy-3,6-disulfonaphthalene-2,7-bisazo)bis-(benzenearsonic acid) (arsenazo III). Murexide has been recognized for a long time as a Ca^{2+} indicator (Gysling and Schwarzenbach, 1949) and has been applied to the measurements of Ca^{2+} transport in isolated sarcoplasmic reticulum (Ohnishi and Ebashi, 1964), mitochrondria (Mela and Chance, 1968), and cell suspension (Scarpa, 1972). As a Ca^{2+} indicator, murexide has the advantage of selectivity and fast response, but its use is limited by the low sensitivity toward Ca^{2+} and by the fact that the Ca^{2+}-induced spectral changes occur in an area of the spectrum where other cellular pigments usually absorb.

Arsenazo III has only recently been recognized as a Ca^{2+} indicator (Michaylova and Ilkova, 1971) and has been applied to the measurements of Ca^{2+} transport in isolated mitochondria (Scarpa, 1974), sarcoplasmic reticulum (Chiu and Haynes, 1977), and chromaffin vesicles (Johnson and Scarpa, 1976), and also for measurements of ionized Ca^{2+} within single cells (DiPolo et al., 1976; Miledi et al., 1977; Thomas and Gorman, 1977; Brown and Pinto, 1978). The major advantage of the use of arsenazo III is its high affinity toward Ca^{2+}, which results in large absorbance changes (Δ_A) following relatively small Ca^{2+} concentration changes, making possible measurements of Ca^{2+} in the nM range. On the other hand, the indicator has relatively poor selectivity and slow rate of complex formation with Ca^{2+} (Scarpa, 1978). Furthermore, the high affinity toward Ca^{2+} is undesirable, because arsenazo III becomes a significant Ca^{2+} buffer in the system, and, at relatively high Ca^{2+} concentrations (>10 μM), the absorbance changes are no longer linear functions of $\Delta_{Ca^{2+}}$ concentrations.

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Bis(4-antipyrylazo)-4,5-dihydroxy-2,7-naphthalenedisulfonic acid (antipyrylazo III) has recently been used for complexometric determination of Ca^{2+} in aqueous systems at high pH (Budesinsky, 1974) but to date has never been used as a metallochromic Ca^{2+} indicator in biological systems. This report describes the characteristics of the antipyrylazo III–calcium complex and, through various examples, shows the potential of this indicator for selective and sensitive kinetic measurements of Ca^{2+} transport in single cells and isolated subcellular organelles. Antipyrylazo III is a "middle range" Ca^{2+} indicator which can be used for measuring, with adequate sensitivity, Ca^{2+} binding and transport in the Ca^{2+} concentration area where arsenazo III and murexide are less effective. In addition, antipyrylazo III has important features of selectivity and response time, so as to promise the widest applicability for measuring Ca^{2+} transients in the presence of biological systems.

Experimental Procedures

Methods

Preparation of Cell Fractions. Rat liver mitochondria were isolated by homogenization and differential centrifugations in 250 mM sucrose and 0.2 mM ethylenediaminetetraacetic acid (EDTA[1]), as described previously (Vinogradov and Scarpa, 1973). EDTA was omitted from the medium used for the last washing and for the final resuspension of mitochondria. Sarcoplasmic reticulum was prepared from white muscle of rabbit hind leg, as described previously (Inesi and Scarpa, 1972). Red cell "ghosts" were prepared from human erythrocytes according to the procedure of Passow (1969). Chromaffin vesicles were isolated from bovine adrenal medulla and purified in a sucrose–Ficoll–D_2O gradient as described previously (Johnson and Scarpa, 1976).

Assay of Functions of Cell Fractions. Respiratory control ratios of rat liver mitochondria were measured in a medium containing 0.25 M sucrose, 15 mM morpholinepropanesulfonic acid (Mops, pH 7.4), 10 mM K_2HPO_4, and 2.2 mg of mitochondrial protein. Sodium glutamate (8 mM) and sodium malate were added to induce state 4 respiration and then 280 μM sodium adenosine 5′-diphosphate (ADP) was added to induce state 3. Oxygen consumption was measured polarographically with a Clark-type electrode in a chamber thermostated at 24 °C and equipped with a stirrer. The oxygen consumption was monitored with a potentiometric recorder. Respiratory control ratios were calculated according to Chance (1959) as the ratio of the rate of respiration in the presence of added ADP and the rate obtained upon ADP expenditure. Sodium adenosine 5′–5′-triphosphate (ATP) dependent Ca^{2+} uptake and ATPase of sarcoplasmic reticulum were measured as described previously (Scarpa et al., 1972). (Na^+, K^+)-dependent ATPase in erythrocyte ghosts was determined by measuring P_i liberated, as described by Sen and Post (1964) in a reaction mixture containing 2 mM Tris–ATP, 100 mM Tris-HCl (pH 7.5), 4 mM $MgCl_2$, 20 mM KCl, and 100 mM NaCl. The (Na,K)ATPase was calculated by subtracting the total ATPase activity in the absence of Na^+ and K^+ plus 10^{-3} M ouabain (Mg-ATPase).

Spectrophotometric Measurements. Dual-wavelength measurements and absolute and differential absorption spectra of antipyrylazo III were obtained with a spectrophotometer designed and built at the Johnson Foundation, University of Pennsylvania. This instrument can be used in the split-beam mode (with two cuvettes and one scanning monochromator) or in dual-wavelength mode (with two monochromators). In either case, it is equipped with a 200-Hz light splitter-chopper, two Bausch and Lomb monochromators with an effective bandwidth of 0.9 nm, used with a 1-nm bandwidth and 1-cm light path. In the scanning mode, the baseline was automatically corrected and clamped by a microprocessor circuitry.

Fast Kinetic Measurements. Temperature-jump measurements were performed using an instrument (built by Messanlagen, Göttingen) similar to that described by Eigen and De Maeyer (1963). A 1-mL reaction mixture was kept at thermal equilibrium at 20 °C and then perturbed in a few microseconds by a 3.5 °C temperature increase obtained through the discharge of a high-voltage capacitor. The changes in absorbance were measured at the indicated wavelengths with a 7-mm light path.

Stopped-flow measurements were obtained by mixing reaction mixtures with the aid of a stopped-flow apparatus designed by Chance (1973) and built in this department. This apparatus mixes reactants with a ratio of 1:160 in less than 1 ms and has an observation chamber with 1-cm path length. Absorbance changes after the mixing and flow velocity were displayed in a storage oscilloscope, as described previously (Inesi and Scarpa, 1972).

Materials

Antipyrylazo III was purchased from K and K, Rare Chemicals Co., Plainview, N.Y., and recrystallized twice in 40% ethanol in water at 60 °C. Chromatography of the indicator results in a single spot if analyzed according to Budesinsky (1974) and in two spots if analyzed according to Kendrik (1976). In the latter case, the contaminant consisted of approximately 15% by densitometric measurements. It was found to have a slightly higher R_f than antipyrylazo III, no toxic effect on the cell function and properties described, and no change in the color following Ca^{2+} addition. Ca^{2+} and Mg^{2+} content was measured by flame atomic absorbance and was found to be 0.07% mole fraction contamination of either ion.

Carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP) was a gift from Dr. P. G. Heytler of DuPont and Co., Wilmington, Del., and A23187 from Dr. R. Hamill, Eli Lilly and Co., Indianapolis, Ind. Sodium adenosine 5′–5′-triphosphate (ATP); sodium adenosine 5′-diphosphate (ADP), grade 1; morpholinepropanesulfonic acid (Mops), and tris(hydroxymethyl)aminomethane were purchased from Sigma.

Results

General Spectral Properties. Figure 1 shows the absorbance spectra of antipyrylazo III in the presence and in the absence of Mg^{2+} and Ca^{2+}. The addition of Ca^{2+} (Figure 1B) induces a large spectral change of antipyrylazo III, consisting of a decrease in absorbance with a λ_{min} at 546 nm and an increase in absorbance with a λ_{max} at 712 nm, with an isosbestic point at 613 nm. Additional spectral shifts are seen in the blue area of the spectrum. The presence of Mg^{2+} induces spectral changes of antipyrylazo III which are significantly different from those produced by Ca^{2+} (Figure 1A). Of particular interest for selective Ca^{2+} measurements is the area of the spectrum above 680 nm, where Ca^{2+}—but not Mg^{2+}—produces large absorbance changes of antipyrylazo III.

Figure 2 shows the differential spectra of antipyrylazo III

[1] Abbreviations used are: Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*′-tetraacetic acid; Tris, tris(hydroxymethyl)aminomethane; ATPase, adenosine triphosphatase.

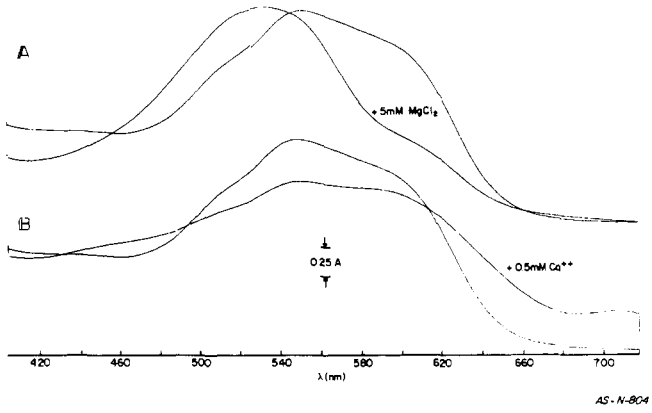


FIGURE 1: Absolute absorbance spectra of antipyrylazo III in the presence of Ca^{2+} and Mg^{2+}. The spectra of the dye in the absence or presence of Ca^{2+} or Mg^{2+} were obtained in the split-beam spectrophotometer described under Methods. The measuring cuvette contained 50 μM antipyrylazo III, 0.05 M KCl, and 20 mM Hepes (pH 7.0), and, where indicated, the concentrations of $CaCl_2$ or $MgCl_2$. The reference cuvette contained the same solution and indicated concentrations of $CaCl_2$ or $MgCl_2$ without the dye. The temperature was 24 °C.

TABLE I: Effect of H^+ and Mg^{2+} on the Dissociation Constant and Extinction Coefficient of the Antipyrylazo III-Antipyrylazo Ca^{2+} Complex.[a]

A.	pH	6.1		6.8	7.5
	K_D (μM)	313		200	140
	Δ_ϵ (mM^{-1} cm^{-1})	9.5		7.4	3.4
B.	[Mg] (mM)	0	1	2.5	5
	K_D (μM)	214	273	335	560
	Δ_ϵ (mM^{-1} cm^{-1})	7.2	6.7	5.9	5.3

[a] The reaction mixture contained 100 mM KCl, 10 mM Tris-maleate, and 50 μM antipyrylazo III. In A, the buffer was adjusted at the pH indicated in the table with additions of KOH. In B, the pH of the buffer was 6.8 and the reaction mixture was supplemented with the concentration of Mg^{2+} indicated in the table.

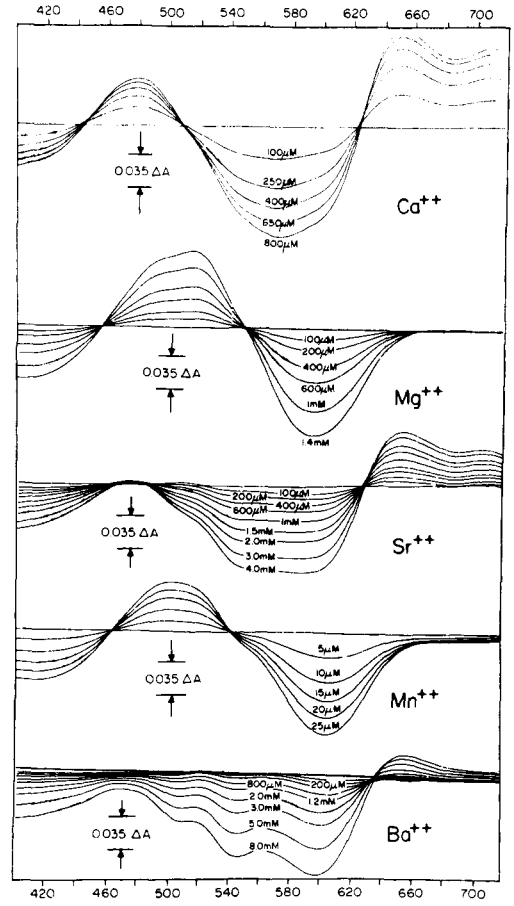


FIGURE 2: Differential absorbance spectra of antipyrylazo III in the presence of various divalent ions. Both the measuring and the reference cuvettes contained 50 μM antipyrylazo III, 0.5 M KCl, and 20 mM Hepes (pH 7.0). Divalent ions were added as chloride salts [Ba^{2+} as $Ba(NO_3)_2$] to the measuring cuvette in the indicated concentrations, and the differential absorption was monitored with split-beam spectrophotometry. The temperature was 25 °C.

vs. antipyrylazo III plus various concentrations of Ca^{2+}, Mg^{2+}, Sr^{2+}, Mn^{2+}, and Ba^{2+}. These differential spectra were obtained in a reaction mixture of high ionic strength, and this results in a slight shift in the λ_{max} and λ_{min} of absorbance and isosbestic points with respect to Figure 1. It is evident from the figure that Ca^{2+} produces absorbance changes in the red area of the spectrum which are qualitatively different from those produced by Mg^{2+}, Mn^{2+}, and Ba^{2+}. Only Sr^{2+} induces spectral changes similar to that of Ca^{2+} but at larger concentrations. Therefore, the intrinsic spectral properties of the antipyrylazo III-calcium complex above 660 nm make possible the use of antipyrylazo III as a selective indicator for free Ca^{2+} concentrations without interference from Mg^{2+} and other divalent cations, which are often present in and transported by cells and cell fractions. For instance, in a dual-wavelength spectrophotometer, only Ca^{2+}—and not Mg^{2+}—produces absorbance changes at 720–790 nm (see also Figure 5).

Similarly to what has been previously reported for other Ca^{2+} indicators (DiPolo et al., 1976), the Δ_ϵ and dissociation constant of the antipyrylazo III-antipyrylazo III plus calcium complex are also affected by the ionic strength of the solution in which antipyrylazo III is dissolved. Figure 3 shows that by raising the ionic strength of the reaction mixture from 0.05 to 0.5 M KCl the dissociation constant of the antipyrylazo III-antipyrylazo III plus calcium complex increases from 95 to 380 μM. The affinity of antipyrylazo III for Ca^{2+} lies just between that of the two most commonly used Ca^{2+} indicators, murexide and arsenazo III.

The dissociation constant of the antipyrylazo III-antipyrylazo III plus calcium complex is also affected by the presence of Mg^{2+} and H^+. Table I shows that an increase in the dissociation constant is produced by increasing H^+ and Mg^{2+} concentrations in the reaction mixture. The dependence of the spectra upon pH occurs with other metallochromic indicators, since they function simultaneously as metal indicators and as acid-base indicators, and consequently the stability constants not only of the metal ion complexes but also of the proton complexes must be considered. Therefore, an effective buffer is needed in the reaction mixture when Ca^{2+} is measured through absorbance changes of antipyrylazo III or other metallochromic indicators. In addition, the changes in Δ_ϵ and K_D which occur under various reaction mixtures stress the need for an internal calibration rather than an absolute calibration of the absorbance changes in terms of Ca^{2+} changes, as will be discussed later.

Suitability for Biological Experiments. Although antipyrylazo III can be used successfully as a sensitive and selective indicator of free Ca^{2+} concentration in buffered solutions, its use for measuring Ca^{2+} binding and/or transport in cells and cell fractions requires at least two additional properties of the dye. Antipyrylazo III should neither bind to nor have side effects on the structure and function of the biological system under study.

The lack of binding of antipyrylazo III to cells or cell fractions was investigated through experiments similar to those

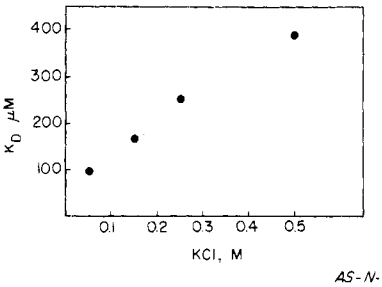


FIGURE 3: Effect of ionic strength on the dissociation constant of Ca-antipyrylazo III. Reference and measuring cuvettes contained 50 μM antipyrylazo III, the indicated concentration of KCl, and 10 mM Hepes (pH 6.8). Ca^{2+} was added in increasing concentrations to the measuring cuvettes and the differences in absorbance (Δ_A) at 675 nm were recorded. The reciprocal of Δ_A was plotted vs. the reciprocal Ca^{2+} concentration for each condition of ionic strength. These plots yielded linear slopes from which the K_D values were calculated.

reported in Figure 4A. Antipyrylazo III at concentrations ranging from 25 μM to 1 mM was added to buffered solutions containing concentrated suspensions of rat liver mitochondria. After a 10-min incubation, the mitochondria were separated from the suspending reaction mixture containing antipyrylazo III by centrifugation, and the supernatant was analyzed for antipyrylazo III content at 613 nm, an isosbestic point for antipyrylazo III *plus* calcium.

A straight line was obtained by plotting the absorbance vs. antipyrylazo III concentration, similar to that obtained by plotting the absorbance of antipyrylazo III in an identical reaction mixture without mitochondria. Identical results were obtained in the presence or absence of Ca^{2+} in the reaction mixture. These data and others obtained with red cell suspensions indicate that antipyrylazo III has nondetectable binding to cell membranes and that when added to a suspension of cells or cell fractions antipyrylazo III remains in the solution external to cells and cell fractions.

Figure 4B shows that antipyrylazo III is without effect on a variety of functions of subcellular organelles such as Ca^{2+}-dependent ATPase of skeletal muscle sarcoplasmic reticulum, respiratory control ratio of rat liver mitochondria, and (Na^+,K^+)ATPase of red cell membranes. The lack of effect on these and other functions was observed even at 1 mM, a concentration of indicator 10–30 times greater than that required for its use as a Ca^{2+} indicator for studies with subcellular organelles. Also, if microinjected at a concentration of 1.2 mM into the axoplasm of giant squid axons, antipyrylazo III had no detectable effect on metabolic and electrical properties of the fiber (not shown).

The time scale of the dissociation of the antipyrylazo III-calcium complex was investigated by a temperature perturbation technique, as described under Methods. A buffered solution containing 50 μM antipyrylazo III and 70 μM Ca^{2+}, 50 mM KCl, and 20 mM Hepes buffer (pH 7) was kept in thermal equilibrium and then perturbed by a rapid increase in temperature within a few microseconds. The time course of the concentration change of the species [antipyrylazo III] and [antipyrylazo III-calcium] to a new equilibrium at higher temperature was measured at 550 or at 650 nm, where the temperature-dependent decrease in the antipyrylazo III-calcium species in the reaction mixture results in an increase or decrease in absorbance, respectively (not shown). The rate of decay of [antipyrylazo III] − [antipyrylazo III-calcium] is characterized by a linear first-order differential equation, and the solution is a linear combination of exponentials. Each exponential term is associated with a relaxation time, τ, which

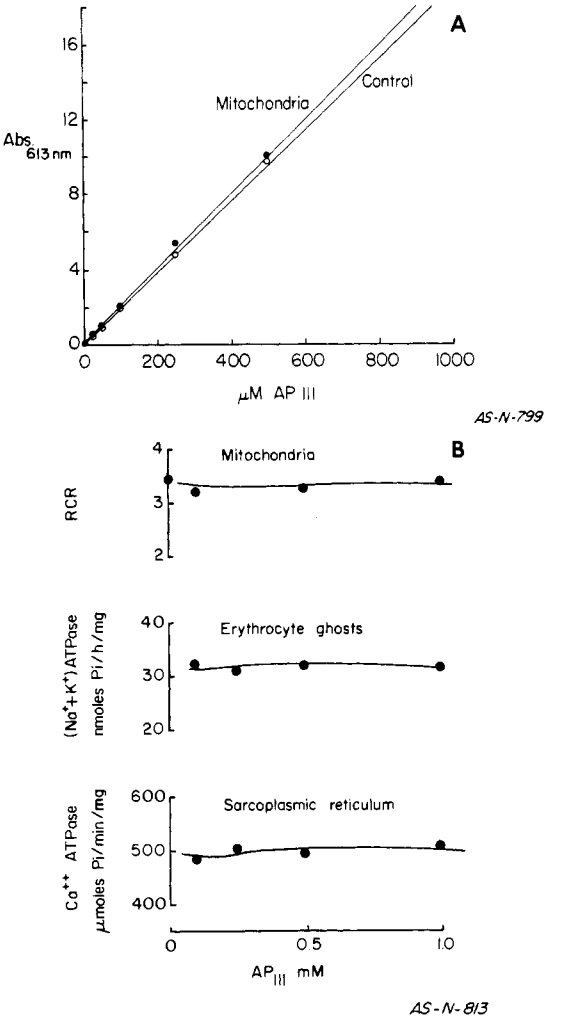


FIGURE 4: (A) A plot of the absorbance at 613 nm vs. the concentration of antipyrylazo III after incubation without (control) or with rat liver mitochondria. The reaction mixture contained 225 mM mannitol, 75 mM sucrose, 0.5 mM EDTA, 5 mM Mops (pH 7.0), and the indicated concentration of dye. (B) Effect of various concentrations of antipyrylazo III on the respiratory control ratio of rat liver mitochondria, (Na^+/K^+)-ATPase activity of erythrocyte ghosts, and Ca^{2+}-ATPase activity of fragmented sarcoplasmic reticulum. Preparation of cellular fractions, assay techniques, and reaction mixtures have been described under Methods.

may be considered as the reciprocal first-order rate constant. Under the above conditions, τ had a value of 180 μs, which indicates that the dissociation of the complex between Ca^{2+} and antipyrylazo III is not rate limiting for measurements of Ca^{2+} transport or binding in the millisecond time ranges. Under similar experimental conditions, the relaxation rates of the antipyrylazo III-calcium complex are one to two orders of magnitude slower than those of several pH indicators or murexide but are over ten times faster than those of arsenazo III (Geier, 1968; Scarpa, 1978).

Kinetic Measurements of Ca^{2+} in Cells and Cell Fractions. Several applications of antipyrylazo III to kinetic measurements of Ca^{2+} transients in cells and cell fractions are illustrated in Figures 5–8. Figure 5 shows the results of an experiment in which the energy-dependent Ca^{2+} transport by rat liver mitochondria was continuously recorded as the changes undergone by antipyrylazo III in the reaction mixture. The addition of Ca^{2+} to the reaction mixture causes an increase in Δ_A at 720–790 nm due to the formation of the calcium-antipyrylazo III complex, which absorbs more light than antipyrylazo III alone. This is followed by a time-resolvable ab-

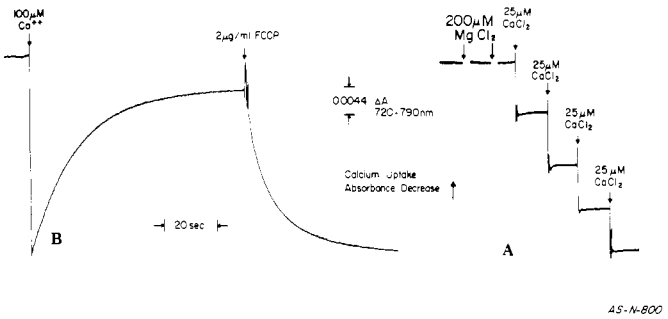


FIGURE 5: Kinetics of Ca^{2+} uptake and release by rat liver mitochondria as monitored by differential absorbance changes of antipyrylazo III. (A) The reaction mixture contained 50 μM antipyrylazo III, 250 mM sucrose, 2 mM $MgCl_2$, 10 mM Mops (pH 7.0), 2 mM succinate, 6 μM rotenone, and 1.1 mg of protein/mL of rat liver mitochondria. The reaction was started by the addition of 100 μM $CaCl_2$, and differential absorbance was recorded with a rotating-wheel spectrophotometer equipped with 720- and 790-nm filters. FCCP was added at the indicated time to cause release of the transported Ca^{2+}. (B) The reaction mixture was similar to that described above but did not contain 2 mM succinate; 2 μg/mL FCCP was added before the calibrating aliquots of Mg^{2+} and Ca^{2+} were added. The temperature was 22 °C.

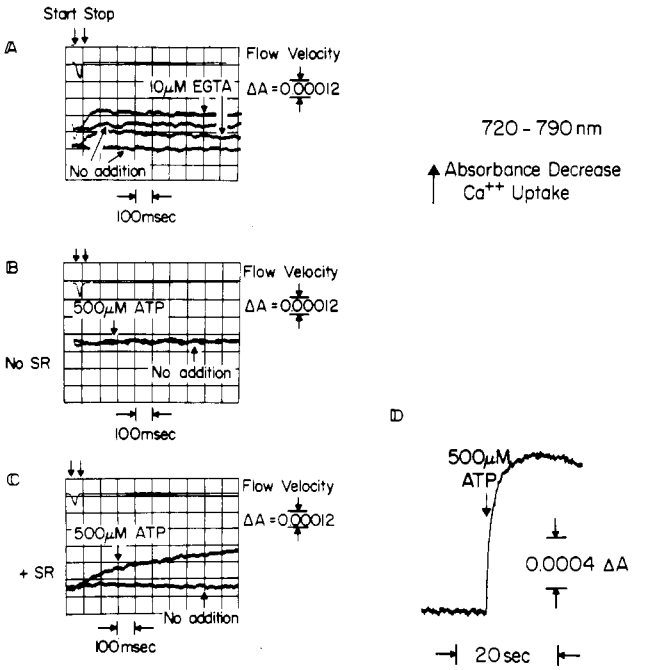


FIGURE 6: Kinetics of Ca^{2+} transport by fragmented sarcoplasmic reticulum as monitored by rapid-flow measurements of antipyrylazo III absorbance changes at 720–790 nm. (A) The large syringe of the stopped-flow apparatus contained 50 μM antipyrylazo III, 50 mM KCl, 10 mM $MgCl_2$, 20 mM Tris–maleate (pH 6.8), and 50 μM $CaCl_2$; the small syringe (1:80 ratio) contained 0.8 mM EGTA (pH 6.8). After rapid mixing, the absorbance changes were measured with a rotating-wheel spectrophotometer at the 720–790-nm pair. (B) The large syringe contained the same reaction mixture; the small syringe contained 40 mM ATP. (C and D) The large syringe contents were supplemented with 0.3 mg of protein/mL of fragmented sarcoplasmic reticulum; the small syringe contained 40 mM ATP. "No addition" refers to discharge of the stopped-flow apparatus without additions of EGTA or ATP. The temperature was 22 °C.

sorbance decrease of antipyrylazo III due to the energy-dependent Ca^{2+} transport in mitochondria and the consequent disappearance of Ca^{2+} from the reaction mixture.

The addition of carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP), an uncoupler of oxidative phosphorylation, prompts a release of Ca^{2+} from mitochondria, and all of the Ca^{2+} previously accumulated is then released. The right trace shows the calibration of the absorbance signal obtained under identical experimental conditions, where the energy-dependent Ca^{2+} transport by mitochondria was prevented completely before Ca^{2+} addition by the presence of FCCP. The trace also shows that the addition of large concentrations of Mg^{2+} is virtually without effect in the differential absorbance of these wavelengths.

These measurements are qualitatively similar to those obtained with murexide (Mela and Chance, 1968; Scarpa and Graziotti, 1973) or arsenazo III (Scarpa, 1974; Crompton et al., 1976). However, antipyrylazo III has intrinsic advantages for the measurements of Ca^{2+} transport by mitochondria. With respect to murexide, antipyrylazo III has a better sensitivity and its Ca^{2+}-dependent absorbance changes can be measured in an area of the spectrum which is at a great distance from the absorbance of cytochromes and other endogenous pigments. On the other hand, the relatively high affinity of arsenazo III toward Ca^{2+} severely limits the use of arsenazo III as a Ca^{2+} indicator for studies of Ca^{2+} transport in mitochondria. Since relatively high Ca^{2+} concentrations (5–100 μM) are generally required in the reaction mixture for measuring mitochondrial Ca^{2+} transport, the presence of arsenazo III produces the undesirable effects of chelating a sizable fraction of Ca^{2+} in the reaction mixture and competing for the mitochondrial Ca^{2+} transport system, which has an apparent K_m similar to that of the arsenazo III–calcium dissociation constant.

Figure 6 shows an example of measurements of Ca^{2+} uptake by fragmented sarcoplasmic reticulum from rabbit skeletal muscle. The Ca^{2+} transport by sarcoplasmic reticulum in the absence of oxalate or other permeant anions is very fast and half of the reaction occurs during the manual mixing of sodium adenosine 5′–5′-triphosphate (ATP) into the cuvette containing the reaction mixture. Therefore, in order to follow kinetically the reaction, ATP was mixed into the reaction mixture with the aid of a stopped-flow apparatus as described previously (Inesi and Scarpa, 1972). The stopped-flow apparatus used for this experiment has a large observation chamber and mixes reagents within 3 ms with a ratio of 1:80, which minimizes dilution artifacts (Chance, 1973). Figure 6A shows the calibration of the absorbance changes in terms of Ca^{2+} concentrations obtained by fast mixing of known concentrations of ethylene glycol bis(β-aminoethyl ether)-*N*,*N*′-tetraacetic acid (EGTA) into the reaction mixture in the absence of sarcoplasmic reticulum. Figure 6B shows that the addition of ATP to the reaction mixture in the absence of sarcoplasmic reticulum does not produce detectable antipyrylazo III absorbance changes, since the formation of sizable concentrations of the ATP–calcium complex is effectively prevented by the presence of 10 mM Mg^{2+}. Figure 6C shows that the addition of ATP to the reaction mixture containing sarcoplasmic reticulum produces an antipyrylazo III absorbance decrease which is due to the disappearance of Ca^{2+} from the reaction mixture consequent to the transport of Ca^{2+} to the vesicles. Figure 6D shows the same experiment of Figure 6C on Ca^{2+} transport by sarcoplasmic reticulum displayed in a strip chart recorder at a longer time span. These results are qualitatively similar to those obtained previously by using murexide as a Ca^{2+} indicator (Inesi and Scarpa, 1972). In this case, however, the Δ_A changes are larger, and consequently the sensitivity of the measurements is largely enhanced.

Figure 7 shows the results of an experiment in which Ca^{2+} transport was measured in isolated bovine chromaffin vesicles using antipyrylazo III. Two 10 μM additions of Ca^{2+} to a

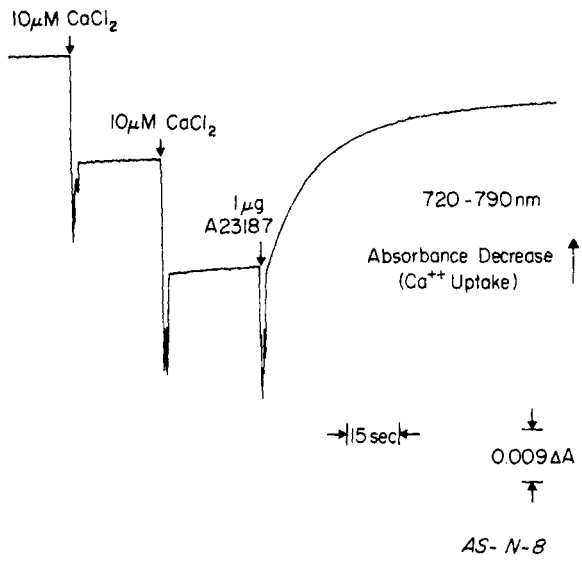


FIGURE 7: Kinetics of ionophore-induced Ca^{2+} uptake by isolated chromaffin granules as measured with antipyrylazo III. The reaction mixture contained 50 μM antipyrylazo III, 270 mM sucrose, 10 mM Tris-maleate (pH 6.9), and chromaffin granules (0.35 mg of protein/mL). Ca^{2+} (20 μM) was added, followed by 1 μg/mL of the divalent cation ionophore A23187. The Ca^{2+} uptake was monitored on the differential absorbance change using the 720–890-nm wavelength pair. The temperature was 22 °C.

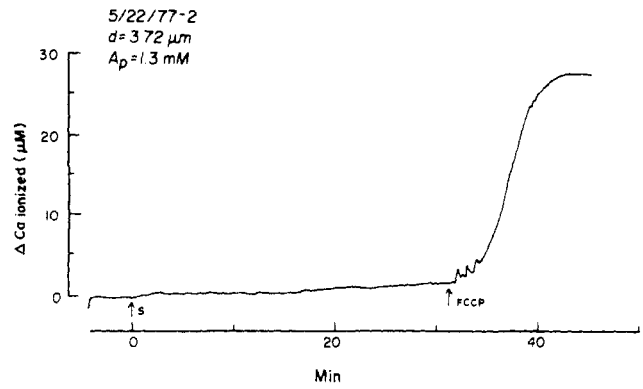


FIGURE 8: Ionized Ca^{2+} in the axoplasm of squid giant axon measured through the detection of the differential absorbance of antipyrylazo III in situ. The axon (372-μm diameter) was dissected, positioned in the measuring chamber, and microinjected with antipyrylazo III, which reached 1.3 mM after diffusional equilibration in the cell. The bathing solution was changed to sea water containing 112 mM $CaCl_2$, and the axon was electrically stimulated 100/s (see arrow S) continuously for the first 32 min. Two microliters of FCCP (2 mg/mL in dimethyl sulfoxide) was added at the top of the axon where indicated. The measurement wavelengths were 675–685 nm, and the temperature was 14 °C.

suspension of chromaffin vesicles produces similar increases in Δ_A at 720–790 nm. The Ca^{2+} added to the reaction mixture remains outside the vesicles during the time of observation until A23187, a divalent cation ionophore, is added. Similar experiments have been carried out with arsenazo III as a Ca^{2+} indicator (Johnson and Scarpa, 1976), but, due to the competition for Ca^{2+} between arsenazo III and the Ca^{2+} binding sites inside the vesicles, the time course of the uptake was distorted by the presence of arsenazo III. Such measurements are not possible with murexide, whose Ca^{2+}-dependent absorbance changes occur in the 400–600-nm area of the spectrum because of the large interference at these two wavelengths by the absorbance changes of adenochrome and other pigments.

Antipyrylazo III can also be successfully used for measuring ionized Ca^{2+} concentrations inside large cells. The details of the experimental setup for measurements of ionized Ca^{2+} and Mg^{2+} using different indicators have been previously described in great detail (Brinley and Scarpa, 1975; DiPolo et al., 1976). Briefly, large cells such as squid giant axons or barnacle muscle fibers are dissected, positioned in a suitable chamber, and microinjected with a known aliquot of metallochromic indicator. After several minutes, the indicator reaches diffusional equilibration in the cytosol, and its absorbance changes, which are functions of the free cation concentrations, are measured by sensitive multiwavelength microspectrophotometry.

Figure 8 shows the results of an experiment in which a freshly dissected squid giant axon was positioned in an observation chamber containing artificial sea water and microinjected with antipyrylazo III. The changes of antipyrylazo differential absorbance were measured in situ at 675–685 nm with a dual-wavelength spectrophotometer and suitable microoptical fibers (Brinley and Scarpa, 1974; Brinley et al., 1977). Following electrical stimulation of the fiber at a high level of extracellular Ca^{2+}, the concentration of ionized Ca^{2+} increases—but only slightly—during the final 30 min in spite of the large amount of Ca^{2+} entering the fiber, because of the intracellular buffering system. The addition of FCCP produced a dramatic increase in axoplasmic Ca^{2+}. This release was from internal axoplasmic stores (most likely mitochondria), because the extracellular bathing fluid had been changed to sea water containing zero concentrations of Ca^{2+} before the FCCP addition to prevent any possible Ca^{2+} entry across the axolemma. The calibration of the absorbance trace was carried out by replacing the axon with a glass capillary of identical internal diameter and flushing it with artificial axoplasm containing the same concentration of antipyrylazo III and various concentrations of $CaCl_2$, as described previously for other indicators (DiPolo et al., 1976; Brinley et al., 1977). Similar measurements have been obtained with arsenazo III, and, due to its higher affinity for Ca^{2+}, the changes in absorbance due to Ca^{2+} are much larger at the intracellular resting Ca^{2+} levels. On the other hand, the high affinity of arsenazo III toward Ca^{2+} limits the interpretation of the data obtained at higher Ca^{2+} concentrations in the cytosol (10–50 μM), since arsenazo III acts as a significant Ca^{2+} buffer in the system. Furthermore, under these conditions most of the arsenazo III exists as a Ca^{2+}-indicator complex, which results in a nonlinear calibration curve of Δ_A vs. ionized Ca^{2+}.

Discussion

Metallochromic Ca^{2+} indicators are substances which undergo large color changes when the concentration of ionized Ca^{2+} in the reaction mixture changes. Common to all of these indicators is the large difference in absorbance between the free indicator and the indicator-calcium complex, which can be used for quantitative measurements of free Ca^{2+} in solution. Based on either empirical observation or application of an increasing knowledge of coordination chemistry, the number of indicators for complexometric titration of Ca^{2+} is rapidly expanding (Budesinsky, 1969; Burger, 1973). In addition to murexide, which has long been known as a metallochromic indicator of Ca^{2+}, 150 compounds that had been evaluated for use as metal ion detectors in inorganic chemical analyses have been reviewed by Budesinsky (1969). To our knowledge, only three of these have been specifically evaluated for their potential as calcium indicators in biological work. These include arsenazo III (Vallieres et al., 1975; Brown et al., 1975; DiPolo et al., 1976), chlorophosphonazo III (Brown et al., 1975; Yoshikami and Hagins, 1978), and antipyrylazo III (present

study). Restrictions on analytical titrations of metals are quite different than for biological measurement of free calcium concentration. For example, in inorganic analyses, pH can vary widely but must be constant and near 7.0 for biological measurements; magnesium is the only commonly interfering cation in biological systems, whereas diverse metals can be contaminants in inorganic analyses. This great difference in the criteria for suitable indicators for a particular ion in the two types of work means that none of the metallochromic indicators listed in Budesinsky's review should be rejected a priori as being unsuitable for the special conditions of biological experiments. A careful survey of these dyes would probably identify one with special properties useful for some biological application in the measurement of free ion concentration.

In the discussion below we consider some criteria which we have found useful in evaluating Ca^{2+} metallochromic indicators for biological work.

(a) The indicator should be readily soluble in water and have minimal or no partition in hydrophobic solvents, which translates into lack of binding and/or penetration of the indicator into the biological membranes under examination. Since a major binding of the indicator to biological membranes usually results in noninterpretable data, it is highly desirable that the indicator remain in the medium surrounding cells and cell fractions (or in cytosol if injected into cells). Under these conditions, the change in absorbance can be related exclusively to Ca^{2+} concentration in the extravesicular solution.

(b) The indicator should have no side effects on cell structure and function.

(c) The indicator should be selective for Ca^{2+} or else the Ca^{2+}-indicator complex should have particular spectral properties different from that of other cations.

(d) The rate constant for the indicator–calcium complex formation and dissociation should be rapid and not rate limiting when fast Ca^{2+} transients are measured. This is one of the major assets of the Ca^{2+} metallochromic indicators, which show faster responses than other techniques, so that the time resolution of Ca^{2+} binding and transport in the micro- or millisecond ranges may be obtained.

(e) Suitable wavelengths should be available at which the Ca^{2+}-related absorbance of the indicator can be detected without interference from nonspecific absorbance changes. Undesirable nonspecific absorbance changes due to swelling or change of the refractive indexes of the membrane under examination occur in a broad area of the spectrum. These can be minimized or abolished with dual-wavelength spectroscopy by electronically subtracting the change of absorbance at the measured λ from the change in a nearby reference λ. Interference from absorbing cellular pigments can be avoided by measuring the absorbance of the indicator in an area of the spectrum where the endogenous pigments do not absorb, usually in the near-red or red area of the visible spectrum.

(f) The differential coefficient of absorbance (Δ_ϵ) between the free indicator and the indicator–calcium complex should be large.

(g) The affinity of the indicator toward Ca^{2+} should be commensurate with the pCa in the reaction mixture. Although the changes in absorbance to be measured are functions of the concentration of the indicator–calcium complex, it is preferable that the equilibrium indicator $\rightleftarrows$ indicator–calcium should be far toward the indicator in the free form. Under these conditions, the calibration curve shows a linearity between Δ_A and $\Delta_{Ca^{2+}}$ and little or no disturbance of the concentration of Ca^{2+} in solution due to the presence of the indicator. It follows that different Ca^{2+} indicators, depending on their affinity toward Ca^{2+}, would have a defined area of Ca^{2+} concentrations in which Ca^{2+} transients can be optimally recorded with respect to sensitivity, linearity of calibration, and minimal disturbance of ionized Ca^{2+}. This condition is similar to that of pH indicators which are more effective at specific pHs and stresses the need for a wide range of Ca^{2+} indicators with different Ca^{2+} affinity for the measurement of Ca^{2+} transients at various pCa.

Table II compares the characteristics of antipyrylazo III with those of murexide and arsenazo III. All three indicators neither bind to cell fractions nor affect cellular properties. There are significant differences in the structure and properties of the calcium complexes, which result in a different applicability of the three indicators for measuring Ca^{2+}, depending on the biological material under study and on the experimental conditions.

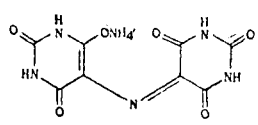
The selectivity for Ca^{2+} of these indicators is quite different. Major interference during Ca^{2+} measurements in biological systems may occur from Mg^{2+}, which is abundant in the cytosol and in cell fractions and often redistributes during various metabolic processes. In this respect, murexide is very selective, since no spectral changes due to Mg^{2+} are observed. Such is not the case with arsenazo III, since the presence of Mg^{2+} induces a major spectral change in the area of the spectrum where Ca^{2+} can be measured. The Mg^{2+} interference can be minimized—but not abolished—by appropriate selection of λ pairs of observation (675–685 nm), but this reduces the Δ_ϵ and therefore the sensitivity of the measurements. Antipyrylazo III is not intrinsically selective toward Ca^{2+} but can be rendered so by appropriate choice of experimental conditions. In the area of the spectrum between 680 and 800 nm, full selectivity is achieved, and Ca^{2+} can be measured virtually without interference from Mg^{2+}.

Temperature-jump experiments obtained under identical conditions show that the rate of dissociation of the murexide–calcium complex is much faster than that of the arsenazo III–calcium complex, which may restrict the use of arsenazo III for fast transients of Ca^{2+} binding and/or transport (1–2 ms or faster). Antipyrylazo III, with a τ of 180 μs, responds slower than murexide to changes of ionized Ca^{2+} concentrations but has an adequately rapid response for monitoring Ca^{2+} transients occurring in biological systems.

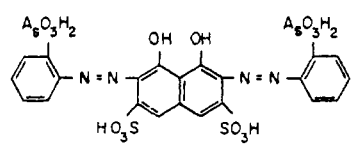
The apparent Δ_ϵ between the free indicator and the indicator–calcium complex is similar for the three indicators. It is larger for arsenazo III but this value is reduced by approximately $\frac{1}{4}$ at 675–685 nm, where the change in absorbance of arsenazo III can be measured with less interference from Mg^{2+}.

The wavelength at which the murexide absorbance changes as a function of the free Ca^{2+} concentration is in the visible area of the spectrum, where most of the cellular pigments absorb light. This requires an accurate selection of the measuring and reference wavelengths, so that the absorbance changes of murexide can be detected without interference from simultaneous absorbance changes of endogenous pigments. Thus, special care should be taken for the selection of wavelengths in the isosbestic points of cytochromes for the measurements of Ca^{2+} transport in mitochondria through murexide absorbance changes. For this reason, the use of murexide is practically excluded for measuring Ca^{2+} transport in erythrocytes, chromaffin vesicles, some bacteria, visual pigments, etc. By contrast, both arsenazo III and antipyrylazo III have Ca^{2+}-dependent absorbance changes in the red area of the spectrum, and Ca^{2+} transport and binding can be measured without nonspecific interference in these organelles. Antipyrylazo III, with measurable Ca^{2+}-dependent absorbance changes at 720–790 nm, offers the best selection of wavelength

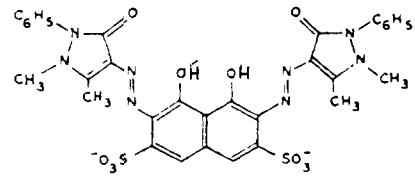
TABLE II[a]



Murexide



Arsenazo III



Antipyrylazo III

	Murexide	Arsenazo III	Antipyrylazo III
Mol wt	284	776	746
Water solubility (mM)	>20	>50	>20
Binding to cells and cell fractions	--	--	--
Side effects on cell structure and function	--	--	--
Indicator-Ca^{2+} complex			
K_D	1-3 mM	15-60 μM	95-380 μM
Δ_ϵ (mM^{-1} cm^{-1})	~6	~25	~7
Relaxation time (τ)	<2 μs	>2.8 ms	180 μs
Suitable differential l (nm)	540-507	675-685; 650-685	670-690; 720-790

[a] Binding and side effects were studied for the three indicators as described in Figure 4A,B. The K_Ds of the indicator-calcium complexes were measured as described in Figure 3. The lower and higher values represent the K_D at 50 and 500 mM KCl, respectively. The Δ_ϵ was measured from spectra obtained in reaction mixtures similar to that of Figure 1. The wavelengths were 540, 650, and 720 nm for murexide, arsenazo III, and antipyrylazo III, respectively. The relaxation time was measured for the three indicators in a reaction mixture containing 50 mM KCl, 50 μM indicator, 20 mM Hepes (pH 7), and a concentration of Ca^{2+} for each experiment which was close to the K_D of the calcium-indicator complex. The suitable wavelengths are wavelength pairs where, for practical considerations (interfering pigments, Mg^{2+} spectrum overlap), Ca^{2+} can be measured in biological systems with little or no interference by nonspecific absorbance changes.

pairs at which Ca^{2+} can be measured without interference by most endogenous pigments, and it is the only indicator so far available which permits the measurement of Ca^{2+} transport in systems such as retinal rods, since the measuring light does not bleach the visual pigments. Also, in the area of the spectrum between 700 and 800 nm other nonspecific absorbance changes due to scattering or change in the volume of the organelles under examination are much smaller than in the visible area of the spectrum.

Table II compares the dissociation constants of the three indicator-calcium complexes obtained at 50 and 500 mM KCl (lower and higher value, respectively). It is evident that the affinity of these three indicators toward Ca^{2+} largely varies and this is probably the most important criterion for the selection of a Ca^{2+} indicator to suit specific experimental conditions.

In view of its low affinity, murexide responds with linear absorbance changes to changes in ionized Ca^{2+} without acting as a significant buffer in the system. On the other hand, the low affinity of murexide toward Ca^{2+} limits the sensitivity of this indicator, and measurements of Ca^{2+} transients smaller than 5 μM are difficult to obtain. In view of these characteristics, murexide is mostly useful when relatively large Ca^{2+} transients are to be measured, especially when large concentrations of Ca^{2+} outside of cells or cell fractions are required. Arsenazo III, with the highest Ca^{2+} affinity, should be used only when Ca^{2+} concentrations are in the nM or low (1-10) μM ranges. Under these conditions, the disturbance of ionized Ca^{2+} in the system due to Ca^{2+} buffering by the indicator is smaller and the calibration curve more linear. Due to its affinity for Ca^{2+}, arsenazo III finds its best application when concentrations of Ca^{2+} in the nanomolar range are the be measured, and it has been successfully used for measuring ionized Ca^{2+} and Ca^{2+} transients in situ in a variety of single cells (DiPolo et al., 1976; Brinley et al., 1977; Miledi et al., 1977; Brown and Pinto, 1978; Yoshikami and Hagins, 1978).

Due to its affinity for Ca^{2+}, antipyrylazo III can be considered a middle-range Ca^{2+} indicator which permits Ca^{2+} measurements in biological systems in areas of Ca^{2+} concentrations where murexide or arsenazo III are, for different reasons, less effective. It is mostly suitable for measurements at concentrations of Ca^{2+} ranging from 1 to 100 μM, where it has adequate sensitivity without compromising calibration linearity or disturbing Ca^{2+} in the system. This range of Ca^{2+} concentrations is mostly suitable for measurements of binding and transport by isolated subcellular organelles, since this is as close to in situ conditions as is possible without the requirements of EGTA-calcium buffers. In addition, antipyrylazo III could be profitably used for measuring Ca^{2+} transport in the presence of suspensions of isolated cells, where the concentration of extracellular Ca^{2+} of a few hundred micromoles is usually required. Under these conditions, the quantitation of the data will be more difficult due to the buffering of Ca^{2+} in the systems by antipyrylazo III, but the sensitivity of the measurements will be much greater than for those obtained with murexide.

Antipyrylazo III is commercially available or it can be synthesized as described by Budesinsky (1974). The commercially available antipyrylazo III should be recrystallized before use as described under Methods. This leaves approximately 15-20% of the contaminants, whose removal is not necessary since the contaminant neither interferes with the Ca^{2+} measurements nor has side effects on cellular properties and functions. The aqueous solutions of the indicator, if kept in brown bottles, are stable for several months (Budesinsky, 1974).

The calibration of the differential absorbance changes of the indicator in solution in terms of differential Ca^{2+} concentrations should be done by adding known aliquots of Ca^{2+} (or EGTA) to the reaction mixture under identical conditions. This "internal calibration" is by far more satisfactory than a more "absolute calibration", because it is independent of the exact knowledge of Δ_ϵ, K_D, absorbance wavelengths (which are variable under different experimental conditions), and the stoichiometry of the antipyrylazo III-calcium complex.

The reaction mixture containing the antipyrylazo III should be well buffered to prevent the changes in Δ_ϵ and K_D of the antipyrylazo III-calcium complex due to pH differences. For practical purposes, the presence of 10-20 mM buffer in the reaction mixture effectively prevents nonspecific absorbance changes arising from pH changes.

The measurements of antipyrylazo III absorbance changes should be carried out at differential wavelengths, which can be obtained through the use of a commercially available or a home-made dual-wavelength spectrophotometer. This is mandatory when Ca^{2+} measurements are carried out in turbid samples containing particulate material, since differential readout at two wavelengths in close proximity is indispensable for minimizing or abolishing nonspecific absorbance changes due to changes in particulate volume and/or refractive indexes.

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