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Indicators of Free Magnesium in Biological Systems†

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ABSTRACT: A technique for measuring kinetically changes of free $[Mg^{2+}]$ in the presence of biological systems is presented. This technique, which is based on the use of the metallochromic indicator Eriochrome Blue SE and dual-wavelength spectroscopy, permits the measurement of Mg^{2+} binding and/or transport to cell or cell fractions without interference by Ca^{2+} and other cations. The difference spectrum of Eriochrome Blue *vs.* Eriochrome Blue plus magnesium exhibits a positive $\Delta\epsilon_{max}$ at 551 nm and a negative $\Delta\epsilon_{max}$ at 572 nm, with an isosbestic point at 563 nm. In contrast, the formation of a complex between Eriochrome Blue and Ca^{2+} or other divalent cations results in a small negative $\Delta\epsilon$ with a broad spectrum between 500 and 680 nm. This allows for selection of wavelength pairs (*i.e.*, 592–554 nm) at which $[Mg^{2+}]$ transients can be measured without interference by Ca^{2+}. At pH 7.1, the Eriochrome Blue–magnesium complex has a $\Delta\epsilon = \sim 5 \times 10^4$ (cm mol)$^{-1}$, a dissociation constant of 1.6 mM and a relaxation time, τ, of 93 msec. In the presence of 30 μM Eriochrome Blue, relatively rapid transients of 0.5 μM $[Mg^{2+}]$ can be measured with high signal-to-noise ratios. At this concentration, Eriochrome Blue was bound insignificantly to biological systems and had no effect on several functions of cells or subcellular organelles. Kinetics of Mg^{2+} release from isolated rat liver are presented. Alternatively, $[Mg^{2+}]$ transients can be measured by the dual-wavelength detection of the absorbance of a second dye, calmagite. With respect to Eriochrome Blue, calmagite has a faster relaxation time, but a much lower $\Delta\epsilon$. The advantages and the limitations of these indicators for Mg^{2+} measurements in biological systems are discussed.

The importance of magnesium in regulation of cellular properties and enzymic functions is well established. Photosynthesis, hibernation, cell adhesion, and metabolism of carbohydrates, proteins, and nucleoproteins all exemplify cellular functions where Mg^{2+} is regarded as a major regulator (Aikawa, 1963; Wacker and Williams, 1968; Wacker and Vallee, 1964).

In spite of its importance, the interaction of Mg^{2+} with biological systems and the regulation of cellular Mg^{2+} homeostasis are poorly understood. Heretofore, simple, rapid, and precise methods for detecting Mg^{2+} at high sensitivity and specificity were unavailable, which accounts for the scarcity of quantitative data on the biological occurrence and function of magnesium.

Several methods have been proposed for Mg^{2+} determination in biological systems. Gravimetric procedures after precipitation of Mg^{2+} with suitable precipitants have the intrinsic limitation for analysis of minute amounts of Mg^{2+}. Colorimetric procedures have been often used which are based either on the formation of an insoluble "lake" between Mg^{2+} and the dye, or on the measurement of the absorption spectrum of a metallochromic dye which forms soluble complexes with Mg^{2+}. In the latter case, however, the indicators fail to discriminate between $[Mg^{2+}]$ and $[Ca^{2+}]$, hence the $[Ca^{2+}]$ must be measured separately and the $[Mg^{2+}]$ obtained by difference (Wacker and Vallee, 1964; Hirschfelder and Serles, 1935). Mg^{2+} radionucleotides are difficult to obtain commercially and their high intensities and short half-time render their usage seldom applicable (Page and Polimeni, 1972). Atomic emission spectroscopy is the principal method presently used for the sensitive and accurate measurement of Mg^{2+}. However, this method requires separation of the material under study through filtration or centrifugation and, often, the solubilization of the biological specimens. This technique is, in some circumstances, precise but is both time consuming and laborious and the long time required for the separation of the material from the surrounding medium renders this method unsuitable for kinetic measurements of Mg^{2+} transients in the presence of biological material. Very recently, the fluorescence of chlorotetracycline has been applied to the measurements of divalent cations within biological membranes (Caswell and Hutchinson, 1971). However, chlorotetracycline does not measure free $[Mg^{2+}]$, but only divalent cations within apolar moieties of biological membranes. Furthermore, the interpretation of the data obtained is made difficult by the lack of specificity toward

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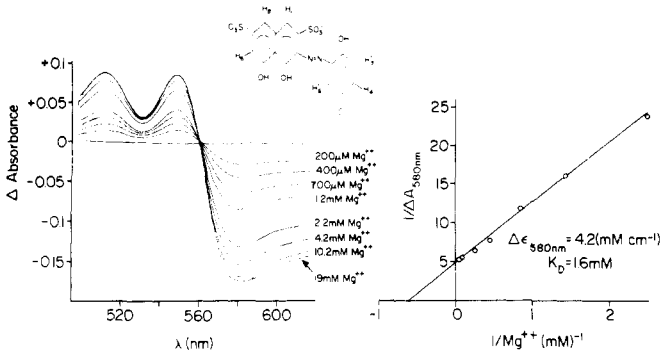

FIGURE 1: Difference spectra of Eriochrome Blue SE *vs.* Eriochrome Blue plus magnesium (A) and double-reciprocal plot of the changes of Eriochrome Blue SE absorbance *vs.* Mg^{2+} concentrations (B). Reference and measuring cuvets contained 200 μM Eriochrome Blue and 40 mM Mops (pH 7.1). The difference spectra and the titration at 580 nm were obtained by adding to the measuring cuvet the concentrations of $MgCl_2$ indicated in the figure; temperature, 22°.

divalent cations and by the diffusion and changes of the environment of the "probe."

The lack of suitable indicators for Mg^{2+} is in sharp contrast with the availability of Ca^{2+} indicators. The measurement of $[Ca^{2+}]$ can be performed by using the easily available isotopes, specific electrodes (Pungor and Toth, 1970; Scarpa and Azzone, 1968), metallochromic (Scarpa, 1972), and photoluminescent indicators (Johnson and Shimomura, 1972), which have provided precise and sensitive measurements of $[Ca^{2+}]$ and have facilitated the understanding of Ca^{2+} interactions with biological systems.

This study presents a simple technique which overcomes most of the limitations of the techniques presently available for measuring Mg^{2+} concentrations. The method is based upon the detection of absorbance changes of suitable metallochromic indicators by dual-wavelength spectroscopy, and provides a way through which Mg^{2+} binding and transport by cellular and subcellular systems can be kinetically measured with high sensitivity and specificity.

Materials and Methods

Preparation of Cell Fractions. Rat liver mitochondria were isolated by homogenization and differential centrifugations in 250 mM sucrose and 0.2 mM EDTA, 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. SR[1] were 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). Ascites tumor cells (Ehrlich-Lettre hyperdiploid) were harvested 6–8 days after inoculation in ICR albino mice and washed and resuspended as described previously (Cittadini *et al.*, 1973).

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 morpholinopropanesulfonate (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 of respiration and then 280 μM ADP, to induce state 3. Oxygen consumption was measured polarographically with a Clark-type electrode in a chamber thermostated at 24° and equipped with a stirrer and 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.

ATP-dependent Ca^{2+} uptake by sarcoplasmic reticulum was measured spectrophotometrically by monitoring the changes in absorbance undergone by murexide, a metallochromic indicator sensitive to the Ca^{2+} concentrations in the medium (Scarpa, 1972; Ohnishi and Ebashi, 1968). The measurements were performed with a dual-wavelength spectrophotometer (Chance, 1972) in a 1-mm light-path cuvet and were continuously recorded. The reaction mixtures contained 20 mM Tris-maleate (pH 6.8), 50 mM KCl, 10 mM $MgCl_2$, 100 μM murexide, and 450 μg of SR protein/ml, temperature 22°. The reaction was started with the addition of 1 mM ATP.

$(Na^+ + K^+)$-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 from the total ATPase activity that in the absence of Na^+ and K^+, plus 10^{-3} M ouabain (Mg-ATPase).

Spectroscopic Techniques. Differential absorption spectra of the dyes were measured with an Aminco DW-2 spectrophotometer with a 1-nm bandwidth and 1-cm light path with baseline compensation.

Dual-wavelength measurements were carried out with a double-beam spectrophotometer (designed at the Johnson Foundation, University of Pennsylvania), equipped with two 250-mm focus Bausch and Lomb monochromators with an effective bandwidth of 0.9 nm.

Atomic absorption measurements were carried out with a Varian Techtron AA-5 atomic absorption spectrophotometer using the resonant line at 2851 Å.

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° and then perturbed in a few microseconds by a 5.2° 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 (1951) and built in this department. This apparatus mixes reactants with a ratio of 1:160 in less than 1 msec 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. Carbonyl cyanide *p*-trifluoromethoxy phenylhydrazone (FCCP) was a gift from Dr. P. G. Heytler of Du Pont and Co., Wilmington, Del. Eriochrome Blue SE (3-[(5-chloro-2-hydroxyphenyl)azo]-4,5-dihydroxy-2,7-naphthalenedisulfonic acid) and calmagite (3-hydroxy-4-[(6-hydroxy-*m*-tolyl)azo]-1-naphthalenesulfonic acid) were purchased from Baker Chemicals and recrystallized twice. Sodium adenosine 5′-diphosphate (ADP) grade 1, morpholinopropanesulfonic acid (Mops), and tris(hydroxymethyl)aminomethane were purchased from Sigma.

Results

Figure 1 shows the differential spectra of Eriochrome Blue and Eriochrome Blue *plus* various concentrations of $MgCl_2$. The additions of $MgCl_2$ to the measuring cuvet produced an in-

[1] Abbreviations used are: SR, sarcoplasmic reticulum; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; Mops, morpholinopropanesulfonic acid.

crease in absorbance with a ΔA_{max} at 510 and 548 mm and a decrease in absorbance with ΔA_{max} at 538 nm. The double-reciprocal plot obtained by plotting the reciprocal of Mg^{2+} concentrations *vs.* the reciprocal of absorbance decreases at 580 nm produced a linear slope from which a $\Delta\epsilon$ (580 nm) of 4.2 mM cm^{-1} and a K_D of 1.6 mM were calculated. Spectra were obtained in a reaction mixture buffered at pH 7.1. Both $\Delta\epsilon$ and K_D were shown to be dependent on pH.

Figure 2 shows the differential spectra of Eriochrome Blue *vs.* Eriochrome Blue *plus* either Mg^{2+} or Ca^{2+}. The addition of $MgCl_2$ to Eriochrome Blue at pH 7.1 produces an increase of absorbance with a ΔA_{max} at 551 nm and a decrease in absorbance with ΔA_{max} at 580 nm, with an isosbestic point at 563 nm. In contrast, the addition of $CaCl_2$ produces a much broader absorbance decrease from 500 to 650 nm. The low affinity of Eriochrome Blue with Ca^{2+} ($K_D > 3.5$ mM) makes possible variations of $[Ca^{2+}]$ in the system without significant effect on the amounts of Eriochrome Blue free in solution. Other divalent cations such as Sr^{2+} and Ba^{2+} produce changes in absorbance similar to that of Ca^{2+}, whereas Mn^{2+} mimics the spectral changes induced by Mg^{2+}. The different spectral response of Eriochrome Blue to the addition of Ca^{2+} and Mg^{2+} makes possible the selection of areas of the spectrum where Mg^{2+} can be detected specifically without Ca^{2+} interference. A and B indicate two examples of wavelength pairs, 592–554 and 578–565 nm, suitable for Mg^{2+} measurements. The data of Figure 2 clearly show that, at either wavelength pair, the ΔA will be large on addition of Mg^{2+} and minimal or absent in the presence of Ca^{2+}.

These intrinsic spectral properties of the Eriochrome Blue–divalent cation complexes and the availability of sensitive dual-wavelength techniques (Chance, 1972) make possible the use of Eriochrome Blue as a specific indicator for the free Mg^{2+} concentration. Figure 3 shows the recording traces of changes in absorbance of Eriochrome Blue induced by the addition of Ca^{2+} and Mg^{2+}, measured with a dual-wavelength spectrophotometer at either 592–554 (Figure 3, arrow A) or 578–565 nm (Figure 3, arrow B). The addition of 25 μM $CaCl_2$ does not produce an appreciable change in the ΔA of Eriochrome Blue at 578–565; in fact, the small decrease in absorbance produced by Ca^{2+} at the measured wavelength, 578 nm, is equal to the decrease in absorbance at the reference wavelength 565 nm. As a result, the differential readout at these two wavelengths is zero. In contrast, the addition of 25 μM $MgCl_2$ produces a significant decrease in ΔA, which is the result of a decrease in absorbance at 578 nm *minus* decrease at 565 nm, the measuring and reference wavelength, respectively. Another pair of wavelengths, 592–554 nm, was used for the recording of the absorbance changes undergone by Eriochrome Blue after Ca^{2+} and Mg^{2+} addition. As shown in Figure 2, arrow A, the changes of ΔA induced by Mg^{2+} are greater than the previous wavelength pair and again, Ca^{2+} addition produces no detectable ΔA changes. Hence, at pH 7.1, two wavelength pairs can be selected for measuring the free Mg^{2+} concentration in the absence of Ca^{2+} interference. The 592–554-nm wavelength couple has the advantage of higher sensitivity, due to the large ΔA obtained. The wavelengths 578–565 nm are much closer. This reduces the sensitivity of Eriochrome Blue–Mg^{2+} ΔA, but offers the advantage of minimizing unrelated changes in absorbance due to changes in volume or refractive index of particles when Mg^{2+} measurements are carried out in the presence of turbid samples.

Although Eriochrome Blue can be used successfully as a sensitive and specific indicator for free Mg^{2+} concentration in buffered solutions, its use for meaningful measurements of

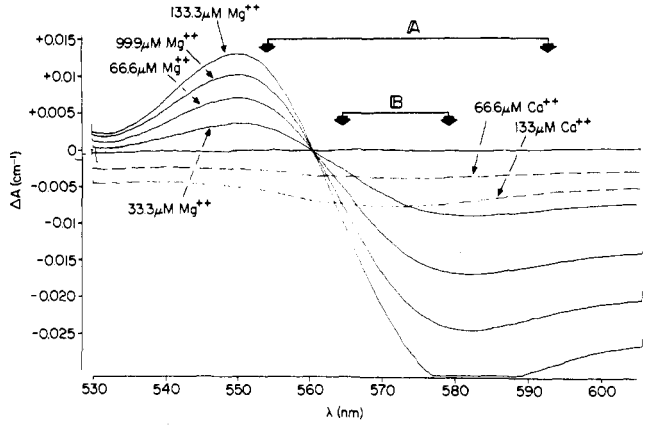

FIGURE 2: The difference spectra of the Eriochrome Blue SE *vs.* Eriochrome Blue SE plus Ca^{2+} or Mg^{2+}. The differential spectrum was obtained by adding to the measuring cuvet the concentrations of $CaCl_2$ or $MgCl_2$ reported in the figure. Both cuvets contained 30 mM Tris-HCl (pH 7.1) and 200 μM Eriochrome Blue; temperature, 24°. A and B indicate regions of the spectra where suitable couples of wavelength pairs are selected for Mg^{2+} measurements without Ca^{2+} interference.

Mg^{2+} transients in the presence of biological systems requires at least two additional properties of the dye. Eriochrome Blue should neither bind to nor have side effects on structure and functions of the biological systems under study.

The lack of major binding and/or transport of Eriochrome Blue by cells and cell fractions is shown in Figure 4. Concentrations of Eriochrome Blue ranging from 8 to 50 μM were added to concentrated suspensions of mitochondria or ascites tumor cells. After incubation times of 2 min, the ascites cells and mitochondria were separated from the suspending medium containing Eriochrome Blue by centrifugation. The supernatant was then analyzed for the Eriochrome Blue content by measuring its absorbance at 562 nm, an isosbestic point for Eriochrome Blue *vs.* Eriochrome Blue *plus* Mg^{2+}. A straight line was obtained by plotting the absorption *vs.* Eriochrome Blue concentrations. A straight line was also obtained by plotting the absorption of Eriochrome Blue in the same medium, but without particles. A small but reproducible decrease in absorbance was observed when the medium contained Eriochrome Blue and particles. These data indicate that adsorption of Eriochrome Blue to the particles did occur to a limited ex-

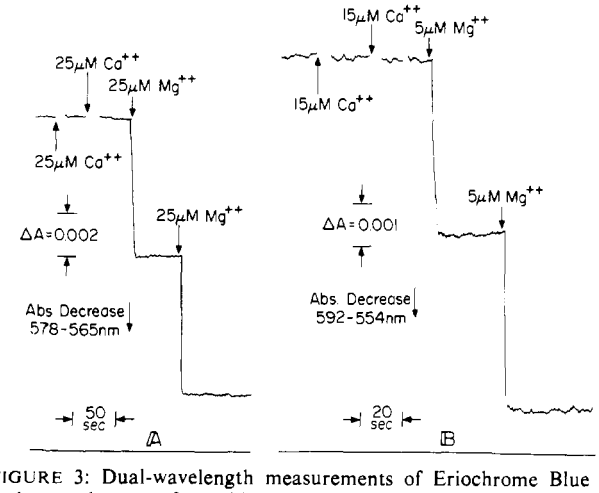

FIGURE 3: Dual-wavelength measurements of Eriochrome Blue absorbance changes after addition of $CaCl_2$ and $MgCl_2$. The reaction mixture contained 30 μM Eriochrome Blue, 100 mM KCl, and 30 mM Mops (pH 7.1). The changes in absorbance were measured in a 1-ml cuvet at the two couple of wavelengths marked with arrows on Figure 2; temperature, 24°.

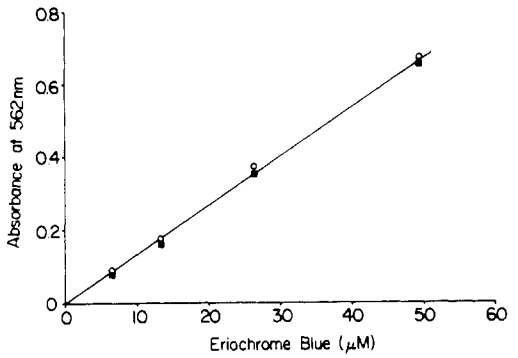

FIGURE 4: A plot of the absorbance at 562 nm *vs.* added Eriochrome Blue concentrations. The reaction mixture contained 250 mM sucrose, 30 mM Mops, 10 μM rotenone, and the concentrations of Eriochrome Blue indicated in the figure. (■) The reaction mixture was supplemented with either 6.8 mg of rat liver mitochondria/ml or 22 mg dry weight of ascites tumor cells. The particles were separated by centrifugation at 15,000*g* (10 min) and the supernatant was assayed for Eriochrome Blue absorbancy. The values obtained with mitochondria and ascites cells were indistinguishable. (○) Values of a control experiment in which Eriochrome Blue was added to the reaction mixture in the absence of either mitochondria or ascites cells; temperature, 21°.

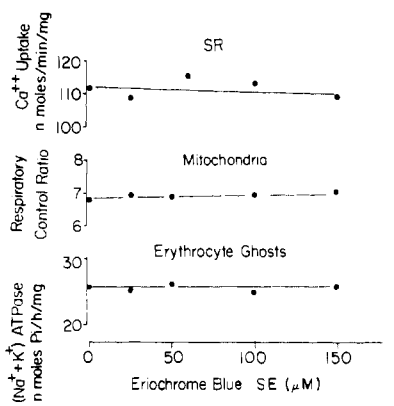

FIGURE 5: Effect of various concentrations of Eriochrome Blue on ATP-dependent Ca^{2+} uptake by fragmented sarcoplasmic reticulum, respiratory control ratio of rat liver mitochondria, and (Na^+/K^+)-ATPase of red cell ghosts. Preparation of cellular fractions, assay techniques, and reaction mixtures are described in the Methods section.

tent, and that the majority of Eriochrome Blue is in the solution external to mitochondria and ascites tumor cells.

Figure 5 shows that Eriochrome Blue has no side effects on a variety of functions of subcellular organelles such as ATP-dependent Ca^{2+} uptake by 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 also at 150 μM Eriochrome Blue, a concentration 3–10 times higher than that required for its use as metallochromic indicator.

Figure 6 illustrates the results of an experiment in which the time scale of the formation of Mg^{2+}-Eriochrome Blue complexes was investigated by stopped-flow or temperature perturbation technique. In Figure 6A, the Eriochrome Blue–magnesium system was kept in thermal equilibrium and then perturbed by rapid increase in temperature within a few microseconds. The recordings at the top of the figure show the time course of the concentration changes of the species [Eriochrome Blue] and [Eriochrome Blue–Mg^{2+}] to a new equilibrium at the higher temperature. The rate of decay of [Eriochrome Blue]:[Eriochrome Blue–Mg^{2+}] is characterized by a linear first-order differential equation, the solutions of which are linear combinations of exponentials. Each exponential term is associated with a relaxation time, τ, which may be considered the reciprocal first-order rate constant. For a single relaxation time $\Delta S = \Delta S_0 e^{-t/\tau}$, ΔS is the difference between the signal at $t = t$ and $t = \infty$. Therefore, the plot obtained by plotting the log of ΔS *vs.* time gives a straight line from which a $\tau = 93$ msec and a $t_{1/2} = 60$ msec can be calculated. The linearity of the plot supports the assumption of a single relaxation process. Figure 6B shows that the addition of either Mg^{2+} or EDTA to a reaction mixture containing Eriochrome Blue and magnesium through a stopped-flow apparatus produces changes in Δ absorbance with a half-time of 70 msec. These results are in good agreement with the above reported rates obtained by temperature-jump techniques and indicate that the changes of absorbance due to the formation of a complex between Mg^{2+} and Eriochrome Blue are quite slow as compared with those of pH indicators or other metallochromic dyes (Geier, 1968).

Figure 7 illustrates some properties of the metallochromic indicator, calmagite (3-hydroxy-4-[(6-hydroxy-*m*-tolyl)azo]-1-naphthalenesulfonic acid). Mg^{2+} and Ca^{2+} produce different ΔA changes when complexed to calmagite, and calmagite can be used for the measurement of Mg^{2+} in the presence of Ca^{2+}, using dual-wavelength spectrophotometry. Figure 7A shows the differential spectra of calmagite free *vs.* calmagite plus either Ca^{2+} or Mg^{2+}. Suitable wavelength pairs can be selected at which free Mg^{2+} concentrations can be measured with minimal or no Ca^{2+} interference. Figure 7B,C show examples of the measurement of calmagite absorbance changes as a function of the time obtained at two suitable wavelength pairs. Similar to what was observed with Eriochrome Blue, Mg^{2+}, but not Ca^{2+}, induces absorbance changes. Figure 7D shows the time scale of the absorbancy changes of calmagite, after a temperature jump obtained under conditions similar to those of Figure 6A. In this case, the half-time for the equilibrium [calmagite] ⇌ [calmagite–magnesium] at higher temperatures was lower than 30 μsec. With respect to Eriochrome Blue, calmagite has the advantage of faster formation of complexes with Mg^{2+} and the disadvantage of smaller ΔA. Calmagite offers another way to measure Mg^{2+} specifically. Its use, however, should be limited to measurements where not only fast, but also large, changes of free Mg^{2+} are expected.

Figure 8 shows kinetic measurements of Mg^{2+} efflux from rat liver mitochondria obtained by recording the absorbance decrease undergone by Eriochrome Blue. In order to minimize the interference due to the changes in the redox state of the mitochondrial respiratory chain components, the measurements were carried out at 540–575 nm, two apparent isosbestic points for the oxidized–reduced form of cytochromes. As reported by various investigators (Kun *et al.*, 1969; Bogucka and Wojtczak, 1971), the addition of uncoupling agents and ADP prompted a release of endogenous Mg^{2+} from mitochondria oxidizing substrates. Figure 8A shows the calibration of the reaction mixture, used for the same experiment but in the absence of mitochondria: two identical additions of Mg^{2+} produce a comparable decrease in the absorbance of Eriochrome Blue. Figure 8B shows that a decrease of absorbance related to Mg^{2+} efflux was observed on addition of ADP and uncoupling agent to rat liver mitochondria oxidizing succinate in the presence of rotenone. As a control, Figure 8C shows that, under identical experimental conditions, similar results were obtained by measuring Mg^{2+} concentrations by flame absorption spectroscopy after separation of the mitochondria by centrifugation.

Discussion

An ideal metallochromic indicator for measuring free [Mg^{2+}] in the presence of a biological system should possess the following properties: (a) a low affinity for magnesium; (b)

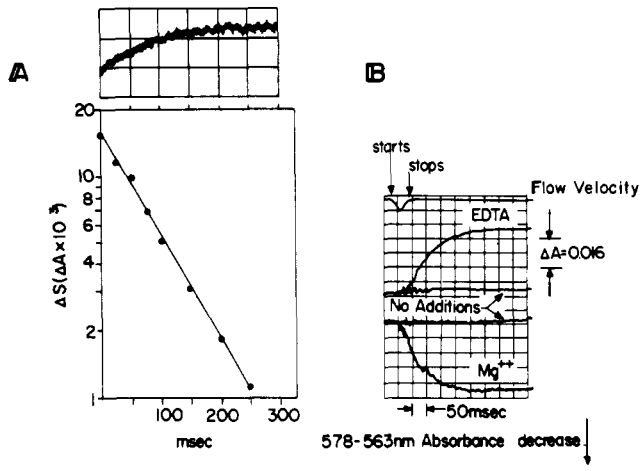

FIGURE 6: Temperature-jump (A) and rapid-flow measurements (B) of the absorbance changes of Eriochrome Blue induced by $MgCl_2$. In part A, the reaction mixture contained 100 mM KCl, 30 mM Mops (pH 7), 100 μM Eriochrome Blue, and 100 μM $MgCl_2$ thermostated at 20°. The top figure shows the recording of the absorbance changes at 580 nm, following a 4° temperature increase with a rising time of 4 μsec. In part B, the large syringe of the stopped-flow apparatus contained 100 mM KCl, 30 mM Mops (pH 7.1), and 100 μM Eriochrome Blue. The smaller syringe (1:160 ratio) contained either 0.1 M $MgCl_2$ or 0.1 M EDTA (sodium salt, pH 7.1). After the rapid mixing, the changes in absorbance were measured in dual-wavelength mode at 578–563 nm. "No addition" refers to the discharge of the reaction mixture through the observation chamber without additions of EDTA or $MgCl_2$; temperature, 20°.

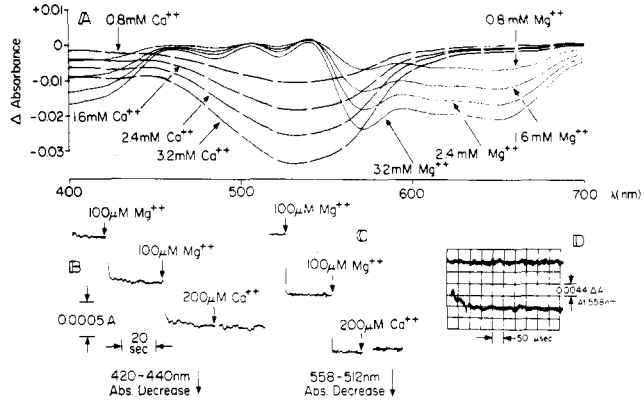

FIGURE 7: Mg^{2+}-induced changes in absorbance of the metallochromic indicator calmagite. Part A shows the differential spectra of calmagite after additions of the concentrations of either Ca^{2+} or Mg^{2+} reported in the figure. Parts B and C show the measurements of changes in absorbance, induced by Ca^{2+} or Mg^{2+} detected at 420–440 or 558–512 nm in dual-wavelength mode. Part D illustrates the absorbance decrease following a temperature jump under conditions similar to those of Figure 6. For all the experiment, the reaction mixtures contained 100 mM KCl and 30 mM Tris-HCl (pH 7.2). Calmagite concentrations were 400 μM in A and D, 30 μM in B and C; temperature, 20°.

a high $\Delta\epsilon$ between the uncomplexed indicator and the indicator complexed with magnesium; (c) a high specificity toward $[Mg^{2+}]$; (d) a lack of binding and/or penetration into the biological material under study; (e) a lack of side effects on the functional properties of the biological system; (f) a suitable wavelength at which the specific absorbance changes of the dye can be obtained without interference by unrelated changes in absorbance of other compounds present; and (g) a high rate constant for the formation of the complex with magnesium.

Some of these requirements are met by Eriochrome Blue SE. Because of the high dissociation constant of the magnesium–Eriochrome Blue complex (K_D = 1.6 mM at pH 7.1 and increases with increasing pH), there is a minimal perturbation of the free $[Mg^{2+}]$ present in the reaction mixture by the dye. From the measured K_D, it was calculated that only a few per cent of the Mg^{2+} was bound to the indicator under the experimental conditions reported. The high $\Delta\epsilon$ results in a large ΔA after addition of micromolar amounts of Mg^{2+}, even at low concentrations of indicator.

The specificity of Eriochrome Blue toward Mg^{2+} is not an intrinsic property of the dye. However, since various divalent cations produce different absorption spectra when complexed with Eriochrome Blue, dual-wavelength spectroscopy makes possible the measurements of Mg^{2+} without interference from Ca^{2+}. Hence, the presence of Ca^{2+}, which is widely distributed in biological materials and the movement of which is often associated with that of Mg^{2+}, does not interfere with Mg^{2+} measurements. Because all the divalent cations tested, with the exception of Mg^{2+} and Mn^{2+}, induce spectral changes similar to that of Ca^{2+}, Eriochrome Blue can be used as a specific indicator for both Mg^{2+} and Mn^{2+}. Owing to the relative scarcity of Mn^{2+} in biological systems, its interference does not create a problem in most of Mg^{2+} measurements. However, when Mn^{2+} movements are expected, measurements of Mg^{2+} without Mn^{2+} interference can still be performed in the presence of EGTA, whose affinity for Mn^{2+} is several orders of magnitude greater than that of Mg^{2+}. Also, changes in pH of the reaction mixture produce changes in Eriochrome Blue absorption and, therefore, well-buffered conditions are mandatory when Mg^{2+} measurements are performed. Most of the measurements reported were carried out in reaction mixtures buffered at pH 7.1–7.2. This was the pH range within physiological limits where the best discrimination between Mg^{2+} and Ca^{2+} was obtained.

At the concentration of Eriochrome Blue used, little, yet reproducible, binding of the indicator to plasma membrane or mitochondria was observed. The limited binding to membrane may be accounted for by the solubility of Eriochrome Blue in aqueous, but not in apolar, environments. Furthermore, even at high concentrations, Eriochrome Blue was shown to have no effect on several functions of cells and subcellular organelles. Another advantage of Eriochrome Blue as an indicator is the availability of several wavelength pairs at which $[Mg^{2+}]$ transients can be spectroscopically measured. Examples of this are the measurements reported of Mg^{2+} release by mitochondria which were performed in a spectral region where interference by the cytochromes was negligible.

A significant limitation of Eriochrome Blue is its slow rate of

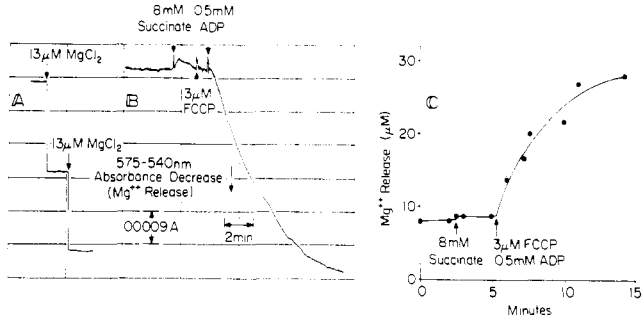

FIGURE 8: Kinetics of Mg^{2+} release by isolated rat liver mitochondria. The reaction mixtures contained 0.20 M sucrose, 20 mM KCl, 30 mM Mops (pH 7.1), 30 μM Eriochrome Blue, and 3 μM rotenone. The reaction mixture was supplemented with 2.8 mg of mitochondrial protein/ml in parts B and C. Mg^{2+} transients were measured in parts A and B through the detection of the absorbance changes of Eriochrome Blue at 540–575 nm. Part C represents a control experiment in which 1-ml aliquots were withdrawn at the times indicated in the figure. The samples were centrifuged 2 min at 15,000g with an Eppendorf desk centrifuge, and the supernatants were analyzed for Mg^{2+} content through absorption flame spectroscopy.

complex formation with Mg^{2+}, the half-time of which is about 60 msec, as compared with less than 3 μsec in the case of murexide–calcium complex formation (Geier, 1968). The slow response of Eriochrome Blue still allows for kinetic measurements of $[Mg^{2+}]$ transients slower than 200 msec. On the other hand, Eriochrome Blue is fully inadequate to resolve kinetically fast reactions involving Mg^{2+} binding and/or transport. In this case, calmagite may be used with the limitation that its low $\Delta\epsilon$ permits only the detection of large $[Mg^{2+}]$ transients.

In summary, the measurements of free $[Mg^{2+}]$ through Eriochrome Blue absorption are limited by the relatively slow response, by some binding to the biological systems and by the interaction with a broad range of cations. However, if suitable wavelength pairs are selected carefully with the proper respect to medium composition and in well-buffered conditions, Eriochrome Blue offers a tool for kinetic measurements of Mg^{2+} binding and transport in biological systems. In addition to the above-described measurements, this technique is now being applied to resolve kinetically the efflux of Mg^{2+} from phospholipid vesicles or mitochondria as facilitated by the divalent cation ionophores. Since this method permits the measurements of the changes of free Mg^{2+} outside most cell or subcellular organelles, it may prove to be of general use for the study of the mechanism of Mg^{2+} binding and transport or for the understanding of the control of intracellular Mg^{2+} homeostasis. In addition, the introduction of Eriochrome Blue into large cells suitable for microinjection offers a realistic approach to the quantitative evaluation of free $[Mg^{2+}]$ present in the cytosol.

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