Fluorescein and carboxyfluorescein have found recent application as probes of intracellular pH. The present study examines several parameters required for interpretation of the spectral information derived from fluorescein and carboxyfluorescein generated intracellularly from their permeant diacetate derivatives. Coefficients were determined for the pH dependence of the difference absorbance, of the absorbance ratios, and of the fluorescence emission intensity ratios at selected wavelength pairs for carboxyfluorescein in aqueous buffers. The effect of light scattering on the apparent pH reported by carboxyfluorescein in dilute cell suspensions was assessed. An apparent intracellular acidification associated with increasing internal dye concentration was found to result probably from interactions of the intracellular probe with itself. Working within the experimental limitations imposed by these considerations, protocols utilizing either direct measurement of absorbance or fluorescence or determination of the null spectral response observed upon release of internal carboxyfluorescein all indicate that the cytosolic space of bovine epididymal sperm is maintained at pH 6.5–6.6.

The monovalent-ion-specific, carboxylic acid ionophores, nigericin and monensin, were utilized to produce transmembrane proton gradients in cells that were allowed to generate intracellular carboxyfluorescein in a preliminary incubation, then resuspended in media buffered at the same pH as the sperm cytosol but of varying cation composition. By interpolation to the null response, the initial internal Na⁺ and K⁺ concentrations in bovine sperm were estimated as 14 ± 2 and 120 ± 5 mM, respectively.

Although a ubiquitous regulatory role of intracellular Ca²⁺ is firmly established, it is not yet clear to what extent and by what means intracellular concentrations and distributions of other ions, particularly K⁺, Na⁺, and H⁺, may either affect internal Ca²⁺ concentrations and distributions (1–5), or directly control diverse cellular processes (6–8). In spermatozoa, for example, increased membrane permeability and entry of extracellular Ca²⁺ promote the membrane fusion component of the exocytic acrosome reaction that is required for fertilization of both mammalian and invertebrate species (for reviews see Refs. 9 and 10). For sperm of several marine invertebrates, polymerization of nonfilamentous actin into an “acrosomal process” is another component of the acrosome reaction that probably results from alkalization of one or more intracellular compartments (6, 11). In addition, entry of extracellular Na⁺ is apparently required for the action of speract, a peptide component released from sea urchin eggs that is responsible for stimulation of sperm motility and metabolism (12), possibly also required for fertilization. In contrast, mammalian sperm do not form an acrosomal process and the physiologically important initiators of the alterations of motility and metabolism and of the acrosome reaction of mammalian sperm have not been identified. However, some evidence indicates that proton release (13–15) and possibly uptake (14) are required for the fusion of acrosomal membranes and other alterations to the sperm that also must precede fertilization in mammals. Clearly, a means of monitoring the intracellular ionic environment would facilitate study of the mechanisms that regulate cellular function in mammalian sperm.

Recently, the pH-dependent spectral properties of the fluorescein chromophore have been employed to estimate internal pH in ascites tumor cells (16), amoeba (17), and macrophages (17–19) following introduction of the conjugate dye either by microinjection (17) or phagocytosis (18, 19) or by generation of the free chromophore from permeant diacetate derivatives (16). This report examines some of the experimental parameters that determine the accuracy with which intracellular fluorescein chromophore reports internal pH of the bovine sperm. In a new application, also described here, the specific Na⁺ for H⁺ and K⁺ for H⁺ exchanges that are promoted respectively by the ionophores monensin and nigericin are coupled to the pH-dependent spectral response of internal carboxyfluorescein, thus allowing examination of intracellular Na⁺ and K⁺ concentrations.

Early work examined the approximate magnitude, duration, and selectivity of proton and metal ion fluxes induced by nigericin (20, 21) and the monensins (22) in isolated mitochondria (20–22) and erythrocytes (21). However, subsequent utilization of these ionophores to increase membrane perme-
FIG. 4. Absorbance responses of extra- and intracellular fluorescein chromophore to alterations in medium pH and membrane permeability. A, 2 \mu M carboxyfluorescein (CFsc) in 75 mM KCl, 40 mM NaCl, 1 mM MgSO\textsubscript{4}, 5 mM fructose, 10 mM Na morpholino propane sulfonate (pH 7.4). B, twice washed bovine epididymal sperm (5 \times 10^6 cells) were incubated for 5 min at 25 °C in 2 ml of the same medium before addition of 4 nmol of carboxyfluorescein and transfer to the assay cuvette. C-E, sperm (5 \times 10^6 cells) were incubated for 15 min at 25 °C in 1.2 ml of 120 mM NaCl, 5 mM KCl, 1 mM MgSO\textsubscript{4}, 30 mM Na morpholinoethane sulfonate (pH 6.1), 5 \mu M carboxyfluorescein diacetate. The cells were collected by centrifugation (8000 \times g, 7 a) and resuspended in 2 ml of the medium used in A and B. After 3 min, the suspension was transferred to a stirred assay cuvette and the optical difference absorbance (A\textsubscript{605} - A\textsubscript{495}) was recorded. F, sperm were prepared in an identical manner except that the preliminary incubation contained fluorescein diacetate rather than carboxyfluorescein diacetate. The pH of each sample was adjusted by addition of 0.1 ml of 1 M Na morpholinoethane sulfonate (pH 5.8). Other additions were made as noted. Final concentrations were: nigericin (NIG), 2.5 \mu M; valinomycin (VAL), 0.5 \mu M; digitonin (DIG), 225 \mu g/ml.

ability in a variety of systems (6, 12, 13, 23, and for reviews see Refs. 24–27) has not provided a predictive model for expected steady state cation and proton distributions. Nor has it been generally recognized that induced cation fluxes depend, in magnitude and direction, upon the initial gradients of both metal ions and protons that exist prior to ionophore addition.

The present study proposes and provides supporting evidence for a simple model that allows calculation of steady state internal cation concentrations from measurement of initial external and internal cation concentrations and of initial and the steady state internal pH achieved after ionophore treatment. In addition, granting the validity of this model, a method is demonstrated for estimation of intracellular proton buffering capacity.

**MATERIALS AND METHODS**

Chemicals were obtained from the following sources: morpholinopropane sulfonate, morpholinoethane sulfonate, and digitonin from Sigma; nigericin, valinomycin, and monensins A, B, and C from Eli Lilly; fluorescein, carboxyfluorescein, and fluorescein diacetate from Eastman Kodak. Dr. John Thomas, University of South Dakota, Vermillion, SD generously provided an initial sample of carboxyfluorescein diacetate. Subsequent material was prepared by the procedure of Bruning et al. (28). Digitonin was recrystallized from ethanol. Stock solutions of fluorescein and carboxyfluorescein (10 or 100 mM) and their deacetates (1 or 10 mM) and of digitonin (40 mg/ml) all in Me\textsubscript{2}SO, and of valinomycin (1 mM), nigericin (1 mM), and the monensins (10 mM) in dimethylformamideethanol (8:2) were stored at −20 °C. Digitonin was added at concentrations previously shown to induce release of cytosolic contents but to leave sperm mitochondria functionally intact (29). Ionophores were added at concentrations determined in preliminary experiments to induce maximal exchange of cations for protons.

Bovine cauda epididymides were a gift of Oscar Mayer Co., Madison, WI. Procedures for sperm preparation were as previously described (30) except that all steps were conducted at room temperature. The medium used to wash sperm consisted of 120 mM NaCl, 5 mM KCl, 10 mM Na morpholino propane sulfonate (pH 7.4). For intracellular generation of fluorescein chromophore, sperm were incubated in a loading buffer: 120 mM NaCl, 5 mM fructose, 1 mM MgSO\textsubscript{4}, 30 mM Na morpholinoethane sulfonate (pH 6.1) and various concentrations of the diacetate derivatives. Intracellular carboxyfluorescein at 1 mM concentration had no detectable effect on cell motility as judged by subjective microscopic examination.

An Aminco DW 2 spectrophotometer and an SLM series 8000 spectrophotometer each equipped with a magnetic stirring device were employed to monitor absorbance and fluorescence changes, respectively. Details of instrumental configurations are found in the Appendix.\textsuperscript{1}

**RESULTS**

**Spectral Properties of the Fluorescein Chromophore in Vitro**—Preliminary experiments described in the Appendix examined the pH dependence of the spectra of fluorescein and carboxyfluorescein (Fig. 1). The spectral coefficients found for absorption and fluorescence emission at selected wavelengths in aqueous media of varying cation composition (Table 1, Fig. 2) form the basis for comparison of the behavior of

\textsuperscript{1} Portions of this paper (including an Appendix, Figs. 1–3, and Table 1) are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are available from the Journal of Biological Chemistry, 9650 Rockville Pike, Bethesda, MD 20814. Request Document No. 82M-203, cite the authors, and include a check or money order for $2.00 per set of photocopies. Full size photocopies are also included in the microfilm edition of the Journal that is available from Waverly Press.
these dyes in cell suspensions. In addition, the effect of increasing dye concentration upon the apparent absorbance also was determined (Fig. 3).

**Intracellular and Extracellular Fluorescein Chromophore in Cell Suspensions**—Examination of dilute carboxyfluorescein alone or of dilute (extracellular) carboxyfluorescein in sperm suspensions demonstrates a comparable decrease in difference absorbance when the pH of the medium is lowered from 7.4 to 6.2 (Fig. 4, A and B, respectively). In this medium, strongly buffered with morpholinopropanesulfonate, neither carboxyfluorescein alone nor extracellular carboxyfluorescein respond upon addition of agents (nigericin, valinomycin, or digitonin) that increase membrane permeability.

In contrast, for suspensions of cells allowed to generate a comparable quantity of internal carboxyfluorescein in a preliminary incubation, a similar change in external pH produces only a small optical response from that chromophore which has leaked from the cells during or following their resuspension in the assay medium (Fig. 4, C and D). A much larger decrease in difference absorbance occurs when the cell interior is acidified by the subsequent addition of valinomycin (promoting H⁺ uptake as the outward-directed K⁺ gradient is dissipated) or when internal chromophore is released to the acidic medium following addition of the membrane-disruptive agent, digitonin. After such digitonin-induced release, difference absorbance does not undergo further change—provided that the preliminary incubation has depleted internal diacetate precursor and that external diacetate has been removed by washing. Otherwise, release of cellular esterase (apparent Vmax of approximately 2 nmol of carboxyfluorescein/10⁶ cells × min at pH 7) rapidly generates more chromophore with associated changes in difference absorbance.

**Intracellular Localization of Fluorescein and Carboxyfluorescein**—The K⁺-specific neutral polycationic ionophore, valinomycin, promotes electrogenic K⁺ uptake with resultant proton efflux from isolated, energized mitochondria (27) and from tumor cell mitochondria in situ (16). In agreement with observations of intact ascites tumor cells (16), Fig. 4, E and F, respectively, show that after addition of valinomycin to sperm suspensions, internal carboxyfluorescein reports a much smaller alkalinization than does internal fluorescein. The secondary alkalization that follows treatment with valinomycin has not been investigated in detail but probably results from secondary anion movements that serve to relax the induced electrochemical gradient. If sperm are loaded with fluorescein, then treated with digitonin to expose their mitochondria, addition of valinomycin produces a smaller response if an oxidative substrate also is provided. No response is observed in the absence of added substrate or with cells that are loaded instead with carboxyfluorescein, then treated with digitonin (data not shown). Fluorescein seems therefore to monitor both cytosolic and mitochondrial pH whereas carboxyfluorescein monitors cytosolic pH exclusively.

Microscopic examination of sperm loaded with carboxyfluorescein showed that the cells are uniformly fluorescent, indicating that the chromophore is distributed throughout the cytosolic space. This suggestion is supported additionally by the release of dye that is induced by the membrane-selective, disruptive agent, digitonin (Table II).

**Cellular Uptake and Hydrolysis of Carboxyfluorescein Diacetate and Chromophore Release**—During intracellular generation of the fluorescein chromophore, its distribution and that of its diacetate precursor between intra- and extracellular compartments depends upon the relative rates of several competing processes that are probably affected to differing extents by intra- and extracellular pH, by initial precursor and cell concentrations, and by temperature (16). We have not explored the separate dependencies of diffusional uptake of the diacetate, its intracellular hydrolysis, and the subsequent partitioning of carboxyfluorescein. However, Table II examines the proportions of internal and external chromophore and precursor found during a standard incubation regimen that was employed with little or no modification in all subsequent experiments reported here.

Under these conditions, the initial rate of diffusional entry apparently exceeds that of the intracellular hydrolysis of the diacetate. However, after 10 min of incubation, nearly half of the total added diacetate has been subjected to intracellular hydrolysis. A constant portion (18-20%) of the free chromophore and precursor found during a standard incubation regimen is released to the extracellular medium as a result of leakage of cellular esterase, loss of free chromophore, or both.

Following washing and resuspension, the retention of carboxyfluorescein by sperm is independent of the pH of the external medium. Half-times for release at 25°C are greater than 60 min at pH 6-8. Rapid and nearly quantitative release of intracellular carboxyfluorescein is induced by concentrations of digitonin (29) that selectively release cytosolic contents but that leave sperm mitochondria functionally intact.

**Sperm Cytosolic pH by Digitonin-induced Null Absorbance Change**—Addition of digitonin to sperm loaded with carboxyfluorescein immediately following their resuspension in media buffered at various pH values produces rapid positive or negative spectral responses as the internal dye is released to media that are respectively more or less alkaline than the intracellular environment of the chromophore (Fig. 5A). Interpolation to the null response indicates the point at which internal and extracellular pH are equivalent and thus allows estimation of the cytosolic pH of sperm (Fig. 5B). For sperm utilizing glycolytic or endogenous substrates and suspended in 120 mM NaCl, an internal pH of 6.50 was found in this experiment. Sperm suspended in 120 mM KCl consistently

### Table II

**Precursor uptake and hydrolysis and release of intracellular carboxyfluorescein**

| Time (min) | Extracellular chromophore | Intracellular chromophore |
|-----------|---------------------------|----------------------------|
|           | Total Amount | Concentration | Free Amount | Concentration |
|           | nmol | μM | % of total extracellular | nmol | mM | % of total intracellular |
| 0         | 14.4 ± 0.7 | 1.2 | 3 | 2.4 ± 0.8 | 0.8 | 58 |
| 5         | 11.8 ± 1.4 | 0.98 | 8 | 8.1 ± 1.4 | 2.9 | 95 |
| 10        | 10.2 ± 0.8 | 0.85 | 13 | 12.5 ± 2.6 | 4.5 | 103 |
| 15        | 9.2 ± 1.2 | 0.77 | 12 | 14.4 ± 3.3 | 5.6 | 101 |
| 20        | 8.3 ± 1.3 | 0.69 | 18 | 16.0 ± 4.2 | 5.7 | 102 |
| 30        | 7.3 ± 1.1 | 0.61 | 22 | 19.0 ± 1.4 | 6.8 | 100 |
| 40        | 6.4 ± 0.8 | 0.53 | 22 | 20.9 ± 0.8 | 7.9 | 100 |
using the ratio of previous applications, intracellular pH was estimated directly from the absorbance of carboxyfluorescein in cell suspensions containing 3 μM carboxyfluorescein diacetate for 20 min at 25 °C. After separation by centrifugation (8000 × g, 10 s) the cells were resuspended in 2 ml of 120 mM NaCl, 1 mM MgCl₂, 5 mM fructose, and 50 mM Na morpholinoethane sulfonate at the indicated pH. Difference absorbance (ΔAₘₐₓ – ΔAᵦₐₐₜ) was monitored in a stirred cuvette at 25 °C with the indicated additions of 100 μg of digitonin (FIG. B). The changes in difference absorbance after digitonin addition determined above (Δ) were fit by a least squares linear regression analysis. Also shown (– – –) are observed absorbance changes from an identical experiment in which 120 mM KCl replaced NaCl in the resuspension medium. The initial internal pH (pHᵢ) corresponding to the interpolated null absorbance response (indicated by the arrows) together with internal chromophore concentrations (c), determined fluorimetrically as described in Table II, were used with coefficients pKᵢ,c, and Cᵢ to calculate the apparent pH change associated with the change in difference absorbance (Δ(A)) induced by addition of digitonin to cells suspended in media containing 120 mM NaCl (Δ) or 120 mM KCl (Δ):

$$
\Delta pH = pH^\prime - pH^0 = pK + \log \frac{C_i + \Delta}{C_i - \Delta} - pH^0
$$

produced a null response 0.03–0.05 pH units higher.

Because difference absorbance does not change in the period prior to addition of digitonin, it may be assumed that sperm internal pH remains constant following resuspension in media buffered between pH 6 and 7. The magnitude of the ΔpH that follows digitonin addition can be calculated from the observed null pH, the observed absorbance change (Fig. 5B), determined internal carboxyfluorescein content (1.7 ± 0.2 μM in this experiment assuming a cytosol volume of 20 μm³ based upon esr determinations of intracellular H₂O space, Ref. 31), and from the observed dependence of Δᵦ upon pH (Table I). Close agreement between calculated ΔpH and the ΔpH expected for release of intracellular dye to the media indicates that absorbance coefficients of the intracellular chromophore closely resemble coefficients in vitro.

Internal pH by Other Protocols, and Artifacts Resulting from Light Scattering and High Internal Dye Concentrations—In previous applications, intracellular pH was estimated directly from the absorbance of carboxyfluorescein in cell suspensions using the ratio A₄₉₀/A₆₆₀ (21) or by observation of the fluorescence emission intensity ratio, A₁₅₃/A₁₅₅, of intracellular fluorescein conjugates (17–19). Fig. 6A shows that the pH reported by absorbance measurements of extracellular carboxyfluorescein in more dense cell suspensions becomes increasingly acidic. The effect is most pronounced in media with pH > 7 (and presumably also at pH < 6) where difference absorbance becomes nonlinear with pH (Fig. 1). Typical of a light-scattering artifact, the effect is readily reversed by removal of the cells by centrifugation, is alleviated by increasing the refractive index of the medium with Ficoll (data not shown), and is much less evident for determinations employing fluorescence emission measurements utilizing perpendicular geometry. In this physically complex system, instrumental limitations (32) also may contribute to aberrant absorbance responses from dense suspensions. Therefore, corrections with a theoretical basis are not available currently. Although an empirical correction conceivably could be derived, for the subsequent experiments reported here we chose the alternative of restricting absorbance measurements to sperm suspension of less than 2 × 10⁶ cells/ml.

The pH reported by carboxyfluorescein contained in dilute sperm suspensions that were allowed to generate increasing quantities of the intracellular chromophore also depends upon the spectral parameter examined (Fig. 6B). Apparent intracellular acidification with increasing internal dye concentra-

![Graph](https://example.com/graph.png)

**Fig. 5.** Utilization of digitonin for null point determination of the cytosolic pH of bovine sperm. A, twice washed sperm (2.5 × 10⁷ cells) were incubated in loading buffer containing 3 μM carboxyfluorescein diacetate for 20 min at 25 °C. After separation by centrifugation (8000 × g, 10 s) the cells were resuspended in 2 ml of 120 mM NaCl, 1 mM MgCl₂, 5 mM fructose, and 50 mM Na morpholinoethane sulfonate at the indicated pH. Difference absorbance (ΔAₘₐₓ – ΔAᵦₐₐₜ) was monitored in a stirred cuvette at 25 °C with the indicated additions of 100 μg of digitonin (FIG. B). B, the changes in difference absorbance after digitonin addition determined above (– – –) were fit by a least squares linear regression analysis. Also shown (– – –) are observed absorbance changes from an identical experiment in which 120 mM KCl replaced NaCl in the resuspension medium. The initial internal pH (pHᵢ) corresponding to the interpolated null absorbance response (indicated by the arrows) together with internal chromophore concentrations (c), determined fluorimetrically as described in Table II, were used with coefficients pKᵢ,c, and Cᵢ to calculate the apparent pH change associated with the change in difference absorbance (Δ(A)) induced by addition of digitonin to cells suspended in media containing 120 mM NaCl (Δ) or 120 mM KCl (Δ):

$$
\Delta pH = pH^\prime - pH^0 = pK + \log \frac{C_i + \Delta}{C_i - \Delta} - pH^0
$$

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The pH reported by carboxyfluorescein contained in dilute sperm suspensions that were allowed to generate increasing quantities of the intracellular chromophore also depends upon the spectral parameter examined (Fig. 6B). Apparent intracellular acidification with increasing internal dye concentra-

![Graph](https://example.com/graph.png)

**Fig. 6.** Interfering effects of light scattering and high internal dye concentrations on pH determinations using the fluorescein chromophore. A, washed bovine sperm (6–500 × 10⁶ cells, as indicated) were suspended in media containing 5 μM carboxyfluorescein (CFₘₚₖ), 120 mM NaCl, 1 mM MgCl₂, and either 50 mM K morpholinoethane sulfonate (pH 6.8) or 50 mM K morpholinoethane sulfonate (pH 7.75). Absorbance spectra (versus 465 nm) were collected and the difference absorbance (ΔAₘₐₓ – ΔAᵦₐₐₜ), determined manually, was used to derive ΔpH and the apparent pH calculated from found in Table I. Apparent pH was also determined from the fluorescence emission intensity ratio of A₄₉₃/₄₅₀ to A₆₃₅/₆₁₅ in the same manner for samples prepared with 0.5 μM carboxyfluorescein. Difference absorbance determination, pH 6.6, C; pH 7.75, O. Fluorescence emission ratio determinations, pH 6.6, □; pH 7.75, ■. A, aliquots of washed sperm (1.5 × 10⁶ cells) were incubated as in Fig. 4 in 1.2 ml of loading buffer containing 0, 2, 5, 10, or 20 nmol of carboxyfluorescein diacetate. After centrifugation (8000 × g, 10 s) cells were resuspended in 2 ml of 120 mM NaCl, 1 mM MgCl₂, 5 mM fructose, and 50 mM morpholinoethane sulfonate at pH 5.8, 6.2, 6.6, or 6.8. Δ, absorbance spectra (450–550 nm, split beam mode) of loaded cells (versus mock-loaded cells) were collected for calculation of apparent pH directly from A₆₃₅/₆₅₀ ratios of the suspensions. A, difference absorbance (ΔAₘₐₓ – ΔAᵦₐₐₜ, dual beam mode) measurements were performed on the same suspensions containing the addition of digitonin (100 μg) to allow calculation of the null point pH as described in Fig. 5. Identical samples were prepared for fluorometric determinations. The fluorescence emission ratios of A₄₉₃/₄₅₀ to A₆₃₅/₆₁₅ were examined before and after addition of digitonin and used to calculate apparent internal pH either directly (○) or by the null point method (□) using coefficients found in Table I. Intracellular and extracellular chromophore content was determined fluorometrically as described in Table II and intracellular concentrations were calculated on the basis of a 20 μm³ H₂O space for the bovine spermatozoan.
tion is more pronounced for determinations made from absorbance than from fluorescence measurements.

Proton liberation from the enzymatic hydrolysis of the diacetae precursor is unable to account for the observed apparent internal acidification because of the high proton buffer concentration of the sperm cytosol (see below). Instead, this effect may arise, at least in part, from an alkaline shift of the pH for the protonation of carboxyfluorescein determined in vitro by difference absorbance measurements at concentrations in excess of 1 mM (Fig. 3) where interactions of the chromophore with itself presumably become important.

Regardless of the complete explanation, it is encouraging that extrapolation to infinitely dilute internal dye concentration provides estimates that are equivalent, within experimental error, for the pH of the sperm cytosol from determinations employing either direct fluorescence or absorbance (ratio) methods or from null point determinations based upon differential measurements of fluorescence or absorbance (Table III). Therefore, subsequent determinations also employed internal chromophore concentrations below 1 mM to minimize this artifact.

**Determination of Internal K⁺ Concentration by Null Point Measurements Employing Nigericin**—The ability of the carboxylic acid ionophore nigericin to promote the transmembrane exchange of H⁺ for K⁺ can be used to lower (Fig. 4) or raise the intracellular pH by placing sperm in media containing, respectively, K⁺ lower or higher than the intracellular concentration. If this medium is buffered at a pH equivalent to the intracellular pH, then the generated transmembrane proton gradient, detected by the intracellular carboxyfluorescein chromophore, provides the basis for a null point determination of internal K⁺ concentration. Fig. 7A demonstrates the difference absorbance response that follows addition of nigericin to sperm loaded with carboxyfluorescein in a preliminary incubation, then suspended in medium at pH 6.6 containing various K⁺ concentrations from 12–115 mM. In these experiments, with various Li⁺ concentrations used to keep the media at constant ionic strength, the rapid change in absorbance was maintained for 1 min or more, indicating that the generated steady state proton gradient is relatively stable. Subsequent addition of digitonin produces a rapid release of cellular chromophore and the return of absorbance to the original value, providing assurance of equivalent initial internal and external pH. By interpolation to the absorbance null point (Fig. 7B), initial internal K⁺ concentration was estimated as 114 mM in this experiment. In four separate experiments, the mean value (±S.D.) was 120 ± 5 mM. The change in internal pH induced by nigericin was calculated by comparison to the spectral response that followed addition of digitonin alone to identical suspensions in media at various pH values (as in Fig. 6). The observed absorbance change

| Method | Cytosolic pH |
|--------|--------------|
| Absorbance ratio ($A_{450}/A_{600}$) | 6.63 ± 0.04 |
| Null absorbance change ($A_{600}$ - $A_{450}$) | 6.59 ± 0.05 |
| Fluorescence emission change ($F_{450}/F_{600}$) | 6.55 ± 0.05 |
| Null fluorescence change ($F_{450}/F_{600}$ - $F_{600}$) | 6.50 ± 0.05 |

(And calculated ΔpH) at high K⁺ concentrations was equivalent in media that contained either Li⁺ or Na⁺. However, at low K⁺ concentrations, in media containing Na⁺, the generated pH gradient was unstable (data not shown). The observed decay towards the initial absorbance probably reflects the countermovement of protons resulting from Na⁺/H⁺ exchange promoted by nigericin, consistent with the transport selectivity $K^+ > Na^+ > Li^+$ observed for nigericin in other systems (25, 27). Despite this instability, maximal absorbance changes and calculated ΔpH provide similar estimates of the null point K⁺ concentration.

**Selectivity of Transport by Monensin and Null Determinations of Internal Na⁺ Concentrations**—The isomeric forms, A, B, and C of the carboxylic acid ionophore monensin, all promote the transmembrane exchange of Na⁺ for H⁺ (22). However, only monensin B possesses sufficient transport selectivity to allow null point determination of intracellular Na⁺ in media utilizing replacement by Li⁺ to maintain ionic strength (Fig. 8). Addition of monensin B to cells loaded with carboxyfluorescein in a preliminary incubation and suspended in media with either high (tracing a) or low (tracing b) initial Na⁺ produced a stable absorbance change that was rapidly and quantitatively reversed by subsequent addition of digitonin. Under identical conditions, addition of monensin C produced an absorbance response that rapidly decayed towards the initial value (tracings c and d), presumably as a result of opposing exchanges of external Li⁺ for internal H⁺ or of internal K⁺ for external H⁺, or both. The stability of the absorbance responses induced by monensin A (tracings e and f) was intermediate between those of isomers B and C. This
Internal Cation Concentrations and Ionophore Action

96 mM Na\(^{+}\), 24 mM Li\(^{+}\)

24 mM Li\(^{+}\), 114 mM Li\(^{+}\)

Fig. 8. Ion transport selectivity of the monensin isomers A, B, and C. Sperm were loaded with carboxyfluorescein in a preliminary incubation as in Fig. 5, then suspended in 2.0 ml of media containing 1 mM MgCl\(_2\), 50 mM Li morpholinoethane sulfonate (pH 6.6), and the indicated concentration of NaCl, KCl, and LiCl. Difference absorbance (A\(_{in} - A_{ex}\)) was monitored during subsequent additions of monensin (MON, 10 nmol) and digitonin (DIG, 100 µg).

ranking of transport selectivity is consistent with that observed previously (22) for Li\(^{+}\) transport in mitochondrial preparations. It should be noted also that even for monensin B, careful selection of ionophore concentration is required to maximize the rate of generation of the proton gradient resulting from exchange for Na\(^{+}\) and to minimize its decay by competing processes.

Fig. 9 summarizes a null point determination of the internal Na\(^{+}\) concentration of bovine sperm utilizing monensin B and media with either Li\(^{+}\) or K\(^{+}\) replacement. An interpolated null absorbance change (and calculated null pH change) were observed at 14 mM Na\(^{+}\) in media containing Li\(^{+}\). In four similar experiments the mean value (+S.D.) was 14 \pm 2 mM.

A Predictive Model for Steady State Cation Distributions Induced by Nigericin or Monensin—The validity of the null point determinations described above do not depend upon the exact stoichiometry of exchange nor upon the steady state distributions of protons and cations achieved after ionophore addition. However, these distributions are of interest for other reasons as, for example, in studies where ionophore-induced increases in membrane permeability are employed as criteria to assess the involvement of cation movements in hormone action or other regulatory phenomena.

Consider, therefore, a simple two-compartment model (Fig. 10) consisting of an extracellular space of infinite volume and an intracellular space bounded by a membrane across which an initial M\(^{+}\) (metal) ion concentration gradient (M\(_{ex}\)/M\(_{in}\)), but no proton gradient exists (H\(_{ex}\) = H\(_{in}\)).\(^{2}\) Introduction of a carboxylic acid ionophore which promotes the 1:1 exchange of M\(^{+}\) (metal) for H\(^{+}\), and an infinite extracellular volume (pK\(_{ox}\) = pH\(_{in}\) and M\(_{Z}\) = M\(_{in}\)), thermodynamic equilibrium after ionophore addition is achieved when the generated proton gradient is equal to the remaining metal ion gradient:

\[
H_{ex}^{+} = 1; \quad i = H_{in}^{+}
\]

\[
M_{ex}^{+} = 10; \quad 100 = M_{in}^{+}
\]

\[
H_{in}^{+} = 1; \quad i = H_{in}^{+}
\]

\[
M_{ex}^{+} = 10; \quad 92 = M_{in}^{+}
\]

\[
100 - x = \frac{M_{in}^{+}}{M_{ex}^{+}} = \frac{H_{in}^{+}}{H_{ex}^{+}} = 1 \times x, \quad x = 8.2
\]

A test was devised for the application of this model to the bovine sperm system under examination. If sperm are suspended in media with no initial transmembrane metal ion

\[^{2}\]The following conventions are employed in super- and subscript notation; \(i\), initial; \(s\), steady state; \(m\), intracellular; \(e\), extracellular.
gradient, imposition of an initial pH gradient and introduction of ionophore should result in a steady state pH gradient equivalent to that observed previously (Fig. 7, 8) with an initial metal ion gradient of equal magnitude. Fig. 11 compares steady state pH gradients generated following addition of ionophore to sperm subjected to such initial gradients of protons, of Na⁺ or of K⁺. Within experimental error the slopes are equivalent, and utilization of the proposed model therefore is supported by measurement of accessible experimental parameters.

In terms of the net molar movements, \( x \), of metal ions and protons, the model depicted in Fig. 10 also indicates that at steady state:

\[
\frac{H_n}{H_m} = \exp (-\Delta pH_{e}^c) = \frac{M_n^x - x}{M_m^x}
\]

Solving for \( x \),

\[ x = M_n^x - (M_m^x \exp (-\Delta pH_{e}^c)). \]

It thus becomes evident that determination of the initial internal pH and metal concentration and the steady state internal pH by the null point methods described above allows calculation of the steady state distribution of cations between intra- and extra-cellular compartments.

Buffering of the internal proton concentration imposes the additional relationship

\[
\lim_{\Delta pH \to 0} \Delta pH_{H^+} = \frac{dx}{dpH} \cdot dx.
\]

Differentiation of the Henderson-Hasselbalch equation (33) provides

\[
\frac{dx}{dpH} \approx \frac{2.3 \left[ K' \cdot [H^{+}] + [H^{+}]^2 + [OH^{-}] \right]}{[B]}
\]

where \([B]\) is the total internal weak acid buffer concentration and \(K'\) its dissociation constant. If we restrict consideration to that region near the pK' of the buffer, then \( \frac{dx}{dpH} \) becomes maximal and

\[
\left( \frac{dx}{dpH} \right)_{\text{max}} = \frac{2.3}{4} [B]
\]

Graphical evaluation of \( \Delta x/\Delta pH \) therefore provides additionally a measure of internal proton buffer capacity.

Typical data for bovine sperm are shown in Fig. 12. The mean value (± S.D.) for total internal proton buffer concentration in three such experiments was 190 ± 20 meq/liter.

**DISCUSSION**

Previous work from other laboratories indicated that either fluorescence or absorbance responses of intracellular fluorescein chromophore are a powerful tool for examination of intracellular pH in cell suspensions (16), cell monolayers (18,
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19) or individual cells (17). We provide here absorbance and fluorescence coefficients for the spectral titration of carboxyfluorescein in vitro (Figs. 1 and 2, Table I) that confirm the relative insensitivity of the spectral properties to otherionic constituents and indicate the suitability of determinations of intracellular pH that utilize intracellular carboxyfluorescein concentrations of less than 1 mM.

For bovine sperm, like ascites tumor cells (16), intracellular carboxyfluorescein, rapidly generated from its permeant diacetate derivative (Table II), is apparently localized to the cytosol where it is retained for long periods in cell suspensions with media buffered between pH 6 and 8. In experiments using limited internal dye and total cell concentrations, good agreement was found (Table III) upon comparison of ratio-metric and null point methods for determination of intracel-lular pH that were, respectively, either dependent or independent of the assumption of equivalent absorbance or fluores-cence coefficients for intra- and extracellular carboxyfluorescein. The assumption of equivalence, therefore, seems justified.

Such estimations of intracellular pH by calculations based upon either direct spectral measurements of internal chromo-mere or upon the spectral changes that follow release of internal chromophore, like estimates obtained by other tech-niques, are qualified by certain considerations concerning intracel-lular compartmentation (94-36). These qualifying considerations are of particular interest for mammalian sperm because some evidence (13-15) suggests that sperm may main-tain a very acidic acrosomal organelle.

Microscopic examination reveals that fluorescence from intracellular carboxyfluorescein is distributed uniformly in the bovine sperm and digitonin induces quantitative release of chromophore (Table II). Therefore, the spectral alterations that follow addition of digitonin to cell suspensions probably contain components derived from each cytosolic compartment of these cells, the relative contributions of which depend upon compartment volume and internal dye concentration. Recent examination of intracellular pH during the sea urchin sperm acrosome reaction revealed an increase of 0.1 in the average internal pH (11), presumably as a consequence of the accom-panying loss of the small and probably acidic acrosomal organelle of these cells. The relatively small acrosome of the bovine sperm may make a similar contribution to the cytosolic pH determined here. However, preliminary determinations in guinea pig sperm, which possess a much larger acrosome, do not indicate a correspondingly more acidic average cyto-solic pH. Furthermore, examination of similar preparations of bovine and hamster sperm by noninvasive 31P-NMR techniques also indicate a cytosolic pH of 6.5-6.6 but do not detect any more acidic cellular compartment containing ATP or P.

If sperm contain an acidic acrosome devoid of ATP and P, then 31P-NMR should report a higher average intracellular pH than the null point method utilized here. The data obtained so far thus do not indicate the presence of a strongly acidic acrosome compartment in epididymal sperm preparations. Perhaps examination of the spectral properties of the fluorescein chromophore within localized subcellular areas may resolve this problem.

Cytosolic pH determined here for bovine sperm utilizing endogenous or glycolytic substrates falls in the range 6.5-6.6. For comparison, the reported pH of rat sperm homogenates is 6.5 (7) and the internal pH of sea urchin sperm calculated from reported (11) distribution ratios of weak bases and acids ranges from 6.6-8.0.

The present study expands the null point concept that was first applied to examination of ion distributions in cellular systems by Hoffman and Laris (37). In addition to null point determinations of internal pH, the techniques developed here also extend utilization of the spectral responses of the fluo-rescein chromophore to examination of other aspects of the intracellular ionic environment. Estimation of intracellular concentrations of K' and Na' is achieved by coupling the pH dependent spectral response of intracellular fluorescein chromophore to the exchange of protons for cations that are promoted by the ionophores nigericin and monensin B (Figs. 7 and 9). These determinations also are subject, in their interpretation, to considerations of compartmentation with additional complications resulting from ionophore action on cation and proton gradients across the mitochondrial mem-brane. Recent determinations of internal pH for rat liver mitochondria in situ (38) and of intramitochondrial Na and K content for muscle tissue (39) are available. Although relative mass densities of K are evenly distributed in sperm (40), absolute Na' and K' contents of sperm mitochondria are unknown. Assuming a ΔpH of 1 across the mitochondrial membrane, and a mitochondrial volume of 2.8 μm3 (41) compared to a total cytosolic volume of 20 μm3 (31), it can be calculated that determined null point K' (120 mM) and Na' (14 mM) concentrations may be respectively as much as 10% higher or lower than the concentrations actually present in the extramitochondrial compartment(s) of the sperm.

Previous estimates (42) of intracellular K' (49 mM) and Na' (21 mM) concentrations for bovine epididymal sperm relied upon chemical determination of total metal content after extensive washing and storage for unspecified periods (with probable deleterious consequences) or employed correction for extracellular medium present in the cell pellet collected by centrifugation. The large correction required for these asymmetrical cells led others (43) to attempt analyses uncorrected for trapped media, with the conclusion that sperm are unable to maintain cation concentration gradients. Data provided in Figs. 7 and 9 clearly indicate that this conclusion is incorrect.

Null point determinations of internal cation concentrations do not require detailed knowledge of the proton and cation fluxes promoted by ionophore addition. However, monovalent and divalent cation ionophores also are employed to promote alterations in intracellular cation concentrations and thereby assess the involvement of ion fluxes as regulatory mediators. For this application, it is appropriate and necessary to consider the ion redistributions that result from ionophore addi-tion.

A model is presented here that is based upon simple ther-modynamic considerations and that allows calculation of steady state cation distributions. This model is supported in its application to bovine sperm by measurement of steady state internal pH reached after ionophore-induced equilibra-tion of initial gradients of either K' or H'. Additional support would require determination by other methods of the steady state internal metal ion concentrations. Given its simple theoretical basis, partial experimental support and the technical difficulty of obtaining full confirmation, it seems reason-able to grant provisional validity to this predictive model. Other interesting relationships then emerge.

One of these is the ability to estimate internal proton buffer capacity utilizing the relationship between dpH/dz and total buffer concentration that was first recognized by Van Slyke (33). The calculated internal buffer capacity for bovine sperm found in these studies (190 meq/liter) is 2-fold or more greater.
than that reported for a variety of other tissues by other methods (36) and is apparently sufficient to prevent significant acidification of the cell interior during the enzymatic generation of intracellular carboxyfluorescein.

It is instructive also to consider the consequences of ionophore addition to systems that contain initial transmembrane gradients of both protons and metal ions. In the model system of Fig. 10, imposition of an inward-directed proton gradient (H<sub>e</sub>, > 1) results in increased proton uptake and metal ion release from the intracellular space. Making the exterior more basic (0.1 < H<sub>e</sub>, > 1) results in less extensive release of metal ions and acidification of the interior. When H<sub>e</sub>, < 0.1, the magnitude of the initial proton gradient exceeds that of the initial metal ion gradient and uptake rather than release of metal ion occurs. Thus, as a practical cautionary note, it should be recognized that metal ion fluxes resulting from addition of nigericin or monensin may not be assumed to follow from estimates of initial metal ion gradients alone.

It is common practice in the study of cellular systems to utilize media buffered between pH 7.5-8.0, whereas intracellular pH is generally in the range 6.5-7.0 (40). The considerations above suggest that interpretation of subsequent responses are greatly simplified if, prior to addition of carboxylic acid ionophores to such systems (in minimal concentrations simple null point determination or by other means.

Acknowledgment—I am deeply indebted to Profs. H. A. Lardy for support and encouragement of this project and for providing access to equipment and materials vital to its completion. I am also grateful for the expert technical assistance of Susan Wielgus.

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SPECTRAL PROPERTIES OF FLUORESCIN AND CARBOXYFLUORESCIN

Appendix to Examination of the Intracellular Ionic Environment and of Ionophore Action by Null Point Measurements Employing the Fluorescein Chromophore

F. Babcock and Glouar Klmp

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Table I. Spectral Coefficients for the Fluorescein Chromophore

| Conditions | K | C | Pk |
|------------|---|---|----|
| 50 nM MES/MOPS | 0.49 | 43.5 | 6.40 |
| 100 nM MES/MOPS | 0.47 | 42.7 | 6.42 |
| 150 nM MES/MOPS | 0.45 | 41.9 | 6.44 |
| 50 nM MES/MOPS | 0.43 | 41.1 | 6.50 |
| 100 nM MES/MOPS | 0.41 | 40.4 | 6.52 |
| 150 nM MES/MOPS | 0.39 | 39.6 | 6.56 |
| 0.25 M KCl | 0.42 | 12.0 | 6.58 |

Table II shows the determined spectral properties of fluorescein and carboxyfluorescein. The absorption coefficient of each dye was determined at 515 nm using a Hitachi U-2000 spectrophotometer. Spectra were corrected for background absorption of buffer determined in the same cuvette. For each dye, the emission wavelength of each dye was measured relative to a reference dye, fluorescein or carboxyfluorescein. The emission intensity was linearly proportional to the concentration of each dye within the range of 0.05 to 0.65 mM. For carboxyfluorescein, the emission intensity increased with increasing pH, while for fluorescein, the emission intensity decreased with increasing pH. The relative emission intensity of each dye was determined at pH 7.4 in 50 mM MES/MOPS buffer. For fluorescein, the emission intensity was 6.5 times greater at pH 6.5 than at pH 7.4. For carboxyfluorescein, the emission intensity was 6.5 times greater at pH 7.4 than at pH 6.5. The results are shown in Table II.

Table II. Spectral Coefficients for the Carboxyfluorescein Chromophore

| Conditions | K | C | Pk |
|------------|---|---|----|
| 50 nM MES/MOPS | 0.42 | 50.0 | 6.10 |
| 100 nM MES/MOPS | 0.40 | 49.0 | 6.06 |
| 150 nM MES/MOPS | 0.38 | 48.0 | 6.02 |
| 50 nM MES/MOPS | 0.36 | 47.0 | 5.98 |
| 100 nM MES/MOPS | 0.34 | 46.0 | 5.94 |
| 150 nM MES/MOPS | 0.32 | 45.0 | 5.90 |

For absorption and fluorescence measurements, carboxyfluorescein was used at concentrations of 0.5 to 5.0 mM in 50 mM MES/MOPS buffer. The emission intensity was linearly proportional to the concentration of each dye within the range of 0.05 to 0.65 mM. For carboxyfluorescein, the emission intensity increased with increasing pH, while for carboxyfluorescein, the emission intensity decreased with increasing pH. The relative emission intensity of each dye was determined at pH 7.4 in 50 mM MES/MOPS buffer. For fluorescein, the emission intensity was 6.5 times greater at pH 6.5 than at pH 7.4. For carboxyfluorescein, the emission intensity was 6.5 times greater at pH 7.4 than at pH 6.5. The results are shown in Table II.
Examination of the intracellular ionic environment and of ionophore action by null point measurements employing the fluorescein chromophore.

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J. Biol. Chem. 1983, 258:6380-6389.

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