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Intracellular pH Measurements in Ehrlich Ascites Tumor Cells Utilizing Spectroscopic Probes Generated in Situ†

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ABSTRACT: The uncharged, colorless molecule fluorescein diacetate diffuses into Ehrlich ascites tumor cells at neutral pH, where intracellular esterases release the chromophore fluorescein. The negatively charged dye is retained by the cell, permitting the intracellular pH to be estimated from the shape of the pH-dependent absorption spectrum. The diacetate derivative of 6-carboxyfluorescein may be used similarly and has the additional advantage of a slower rate of leakage out of the cell but requires incubation at pH 6.2 to facilitate initial entry into the cell. After removal of external dye by centrifugation, 80–92% of the remaining dye is unresponsive to external pH changes. Calibration of the intracellular fluorescein spectra is obtained by equilibration of the internal and external pH with nigericin in K^+ buffers. Results of intracellular pH measurements by this method are in good agreement with those obtained by measuring the distribution ratio of the weak acid 5,5-dimethyl[2-^{14}C]oxazolidine-2,4-dione, under a variety of metabolic conditions. Besides the accurate estimation of intracellular pH, the method permits the kinetics of intracellular pH changes as small as 0.01 to be followed. Intracellular fluorescein reports pH changes occurring in both the cytoplasmic and the mitochondrial compartments, whereas 6-carboxyfluorescein reports only the cytoplasmic compartment. At equivalent concentrations, nigericin is more effective than valinomycin plus the protonophore 1799 in dissipating plasmalemma pH gradients. Either is effective at lower concentrations in dissipating mitochondrial pH gradients. Addition of glucose to Ehrlich ascites cells results in a transient acidification of the cytoplasm in close correspondence to the intracellular lactate levels. The transient acidification can be explained by the initial rapid rate of glycolysis exceeding the rate of lactate export.

Diacyl derivatives of fluorescein have been used as sensitive indicators for determining the presence of intracellular esterases. The colorless, neutral diacetate compound diffuses into the cell, where intracellular enzymes release the strong chromaphore fluorescein ($E_{490}^{mM} = 90$; quantum yield ≈ 0.9) by hydrolytic removal of the acetate groups. The intracellular retention of fluorescein has been used to monitor the intactness of cell membranes (Rotman & Papermaster, 1966) and to distinguish live from dead cells in cell sorting devices (Bonner et al., 1972). In addition, fluorescence polarization measurements on intracellular fluorescein have been used to determine the viscosity of the cell cytosol in *Euglena* and yeast (Burns, 1969; Cercek & Cercek, 1972).

Since the spectrum of fluorescein is highly dependent on pH (Fothergill, 1964), a natural extension of these previous

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studies is to utilize the trapped fluorescein to determine intracellular pH. We have previously reported (Thomas et al., 1976) the use of the fluorescence excitation spectrum of fluorescein to estimate the internal pH of a procaryote, *Bacillus acidocaldarius*. In this report, the absorption spectra of intracellular fluorescein and its 6-carboxy derivative are used to monitor the internal pH of Ehrlich ascites tumor cells.

Materials and Methods

Cells. Ehrlich ascites tumor cells were maintained in mice, as previously described (Scholnick et al., 1973). Cells were harvested after 7–10 days of growth and washed twice with a buffer a pH 7.4 containing 110 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 4 mM sodium phosphate, and 50 mM Hepes (*N*-2-hydroxyethylpiperazine-*N'*-ethanesulfonic acid).

Synthesis of the Diacetate Derivative of 6-Carboxyfluorescein. The diacetate derivative of 6-carboxyfluorescein was synthesized by a modification of the method for the synthesis of fluorescein diacetate (Orndorff & Hemmers, 1927). Five grams of 6-carboxyfluorescein was refluxed with 30 mL of acetic anhydride for 3 h. The excess acetic anhydride was then removed under reduced pressure by rotary evaporation at 40 °C. The residue was dissolved in 50 mL of tetrahydrofuran, and water was added until the onset of turbidity. The turbidity was cleared with additional tetrahydrofuran, and the solution was stirred 4 h at room temperature. After addition of 50 mL of water, the solution was extracted twice with 60 mL of chloroform; 0.5 g of charcoal and 1.0 g of silica gel were added to the chloroform extract and the mixture was stirred overnight. After filtration, the solvent was removed under reduced pressure, and the resultant oil was dissolved in a small amount of benzene at room temperature. Crystals of 6-carboxyfluorescein diacetate formed upon standing for several days at room temperature: yield, 70%. The sample was recrystallized in chloroform–acetone (1:1): mp 145 °C (dec).

Synthesis of the Ethyl Ester of 6-Carboxyfluorescein Diacetate. One gram of 6-carboxyfluorescein was refluxed with 15 mL of acetic anhydride for 90 min. The reaction mixture was evaporated to dryness on a rotary evaporator. The product was dissolved in 20 mL of chloroform and washed four times with 40 mL of water in a separatory funnel. The chloroform layer was dried over anhydrous $MgSO_4$, and the solvent was removed by rotary evaporation. The residue was refluxed with 20 mL of $SOCl_2$ for 1 h. $SOCl_2$ was removed by rotary evaporation, and the residue was dissolved in 5 mL of tetrahydrofuran. The tetrahydrofuran solution was added dropwise with stirring to a stoichiometric amount (based on the starting amounts of 6-carboxyfluorescein) of sodium ethoxide in excess ethanol. The resulting solution was centrifuged, and the solvent was removed in a rotary evaporator. The remaining oil was dissolved in 30 mL of chloroform and washed several times with water in a separatory funnel. The chloroform solution was stirred with 0.5 g of charcoal overnight, filtered, and dried over $MgSO_4$, and the residual solvent was removed by rotary evaporation. The resulting yellow oil (yield, 55%) showed characteristic triplet and quartet signals for the ethoxy group (CH_2 = 4.35 ppm and CH_3 = 1.40 ppm downfield from Me_4Si) in ^1H NMR which were absent in the spectrum of the starting material.

Thin-Layer Chromatography of Fluorescein Derivatives. The fluorescein compounds (1 μg) dissolved in dimethyl sulfoxide were spotted on plastic sheets coated with silica gel 60. The plates were developed in toluene–acetic acid (9:5). Locations of the fluorescein derivatives were determined under long-wavelength UV light; the colorless acetylated derivatives required incubation in an ammonia atmosphere to make them visible.

Loading of Cells with Fluorescein or 6-Carboxyfluorescein. To obtain Ehrlich ascites cells with either fluorescein (F cells)[1] or 6-carboxyfluorescein (CF cells) trapped within, cells were suspended at 6 mg of protein/mL in 50 mM Mes [2-(*N*-morpholino)ethanesulfonic acid] buffer, pH 6.2, containing 110 mM NaCl, 5 mM KCl, and 1 mM $MgCl_2$. Fluorescein diacetate (final concentration 6 μM) or 6-carboxyfluorescein diacetate (final concentration 35 μM) was added from a 10 mM stock solution in dimethyl sulfoxide. Control cells were incubated simultaneously with an equivalent amount of dimethyl sulfoxide. After incubation for 15 min on ice, the cells were diluted with two volumes of ice-cold buffer at pH 7.4, containing 40 mM Hepes, 110 mM NaCl, 5 mM KCl, and 1 mM $MgCl_2$, and centrifuged at 2000*g* for 5 min at 4 °C. The supernatant was decanted, and the cells were washed once more with pH 7.4 buffer. The washed cells were finally suspended at 25 mg of protein/mL and kept on ice until use.

Spectrophotometric Measurements. Absorbance measurements were made in an Aminco DW-2 spectrophotometer with a thermostated cell compartment. The absolute spectra of the fluorescein- or 6-carboxyfluorescein-loaded cells were determined vs. control cells at the same concentration, generally at 2.5 mg of protein/mL. Unless otherwise stated, measurements were made at 20 °C.

Measurements of Intracellular pH by the Distribution Ratio of a Weak Acid or Base. Intracellular pH was measured by the distribution of the weak acid DMO (5,5-dimethyl[2-^{14}C]oxazolidine-2,4-dione) by a modification of published methods (Poole et al., 1964), by using [^3H]inulin to correct for the extracellular water space. The internal water space was determined in duplicate experiments with ^3H_2O, by using [^{14}C]inulin to correct for extracellular water. After incubation of the cells for 5 min, 0.2-mL aliquots were layered on microcentrifuge tubes which contained 0.1 mL of a silicone fluid mixture [5.2 parts Versiube F 50:1 part SF 96(50)], floating on 0.1 mL of 5% trichloroacetic acid in 8% glycerol (Portis & McCarty, 1973). The tubes were immediately centrifuged in a Coleman microfuge for 30 s; aliquots of both the supernatant and the pellet were counted.

Scintillation Counting. All samples were counted in ACS (Amersham Corp., Arlington Heights, IL) in a Beckman L.S. 7000 scintillation spectrometer, by using the automatic quench compensation.

pH Measurements. pH changes in the external buffer were followed with a Corning Model 12 research pH meter with a Sargent Welch combination pH electrode (Model S-36070–10) and a Heath recorder.

Assays. Lactate was assayed enzymatically (Hohorst, 1962), and protein was assayed colorimetrically (Lowry et al., 1951).

Assay for Esterase Activity. Esterase activity was measured at 490 nm vs. 464-nm reference in the Aminco DW-2 spectrophotometer. One aliquot of enzyme was added to a cuvette containing 6 μM fluorescein diacetate, 50 mM Hepes, pH 7.4, 110 mM NaCl, 5 mM KCl, and 1 mM $MgCl_2$ in a total volume of 3 mL. One unit of activity was equal to 1 nmol of fluorescein formed per min at 20 °C.

Chemicals. Fluorescein diacetate, ouabain, rotenone, valinomycin, NAD^+, L-lactate dehydrogenase (EC 1.1.1.27), L-lactic acid, quercetin, Hepes, Mops, and Mes were purchased

[1] Abbreviations used: F cells and CF cells represent respectively cells with fluorescein or 6-carboxyfluorescein trapped within.

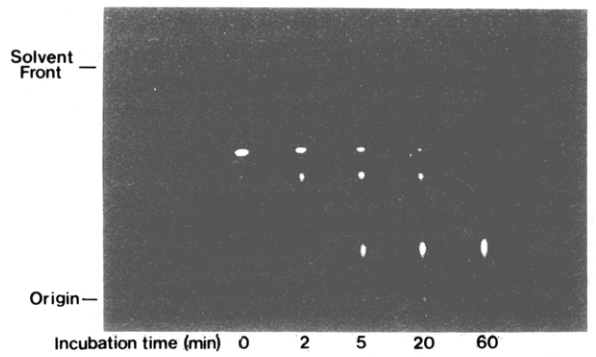

FIGURE 1: Thin-layer chromatography of enzymatic hydrolysis products obtained from 6-carboxyfluorescein diacetate. 6-Carboxyfluorescein diacetate (1 mM) was incubated with Ehrlich ascites cells (22.5 mg/mL) in a 50 mM Hepes buffer (pH 7.4) containing 110 mM NaCl, 5 mM KCl, and 1 mM $MgCl_2$. At the indicated time intervals, 10-mL aliquots of these cell suspensions were spotted directly on the TLC plates and dried. The plates were developed as described under Materials and Methods, by relying on the solvents to extract the fluorescein derivatives.

from Sigma Chemical Co., St. Louis, MO. 6-Carboxyfluorescein was a project of Eastman Kodak, Rochester, NY. [^{3}H]Methoxyinulin (199 mCi/g), [^{14}C]inulin (2.29 mCi/g), [^{14}C]DMO (49.9 mCi/mmol), and ^3H_2O (25 mCi/mg) were obtained from New England Nuclear, Boston, MA; fluorescein (free acid) was purchased from J. T. Baker (Kromex reagents), Phillipsburg, NJ; SITS was from Pierce Chemical Co., Rockford IL; and phloretin was from K & K Laboratories, Plainview, NY. Nigericin was a gift from Dr. R. Hosley of Eli Lilly Co., Indianapolis, IN, and the uncoupler 1799 was a gift from Dr. P. Heytler, DuPont, Wilmington, DE. Versilube F 50 and SF 96(50) silicone oil were obtained from General Electric Co., Waterford, NY.

Results

Thin-Layer Chromatography of 6-Carboxyfluorescein Diacetate and Its Enzymatic Hydrolysis Products. In thin-layer chromatograms the 6-carboxyfluorescein diacetate synthesized as described under Materials and Methods contained one major component. The R_f for 6-carboxyfluorescein diacetate was 0.70, with a minor spot (less than 10%) representing the monoacetate derivative at R_f 0.55. These R_f values are much higher than that of 6-carboxyfluorescein (0.25), as expected for less polar acetate derivatives in this solvent system. Figure 1 illustrates the time course of conversion of 6-carboxyfluorescein diacetate to 6-carboxyfluorescein by ascites tumor cells. The spot identified as the diacetate gradually decreased in intensity as the incubation time increased. The monoacetate spot first increased in intensity and then disappeared. Eventually both mono- and diacetate derivatives were replaced by a single spot which cochromatographed with 6-carboxyfluorescein.

The ethyl ester of 6-carboxyfluorescein diacetate had an R_f of 0.79, with a minor spot at R_f 0.70. After incubation with ascites tumor cells, the spot at R_f 0.79 was replaced by a new spot appearing at R_f 0.42, while the minor spot at R_f 0.70 remained.

Spectral Characteristics of the Internal pH Indicator. The loading procedure described under Materials and Methods resulted in approximately equal amounts of either 6-carboxyfluorescein (CF cells) or fluorescein (F cells) being trapped within the cell, as determined spectrophotometrically (Figure 2). Based on a molar extinction coefficient of 9×10^4 at 490 nm and an internal volume of 5 μL/mg of protein, the intracellular concentration of either indicator was about 0.2 mM. The spectra of the intracellular dyes were slightly red-shifted as compared to the spectra obtained in solution. The fluorescein spectrum was shifted about 2 nm, while the 6-carboxyfluorescein spectrum was shifted about 4 nm. These shifts may reflect a small degree of indicator binding within the cell.

The spectra of fluorescein and 6-carboxyfluorescein solutions are pH dependent (Figure 3). Lowering the pH decreased the absorbance at 490 nm, with an isosbestic point occurring at about 465 nm at pH 6 or higher. In contrast, the spectra of the intracellular indicators were relatively insensitive to external pH changes (Figure 4). In this experiment, CF and F cells were suspended in a weakly buffered isosmotic medium at pH 7.1, and the absorbance at 490 nm (vs. 465-nm reference) was followed as a function of time. Addition of sufficient 1 M Mes to rapidly decrease the medium pH from 7.1 to 5.8 caused a rapid but small decrease in the absorbance

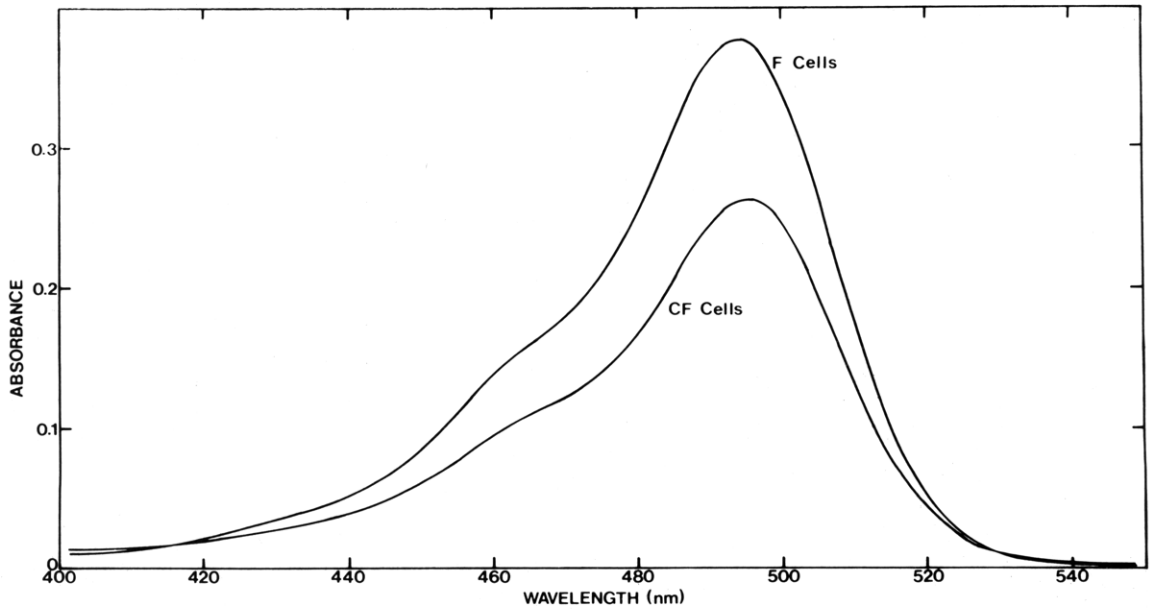

FIGURE 2: Spectra of CF and F cells. Cells were loaded with 6-carboxyfluorescein or fluorescein as described under Materials and Methods. Spectra were measured at 2.5 mg of protein/mL in a 50 mM Hepes buffer (pH 7.4) containing 110 mM NaCl, 5 mM KCl, and 1 mM $MgCl_2$. An equivalent concentration of untreated cells was used in the reference cuvette.

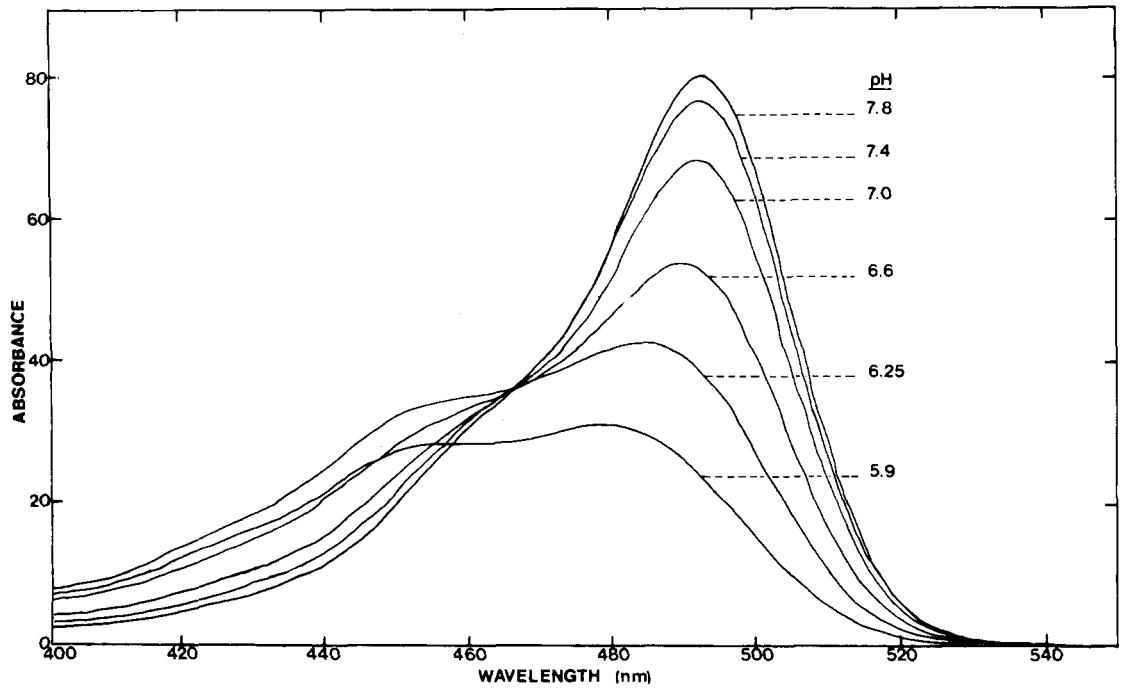

FIGURE 3: pH dependence of 6-carboxyfluorescein spectra. The spectra of 9 μM 6-carboxyfluorescein were measured in 100 mM Mops buffers at the indicated pH.

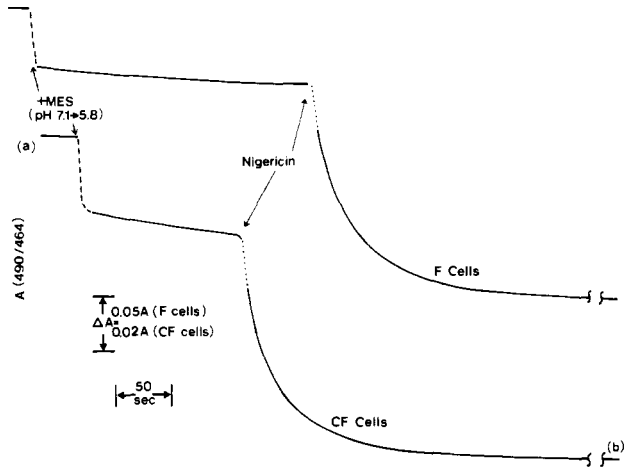

FIGURE 4: Kinetics of spectral responses to external and internal pH changes. CF or F cells (2.5 mg/mL) in 10 mM Mes buffer (pH 7.1) containing 140 mM NaCl, 5 mM KCl, and 1 mM $MgCl_2$ were monitored at 490 nm vs. 465-nm reference wavelength. At the indicated point, 1 M Mes (acid form) was added with stirring to shift the external pH to 5.8, followed by nigericin (10 μg/mL) to equilibrate the internal and external pH.

at 490 nm. The amount of this change varied between 8 and 20% of the absorbance decrease observed when the same concentration of indicator was present outside the cells. Thus, a small proportion of the indicator is apparently accessible to external pH changes. Addition of nigericin to equilibrate the external and internal pH resulted in a large drop in the absorbance and a blue spectral shift consistent with acidification, as shown in Figure 5.

Besides demonstrating that most of the indicator is apparently inside the Ehrlich ascites cell, these experiments demonstrate that the internal pH of these cells is relatively unaffected by external pH changes, at least over short time intervals. Addition of nigericin (10 μg/mL) caused rapid equilibration of the external and internal pH through an exchange of internal K^+ for external H^+, demonstrating that the internal indicator was responsive to pH changes. These experiments were performed in a buffer containing approximately the intracellular K^+ concentration (130 mM) so that a K^+ gradient would not drive the formation of a pH gradient (Lassen et al., 1971). Valinomycin (5 μg/mL) slightly accelerated the rate of pH equilibration observed with nigericin without affecting the spectrum observed at equilibrium, indicating that the K^+/H^+ exchange induced by nigericin was not limited by the internal K^+ concentration.

Table I: Effect of pH during Loading on the Amount of Indicator Trapped within the Cell[a]

	intracellular concn (mM)			
incubation pH	8.4	7.4	6.3	5.3
fluorescein diacetate	0.21	0.25	0.26	0.32
6-carboxyfluorescein diacetate	0.02	0.06	0.20	0.45
6-carboxyfluorescein diacetate ethyl ester		0.11	0.11	

[a] Cells were incubated for 5 min at room temperature with either 6 μM fluorescein diacetate, 30 μM 6-carboxyfluorescein diacetate, or 30 μM 6-carboxyfluorescein diacetate ethyl ester in the following 50 mM buffers containing 110 mM NaCl, 5 mM KCl, and 1 mM $MgCl_2$: Tricine (pH 8.4); Hepes (pH 7.4); and Mes (pH 6.3 and 5.3). The protein concentration was 3 mg/mL. After loading, we centrifuged and resuspended the cells in the pH 8.4 buffer at 3 mg/mL. Spectra were measured vs. untreated control cells. The intracellular concentrations were calculated on the basis of $E_{490}^{mM} = 90$ and an intracellular water space of 5 μL/mg of protein.

Effect of External pH on the Intracellular Accumulation of Indicator. The amount of 6-carboxyfluorescein which accumulated intracellularly was dependent on the pH of the medium, while the loading of fluorescein was comparatively independent of pH (Table I). Between pH 8.4 and 5.3 the fluorescein loading increased by only 50%, while the amount of intracellular 6-carboxyfluorescein increased more than 20-fold. Since fluorescein diacetate has no ionizable group, its entry into the cell should be relatively pH independent. However, 6-carboxyfluorescein diacetate has a free carboxyl group; the increased efficiency of loading of this compound observed at lower pH indicates that it enters the cell in the protonated form, as is characteristic for other organic acids (Waddell & Bates, 1969). The ethyl ester of 6-carboxy-

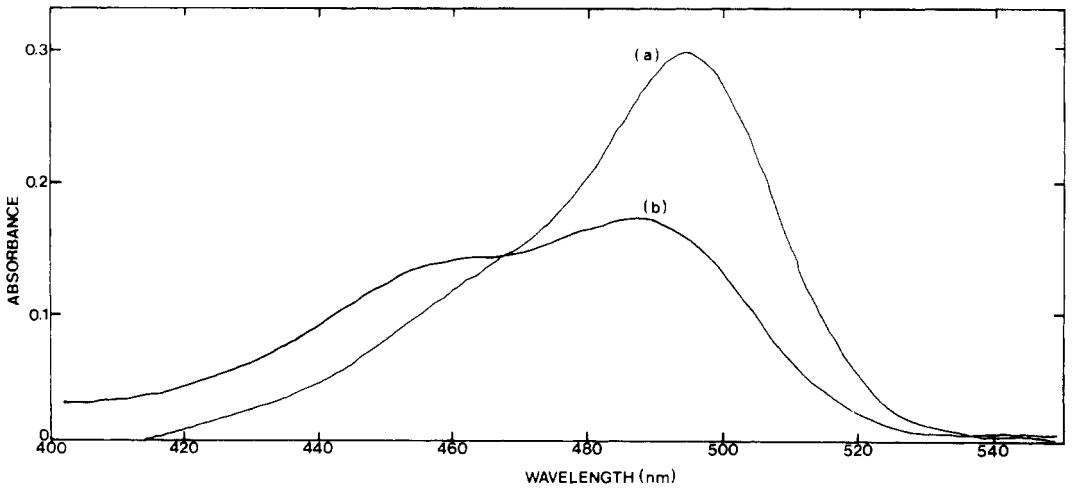

FIGURE 5: Effect of internal pH changes on the spectra of CF cells. CF cells were treated as described in Figure 4. Spectra were measured vs. control cells in pH 7.1 buffer [prior to (a) on Figure 4] and after equilibration of the internal and external pH with nigericin at pH 5.8 [at (b) on Figure 4].

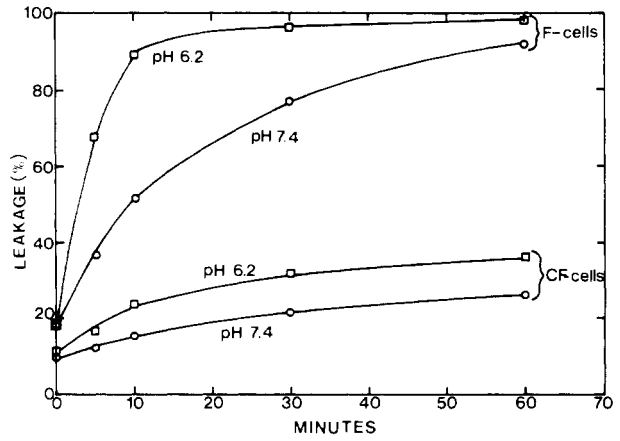

FIGURE 6: Rate of leakage of indicator from CF or F cells at 20 °C. Cells were suspended at 2.5 mg of protein/mL in either 50 mM Hepes (pH 7.4) or Mes (pH 6.2) containing 110 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, and 10 μg of nigericin/mL. Two-milliliter aliquots were discharged at the indicated times into 13 × 100 mm test tubes on ice and immediately centrifuged at 2000g for 2 min at 4 °C. The amount of indicator in the supernatant was measured spectrophotometrically at 490 nm after adjustment to pH 10. The calculation of the percent leakage was based on the total amount of indicator initially present in the cells.

fluorescein diacetate, like fluorescein diacetate, has no ionizable group and also entered the cell independently of external pH.

Indicator Efflux Rates. The rate of leakage out of the cells was determined from the amount of indicator present in supernatants after centrifugation of washed cells. The leakage rate for both 6-carboxyfluorescein and fluorescein was temperature dependent. At 20 °C the leakage was appreciable (Figure 6), while less than 10% leakage was observed after 2 h at 4 °C with either CF or F cells. For measurement of leakage at 20 °C, the aliquots were dispersed into ice-cold tubes and immediately centrifuged at 4 °C.

Figure 6 shows that the rate of fluorescein leakage was dependent on the internal pH, a faster rate being observed at more acidic internal pH values. In the absence of nigericin, the rate of leakage in external pH 6.2 buffer was essentially the same as in pH 7.4 buffer. Addition of nigericin, which equilibrated internal and external pH, accelerated the leakage with pH 6.2 but not with pH 7.4 buffer. Thus, the efflux of the indicator is probably via the protonated species.

In contrast to fluorescein, the leakage rates of 6-carboxyfluorescein were not affected significantly by nigericin in either pH 6.2 or pH 7.4 buffers. Moreover, the leakage rate of 6-carboxyfluorescein was much slower than fluorescein for all cases, presumably due to the presence of the extra negative charge.

Table II: Subcellular Location of Fluorescein Diacetate Esterase[a]

fraction	total act.	total protein (mg)	sp act.
intact cells	338	41.9	8.1
crude homogenate	255	34.0	7.5
121g pellet	235	22.6	10.4
12100g pellet	3.6	1.74	2.1
113000g pellet	4.2	1.15	3.6
113000g supernatant	49.1	2.87	17.1

[a] Ehrlich ascites cells were homogenized with a Dounce homogenizer in 0.25 M sucrose plus 1 mM Na_2EDTA, pH 4 (Wu & Sauer, 1967). The crude homogenate was centrifuged at 1000 rpm for 5 min in a Sorvall RC-2B centrifuge with an SS-34 head (121g pellet), and the resulting supernatant was centrifuged for 10 min at 10000 rpm (12100g pellet). The 113000g pellet and supernatant fractions were obtained by centrifuging the 10000g supernatant for 1 h at 40000 rpm in a Beckman L3-50 ultracentrifuge (60 Ti head). Enzyme activity was measured in a 50 mM Hepes buffer (pH 7.4), containing 110 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, and 10 μM fluorescein diacetate. Absorbance was followed at 490 nm (465-nm reference) in the Aminco DW-2 spectrophotometer. One unit of activity equals 1 nmol of fluorescein formed per min.

The leakage rates were not affected by several nonspecific transport inhibitors, including SITS (4-acetamido-4′-isothiocyano-2,2′-disulfonylstilbene), quercetin, or phloretin.

Subcellular Location of Ester Hydrolysis. Ehrlich ascites cells were homogenized and fractionated by differential centrifugation in an attempt to find out in which subcellular compartment the enzyme hydrolyzing fluorescein diacetate was located. Table II shows that most of the activity was in the fraction containing unbroken cells and nuclei after breaking the cells with the Dounce homogenizer (Wu & Sauer, 1967). The 113000g supernatant contained about 20% of the homogenate activity and about twice the specific activity. Very little activity was found in either the mitochondrial or microsomal fractions.

Estimation of the Internal pH of Ascites Cells. The intracellular pH of ascites cells may be estimated from the shape of the absorption spectrum of the internal indicator (see Figure 3). A calibration curve for 6-carboxyfluorescein was con-

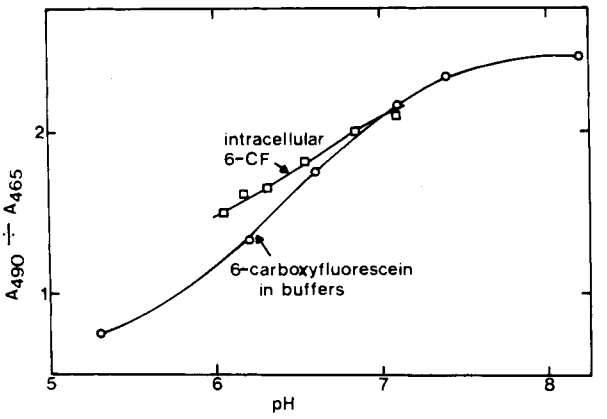

FIGURE 7: Calibration curves relating spectral shape (A_{490}/A_{465}) to pH. (○) 6-Carboxyfluorescin (6 μM) in the indicated buffers (50 mM) containing 110 mM NaCl, 5 mM KCl, and 1 mM $MgCl_2$: Tricine (pH 8.2); Hepes (pH 7.1); Mops (pH 7.1); Pipes (pH 6.6); and Mes (pH 6.2 and 5.3). (□) Ascites cells loaded with 6-carboxyfluorescein in 30 mM buffers containing 130 mM KCl and 1 mM $MgCl_2$ after 5-min equilibration with 10 μg/mL of nigericin: Mes (pH 6.54 and below) and Mops (above 6.54); 20 °C.

structed by plotting the ratio of absorbances at 490 vs. 465 nm (isosbestic point) as a function of pH (Figure 7). The shape of this calibration curve indicates that a pK of approximately 6.5 is associated with the spectral change. The smooth line represents a theoretical curve calculated on the basis of a pK of 6.5. The calibration curve for 6-carboxyfluorescein closely resembles the calibration curve for fluorescein (not shown).

This calibration curve is for the external indicator rather than for the indicator within the cell. The spectral dependence on pH within the cell could be considerably altered from that of the external indicator. To obtain the calibration curve for the internal indicator, the spectra of CF cells were determined in buffers after equilibration of the internal and external pH with nigericin. The isosmotic buffers used in these experiments contained 130 mM K^+ to prevent the transmembrane K^+ gradient from affecting the pH gradient. A 5-min incubation time with nigericin (10 μg/mL) was sufficient to give better than 90% equilibration of the internal and external pH, as determined by both the magnitude of the indicator response (percent completion) and the DMO measurements. After this equilibration period, less than 15% of the 6-carboxyfluorescein was found to be outside the cell, as determined by centrifugation experiments. The calibration curve (A_{492}/A_{465} vs. pH) obtained from these experiments is also shown in Figure 7. The two calibration curves are similar, although the spectral responsiveness of the internal indicator to pH changes is somewhat suppressed compared to the indicator in buffer solutions. The similarity of these two curves allows us to ignore the effect of leakage (≤15%) on the shape of the calibration curve for the internal indicator, which was obtained under conditions of internal and external pH equilibration.

In utilization of the calibration curve to determine an unknown internal pH, however, the effects of leakage need to be considered. If the internal and external pH are close, the calibration curve may be used directly. When the internal and external pH are significantly different, the contribution by the external indicator would result in an underestimation of the transmembrane ΔpH. Since the percentage of indicator outside the cells is easily determined by centrifugation experiments, the corrections are relatively simple. For example, if the indicator spectrum corresponds to an internal pH of 6.5 when the external pH is 7.0 and 20% of the indicator is found to be outside, the actual internal pH is lower by 0.1 (20% of 0.5), namely, 6.4. The internal pH of Erlich ascites cells under various metabolic conditions was measured with CF cells and by the distribution ratio method (Table III). For both methods, the samples were taken after 5-min incubation under the indicated conditions. The two methods are seen to be in good agreement.

Table III: Comparison of Intracellular pH Values Determined by the Spectroscopic Method and Distribution Ratio Method (DMO)[a]

incubation conditions	pH (DMO)	pH (spectrophotometric)
pH 7.4	7.45	(7.4)[b]
pH 6.2	7.15	7.1
pH 6.2 + 10 mM lactate	6.65	6.8
pH 7.4 + rotenone (10 μM)	7.35	(7.3)[b]
pH 7.4 + rotenone + glucose (10 mM)	7.36	(7.3)[b]
pH 6.2 + rotenone	7.17	7.1
pH 6.2 + rotenone + glucose	6.79	6.8
pH 6.2 + 1799 (0.5 μM)	7.15	7.1
pH 6.2 + valinomycin (1 μM)	7.12	(7.2)[b]
pH 6.2 + 1799 + valinomycin	7.04	7.1
pH 6.2 + nigericin (5 μM)	6.35	6.4

[a] For the distribution ratio method, CF cells were incubated at 6 mg/mL with the indicated additions in either a 50 mM Mes buffer (pH 6.2) or a 50 mM Hepes buffer (pH 7.4) containing 110 mM NaCl, 5 mM KCl, and 1 mM $MgCl_2$. After 5-min incubation at 20 °C, an aliquot was processed as described under Materials and Methods. For the spectrophotometric method, cells were incubated at 2.5 mg/mL for 5 min before spectra were measured against control cells in the reference cuvette. [b] Values in parentheses were obtained by extrapolation of the calibration curve (Figure 7) by assuming that the curve obeys the Henderson-Hasselbalch equation with a pK of 6.5 for the chromophore.

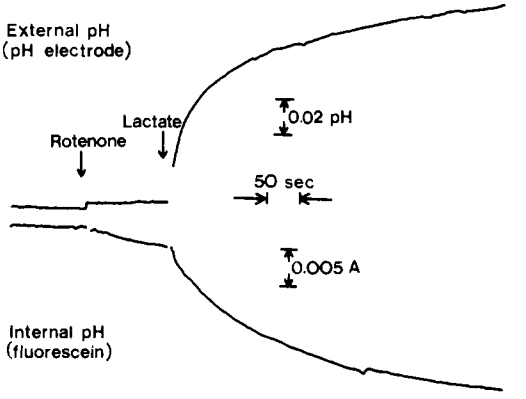

FIGURE 8: Intracellular and extracellular pH changes accompanying lactate transport. F cells were suspended in 4 mM sodium phosphate buffer (pH 6.3) containing 130 mM NaCl, 5 mM KCl, and 1 mM $MgCl_2$. Protein concentration was 2.5 μg/mL. External pH changes were monitored with a pH electrode (top trace) and internal pH changes were monitored spectrophotometrically (bottom trace) at 490 nm (464-nm reference). Final concentrations: rotenone, 10 μM, and lactate, 10 mM. Temperature was 15 °C.

Internal pH Changes Accompanying Lactate Transport. Spencer & Lehninger (1976) have shown that lactate is cotransported 1:1 with protons in Ehrlich ascites cells. Figure 8 shows that the intracellular indicator can be used to monitor intracellular pH changes accompanying lactate transport. F cells were suspended in a solution weakly buffered at pH 6.5. The shape of the intracellular fluorescein spectrum under these conditions corresponded to an internal pH of 7.0. The addition of rotenone caused a slow rate of internal acidification without any external responses being observed from the pH electrode. Since rotenone prevents ATP formation coupled to respiration, the observed changes are probably due to net ATP hydrolysis. Addition of sodium lactate (pH 7.0) caused an increase in the external pH and a decrease in the internal pH, corresponding to the net influx of protons with lactate.

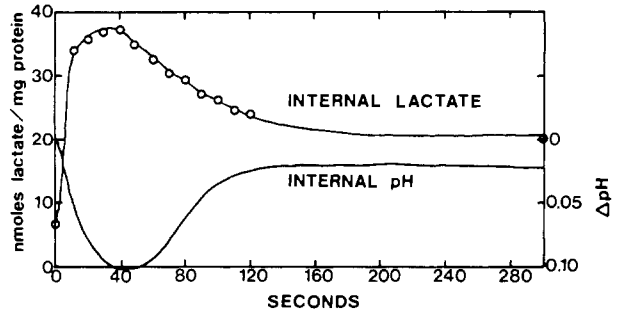

FIGURE 9: Kinetic comparison of internal pH changes and lactate concentrations following the addition of glucose to Ehrlich ascites cells. F cells were suspended at 2.5 mg/mL in 50 mM Tricine buffer (pH 7.4) containing 110 mM NaCl, 5 mM KCl, and 1 mM $MgCl_2$. After 1 min at 20 °C, rotenone (10 μM) was added, followed 1 min later by glucose (10 mM) at $t = 0$. Spectrophotometric responses were followed at 490 nm (465-nm reference). A duplicate experiment was performed for lactate determinations with 2.25-mL aliquots removed at the indicated time intervals after glucose addition and added to test tubes on ice containing 0.25 mL of 10 mM $HgCl_2$. The cells were centrifuged for 10 min at 3000g, resuspended in 1 mg of H_2O, deproteinized with 0.4 mL of 2 N $HClO_4$, and centrifuged at 1800g for 5 min. An aliquot (0.8 mL) was neutralized with 0.2 mL of solution containing 2 M KOH, 0.4 M KCl, and 0.4 M imidazole. The precipitate was removed by centrifugation, and an aliquot of the supernatant was assayed enzymatically for lactate.

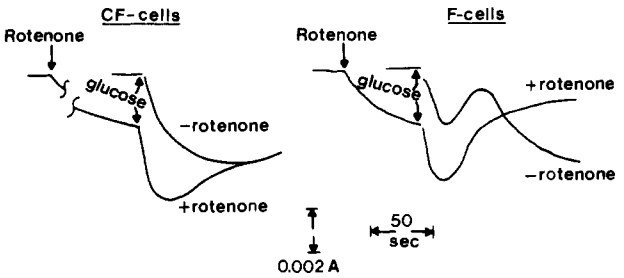

FIGURE 10: Comparison of spectral responses of CF and F cells to glucose addition ± rotenone. CF or F cells were suspended at 2.5 mg of protein/mL in 50 mM Hepes buffer (pH 7.4) containing 110 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, and 4 mM sodium phosphate. Absorbance changes were followed at 490 nm (465-nm reference). Final concentrations: rotenone, 12 μM, and glucose, 10 mM.

Internal pH Changes Accompanying Glycolysis. One of the advantages of using the spectrophotometric method for internal pH changes is that rapid kinetic changes may be easily followed. Figure 9 shows the changes in internal pH on addition of glucose to F cells. Glucose caused a transient decrease of about 0.1–0.2 in the intracellular pH, followed by a recovery to almost the original pH. No internal pH responses were observed with 3-*O*-methylglucose, which is not metabolized. Also shown in Figure 9 are the internal lactate concentrations measured over the same time period. It can be seen that the transient pH changes observed on addition of glucose can be accounted for by the level of intracellular lactate. The transient accumulation of lactate in the cells may be explained by the glycolytic rate exceeding the rate of lactate export during the first minute after glucose addition. It is well-known that glucose induces a rapid glycolysis in Ehrlich ascites cells, which is inhibited several-fold about 1 min later (Hess & Chance, 1961).

Comparison of Spectrophotometric Responses of CF and F Cells. CF and F cells gave similar intracellular pH responses to glucose addition as long as mitochondrial inhibitors were present during the assay (Figure 10). However, the responses differed greatly in the absence of mitochondrial inhibitors. As shown in Figure 11, F cells responded to the addition of valinomycin and rotenone, where CF cells did not. Rotenone

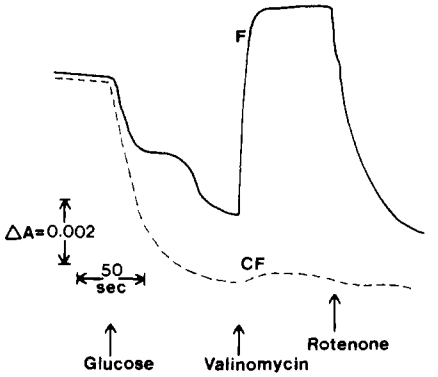

FIGURE 11: Spectral responses of CF and F cells to mitochondrial pH changes. CF or F cells were suspended at 2.5 mg of protein/mL in 50 mM Mes buffer (pH 6.4) containing 110 mM NaCl, 5 mM KCl, and 1 mM $MgCl_2$. Absorbance was monitored at 490 nm (464-nm reference). Final concentrations: glucose, 10 mM; valinomycin, 1 μM; and rotenone, 12 μM.

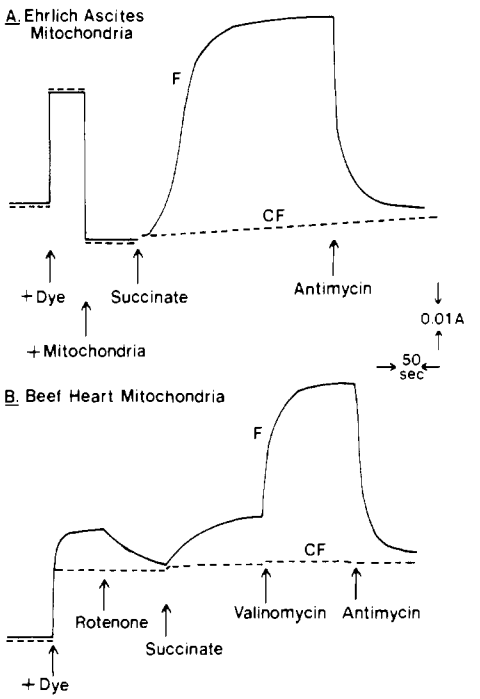

FIGURE 12: Spectral responses of fluorescein and 6-carboxyfluorescein to pH transitions in isolated mitochondria. (A) To ascites cell mitochondria at 0.5 mg/mL in 50 mM Mes buffer (pH 6.1) containing 110 mM KCl and 1 mM $MgCl_2$, additions were made as indicated in the figure. (B) Fresh heavy bovine heart mitochondria were suspended at 1 mg/mL in the same buffer as in (A). Final concentrations of fluorescein (F) and 6-carboxyfluorescein (CF) were 2 μM. Absorbance was monitored at 490 nm (464-nm reference). Final concentrations for (A) and (B): succinate, 10 mM; valinomycin, 0.5 μM; antimycin, 10 μM; and rotenone, 12 μM.

or 1799 (0.5 μM) reversed the effect of valinomycin. At the same concentrations, 1799 and valinomycin had little effect on pH gradients across the cell membrane (Table III). These observations indicate that a mitochondrial response was recorded in F cells. It is known that valinomycin increases the pH gradient generated in mitochondria, where 1799 eliminates it and rotenone prevents its formation.

The results can be explained if fluorescein diffuses into the mitochondria after it is initially released in the cytosol. In the presence of valinomycin, the weak acid fluorescein tends to accumulate in the basic mitochondrial matrix, in the same manner as other weak acids (Waddell & Bates, 1969), including DMO. Figure 12 supports this interpretation. When fluorescein was added to a suspension of isolated mitochondria

from Ehrlich ascites cells (Figure 12A) or bovine heart (Figure 12B), the same energy-dependent pH changes were seen as with intact ascites cells. Addition of substrates in the presence of valinomycin resulted in alkalinization of the matrix; inhibitors of respiration abolished the effect. With 6-carboxyfluorescein, no energy-dependent responses were observed with either ascites or beef heart mitochondria. Apparently, fluorescein can enter the mitochondrial matrix, while 6-carboxyfluorescein cannot.

Discussion

A variety of methods have been used to measure intracellular pH. Recent reviews are available (Waddell & Bates, 1969; Rottenberg, 1975) which describe in detail the principles and disadvantages of current methods. The use of microelectrodes is the most direct but also the most difficult method experimentally. The most popular method is to measure the distribution ratio of a weak acid across the cell membrane. Waddell and Butler introduced the use of DMO (5,5′-dimethyloxazolidine-2,4-dione) for this purpose (Waddell & Butler, 1959). The method assumes that the unionized form of the weak acid is freely permeable across the membrane, while the ionized form is impermeant. At equilibrium, the concentration of the unionized form is equal on either side of the membrane, while the concentration of the ionized form depends on the pH of the compartment, as determined by the Henderson–Hasselbalch equation. Since this is an equilibrium method, it is not very suitable for following rapid changes in intracellular pH. Moreover, the intracellular pH value obtained represents a statistical average for all cell compartments.

For kinetic investigations of intracellular pH, the use of internal pH indicators offers the advantage of continuous spectroscopic monitoring with great sensitivity. Theoretically, such measurements require that the indicator be located exclusively within the compartment to be studied. None of the indicators currently used fulfills this criterion (Rottenberg, 1975). We believe the technique presented in this paper overcomes the objections of other indicator methods. In this method, an impermeant pH indicator is generated in situ by intracellular enzymes. Calibration is accomplished by measuring the spectrum after equilibration of the external and internal pH with nigericin, with the indicator within the cell thereby compensating directly for spectral artifacts due to the binding of the indicator by cellular components. The spectra of the indicator within the cell are similar to those obtained in solution, indicating that binding artifacts are not a large problem with these fluorescein compounds. Polarization measurements have indicated that fluorescein binding to cell components is not significant in other eucaryotic organisms (Celada & Rotman, 1967). The rapid release of fluorescein from cells following damage to the cell membrane is consistent with this interpretation (Rotman & Papermaster, 1966).

The intracellular accumulation of fluorescein and 6-carboxyfluorescein is consistent with the general model proposed by Rotman & Papermaster (1966). Fluorescein diacetate, being an uncharged compound, diffuses into the cell where intracellular esterases release the more polar fluorescein. Because the latter has two additional ionizable groups with pK values below 7, it cannot readily exit the cell. The low-pH requirement for the entry of 6-carboxyfluorescein diacetate, which has one ionizable group, is consistent with this model, as is its slower exit rate after enzymatic hydrolysis, when it has three ionizable groups. The rate of leakage of 6-carboxyfluorescein from liposome vesicles has been reported to be on the order of weeks, several orders of magnitude slower than fluorescein leakage rates in the same system (Weinstein et al., 1977).

The entry of the ethyl ester of 6-carboxyfluorescein diacetate was not dependent on the medium pH (Table I), but unfortunately, the appropriate enzyme is apparently not present in the Ehrlich ascites cell to hydrolytically remove the ethyl ester group after entry. Thus, the ethyl ester of 6-carboxyfluorescein diacetate offers no advantage over fluorescein diacetate, since both have the same net charge after hydrolysis.

Although the enzyme(s) responsible for hydrolysis of the fluorescein esters in Ehrlich ascites cells has not been identified, we have shown that considerable esterase activity released by homogenization is recovered in the cytosolic fraction, with little activity present in the mitochondrial fraction. After release in the cytoplasm, fluorescein can diffuse into the mitochondria, while 6-carboxyfluorescein cannot, presumably because of its extra ionizable group. Cells loaded with the ethyl ester of 6-carboxyfluorescein, which has the same charge as fluorescein, gave mitochondrial responses similar to those of F cells. Thus, it is the charge on the fluorescein derivative that determines entry into the mitochondrion and not the structural differences between fluorescein and 6-carboxyfluorescein. The use of fluorescein and 6-carboxyfluorescein as intracellular pH probes offers the possibility of distinguishing between pH changes occurring in the mitochondrial and in the cytosolic compartments. An alternative approach to detect the pH difference between the cytosol and the mitochondria in intact rat liver cells by ^{31}P nuclear magnetic resonance measurements was recently described (Cohen et al., 1978).

As in the distribution ratio method, the distribution of intracellular fluorescein between the cytoplasmic and the mitochondrial compartments probably depends on the extent of the pH gradient across the mitochondrial membrane. Since fluorescein has two ionizable groups with pK values below 7, the distribution ratio expected from a ΔpH of 1 is greater than 10. This makes it especially sensitive for detecting small mitochondrial pH gradients. However, the magnitude of the spectral response is limited by the pK of the indicator (6.5). Thus, at intracellular pH values of 7.5 or above, large changes in the mitochondrial pH gradient affect the total absorbance only slightly, even when the distribution of the dye between the cytosol and the mitochondria changes dramatically.

The technique presented here of utilizing enzymes present within the cell to generate impermeant molecular probes presents the possibility for investigating other subcellular locations. Probes could be designed to take advantage of enzymes located in the particular compartment to be studied.

Fluorescein isothiocyanate labeled dextran has recently been used to monitor the intralysosomal pH in intact mouse peritoneal macrophages (Ohkuma & Poole, 1978). Fluorescein isothiocyanate labeled cytochrome c has been used to monitor the internal pH in sonic submitochondrial particles (Thomas & Johnson, 1975).

Although we have used absorbance measurements by the dual-wavelength technique to monitor the internal pH changes, the fluorescence properties of fluorescein and its carboxy analogue can also be used. The pK of the fluorescence spectral transition of fluorescein is also about 6.5. Fluorescence is usually considered to offer greater sensitivity and has the additional advantage of not requiring a reference sample of similar turbidity, as required for absorbance measurements. However, with the Aminco DW-2 spectrophotometer, sensitivity has not been any problem. Moreover, with the fluorescence method small changes in fluorescence are measured against a large background signal, thereby reducing

the sensitivity. Thus, the dual-wavelength technique, which measures deviations from a null-balance point, offers equivalent or greater sensitivity for the detection of small pH perturbations.

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Chemical, Physical–Chemical, and Immunological Properties of Papain-Solubilized Human Transplantation Antigens†

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ABSTRACT: Papain-solubilized HLA-A, -B, and -C antigens have been isolated from cadaveric spleens. The isolated material was homogeneous and comprised subunits with the apparent molecular weights 33 000 and 12 000. Amino acid analyses of a mixture of HLA antigen heavy chains obtained from a great number of spleens with different HLA antigen phenotypes revealed a composition that is very similar to that of individual HLA-A and -B antigens. Likewise, the NH_2-terminal 30 residues of the HLA-antigen heavy chain mixture were virtually identical with that recorded for individual specificities. The circular dichroism spectra for the isolated HLA antigens and for free β_2-microglobulin revealed similarities with spectra recorded for immunoglobulin chains and domains. The HLA-antigen heavy chain may contain an appreciable amount of β structure. Antibodies raised against free β_2-microglobulin react better with β_2-microglobulin in free form than when bound to the HLA-A, -B, and -C antigen heavy chains. This is due to the fact that free β_2-microglobulin can bind a maximum of four Fab fragments simultaneously, whereas the HLA-antigen-associated β_2-microglobulin can bind only two Fab fragments without dissociating from the heavy HLA-antigen subunit.

The HLA[1] antigens are recognized by T-killer cells in the graft vs. host reaction (see Thorsby, 1974). It is the hydrophilic portion of the membrane-integrated HLA antigens that directly participates in this recognition event. To obviate the need for detergents in dealing with biologically active transplantation antigens, several groups of workers have shown that limited proteolysis yields a water-soluble fragment which can be isolated (Sanderson & Batchelor, 1968; Mann et al., 1969; Miyakawa et al., 1971; Cresswell et al., 1973; Peterson et al., 1974). Miyakawa et al. (1971) and Turner et al. (1975) have previously detailed methods to isolate papain-digested HLA antigens. In both studies the starting materials consisted of large quantities of in vitro grown lymphoblastoid cells. Because of the high cost of cultured cells, we have developed methods to obtain papain-solubilized HLA antigens in

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[1] For brevity, HLA-A, -B, and -C antigens are called HLA antigens throughout this article.

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