CHLOROTETRACYCLINE AS A FLUORESCENT Ca^{2+} PROBE IN PANCREATIC ISLET CELLS

Methodological Aspects and Effects of Alloxan, Sugars, Methylxanthines, and Mg^{2+}

I.-B. TÄLJEDAL

From the Department of Histology, University of Umeå, S-901 87, Umeå 6, Sweden

ABSTRACT

Pancreatic islets, or suspensions of islet cells, from noninbred *ob/ob*-mice were incubated with chlorotetracycline and analyzed for Ca^{2+}-dependent fluorescence in a microscope. Unless logarithmically transformed, signals from islets were asymmetrically distributed with unstable variance. Signals from cells pelleted in glass capillaries were more homogeneous and depended linearly on the thickness of the sample. The effect of sample thickness and a significant enhancement of fluorescence by alloxan suggest that β-cells were involved in producing the signal from whole islets. The signal from dispersed cells was probably diagnostic of Ca^{2+} in β-cell plasma membranes because it was suppressed by La^{3+} and had a spectrum indicative of an apolar micromilieu; fluorescent staining of cell surfaces was directly seen at high magnification. Fluorescence from cells was enhanced by 0.5–10 mM Ca^{2+} in a dose-dependent manner, whereas less than 0.5 mM Ca^{2+} saturated the probe alone in methanol. The signal from islets or dispersed cells was suppressed by 5 mM theophylline; that from cells was also suppressed by 0.5 mM 3-isobutyl-1-methylxanthine, 1.2 or 15 mM Mg^{2+}, 3–20 mM D-glucose, and, to a lesser extent, 20 mM 3-*O*-methyl-D-glucose. D-glucose was more inhibitory in the absence than in the presence of Mg^{2+}, as if Mg^{2+} and D-glucose influenced the same Ca^{2+} pool. L-glucose, D-mannoheptulose, or diazoxide had no noticeable effect, and 20 mM bicarbonate was stimulatory. The results suggest that microscopy of chlorotetracycline-stained cells can aid in characterizing calcium pools of importance for secretion. Initiation of insulin release may be associated with an increased affinity of certain membrane ligands for Ca^{2+}.

KEY WORDS calcium ion · chlorotetracycline · fluorescence microscopy · islets of Langerhans · lanthanum ion · pancreatic β-cells · pancreatic islets

Insulin secretion is highly dependent on extracellular Ca^{2+}. The secretory response in vitro to the physiological stimulus, D-glucose, is rapidly abolished on withdrawal of Ca^{2+}; on reintroduction of Ca^{2+}, secretion is promptly restored (11, 17). Some thiol-blocking agents can induce insulin release into a Ca^{2+}-free medium, but their effects are diminished by Ca^{2+} deficiency (3, 18).

The rapid and reversible effects of Ca^{2+} have

drawn attention to the possible role of Ca^{2+} as a mediator of the insulin-releasing signal. D-glucose appears to induce a movement of the tracer, $^{45}Ca^{2+}$, from the extracellular fluid to islet cells (22, 26, 27, 38). Because the movement of tracer can be observed under approximate isotopic equilibrium and in islets containing more than 90% β-cells (26), it is likely to reflect a real uptake of Ca^{2+} by β-cells. The mechanisms responsible for Ca^{2+} uptake and their significance for insulin release are unclear, however; important problems to be elucidated concern the regulation of flux kinetics, the likelihood of Ca^{2+} being heterogeneously distributed in different cellular pools, and the possibility of Ca^{2+} serving more than one role in insulin release. For example, it is unknown whether the glucose-stimulated Ca^{2+} uptake in β-cells is predominantly due to enhanced influx or inhibited efflux (25, 26, 36), and to what extent the Ca^{2+} uptake is causing, or is otherwise associated with the enhancement of insulin release (21).

Because the study of Ca^{2+} in β-cells appears both important and difficult, analytical principles other than that of the isotopic tracer may be worth considering. Ultrastructural studies of pyroantimonate precipitation in islet sections have suggested a preferential localization of glucose-sensitive Ca^{2+} pools to the β-cell plasma membrane and insulin secretory granules (29, 41, 43); radioactive labeling of lanthanum-displaceable calcium in microdissected islets emphasized the possible importance of membrane-located Ca^{2+} in insulin release (25). Fluorescence probing with chlorotetracycline is a methodological principle which has the attraction of promising quantitative information on the interaction of nonradioactive Ca^{2+} with biological material, notably hydrophobic structures such as membranes. The light emission from chlorotetracycline varies with the amount and nature of divalent cation chelated, as well as with the properties of the chemical microenvironment surrounding the probe (7). Fluorescence measurements have thus been helpful in characterizing the association of Ca^{2+} with mitochondria (6, 35), sarcoplasmic reticulum fragments (8), and synaptosomes (44). By spectrum analyses, attempts have been made to clarify such parameters as rotatory restrictions, hydrophobicity of Ca^{2+} solvent, and nearness of Ca^{2+} to aromatic residues (6, 8).

The potential value of chlorotetracycline as a Ca^{2+} probe in the complex system represented by intact islets or islet cells has so far remained unexplored, except that the probe was used to demonstrate interactions between Na^+ and Ca^{2+} in microdissected islets (48). The present paper considers certain methodological problems arising from the anatomy of islets and describes a simple technique for microscopic measurements of fluorescence from dispersed islet cells. The technique is used to show striking influences of D-glucose, methylxanthines, or Mg^{2+} on a Ca^{2+}-dependent fluorescence signal which, in view of its being suppressed by La^{3+}, presumably originates in β-cell plasma membranes.

MATERIALS AND METHODS

Animals and Isolation of Whole Islets and Dispersed Islet Cells

Adult noninbred *ob/ob*-mice from the Umeå colony were starved overnight. For each experiment with intact pancreatic islets, about 25 islets were isolated by free-hand microdissection without the use of collagenase (13). Microdissection was carried out at room temperature (approximately 22°C) in a medium composed as Krebs-Ringer bicarbonate buffer (9) except for being equilibrated with ambient air and buffered (pH 7.4) with 20 mM *N*-2-hydroxyethylpiperazine-*N'*-yl-ethane sulfonic acid (Hepes). For each experiment with dispersed islet cells in suspension, about 400 islets were isolated by collagenase digestion and broken up by shaking in Ca^{2+}-free tissue culture medium 199 supplemented with 1 μg/ml DNase and, unless otherwise stated, 1 mM ethyleneglycolbis [β-aminoethyl ether]*N*,*N'*-tetraacetic acid (EGTA); cells were cleansed from tissue debris by centrifuging through dense albumin (33). The viability and functional characteristics of dispersed islet cells have been described (10, 30, 33, 34). In some experiments, pancreatic glands were digested with collagenase for only half as long as when preparing islets. Islet-free fragments were isolated and shaken in Ca^{2+}-free medium as described above to prepare suspensions of exocrine pancreas cells.

Incubations

Incubations were performed at 37°C in media the basal composition of which was the Krebs-Ringer bicarbonate formula (9) supplemented with 20 mM Hepes. The media were equilibrated with ambient air and, unless otherwise stated, the bicarbonate of the original formula was reduced by one-fifth (islet experiments) or was entirely omitted (dispersed cells experiments); the effect of bicarbonate was experimentally investigated as described in Results. In experiments aimed at studying the influence of La^{3+}, a Tris-buffered medium balanced in cations but lacking anions other than Cl^- was used; this medium has previously been employed in studies of $^{45}Ca^{2+}$ handling and insulin release (26). The length of

incubations and the additions of test substances, notably 10 μM chlorotetracycline as Ca^{2+}-dependent fluorophore, are described in the legends to the figures and tables.

Fluorescence Microscopy

Islets that had been incubated at 37°C were prepared for fluorescence measurements by being individually transferred to small wells (about 2-mm diameter and 1-mm depth) drilled in a plate of gray polyvinylchloride. By using a braking-pipette, each islet was transferred together with a volume of incubation fluid that was sufficiently large to protrude above the surface of the plate and yet sufficiently small not to spread on the plate beyond the margin of the drilled well. A thin circular rim of exsiccator grease had been placed around each well with the aid of a piece of tubing. A tightly sealed mount of islet was obtained by gently placing a cover glass on the protruding medium drop and pressing against the grease. Certain care and exercise was necessary to avoid trapping air bubbles under the cover, and to place each islet on the bottom of the well without disturbance from surface tension.

After incubation of dispersed islet cells, the suspensions were centrifuged for 5 min at about 50 *g*. Most of the supernatant medium was discarded, and the cells were resuspended and taken up in disposable micropipettes (Microcaps 0.5, 1, 2, 3, 5, or 10 μl; Drummond Scientific Co., Broomall, Pa.). The filled capillary micropipettes were dipped in a commercially available paste intended for sealing blood sampling capillaries (sealing wax D553; Radiometer Co., Copenhagen, Denmark); they were placed in a test tube and centrifuged as described above. The capillaries were then mounted between object and cover glasses. Immersion oil was gently applied at the rim of the cover and allowed to fill the space between the two glasses and the capillaries. Thus, the cell sediments became embedded in a sandwichlike mount that had plane surfaces and was almost homogeneous with respect to refractive index. Fluorescence from the sedimented cell pellet was measured under incident excitation in the same microscope that was used for measuring whole islets. Experiments aimed at defining the method are reported in Results. For some of those experiments, the inner diameter of the glass capillaries had to be known fairly accurately. This parameter was therefore determined by filling the capillaries with an alkaline solution of *p*-nitrophenol and measuring A_{410} across the capillaries in a Zeiss microscope photometer (×6 objective) as well as in an ordinary spectrophotometer cuvette with a 10-cm optic path. The circular measuring area in the microscope had a diameter of only 15 μm and was carefully positioned over the capillary axis. Photometrically determined diameters agreed well with those calculated from the length of the capillaries and the volume stated by the manufacturer; for the various capillaries, the optically determined diameter was in micrometers (calculated diameter in parentheses): 0.5 μl, 143 (138); 1 μl, 209 (200); 2 μl, 277 (280); 3 μl, 342 (342); 5 μl, 434 (446); 10 μl, 532 (556).

Islets mounted in polyvinylchloride wells, or dispersed cells pelleted in capillaries, were placed under a Leitz Ultropak objective (×11) in a light microscope equipped with the phototube arrangement of a Leitz MPV I microscope photometer. The islets or cell pellets were excited by epi-illumination from a xenon lamp attached to a Leitz prism monochromator. The thin rectangular beam from the monochromator slit was made approximately circular by a simple system of intervening lenses and was projected onto an islet or cell pellet via the ring-shaped mirror in the objective; the degree of polarization due to the angular mirror is not known. The emitted light passed through the center of the objective and through a Leitz Veril B 60 interference filter with 25-nm bandwidth at 550 nm before reaching the phototube. The monochromator had a mechanical slit width of 0.4 mm, corresponding to a spectral width of 4 nm at 390 nm excitation. The emitted light to be measured was restricted by a diaphragm, the aperture of which corresponded to a circle with a diameter of the order of 0.1 mm in the object plane (measuring area); the influence of this diameter on the fluorescence signal was systematically analyzed as described in Results. Fluorescence readings are presented in arbitrary units: the unit is the same within each series of experiments but differs between series as a result of adjustments of the xenon lamp and for reasons of convenience in preparing graphs. Unless otherwise stated, the unit is proportional to the actual meter readings. Excitation was at 390 nm and emission at 530 nm except when taking spectra.

In some experiments aimed at elucidating the subcellular origin of chlorotetracycline fluorescence signals, aliquots of incubated islet cell suspensions were spread on object glasses, covered with slips, and sealed with a rim of immersion oil. The cells were immediately inspected under an immersion oil lens (×100, NA = 1.30) in an ordinary fluorescence microscope equipped with a Zeiss halogen lamp, filters, and epicondensor.

Measurements of Insulin Release

Batches of two microdissected islets were incubated in the modified Krebs-Ringer bicarbonate buffer (5 mM $NaHCO_3$, 20 mM Hepes) equilibrated with air and containing 0 or 1 mg/ml bovine serum albumin as indicated. After preliminary incubation for 40 min in medium containing 3 mM D-glucose, the release of insulin during 60 min of incubation with 3 or 20 mM D-glucose was measured in the presence or absence of chlorotetracycline. Insulin released to the medium was radioimmunologically assayed; the incubated islets were freeze-dried (−40°C, 0.1 Pa) overnight and weighed on a quartz-fiber balance.

Mathematics

To evaluate the method of measuring fluorescence

from cells sedimented in glass capillaries, it was necessary to consider the volume of the sample under the measuring area. The measuring area was defined by an approximately circular diaphragm, and the cell-containing capillaries were positioned at right angles to the optic axis of the microscope. The sample analyzed can therefore be seen as the body circumscribed by two straight cylinders with radii, R (capillary) and r (measuring area), and with their axes intersecting at right angles. The volume of such a body is given by the expression,

$$V = 8 \int_0^r \sqrt{(r^2 - x^2)(R^2 - x^2)}\, dx$$

This integral was solved for various values of the ratio, R/r, by numerical integration on a digital computer.

For statistical analysis of experimental data, the tests and principles described by Snedecor and Cochran (47) were used.

Chemicals

Chlorotetracycline from American Cyanamid Co., Pearl River, N.Y., was donated by Cyanamid Svenska AB, Stockholm, Sweden. D-Glucose, $LaCl_3 \cdot 7H_2O$ in crystalline form, and $CaCl_2$, as a standardized stock solution in water, were bought from BDH Chemicals Ltd., Poole, Eng. $MgCl_2 \cdot 6H_2O$ was obtained from E. Merck AG, Darmstadt, Germany, alloxan monohydrate from Nutritional Biochemicals Corporation, Cleveland, Ohio, diazoxide (7-chloro-3-methyl-1,2,4-benzothiadiazine 1,1-dioxide) from Schering Corporation, Bloomfield, N.J., and 3-isobutyl-1-methylxanthine from Aldrich-Europe, Janssen Pharmaceutical, Beerse, Belgium. Hepes, theophylline, bovine serum albumin (fraction 5), 3-*O*-methyl-D-glucose, L-glucose, and D-mannoheptulose were from Sigma Chemical Co., St. Louis, Mo., Collagenase and DNase (2 U/μg) were obtained from Worthington Biochemical Corp., Freehold, N.J.

RESULTS

Statistical Aspects of Fluorescence Measurements on Whole Islets

The fluorescence signal from islets incubated with chlorotetracycline exhibited a non-Gaussian frequency distribution. A disproportionately large number of low fluorescence values were recorded under a variety of experimental conditions, including incubation with different concentrations of Ca^{2+}. This phenomenon is illustrated in Fig. 1, which shows the distribution of fluorescence intensities from islets incubated with 0 or 20 mM D-glucose in combination with 0 or 2 mM alloxan. Fig. 1*a* gives the values as recorded with the microscope fluorometer. The coefficient of skewness was significant in all four groups (g_1 = 0.958–1.308 with $P \leq 0.02$, two-tailed). Associated with the skewness was a conspicuous ten-

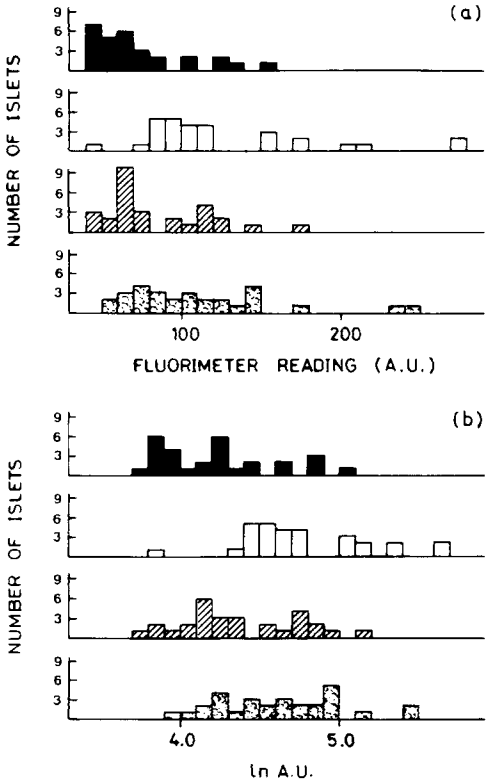

FIGURE 1 Frequency distributions of fluorescence signals from whole islets. In Fig. 1*a* the actual fluorometer readings are given without any nonlinear transformation of data; they are expressed in arbitrary units (A.U.) obtained by standardizing the meter readings against fluorescence from a piece of plastic carried through all experiments. In Fig. 1*b* the same results are also shown after transformation to natural logarithms. From top to bottom in each panel, results are given for islets incubated without alloxan or glucose (solid bars), with 2 mM alloxan in glucose-free medium (open bars), with 20 mM D-glucose in alloxan-free medium (hatched bars), and with 2 mM alloxan plus 20 mM D-glucose (stippled bars). After 40 min of incubation without alloxan or chlorotetracycline, islets were incubated for 10 min with alloxan added from a fresh stock in 1 mM HCl; control media were supplemented with a corresponding volume of HCl alone. Incubation was then continued for 60 min in alloxan-free media supplemented with 10 μM chlorotetracycline. All media contained 2.56 mM Ca^{2+} and 0 or 20 mM glucose throughout. For each type of treatment, altogether 29 islets were studied in six separate experiments.

dency for the standard error to increase with the mean value if calculated directly on the original data (Table I). Logarithmic transformation made the distribution nearly symmetrical and the variance almost homogeneous in the different groups (Fig. 1 *b*, Table I). Practically no further improvement was obtained by a second logarithmic transformation; square root transformation was less effective in decreasing the asymmetry and heterogeneity of variance.

The data in Fig. 1 were collected in six separate experiments of identical design. Within each experiment, islets from one animal were incubated and fluorometrically analyzed in parallel. To examine how much of the random error was the result of differences between islets, as distinct from differences between experiments, the once logarithmically transformed data were subjected to an analysis of variance (Table II). Although moderate, the between-experimental source or error cannot be ignored when studying effects of various treatments, because it was significant for islets incubated with 20 mM D-glucose.

Effects of Alloxan and D-Glucose on Chlorotetracycline Fluorescence from Whole Islets

In mouse and rat islets, α_1-cells and α_2-cells are preferentially located in the periphery of the islets (14). They make up less than 10% of the endocrine cells in *ob/ob*-mouse islets (15), but their location is a potential source of systematic error in attempts to measure signals from β-cells. Morphological studies of several species (42) including *ob/ob*-mice (40) indicate that β-cells are especially, and virtually selectively, affected by the cytotoxic action of alloxan. It was therefore felt that studying the influence of alloxan might help to elucidate the contribution of β-cells to the fluorescence signal from whole islets. Measurements of Rb^+ accumulation (31) and trypan blue uptake (10) have demonstrated the sensitivity of *ob/ob*-mouse β-cells to alloxan in vitro.

Experiments were designed to simultaneously test the influence of alloxan and D-glucose. This was done because the β-cytotoxic action of alloxan

TABLE I

Effects of Various Transformations on Stability of Variance in Microscope Fluorometric Data for Whole Islets

Transformation	Fluorescence (arbitrary units)			
	No D-glucose		20 mM D-glucose	
	No alloxan	2 mM alloxan	No alloxan	2 mM alloxan
None	73.4 ± 5.5	126.6 ± 10.5	84.4 ± 6.2	110.0 ± 8.8
ln	4.224 ± 0.068	4.759 ± 0.075	4.367 ± 0.069	4.621 ± 0.074

Mean values ± SEM of data shown in Fig. 1 before and after transformation to natural logarithms. The number of observations in each group is 29.

TABLE II

Comparison of the Sources of Variance between Islets and between Experiments

Incubation conditions	Mean square (arbitrary units)		*F*-value
	Between experiments	Within experiments	
No glucose, no alloxan	0.274	0.112	2.45
No glucose, 2 mM alloxan	0.196	0.156	1.26
20 mM glucose, no alloxan	0.368	0.067	5.49*
20 mM glucose, 2 mM alloxan	0.418	0.106	3.94‡

The ln data of Fig. 1 and Table I were subjected to an analysis of variance. For each incubation condition there were six separate experiments, each of which comprised individual measurements on four to five islets. To facilitate the calculations, four islets in each experiment were included in the analysis of variance; the odd islets to be excluded were selected at random.

* $P < 0.01$.

‡ $P < 0.05$.

is counteracted by D-glucose (2, 10, 31, 45), and because D-glucose has been shown to induce Ca^{2+} uptake by *ob/ob*-mouse islets as measured with the aid of isotopic tracer (25, 26).

Table III summarizes results obtained with 1, 2, and 4 mM alloxan in the presence or absence of 20 mM D-glucose. Each concentration of alloxan was tested in a separate series of experiments. Each of these series comprised parallel incubations of an equal number of islets in four different media (neither alloxan nor glucose, alloxan alone, glucose alone, alloxan plus glucose). Analysis of variance revealed that a highly significant difference existed between the group means in the series of experiments with 2 mM alloxan ($F = 11.49$, $F_{0.005} < 4.73$) and 4 mM alloxan ($F = 6.11$, $F_{0.005} < 4.73$); no significant difference was observed between the groups in experiments with 1 mM alloxan ($F = 0.86$).

In the experiments with 2 mM alloxan, the least significant difference between two group means was 0.200 at the 5% level and 0.264 at the 1% level, indicating that the enhancing effect of alloxan on chlorotetracycline fluorescence was significant in the absence ($P < 0.01$) as well as in the presence ($P < 0.05$) of 20 mM D-glucose. The effects of 4 mM alloxan exceeded the least significant difference for the 1% level (0.230) whether or not glucose was present.

TABLE III

Effects of Various Concentrations of Alloxan on Chlorotetracycline Fluorescence from Whole Islets

Concentration of alloxan	Fluorescence (arbitrary ln units)	
	No D-glucose	20 mM D-glucose
mM		
0	4.197 ± 0.049 (68)	4.295 ± 0.051 (68)
1	4.142 ± 0.110 (20)	4.085 ± 0.097 (20)
2	4.759 ± 0.075 (29)	4.621 ± 0.074 (29)
4	4.679 ± 0.049 (19)	4.603 ± 0.055 (19)

For each concentration of alloxan, four to six separate experiments were performed, each of which comprised parallel incubations of islets with and without alloxan in combination with 0 or 20 mM D-glucose. After preliminary incubation for 40 min without alloxan or chlorotetracycline, half the number of islets were incubated for 10 min in the same media supplemented with alloxan; the drug was added from a fresh stock in 1 mM HCl. The other half number of islets were incubated for 10 min in media supplemented with the same volume of HCl alone. Incubation was then continued for 60 min in alloxan-free media containing 10 μM chlorotetracycline. The concentrations of D-glucose were kept constant throughout, and all media contained 2.56 mM Ca^{2+}. Mean values ± SEM are given for the numbers of islets shown in parentheses. Data are natural logarithms of recorded values. Results in the absence of alloxan have been brought together. In the statistical testings described in the text, however, only the results of strictly parallel incubations are compared.

Effects of D-glucose alone were not significant. However, D-glucose probably counteracted the effect of 2 mM alloxan, since the difference between the alloxan effects at 0 and 20 mM D-glucose was 0.281 ± 0.143 (SEM) and the probability of this difference being zero is approximately 5% ($t = 1.97$, $t_{0.05} = 1.98$, $t_{0.1} = 1.66$). The effect of 4 mM alloxan was not demonstrably counteracted by D-glucose, because in this case the difference between effects at 0 and 20 mM D-glucose was only 0.035 ± 0.123 (SEM).

Control incubations revealed no effect of alloxan on the background recordings from islets incubated without chlorotetracycline (not shown), and alloxan did not enhance the fluorescence of the chlorotetracycline-Ca^{2+} chelate in cell-free methanolic solution (Fig. 2).

Effect of Methylxanthine on Chlorotetracycline Fluorescence from Whole Islets

Because methylxanthines potentiate glucose-stimulated insulin release (20), their effect on chlorotetracycline fluorescence was studied. Table IV shows that 5 mM theophylline partially decreased the Ca^{2+}-dependent fluorescence, whereas no significant effect was observed with 0.5 mM 3-isobutyl-1-methylxanthine. Neither theophylline nor 3-isobutyl-1-methylxanthine had any effect on the fluorescence of Ca^{2+}-chlorotetracycline in methanol (Fig. 2).

Reversibility of the Fluorescence Signal from Whole Islets

Although β-cells are in the vast majority among the endocrine cells in *ob/ob*-mouse islets, the complex microanatomy of a whole islet makes it difficult to decide how much of the fluorescence signal derives from different tissue compartments, e.g. the interior of cells, cell surfaces, and the extracellular space between cells. By controlled washings of $^{45}Ca^{2+}$-labeled islets, it has been possible to delineate two pools of glucose-sensitive calcium, one of which was assumed to be associated with β-cell plasma membranes (25).

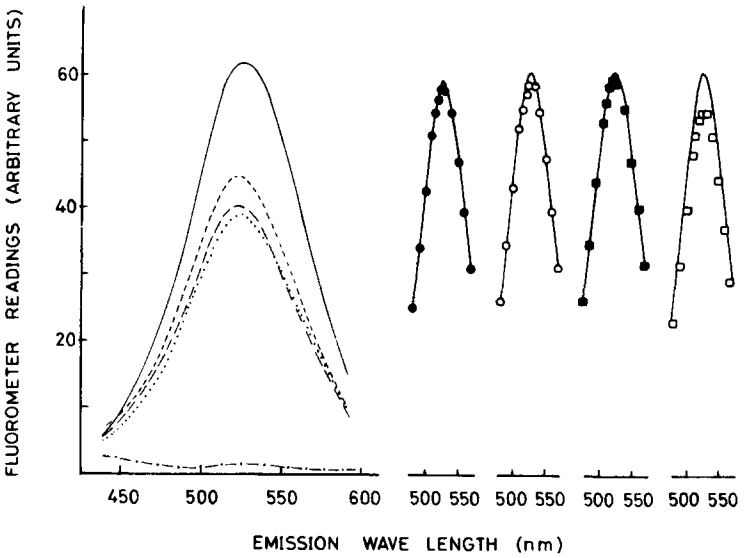

FIGURE 2 Effects of various agents on the emission spectrum of 10 μM chlorotetracycline in 90% methanol buffered with 20 mM HEPES (pH 7.4). Uncorrected spectra were taken in an Aminco-Bowman spectrofluorophotometer with 390 nm excitation. The main diagrams to the left show results obtained in Ca^{2+}-free solution containing 1 mM EGTA (—·—·—), and in solutions containing 2 mM $CaCl_2$ (——), 2 mM $LaCl_3$ (- - - -), 2 mM $CaCl_2$ and 2 mM $LaCl_3$ (· · · · · ·), or 5 mM $CaCl_2$ and 2 mM $LaCl_3$ (------). In the contracted diagrams to the right, the unbroken line shows the spectrum obtained with 10 μM chlorotetracycline and 2 mM $CaCl_2$ alone. The experimental points show results obtained when also adding 20 mM D-glucose (●), 0.5 mM 3-isobutyl-1-methylxanthine (○), 5 mM theophylline (■), or 4 mM alloxan (□).

TABLE IV

Effect of Theophylline, 3-Isobutyl-1-Methylxanthine, and Ca^{2+} Deficiency on Chlorotetracycline Fluorescence from Whole Islets

Modification of medium	Fluorescence (arbitrary ln units)
None (control)	4.87 ± 0.07
5 mM theophylline	4.59 ± 0.08*
0.5 mM 3-isobutyl-1-methylxanthine	5.05 ± 0.07
1 mM EGTA, no Ca^{2+}	4.29 ± 0.06‡

After preliminary incubation for 40 min without chlorotetracycline or other test substances, islets were incubated for 60 min in media containing 10 μM chlorotetracycline and modified as indicated. Ca^{2+}, 2.56 mM, was included throughout, except in one group of islets which were exposed to 1 mM EGTA in Ca^{2+}-free medium during the final 60 min. In each of three separate experiments, parallel incubations of five islets in each medium were performed. Mean values ± SEM for the 15 islets in each group are given.

* $P < 0.01$, and ‡ $P < 0.001$ for difference from control (two-tailed t test). The arbitrary units are not directly comparable to those in Tables I–III due to adjustment of the xenon lamp between the series of experiments.

Table V shows that the fluorescence from islets incubated with Ca^{2+} and chlorotetracycline was reversed during subsequent incubation in the absence of Ca^{2+} or chlorotetracycline or both. Studying the decrease of fluorescence with time could therefore in theory help to map out the localization of the probe in whole islets. So far, however, the microscope procedures have hampered such exact timing of the washing periods as is necessary for reliable studies of fractional Ca^{2+} release.

Development of a Technique for Measuring Chlorotetracycline Fluorescence from Dispersed Islet Cells

To circumvent problems associated with the anatomy of islets, it was decided to analyze the possibilities of measuring chlorotetracycline fluorescence from dispersed islet cells in suspensions lacking a tissue extracellular space. In pilot experiments the usefulness of an ordinary Aminco-Bowman spectrofluorophotometer was examined. The liability of cells to sediment in the cuvette made it difficult to obtain steady fluorescence

TABLE V

Reversibility of Chlorotetracycline Fluorescence in Whole Islets

Concentration of Ca^{2+} during first incubation (mM)	Conditions during second incubation	Fluorescence (arbitrary ln units)
Experimental series A		
2.56	2.56 mM Ca^{2+}, 10 μM CTC	4.33 ± 0.09 (5)
2.56	0* mM Ca^{2+}, 0 μM CTC	2.68 ± 0.08 (5)
0	0 mM Ca^{2+}, 10 μM CTC	3.67 ± 0.04 (5)
0	0* mM Ca^{2+}, 0 μM CTC	2.45 ± 0.02 (5)
Experimental series B		
2.56	2.56 mM Ca^{2+}, 10 μM CTC	4.63 ± 0.06 (19)
2.56	0* mM Ca^{2+}, 10 μM CTC	4.18 ± 0.05 (19)

Islets were first incubated for 60 min in glucose-free media containing 10 μM chlorotetracycline (CTC) and Ca^{2+} as indicated in the left column. They were then incubated for 60 min in media modified as described in the middle column. In series A no alloxan was used, whereas in series B the islets were exposed to 4 mM alloxan during the first incubation. Mean values ± SEM are given for the number of islets stated in parentheses. The number of independent experiments was one in series A and four in series B.

* 1 mM EGTA was present.

signals. More important, the small mass of islets available for one experiment resulted in cell suspensions so diluted as to give a poor cell-to-medium signal ratio even in 100-μl microcuvettes. A simple microscope method was therefore developed.

Fig. 3 shows light recordings along two different micropipettes (1 and 3 μl) in which cells incubated with 5 mM Ca^{2+} and 10 μM chlorotetracycline had been sedimented as described in Materials and Methods. In both cases the values recorded for packed cells were severalfold higher than those recorded for the aqueous chlorotetracycline-containing medium in the same capillary. The variation of fluorescence along the column-shaped sediment within one capillary appeared to be approximately random; the coefficient of variation was 9.4% in the 1-μl capillary and 7.3% in the 3-μl capillary. The total random error associated with the measuring technique was estimated from single measurements on duplicate 3-μl capillaries; each duplicate was made with cell samples from one suspension. When cells had been incubated with 5 mM Ca^{2+}, 10 μM chlorotetracycline and various concentrations of D-glucose (0–30 mM), the coefficient of variation between capillaries was 8.3%. This value is only marginally greater than the estimated random error of measurements on a single 3-μl capillary. It is therefore concluded that the technique of sedimenting islet cells in capillaries for measuring cell fluorescence is highly reproducible.

For the method to be practical, correct measurements must not require too great a precision in adjusting the cell sample in the microscope optic path. The main diagram in Fig. 3 shows that the adjustment of the measuring area along the cell column is not critical; measurements reported below were routinely performed approximately 0.1–0.5 mm from the bottom of the centrifuged cell pellet.

Because the depth of the cell pellets in the optic path (inner diameter of capillaries) was greater than the depth of focus, the effect of focusing was studied. The *inset* of Fig. 3 shows that the fluorometer recordings changed very little within a wide latitude of focusing, indicating that high focusing precision is not essential; to standardize the routine, it was decided to focus on the center of the cell pellet so that the borders of the pellet appeared sharp in visible light.

Inasmuch as the cell pellets were horizontally oriented cylinders, it was natural to place the center of the measuring area on the axis of the cylinder. The need for precision in this orientation was studied with three differently sized capillaries. As shown in Fig. 4, rather drastic mispositioning of the cell pellet had small effects on the fluorometer reading. Placing the edge, instead of the center, of the measuring area on the cylinder axis decreased the recorded value by only 5% in the 10-μl capillary, 8% in the 3-μl capillary, and 16% in the 1-μl capillary. Such a drastic mispositioning far exceeds the unavoidable mispositioning that occurs in routine work. Consequently, the orientation of the measuring area across the cylin-

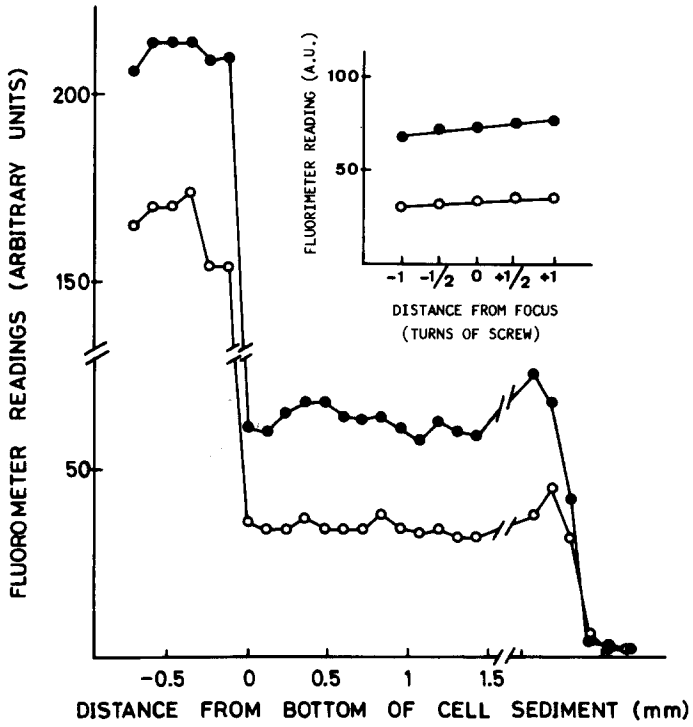

FIGURE 3 The main diagram shows light recordings along glass capillaries used for sedimenting islet cells. After incubating suspensions of dispersed islet cells for 60 min with 5 mM Ca^{2+} and 10 μM chlorotetracycline, cells were taken up and sedimented in a 1-μl (○) or a 3-μl (●) micropipette. Light recordings were then made in the microscope fluorometer with the measuring area (about 110-μm diameter) centered on the axis of the capillary. The values on the abscissa indicate the longitudinal position of the measuring area in millimeters from the bottom of the cell sediment; the direction toward the wax plug sealing capillaries is shown by negative numbers, whereas positive numbers indicate the direction toward the supernate. The drastic fall of light intensity around position zero shows the transition from opaque sealing wax to cells; the drastic fall to the very right shows the transition from cell sediment to supernate. The sediments in the two capillaries were not equally long; to align the zones of transition, the abscissa has been cut differently in the two cases. The *inset* shows how recordings from one longitudinal position were affected by turning the focusing knob. Zero on the abscissa indicates a position of the knob to give sharp outer borders of the sediments in visible light. The other values indicate numbers of full turns of the knob in the direction of focusing above (negative) or under (positive) the zero plane.

drical cell pellet is not a serious source of error.

Fig. 4 indicates that the fluorescence recordings depended on the thickness of the cell pellet. To perform a more careful analysis of this relationship, six types of micropipettes of different sizes were used. For the fluorescence signals from the different capillaries to be compared, the relative volumes of the samples under the measuring area must be known. This volume is not strictly proportional to the capillary diameter, because the capillary walls are circular. However, the volume under a measuring area with the same diameter as that of the capillary is only 15% smaller than the volume of a cylinder with diameter and height equal to the capillary diameter. Moreover, the difference in volume between a sample contained in a capillary and one contained between plane surfaces rapidly decreases with the diameter of the measuring area (*inset* of Fig. 5*a*). Therefore, as shown for a great range of measuring areas (Fig. 5*a*), the relationship between the sample volume in a capillary and the capillary diameter exhibits such a small deviation from linearity as to be of no practical consequence.

In Fig. 5*b*, actual fluorescence recordings from islet cells incubated with 10 μM chlorotetracycline and sedimented in various capillaries have been plotted against the capillary diameter. A linear

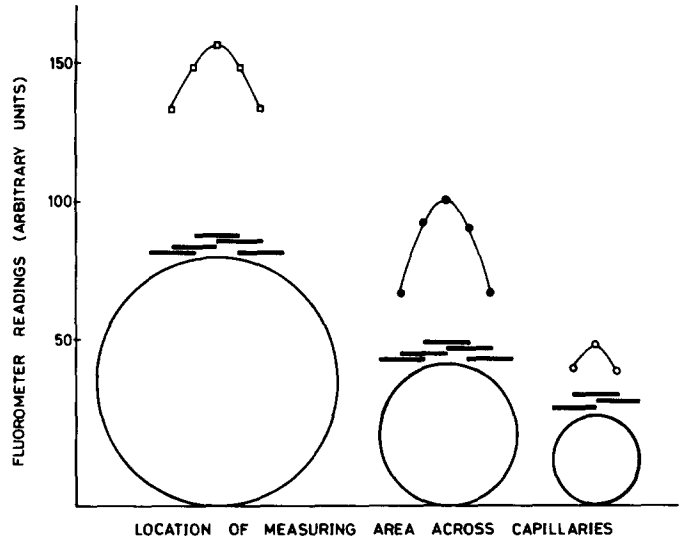

FIGURE 4 Fluorescence measurements at various positions across cylindrical cell sediments. Dispersed islet cells were incubated as in Fig. 3 and sedimented in 10-μl (□), 3-μl (●), and 1-μl (○) micropipettes. At a constant longitudinal position, the measuring area was placed at various positions across the capillaries, as indicated by the horizontal bars above the cross sections of cell sediments. The diameter of the cell sediments (inner diameter of capillaries) was about 0.21 mm (1 μl), 0.34 mm (3 μl), or 0.53 mm (10 μl); the diameter of the measuring area was about 0.11 mm.

relationship was obtained whether the cells had been incubated in 5 mM Ca^{2+} or in Ca^{2+}-free medium. It is therefore concluded that the fluorescence probably arose from all depths of the cell pellet and was not significantly quenched by the thickness of the sample. For comparison, Fig. 5*b* shows recordings from the same types of capillaries filled with 5 mM Ca^{2+} and 100 or 500 μM chlorotetracycline in 50% (vol/vol) methanol. The relationship was again linear for 100 μM chlorotetracycline, whereas with 500 μM chlorotetracycline quenching was apparent in the widest capillaries.

The range of capillary diameters covered the diameters of microdissected *ob/ob*-mouse islets. Therefore, it is also inferred that the signals from whole islets originated at all depths of the islets and not predominantly in the peripheral zone enriched in non-β-cells.

Effect of Ca^{2+} on Chlorotetracycline Fluorescence from Islet Cells

Fig. 5 illustrates the stimulatory effect of 5 mM Ca^{2+} on chlorotetracycline fluorescence from islet cells sedimented in differently sized capillaries. To study the effect of Ca^{2+} in more detail, cells were incubated with various concentrations of Ca^{2+} and 10 μM chlorotetracycline; all samples were then sedimented in 3-μl capillaries. Fig. 6 shows that the fluorescence from islet cells increased with Ca^{2+} concentration in an almost linear fashion up to 10 mM. Because this concentration of Ca^{2+} is 1,000 times greater than the concentration of chlorotetracycline, the dose-response relationship probably reflects the interaction of Ca^{2+} with islet cells rather than the affinity of Ca^{2+} for the fluorescent probe as such. This conclusion was strongly supported by titrating chlorotetracycline with Ca^{2+} in cell-free solution. As shown in Fig. 6, 10 μM chlorotetracycline was fully saturated by 0.5 mM Ca^{2+} in both 70 and 90% methanol.

Effect of Chlorotetracycline Concentration on Fluorescence from Islet Cells

In Ca^{2+}-free medium the fluorescence from islet cells increased about linearly with chlorotetracycline concentration in the range of 1–40 μM (Fig. 7). With 5 mM Ca^{2+} present, the concentration dependence was slightly sigmoidal. Because the corresponding curve for chlorotetracycline in methanol showed no sigmoidicity, the more complex dose-response relationship in islet cells is interpreted as reflecting the probe's interaction with cells as well as with Ca^{2+}. Fig. 7 indicates that 10 μM was the lowest concentration of

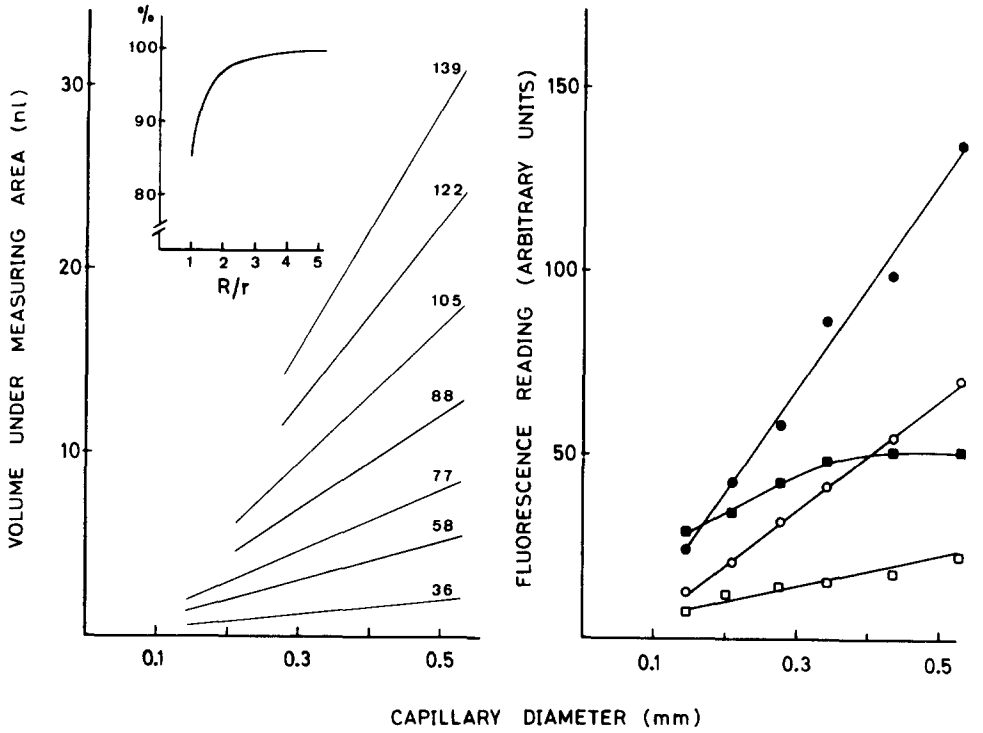

FIGURE 5 Effect of the inner diameter of capillaries used for sedimenting cells on the theoretical volume under the measuring area (left panel) and the fluorescence measured (right panel). The left main diagram shows relationships between theoretical volume and capillary diameter for a number of different measuring areas; the radius of the measuring area in micrometers is stated near each curve. The *inset* shows how the theoretical volume is influenced by the ratio between the radii of the cell-containing capillary, R, and the measuring area, r. The volume is expressed as the percentage of that of a true cylinder with radius, r, and height, R. The right panel shows actual fluorescence readings from cells sedimented in variously sized capillaries after being incubated for 60 min with 0 (○) or 5 (●) mM Ca^{2+} in medium containing 3 mM D-glucose and 10 μM chlorotetracycline. The same relationship is also indicated for capillaries without cells but containing 100 (□) or 500 (■) μM chlorotetracycline together with 5 mM Ca^{2+} in 50% aqueous solution of methanol. To obtain measurable signals from the cell-free capillaries, the amplification of the recording unit had to be considerably increased; this has been taken into account when drawing the diagram, but caution should nonetheless be exercised when comparing the arbitrary units for chlorotetracycline in cells and methanol.

chlorotetracycline that produced Ca^{2+}-dependent fluorescence signals of sufficient intensity to be practical in routine work. At high concentrations, chlorotetracycline may behave as an ionophore. However, 10 μM is not even 50% of the concentration shown to lack significant ionophoric activity in synaptosomes (44).

Reversibility and La^{3+}-Sensitivity of the Ca^{2+}-Induced Chlorotetracycline Fluorescence from Islet Cells

The Ca^{2+}-chlorotetracycline fluorescence from islet cells was highly reversible (Table VI). When cells had been loaded with 5 mM Ca^{2+} and 10 μM chlorotetracycline for 40 min, subsequent incubation for 40 min without Ca^{2+} (in chlorotetracycline-containing medium) reduced the fluorescence to the same low level as that observed with cells incubated in Ca^{2+}-free medium throughout. This fall of fluorescence on removal of Ca^{2+} could not be prevented by including La^{3+} in the medium during the second incubation. Removing chlorotetracycline during the second incubation, while keeping 5 mM Ca^{2+} throughout, caused fluorescence to fall below the value recorded for cells incubated with 10 μM chlorotetracycline in Ca^{2+}-free medium.

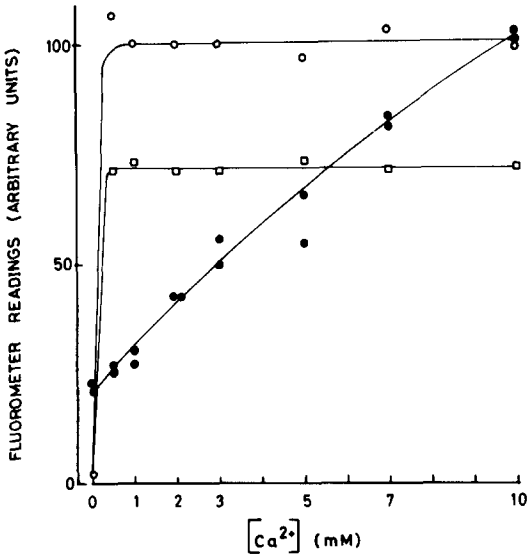

FIGURE 6 Effect of Ca^{2+} concentration on the fluorescence from chlorotetracycline in islet cells or methanol. After incubation for 60 min in glucose-free medium containing 10 μM chlorotetracycline and Ca^{2+} as indicated (1 mM EGTA at zero Ca^{2+}), dispersed islet cells were sedimented in 3-μl capillaries and analyzed by microscope fluorometry; results of two separate experiments are shown for each Ca^{2+} concentration (●). In addition, fluorescence from 10 μM chlorotetracycline in 70% (□) or 90% (○) methanol buffered with 20 mM Hepes (pH 7.4) was studied in an Aminco-Bowman spectrofluorophotometer (390 nm excitation, 530 nm emission). The arbitrary units for measurements in the microscope are not directly comparable with those for measurements in the spectrofluorophotometer.

Isotopic tracer experiments have indicated that La^{3+} ions inhibit transmembrane fluxes of Ca^{2+} in various cells, including those of pancreatic islets (25, 26). Therefore, the failure of La^{3+} to prevent fluorescence from falling on removal of extracellular Ca^{2+} (Table VI) suggests that the fluorescent Ca^{2+}-chlorotetracycline chelate was predominantly associated with the plasma membranes of islet cells.

When islet cells were transferred from a Ca^{2+}-free medium to one containing 5 mM Ca^{2+} and 10 μM chlorotetracycline, the inclusion of 2 mM La^{3+} in the second medium largely prevented ($P < 0.01$) the formation of fluorescent chelate in the islet cells (Table VII). Moreover, cells incubated for 80 min with 5 mM Ca^{2+} fluoresced much more than those incubated equally long without Ca^{2+}; most of this difference disappeared if 2 mM La^{3+} was included in the medium during the final 40 min of exposure to Ca^{2+} (Table VIII), indicating that La^{3+} diminished the concentration of Ca^{2+}-chlorotetracycline chelate in the plasma membranes. The La^{3+}-induced suppression of Ca^{2+}-chlorotetracycline fluorescence in methanol (Fig. 2) shows that La^{3+} can bind strongly to the probe. The effects of La^{3+} on Ca^{2+}-chlorotetracycline fluorescence in islet cells may therefore express either competition of the cations for membrane sites or a dissociation of the Ca^{2+} chelate due to decreased concentration of free chlorotetracycline in the membrane. In either case, the effect suggests that the Ca^{2+}-dependent fluorescence signal arises mainly from the membrane, if it is accepted that La^{3+} blocks transmembrane transport without entering the cells. That La^{3+} inhibits Ca^{2+} transport in islet cells has been

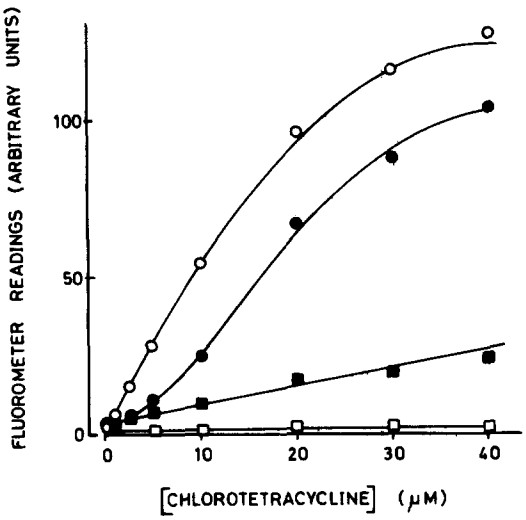

FIGURE 7 Effect of chlorotetracycline concentration on the fluorescence from chlorotetracycline in islet cells or methanol. After incubation for 60 min in glucose-free medium containing 0–40 μM chlorotetracycline, dispersed islet cells were sedimented in 3-μl capillaries and analyzed by microscope fluorometry; results are given as mean values of experiments with one to four (1, 30, and 40 μM) or five to nine (0, 2.5–20 μM) different preparations of cells incubated with 0 (■) or 5 (●) mM Ca^{2+}. In addition, the graph shows fluorescence from 0–40 μM chlorotetracycline in 90% methanol buffered with 20 mM Hepes (pH 7.4) and containing 0 (□) or 2 (○) mM Ca^{2+}; measurements were performed as in Fig. 6, and means of three different experiments are given. SEM values are not indicated because they were comparable to the widths of dots symbolizing the means. Ca^{2+}-free media contained 1 mM EGTA throughout.

TABLE VI

Reversibility of Chlorotetracycline Fluorescence in Dispersed Islet Cells

Concentration of Ca^{2+} during first incubation (*mM*)	Conditions during second incubation	Fluorescence (arbitrary units) Exp. I	Exp. II
Experimental series A			
0	0 mM Ca^{2+}, 10 μM CTC	0.74 ± 0.04	1.13 ± 0.04
5	5 mM Ca^{2+}, 10 μM CTC	2.63 ± 0.20	4.50 ± 0.10
5	0 mM Ca^{2+}, 10 μM CTC	0.89 ± 0.02	1.44 ± 0.04
5	0* mM Ca^{2+}, 10 μM CTC	0.80 ± 0.04	1.59 ± 0.02
5	5 mM Ca^{2+}, 0 μM CTC	0.39 ± 0.02	0.63 ± 0.02
Experimental series B			
0*	0 mM Ca^{2+}, 10 μM CTC	1.06/0.93	1.33/1.23
0*	0 mM Ca^{2+}, 2 mM La^{3+}, 10 μM CTC	1.20/1.00	1.27/1.33
5	5 mM Ca^{2+}, 10 μM CTC	4.00/4.20	4.30/4.60
5	0 mM Ca^{2+}, 10 μM CTC	1.00/0.93	1.53/1.57
5	0 mM Ca^{2+}, 2 mM La^{3+}, 10 μM CTC	1.40/1.27/1.27	1.23/1.33

Islet cells were preliminarily incubated for 30 min in basal medium lacking Ca^{2+} (no EGTA present). They were then incubated for 40 min in basal medium supplemented with 10 μM chlorotetracycline (CTC) and Ca^{2+} as indicated in the left column. During a subsequent period of 40 min the cells were incubated in media modified as described in the middle column; in experimental series B oligo-anionic Tris buffer was used during the second 40-min incubation to avoid precipitation of La^{3+}. In each series, results for two separate experiments are shown. Individual values for duplicate cell sediments (series B) or mean values ± SEM of triplicate sediments (series A) are given. Untransformed data are given as read in the microscope fluorometer on cells sedimented in 3-μl capillaries.

* 1 mM EGTA was present.

TABLE VII

Inhibitory Effect of La^{3+} on Ca^{2+} Uptake by Islet Cells as Visualized by Chlorotetracycline Fluorescence

Concentration of additions to medium			Fluorescence (arbitrary units)
Ca^{2+}	La^{3+}	CTC	
mM	*mM*	μM	
0	0	0	0.129 ± 0.012
0	0	10	0.741 ± 0.030
0	2	10	0.728 ± 0.036
5	0	10	1.613 ± 0.079
5	2	10	0.988 ± 0.071*

After preliminary incubation as in Table VI, dispersed islet cells were incubated for 40 min in oligo-anionic Tris buffer supplemented with Ca^{2+}, La^{3+}, and chlorotetracycline (CTC) as indicated. Mean values ± SEM of four separate experiments are given. Data are untransformed values as read in the microscope fluorometer on cells sedimented in 3-μl capillaries.

* $P < 0.01$ for difference from 5 mM Ca^{2+} and 10 μM chlorotetracycline in the absence of La^{3+} (two-tailed t test).

shown (26). That La^{3+} does not deplete the cells of chlorotetracycline is shown by the unaltered fluorescence in Ca^{2+}-free medium (Tables VI–VIII). As described below, evidence against the permeation of La^{3+} in islet cells was obtained by microscope inspection at high magnification.

Although fluorescence from a La^{3+}-chlorotetracycline chelate was readily observed in methanol (Fig. 2), La^{3+} did not noticeably enhance fluorescence from chlorotetracycline in dispersed islet cells (Tables VI–VIII). This difference between Ca^{2+} and La^{3+} may appear puzzling in view of the above conclusion that Ca^{2+} mainly interacted with chlorotetracycline in the plasma membranes. However, plausible explanations can be derived from the greater charge density of La^{3+}; perhaps La^{3+} was prevented from reaching those hydrophobic regions of the membrane where intensely emitting Ca^{2+}-chlorotetracycline resided, or perhaps La^{3+} reached the same regions as Ca^{2+} but was so strongly coordinated to membrane electronegative groups as to interact only weakly with the fluorescent probe.

Effects of Sugars on Chlorotetracycline Fluorescence from Islet Cells

Fluorescence from dispersed islet cells was inhibited by 3–20 mM D-glucose in a dose-dependent fashion (Fig. 8, Table IX). A significant, though smaller, effect was also obtained with 3-*O*-methyl-D-glucose, whereas L-glucose and D-

TABLE VIII

La^{3+}-Induced Suppression of Ca^{2+}-Dependent Chlorotetracycline Fluorescence in Islet Cells

Concentration of Ca^{2+}	Concentration of La^{3+} during second incubation	Fluorescence (arbitrary units)		
		Exp. I	Exp. II	Exp. III
mM	*mM*			
0	0	1.28 ± 0.02	0.73/0.73	1.17 ± 0.03
0	2	1.31 ± 0.06	0.70 ± 0.05	1.37/1.30
5	0	3.80/3.00	2.06 ± 0.15	2.37 ± 0.05
5	2	1.83 ± 0.09	0.82 ± 0.04	1.67 ± 0.04

After preliminary incubation as in Table VI, dispersed islet cells were incubated for 40 min in oligo-anionic Tris buffer containing Ca^{2+} as indicated in the first column. They were then incubated for a second 40-min period in the same concentrations of Ca^{2+} with or without 2 mM La^{3+}. All media contained 10 μM chlorotetracycline throughout. Results are given for three separate experiments. Individual values are given for duplicate sediments and mean values ± SEM for triplicates. Data are untransformed values as read on cells sedimented in 3-μl capillaries.

mannoheptulose appeared to be ineffective. The inhibitory action of D-glucose was observed whether the Ca^{2+} concentration was 5 mM (Fig. 8, Table IX) or 2.56 mM (Table X). However, it was not observed in cells incubated without Ca^{2+} in chlorotetracycline-containing medium (Table IX) or with Ca^{2+} in cell-free methanolic solutions of chlorotetracycline (Fig. 2). It is concluded, therefore, that D-glucose inhibited that Ca^{2+} signal which, in view of its sensitivity to La^{3+}, presumably originated in the β-cell plasma membrane.

Effects of Methylxanthines, Diazoxide, Mg^{2+}, Bicarbonate, and Albumin on Chlorotetracycline Fluorescence from Islet Cells

Table X shows that the Ca^{2+}-dependent fluorescence from dispersed islet cells was significantly and about equally inhibited by 5 mM theophylline or 0.5 mM 3-isobutyl-1-methylxanthine. The chlorotetracycline fluorescence from cells incubated without Ca^{2+} was completely unaffected by 3-isobutyl-1-methylxanthine, the only xanthine tested for effect on Ca^{2+}-independent fluorescence.

Diazoxide, a potent inhibitor of insulin release with reported effects on the $^{45}Ca^{2+}$ handling by islets (22, 25, 38), had no significant effect on the fluorescence (Table X).

The normal incubation medium contained 1.2 mM Mg^{2+}. Omitting Mg^{2+} caused a marked and highly significant enhancement of fluorescence (Table X). This effect was distinctly Ca^{2+}-dependent since cells incubated in Ca^{2+}-free medium showed the same fluorescence whether or not 1.2 mM Mg^{2+} was present. The fact that the fluorescence intensity did not fall on removing Mg^{2+} from a Ca^{2+}-free medium shows that the signals measured in normal medium were not the result of chelation of exogenous Mg^{2+}.

The marked Ca^{2+} signal from Mg^{2+}-deficient cells was at least as sensitive to D-glucose as were the signals recorded in the presence of 1.2 mM Mg^{2+}. In fact, in six out of six experiments, the inhibitory action of D-glucose was greater in the absence than in the presence of 1.2 mM Mg^{2+} ($P < 0.01$, two-tailed rank test). Expressed in the percentage units employed in Table X, the difference between the glucose effects (fluorescence at 0 mM D-glucose minus fluorescence at 20 mM D-glucose) at 0 and 1.2 mM Mg^{2+} was 21.7 ± 2.8 (SEM) with $P < 0.001$ (two-tailed t test, justified by small SEM).

Because the present microscope methods involved repeated transfers of islets and cells in open vials, buffering with bicarbonate alone was not considered a reliable method of keeping a stable pH. Hepes was therefore included in the media, and in experiments with cells bicarbonate ions were routinely omitted. Because the islet uptake of $^{45}Ca^{2+}$ is greater in bicarbonate than in Tris buffer (25, 26) and bicarbonate deficiency has been reported to decrease insulin secretory rates (26, 28), the effect of bicarbonate on chlorotetracycline fluorescence was studied. Table X

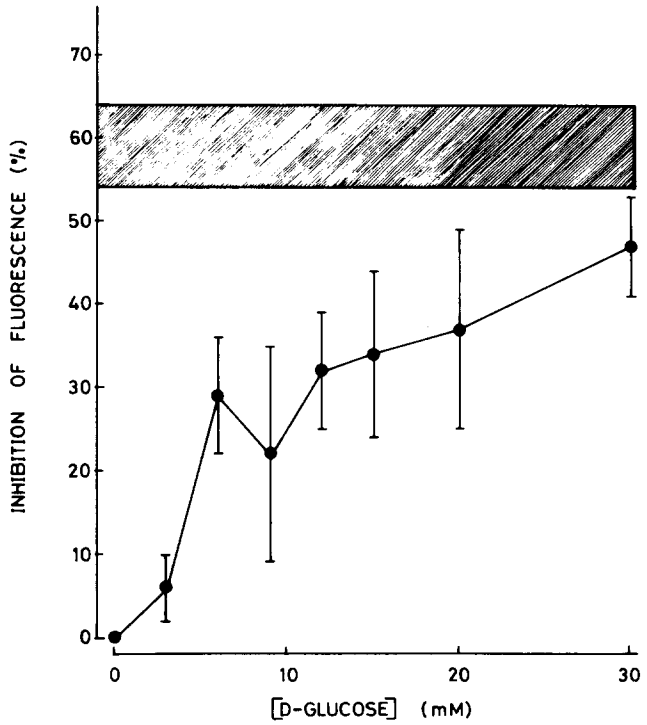

FIGURE 8 Dose-response relationship between D-glucose concentration and inhibition of Ca^{2+}-dependent chlorotetracycline fluorescence from islet cells. Dispersed cells were incubated for 60 min in media containing 5 mM Ca^{2+} and D-glucose as indicated, or in medium lacking both Ca^{2+} and D-glucose but containing 1 mM EGTA; all media contained 10 μM chlorotetracycline. Results are expressed as the percentage inhibition of fluorescence in glucose-free medium containing 5 mM Ca^{2+}. Each point shows the mean value of six separate experiments, in two of which cells were sedimented in 2-μl capillaries and in four of which they were sedimented in 3-μl capillaries; there was no noticeable difference between results obtained with the different sizes of capillaries. Vertical bars denote SEM. The upper shaded area indicates the mean ±SEM of fluorescence from cells incubated with chlorotetracycline in Ca^{2+}-free medium. Already, on the simplified assumption that the regression of inhibition on glucose concentration was not curvilinear but straight, correlation is significant ($r = 0.91$ with equal weights in each point, $P < 0.01$).

shows that bicarbonate significantly increased the Ca^{2+}-dependent, but not the Ca^{2+}-independent, fluorescence from islet cells.

Serum albumin, 1 mg/ml, had no effect whether or not Ca^{2+} was present.

Emission Spectra of Chlorotetracycline Fluorescence from Islet Cells

Fig. 9 shows emission spectra taken in the microscope with the aid of the Veril interference filter. No disturbing peak of fluorescence was recorded from islet cells incubated without chlorotetracycline. In the presence of the probe, typical chlorotetracycline spectra were readily observed with peak emission in the region of 520–540 nm. Similar spectra have previously been reported for microdissected islets without detection of any effect of exogenous Ca^{2+} on the peak position (48). As shown in Fig. 9 for dispersed islet cells, however, the spectrum recorded in the presence of Ca^{2+} was clearly red-shifted by about 5 nm as compared with that recorded for cells incubated in Ca^{2+}-free medium. This spectral shift suggests that chlorotetracycline existed in at least two different conformations in the β-cells: a Ca^{2+}-chelate in apolar surrounding predominating in the presence of extracellular Ca^{2+}, and another conformation more closely related to the Mg^{2+}-chelate type (Ca^{2+}-chelate in polar surrounding) predominating in cells incubated without Ca^{2+} (cf. 7). Whether these conformations of the probe

TABLE IX

Effects of Various Sugars on Chlorotetracycline Fluorescence from Islet Cells

Sugar tested	Concentration of Ca^{2+}	Fluorescence
	mM	*% control*
None	5	96.0 ± 1.2 (3)
3 mM D-glucose (control)	5	100
10 mM D-glucose	5	70.0 ± 4.8* (4)
20 mM D-glucose	5	62.8 ± 2.6‡ (14)
20 mM 3-*O*-methyl-D-glucose	5	79.3 ± 5.6§ (6)
20 mM L-glucose	5	95.3 ± 5.5 (3)
20 mM D-mannoheptulose	5	94.5 ± 2.2 (4)
10 mM D-glucose plus 20 mM D-mannoheptulose	5	75.0 ± 4.0 (4)
3 mM D-glucose	0	56.5 ± 2.3 (15)
20 mM D-glucose	0	54.2 ± 5.4 (5)

After preliminary incubation as in Table VI, dispersed islet cells were incubated for 60 min in Hepes-stabilized Krebs-Ringer buffer containing 0 or 5 mM Ca^{2+} and sugar as indicated; Ca^{2+}-free media also contained 1 mM EGTA. In each experiment, at least one sample of cells was incubated with 3 mM D-glucose and 5 mM Ca^{2+}. To standardize the measurements over a long series of experiments, all values have been expressed as percentages of the fluorescence read for the cells thus incubated. Cells were sedimented in 10-μl capillaries throughout. Mean values ± SEM are given for the numbers of separate experiments stated in parentheses. * $P < 0.01$, ‡ $P < 0.001$, and § $P < 0.02$ for difference from control; § $P < 0.01$ for difference from 20 mM D-glucose (two-tailed t tests).

in islet cells differed with respect to the species of the cation chelated, or to the polarity of microenvironment, or both, is so far unknown. The spectrum differences were too small for any effect of D-glucose or 3-isobutyl-1-methylxanthine to be discerned.

Effect of the Cell Dispersion Technique on Chlorotetracycline Fluorescence from Islet Cells

The technique routinely used for preparing cell suspensions involved shaking the islets with 1 mM EGTA followed by preliminary incubation in EGTA-free buffer to remove the chelator (33). Cells thus prepared are viable (10, 30, 33, 34), but it is not known whether EGTA is permanently incorporated into the cells in amounts interfering with the behavior of membrane-located Ca^{2+} stores. Shaking the islets without any chelator resulted in a poor yield of dispersed cells and could not be used for routine purposes. However, as shown by a limited set of experiments, cell suspensions prepared without chelator exhibited about the same Ca^{2+}-dependent chlorotetracycline fluorescence as cells prepared with the aid of EGTA (Table XI). Moreover, good yields of cells were obtained by shaking the islets with 5 mM, but not 2 mM, sodium citrate, a chelator that has perhaps less drastic effects in being a naturally occurring metabolite. The chlorotetracycline fluorescence from cells dispersed by citrate was virtually identical to that from cells dispersed by EGTA, as shown by experiments with 0, 2.56, and 5 mM Ca^{2+}. The inhibitory effect of 20 mM D-glucose on fluorescence was obtained whether citrate or EGTA was used to break up the islets (Table XI).

Chlorotetracycline Fluorescence from Exocrine Pancreas Cells

Cell suspensions prepared by treating fragments of exocrine pancreas in the same way as islets responded to Ca^{2+} with enhanced chlorotetracycline fluorescence. In contrast to the islet cells, however, the exocrine pancreas cells showed no reduction of the fluorescence when incubated with 20 mM D-glucose (Table XI).

Microscope Localization of Chlorotetracycline in Islet Cells

Islet cells incubated for 60 min without chlorotetracycline were nearly invisible under the ×100 oil lens in blue excitation light. When 50 μM chlorotetracycline was included in Ca^{2+}-free medium (1 mM EGTA present), the cells appeared weakly yellow but emission was too dim for fine details to be observed. Weak staining was also obtained with 10 μM chlorotetracycline in the presence of 5 mM Ca^{2+}. However, the cells were brighter than those incubated without chlorotetracycline, and in the most intensely emitting cells a pattern of very fine shining points could be seen. The exact localization of these bright spots to the surface or interior of cells could not be decided. By comparison with the cell size, their diameter was estimated to be of the order of 0.1 μM; they appeared smaller than the details clearly resolved by the ×100 lens when used for phase contrast microscopy in visible light.

Incubating the islet cells with 50 or 100 μM chlorotetracycline and 5 mM Ca^{2+} produced a succinct staining pattern. The intensity varied

TABLE X

Effects of Methylxanthine, Diazoxide, Mg^{2+}, D-Glucose, Bicarbonate, and Albumin on Chlorotetracycline Fluorescence from Islet Cells

Alteration of medium constituent other than Ca^{2+}	Concentration of Ca^{2+}	Fluorescence
	mM	*% control*
None (control)	2.56	100
None	0	51.9 ± 1.7 (15)
No chlorotetracycline	2.56	19.3 ± 1.3 (10)
5 mM theophylline	2.56	68.6 ± 2.7* (5)
0.5 mM 3-isobutyl-1-methylxanthine	2.56	68.2 ± 4.7‡ (5)
0.5 mM 3-isobutyl-1-methylxanthine	0	47.6 ± 2.0 (5)
125 μg/ml diazoxide	2.56	88.8 ± 4.9 (5)
No Mg^{2+}	2.56	140.1 ± 2.2* (10)
No Mg^{2+}	0	53.0 ± 3.4 (4)
15 mM Mg^{2+}	2.56	39.5 ± 2.8 (4)
20 mM D-glucose	2.56	74.8 ± 1.5* (6)
20 mM D-glucose, no Mg^{2+}	2.56	92.2 ± 3.8 (6)
1 mg/ml bovine serum albumin	2.56	97.0 ± 3.8 (3)
1 mg/ml bovine serum albumin	0	49.3 ± 4.3 (3)
None (control)	5	100
None	0	39.5 ± 5.2 (4)
20 mM $NaHCO_3$	5	174.3 ± 5.5* (4)
20 mM $NaHCO_3$	0	42.5 ± 3.5 (4)
No chlorotetracycline	5	9/6
No chlorotetracycline, 20 mM $NaHCO_3$	5	8/5

Dispersed islet cells were incubated and results are presented as in Table IX, except that values were standardized against those obtained with 2.56 or 5 mM Ca^{2+} in glucose-free medium. Mean values ± SEM for the numbers of separate experiments are stated in parentheses; the effect of omitting chlorotetracycline in the presence of 5 mM Ca^{2+} was tested in only two experiments for which the individual results are presented.

* $P < 0.001$, ‡ $P < 0.01$ for differences from control (two-tailed t tests).

among cells, but the following three subcellular localizations were striking. Firstly, the cell periphery was outlined by a thin zone that shone more brightly than the rest of the cell. This peripheral staining was not smooth and continuous but gave a patchy impression. By carefully focusing at various depths of the cells, the interrupted peripheral outline of the equator could be followed into a pattern of small shiny granules, or somewhat elongated grains, on the top of the intact cells. These granules had about the same size as the fluorescent points observed after incubation with only 10 μM chlorotetracycline and are likely to be identical with those. Because of its clearly peripheral localization, the patchy fluorescence is assumed to arise from chlorotetracycline associated with plasma membranes. As this membrane staining was not noticed in cells incubated with 50 μM chlorotetracycline in Ca^{2+}-free buffer, the patches are thought to represent Ca^{2+} deposits in, or in close proximity to, the plasma membranes. Cytoplasmic background fluorescence in combination with fading under the strong excitation light made it virtually impossible to document the thin peripheral zone of Ca^{2+}-dependent fluorescence by photography. However, the localization was similar to that described below for La^{3+} fluorescence.

Secondly, whether or not 5 mM Ca^{2+} was present, cells incubated with 50–100 μM chlorotetracycline exhibited a smooth cytoplasmic background fluorescence, against which the nucleus stood out as a conspicuous dark area (Fig. 10).

Thirdly, after incubation with 50–100 μM chlorotetracycline in combination with 5 mM Ca^{2+}, extremely delicate zones of intense fluorescence were seen to surround intracellular bodies. These bodies were bigger than the peripheral fluorescent patches but much smaller than the cell nucleus. They were approximately spherical, and their intracellular localization was evident from focusing at various depths. Because the zones of intense

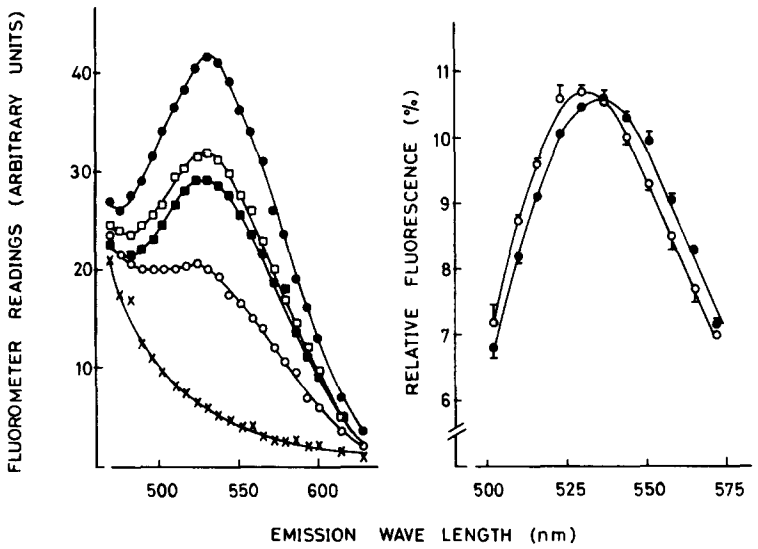

FIGURE 9 Emission spectra for chlorotetracycline fluorescence from islet cells. The left panel shows results obtained with dispersed islet cells incubated for 60 min with 10 μM chlorotetracycline in medium also containing 2.56 mM Ca^{2+} (●), 2.56 mM Ca^{2+} and 20 mM D-glucose (□), 2.56 mM Ca^{2+} and 0.5 mM 3-isobutyl-1-methylxanthine (■), or 1 mM EGTA but not Ca^{2+} (○). In addition, cells were incubated without chlorotetracycline in medium containing 2.56 mM Ca^{2+} (×). To facilitate comparison of peak positions, the differences above the chlorotetracycline-free background were calculated in each experiment. The differences obtained in the range 505–572 nm were summed up and designated as 100%, and the values for the individual wavelengths re-expressed as percentages. The right panel shows values ±SEM of such relative values obtained in four separate experiments without Ca^{2+} (○) or with 2.56 or 5 mM Ca^{2+} (●); no difference was noticed between 2.56 and 5 mM Ca^{2+} as regards the peak position.

fluorescence were very thin (certainly below the resolution power of an ordinary light microscope), the intracellular bodies gave the impression of being membrane delimited. Conceivably, they were mitochondria, perhaps slightly swollen, or lysosomes, or vacuoles of endoplasmic reticulum. The dark nuclear region was also sometimes seen to be delineated by a similar delicate zone of intense fluorescence. The contrast of these intracellular structures against the background was too poor to allow demonstration by photography.

The appearance of islet cells incubated with 50–100 μM chlorotetracycline and 2 mM La^{3+} was also examined. The cells looked the same whether or not 5 mM Ca^{2+} was included in the medium. They were more aggregated than in La^{3+}-free media but not so much as to preclude inspection of individual cells. Virtually no intracellular structures were seen, and the cytoplasmic background fluorescence was so weak as to abolish the negative contrast of nuclei. Instead, there was a very succinct fluorescent demarcation of the cell periphery (Fig. 11). This peripheral fluorescence differed slightly, though clearly, from that induced by Ca^{2+} in the absence of La^{3+}: although the general localization was the same, the fluorescent outline after La^{3+} was smoother and did not give the patchy and interrupted appearance seen after incubation with Ca^{2+} in La^{3+}-free medium.

The above-described visual observations indicate that Ca^{2+}-chlorotetracycline binds to islet cell membranes and that La^{3+} prevents this binding without entering the cells. The observations also show that chlorotetracycline at a concentration of 50 μM or more is capable of entering islet cells. However, whether intracellular formation of Ca^{2+} chelate occurs when the concentration of the probe is only 10 μM cannot be decided from the visual inspection of cells.

Insulin Release

Table XII shows that 10–100 μM chlorotetracycline did not influence the release of insulin from islets incubated with 3 or 20 mM D-glucose in the presence of 1 mg/ml of serum albumin, an

TABLE XI

Chlorotetracycline Fluorescence from Islet Cells not Exposed to EGTA, and from Exocrine Pancreas Cells

Chelator used when dispersing cells	Concentration of Ca^{2+}	Concentration of D-glucose	Fluorescence
	mM	*mM*	*% control*
Experiments with islet cells			
1 mM EGTA	5	0	100
5 mM citrate	5	0	102.3 ± 4.4 (6)
None	5	0	90.3 ± 6.8 (3)
1 mM EGTA	2.56	0	61.3 ± 3.5 (3)
5 mM citrate	2.56	0	61.3 ± 5.6 (3)
None	2.56	0	58/51
1 mM EGTA	0	0	46.1 ± 3.6 (9)
5 mM citrate	0	0	48.2 ± 3.1 (6)
None	0	0	37/33
1 mM EGTA	5	20	67.0 ± 6.1 (3)
5 mM citrate	5	20	64.3 ± 2.0 (3)
Experiments with exocrine pancreas cells			
1 mM EGTA	5	0	100
1 mM EGTA	5	20	101.0 ± 2.0 (4)
1 mM EGTA	0	0	30.3 ± 2.7 (4)

Cell suspensions were prepared by shaking islets or fragments of exocrine pancreas with 1 mM EGTA or 5 mM sodium citrate, or without calcium chelator. After preliminary incubation for 30 min in medium lacking both Ca^{2+} and chelator, the cells were incubated for 60 min with 10 μM chlorotetracycline as well as Ca^{2+} and D-glucose as indicated; Ca^{2+}-free incubation media contained 1 mM EGTA whether or not EGTA had been used when preparing the cell suspensions. Results are expressed as in Table IX except that the values were standardized against those obtained with islet or exocrine pancreas cells dispersed by EGTA and subsequently incubated with 5 mM Ca^{2+} in glucose-free medium. The yield of dispersed cells was minute when islets were broken without chelator; the individual results of two separate experiments with 2.56 or 5 mM Ca^{2+} are shown.

albumin concentration that does not affect the chlorotetracycline fluorescence from islet cells (Table X). Albumin is required to prevent adsorption of insulin to the incubation vials. Nonetheless, because the fluorescence studies were generally performed in the absence of albumin, the release of insulin was also measured in albumin-free media. At a concentration of 100 μM, chlorotetracycline brought about an apparent enhancement of insulin release. This effect could signify a real stimulation of secretion or a prevention of insulin adsorption by chlorotetracycline and should be further analyzed elsewhere. Here, it is important that 10 μM chlorotetracycline influenced neither the basal secretory rate nor the response to 20 mM D-glucose in the absence of albumin. It is concluded that 10 μM chlorotetracycline is a fairly inert probe as regards maintenance of the insulin secretory machinery.

DISCUSSION

A previous, more limited study on whole islets (48) suggested the usefulness of fluorescence probing with chlorotetracycline for exploring roles of cations in insulin release. Reservations were made for uncertainties regarding the exact localization of the probe. A major purpose of the

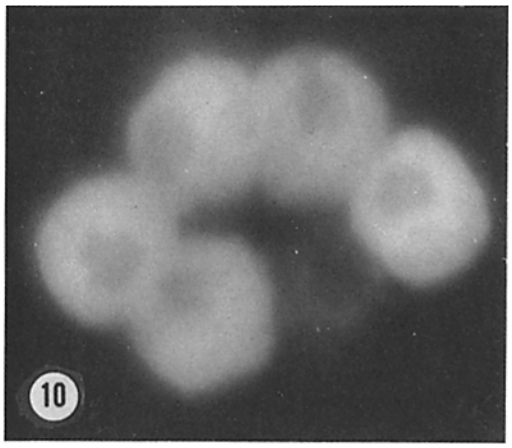

FIGURE 10 Fluorescence microscope appearance of dispersed islet cells after incubation for 60 min with 50 μM chlorotetracycline in Ca^{2+}-free medium containing 1 mM EGTA. Note intracellular dark area representing the nucleus. ×1,200.

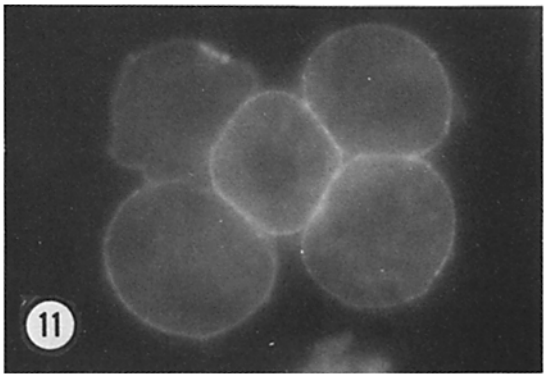

FIGURE 11 Fluorescence microscope appearance of dispersed islet cells after incubation for 60 min with 50 μM chlorotetracycline in medium containing 2 mM La^{3+}. Note relative absence of cytoplasmic staining and presence of a distinct outline of the cell periphery. × 1,200.

TABLE XII

Insulin Release in the Presence of Chlorotetracycline

Concentration of chlorotetracycline (μM)	Immunoreactive insulin in medium (ng/μg dry weight of islets)		
	3 mM glucose (*a*)	20 mM glucose (*b*)	Effect of glucose (*b*) − (*a*)
Serum albumin present			
0	0.20 ± 0.10	1.98 ± 0.33	1.78 ± 0.28*
10	0.27 ± 0.10	2.38 ± 0.63	2.11 ± 0.55‡
50	0.35 ± 0.17	2.15 ± 0.36	1.81 ± 0.27*
100	0.33 ± 0.16	2.04 ± 0.30	1.71 ± 0.27*
No serum albumin			
0	0.025 ± 0.005	0.101 ± 0.035	0.075 ± 0.036
10	0.026 ± 0.009	0.128 ± 0.019	0.102 ± 0.015§
100	0.154 ± 0.035	1.804 ± 0.194	1.651 ± 0.204§

Amounts of insulin in the medium after incubating islets for 60 min were radioimmunologically assayed with crystalline mouse insulin as reference. Each experiment comprised parallel incubations with or without the indicated concentrations of chlorotetracycline in combinations with 3 or 20 mM D-glucose. Mean values ± SEM are given for six separate experiments with incubation media containing 1 mg of serum albumin per milliliter, and for seven separate experiments with albumin-free media.

* $P < 0.01$, ‡ $P < 0.02$, and § $P < 0.001$ for differences between 20 and 3 mM D-glucose (two-tailed *t* tests on paired data). The effect of 100 μM chlorotetracycline in the absence of albumin was significant with $P < 0.02$ at 3 mM D-glucose and $P < 0.001$ at 20 mM D-glucose.

more penetrating investigation reported here has been to ease this uncertainty. The influence of alloxan, and the relationship between fluorescent signal and sample thickness, strongly suggest that chlorotetracycline in β-cells contributed significantly to the fluorescence from whole islets. Thus, it is plausible that the inhibitory effects on islet fluorescence previously observed with Mg^{2+} (48), and here with theophylline, are attributable to interactions with β-cells. This interpretation is supported by the demonstration of Mg^{2+} and methylxanthine effects with suspensions of islet cells, among which more than 90% were β-cells. Obviously, any disproportionate contribution of fluorescence from peripherally located α-cells would be eliminated by the more random distribution of cells in suspensions.

The simple technique described here for measurements on dispersed islet cells made it possible to detect influences of D-glucose on the fluorescence signal, an important advance in comparison with measurements on whole islets. The system of dispersed cells also justified identification of the Ca^{2+}-dependent signal as one predominantly arising from probe molecules located in plasma membranes. As support for this interpretation, the accretion of chelate to the periphery of cells was clearly seen at high magnification. Visual inspection also revealed the occurrence of intracellular Ca^{2+} chelate at high concentrations of chlorotetracycline. However, the quantitative experiments with La^{3+} appear incompatible with any major contribution to the Ca^{2+} signal from intracellular sources when only 10 μM of the probe was used.

The possibility of fairly selectively measuring Ca^{2+} signals from islet cell plasma membranes is attractive in view of previous experiments which have implicated the membrane-located Ca^{2+} with important functions in insulin secretion. Both ultrastructural studies of pyroantimonate precipitates (29, 41, 43) and measurements of $^{45}Ca^{2+}$ fluxes (25) have revealed stimulatory effects of D-glucose on the association of Ca^{2+} with β-cell plasma membranes. Moreover, the radioactively labeled Ca^{2+} was found to have sufficient mobility to meet the kinetic requirements of a mechanism participating in acute stimulus-secretion coupling (25). The results reported here show that the fluorescent Ca^{2+} signal was inhibited by D-glucose in a dose-dependent manner within the range of concentrations to which insulin secretion is responsive (19, 20). This effect must be due to an influence of D-glucose on the islet cells, and not to an effect on the Ca^{2+}-chlorotetracycline chelate as such. As evidence for this conclusion, the fluorescence signal from cells depended on Ca^{2+} concentration in quite another fashion than did the fluorescence from chlorotetracycline in cell-free methanolic solution; moreover, D-glucose had

no effect on the Ca^{2+}-chlorotetracycline chelate in methanol or in exocrine pancreas cells.

The observation of a decreased Ca^{2+} signal from the membranes of islet cells was unexpected in view of the previous demonstrations of enhanced $^{45}Ca^{2+}$ deposition or pyroantimonate precipitation in response to D-glucose. This apparent discrepancy may have two entirely different explanations, both of which raise important new questions for further exploration.

Firstly, an increased deposition of Ca^{2+} in the membrane may occur concomitantly with a decreased fluorescence signal from the same pool. This phenomenon could result from an increased polarity of the Ca^{2+} solvent or a tighter coordination of Ca^{2+} to fixed ligands in the membrane. The mechanism may be illustrated in the present study by the absence of any measurable fluorescence from La^{3+}-chlorotetracycline in islet cells, in spite of which La^{3+} clearly interacted with islet cell membranes, on the one hand, and with chlorotetracycline in methanol, on the other. The present results would seem to have laid the foundation for directly tackling questions concerning the chemical microenvironment of membrane-located Ca^{2+} in β-cells by expanded spectral analyses, including polarization spectra.

Secondly, it is theoretically possible that D-glucose inhibits the deposition of Ca^{2+} in the regions probed by chlorotetracycline in dispersed islet cells. Although this possibility appears more speculative and unlikely in view of previous $^{45}Ca^{2+}$ experiments, admittedly those experiments have so far been carried out only with intact islets and not with dispersed islet cells. The release of insulin from dispersed islet cells is clearly stimulated by D-glucose but not so effectively as that from intact islets (30, 33). If D-glucose has both inhibitory and stimulatory influences on the Ca^{2+} uptake by β-cells, the balance between these effects could be different in dispersed cells as compared with islets. Experiments with ionophores have, indeed, led to the proposal that the β-cell plasma membrane contains both stimulatory and inhibitory Ca^{2+} pools (16).

The relative specificity of the inhibitory action for D-glucose, among the sugars tested, is against interpreting the effect as a kind of trivial physical influence of sugar. Sugars are known to decrease the binding of Ca^{2+} to cellulose acetate filters, but L-glucose is at least as effective as D-glucose in that respect (J. Sehlin, personal communication). On the other hand, the effects of various sugars on the fluorescent Ca^{2+} signal from islet cells did not show a perfect correlation with the known effects of the same sugars on insulin release. Thus, 3-*O*-methyl-D-glucose exerted a slight inhibitory action on fluorescence, although this nonmetabolizable sugar has not been found to influence the release of insulin (19, 39). Moreover, contrasting the effect of D-glucose on insulin release (32), that on fluorescence was not noticeably counteracted by D-mannoheptulose. The effectiveness of sugars reported here most closely corresponds to their effectiveness as substrates for the hexose transport system in the β-cells (23, 24). Although Ca^{2+} deficiency has only a marginal, if any, effect on sugar transport in the β-cells (19), some kind of connection between sugar transport and membrane-located Ca^{2+} is suggested by the present data. Further studies are necessary to clarify the nature of this connection and its possible relevance for the regulation of insulin release. That the effect of D-glucose on fluorescence may be related to secretory control is suggested by the fact that the D-glucose-inhibitable Ca^{2+} signal was also suppressed by Mg^{2+}; Mg^{2+} is thought to inhibit glucose-stimulated insulin release by competing with Ca^{2+} (12), and evidence for Ca^{2+}:Mg^{2+} competitions in β-cells has also been obtained by measuring the membrane electric activity with impaled microelectrodes (1).

The potentiating effect of methylxanthines on glucose-stimulated insulin release is generally attributed to an ability of these drugs to raise the concentrations of cyclic AMP and dissociated Ca^{2+} in β-cells; assumed changes of the cytosolic Ca^{2+} concentration are often thought to result from cyclic-AMP-mediated mobilization of Ca^{2+} from intracellular stores (4, 5, 32). Supporting the idea that methylxanthines can directly or indirectly mobilize bound Ca^{2+} in β-cells, Sehlin (46) reported an inhibitory effect of theophylline on the binding of $^{45}Ca^{2+}$ by a microsomal fraction of homogenized *ob/ob*-mouse islets. The inhibitory effects of theophylline and 3-isobutyl-1-methylxanthine on Ca^{2+}-dependent chlorotetracycline fluorescence described here suggest that methylxanthines influence the binding of Ca^{2+} to the plasma membranes of β-cells. Whether the effect is mainly to release bound Ca^{2+} or, conversely, to enhance liganding of the ion is not known, although the former alternative would seem most likely in view of the $^{45}Ca^{2+}$ experiments with microsomes (46). Both alternatives should be considered in further experiments, however, be-

cause studies of the $^{45}Ca^{2+}$ handling by whole islets have suggested that theophylline affects β-cells in several ways, some of which resemble and some of which are different from those of glucose (37).

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