MOLECULAR PHARMACOLOGY, 27:366–374

Mechanism of Action of Diabetogenic Zinc-Chelating Agents

Model System Studies

R. M. EPAND, A. R. STAFFORD, M. TYERS, AND E. NIEBOER

Department of Biochemistry, McMaster University Health Sciences Centre, Hamilton, Ontario L8N 3Z5 Canada

Received July 31, 1984; Accepted November 28, 1984

SUMMARY

Using model systems, we have studied the properties of a number of zinc-chelating agents which are known to cause diabetes in laboratory animals. The abilities to permeate membranes and to complex zinc inside liposomes with the release of protons are suggested as chemical properties that can enhance diabetogenicity. When such complexing agents are added to lipid vesicles at pH 6 containing entrapped zinc ions, they acidify the contents of these vesicles. We have demonstrated this effect by measuring intravesicular pH both with a fluorine-containing F NMR probe as well as with the fluorescent probe, quinine. For example, using quinine, we observed that 0.1 mM 8-hydroxyquinoline reduced the intravesicular pH of sonicated phospholipid vesicles containing entrapped Zn^{2+} (as sulfate) from pH 6.0 to 2.8. These diabetogenic chelating agents also solubilized zinc-insulin precipitates from unbuffered suspensions at pH 6.0. The solubilization results from the acidification of these suspensions. Dithizone and 8-hydroxyquinoline at 4 mM solubilized 97 and 42%, respectively, of the suspended insulin. We suggest that if such proton release occurs within the zinc-containing insulin storage granules of pancreatic β-cells, solubilization of insulin would be induced. Such an event would lead to osmotic stress and eventually to rupture of the granule. The effects of diethyldithiocarbamate (DDC), an agent that has been found to protect rabbits against the induction of diabetes by some other zinc-chelating agents, were also studied. DDC caused a decrease of 3.5 units in the intravesicular pH of zinc-containing vesicles by a mechanism not involving the release of protons upon chelation of zinc. We have demonstrated several properties of DDC which may contribute to its ability to protect against the induction of diabetes. These include its ability to store zinc as a hydrophobic complex in membranes, its consumption of protons upon spontaneous decomposition, and the ability of one of its decomposition products, diethylamine, to accelerate the dissipation of pH gradients across lipid bilayers. Diethylamine is particularly effective in stimulating a rapid dissipation of such pH gradients, even at micromolar concentrations. We have attempted to estimate quantitatively the extent of proton liberation by various zinc-chelating agents. This analysis demonstrated that partitioning of the ligand between organic and aqueous phases, ligand acidity, and zinc complex stability determine the extent of proton release.

INTRODUCTION

Many agents known to induce diabetes are also zinc-chelating agents and include dithizone (1, 2), oxine (2), quinaldic acid (3), and derivatives of quinoline (4). The role of zinc in the storage of insulin in the pancreas (5) coincides with the suggestion that zinc chelation is important to the mechanism of action of these diabetogenic agents (2). This suggestion is also supported by the fact that the induction of chemical diabetes is accompanied by a loss of histochemically detectable zinc from pancreatic β-cells (6) and that zinc protects animals against chemical diabetes (7, 8). In addition, the guinea pig, which has much less zinc in its pancreatic hormone storage granules, is less susceptible to chemical diabetes (1). Insulin secretagogues also stimulate the release of zinc from pancreatic islets (9). However, since about 1956 when it was first suggested that one of the most commonly used chemical diabetogenic agents, alloxan, did not chelate zinc (10), interest in the role of zinc in chemical diabetogenesis has declined. Nevertheless, there is a great deal of circumstantial evidence to indicate that zinc is important in the diabetogenic action of a number of other zinc-chelating agents such as dithizone and oxine.

This research was supported by a grant from the Juvenile Diabetes Foundation and by the Natural Sciences and Engineering Research Council of Canada.

0026-895X/85/030366-09$02.00/0

One of the features that remains to be explained about the diabetogenic action of zinc-chelating agents is that not all zinc-chelating agents are diabetogenic, and in some cases, structurally very similar compounds can vary widely in their ability to induce diabetes. Thus, for example, although oxine is diabetogenic, neither 7,8-dihydroxyquinoline (4), 5,8-dihydroxyquinoline (11), nor 8-hydroxyquinoline-5-sulfonic acid (12) are diabetogenic, despite their similar chemical structure and their ability to chelate zinc. In addition, it has been shown that one zinc-chelating agent, DDC,[1] can protect rabbits against the diabetogenic action of another zinc-chelating agent, dithizone (13).

We have developed a hypothesis that can explain the diverse actions of different zinc-chelating agents, and in this work we test several predictions of this hypothesis using model systems. Our hypothesis is based upon the fact that insulin storage granules of the pancreatic β-cell contain a dense core of material containing insulin (14) as well as zinc (15). This material is believed to be initially present as an amorphous precipitate but may eventually mature into a crystalline array (5). There is general agreement regarding the size of β-cell storage granules, which have been found to have a diameter of 290–342 nm (16–18). For a spherical granule, this corresponds to a volume of about 1.4×10^{-17} liters. The insulin content of the granule recently has been estimated at 10^6 molecules of insulin per granule (18). Thus, the effective concentration of insulin alone in the granule is of the order of 0.1 M. A somewhat lower estimate of the concentration of insulin of 10 to 30 mM has been made (5) on the basis of granule volume determined by electron microscopy of islet cells (16). It is conceivable that the higher estimate derives from the fact that the purified granules represent a subpopulation containing more insulin. The osmotic pressure within the granule is kept low as a result of the insolubility of the zinc-insulin complex. However, the solubility of the zinc-insulin complex is highly dependent on pH, and its lowest solubility (19) occurs in the pH range from 5 to 6. This pH range is also the same as the intragranular pH of pancreatic storage granules (20). Consequently, the insulin would be close to the point of its minimum solubility under physiological conditions. This low intragranular pH is maintained by an ATP-driven proton pump (21). Conceivably, insulin could be solubilized by the acidification of the granule contents. The resultant dissolution of the granule content would lead to a marked increase in osmotic pressure causing a rupture of the granules. This would also explain the finding that isolated insulin secretory granules are stable only in the pH range 5 to 7 but burst at more extremes of pH (22). A similar type of pH-induced osmotic stress has been implicated as possibly being involved in physiological mechanisms of insulin secretion (21, 23, 24). Since proton gradients across membranes are implicated in the regulation of many biochemical processes, including the translocation of ions and neutral metabolites, it is not surprising that intragranular acidification has serious consequences.

[1] The abbreviations used are: DDC, diethyldithiocarbamate; PIPES, piperazine-*N*,*N*′-bis(2-ethanesulfonic acid); oxine, 8-hydroxyquinoline; NMR, nuclear magnetic resonance.

EXPERIMENTAL PROCEDURES

Materials

$^{65}ZnCl_2$ (3.4 mCi/mg in 0.5 N HCl) was purchased from New England Nuclear (Boston, MA). Ammonium dithiocarbamate was prepared from ammonia gas and carbon disulfide according to the method of Mathes (25). The NMR probe 2,2,2-trifluoroethylamine hydrochloride (Aldrich Chemical Co., Milwaukee, WI) was converted into its sulfate salt by passage through an anion exchange column (Dowex 1-X8), while ammonium dithiocarbamate was converted into its sodium salt by passage through a cation exchange column (Bio-Rad AG-50W-X12). Absence of chloride in the sulfate salt of 2,2,2-trifluoroethylamine was verified with a silver nitrate test. Quinine, monensin, and insulin (bovine, 0.5% zinc, 25.6 IU/mg) were purchased from Sigma Chemical Co. (St. Louis, MO).

Methods

Rate of efflux of Zn^{2+} from phospholipid vesicles. Sonicated unilamellar vesicles were prepared from egg lecithin:cholesterol (molar ratio, 4:1; 10 mg of total lipid/ml) in 4 ml of 10 mM $ZnCl_2$, 10 mM PIPES (pH 6.0), and 100 μl of $^{65}ZnCl_2$ stock solution (1 mCi/ml in 0.02 N HCl). The sonicated preparation was passed through a 1.5 × 25 cm column of Sephadex G-50, eluted with 10 mM PIPES containing 15 mM KCl at pH 6.0. Vesicles containing 10,000 cpm/ml of entrapped $^{65}Zn^{2+}$ were eluted in the void volume. The ability of chelating agents to remove entrapped Zn^{2+} was evaluated by incubating chelating agents at a final concentration of 1 mM with 1 ml of vesicle stock suspension. Incubation was carried out at room temperature in foil-covered test tubes. Transport was stopped at 15 and 60 min by passing 0.5 ml of incubated vesicles through a Sephadex G-50 column (0.5 × 12 cm) and collecting 7-drop fractions. The fractions were then counted on a Beckman gamma counter (model Gamma 300). Transport was assessed by the fraction of $^{65}Zn^{2+}$ left in vesicles (void volume fraction) relative to the amount initially present in zero time controls. In separate experiments, the chelating agents did not cause the release of entrapped carboxyfluorescein from vesicles (experiment not described here), indicating that these agents promote the efflux of zinc from *intact* vesicles. Other ion fluxes, such as the efflux of anions or the influx of protons, required to maintain electrical neutrality, were not studied in this experiment.

Fluorine NMR pH measurements. The method employed was adapted from work on red blood cells by Taylor *et al.* (26). Multilamellar vesicles composed of egg lecithin:cholesterol:dicetylphosphate (7.5:2:0.5) at a concentration of 50 mg/ml were suspended in 10 mM $CF_3CH_2NH_2$ (as a solution of the sulfate salt adjusted to pH 6), plus 10 mM $ZnSO_4$. The pH of the buffer was adjusted so that the final pH, after neutralization of the dicetylphosphate, would be 6.0. Preparations were twice centrifuged at 6000 × *g* for 5 min to remove extravesicular zinc and resuspended in 5 mM $CF_3CH_2NH_2$, 6.7 mM K_2SO_4, pH 6.0. Chelating agents at final concentrations of 1 mM were added to 1.5-ml aliquots of the vesicle suspensions. Some of the chelating agents such as dithizone were not sufficiently soluble in water to be added as aqueous solutions to achieve a final concentration of 1 mM. We therefore introduced the chelating agents in the form of a separate sonicated suspension of egg phosphatidylcholine which had been prepared from a lipid film containing 5–10% chelating agent by weight. Spectra were taken at 4° after various incubation times at 4°. All spectra were recorded on a Brucker WH 90 spectrometer using 10-mm sample tubes. Fourier transform of the pulse free induction decay was calculated by averaging the results of at least 500 pulses. Chemical shifts were measured relative to CF_3COOH, which was used as an external standard with a chemical shift of −78.9 ppm. ^1H decoupling was found not to be required. The dependence of the chemical shift of $CF_3CH_2NH_2$ on pH was measured at 4° in a separate experiment using 10 mM $CF_3CH_2NH_2$ and 10 mM $ZnSO_4$. The presence of $ZnSO_4$ had no effect on the observed

chemical shift. In all NMR measurements with the liposomes, zero time control spectra were run prior to addition of the chelating agent. The NMR assay was particularly useful for studying the water-insoluble chelating agent dithizone, as this agent could be added in the form of a separate vesicle population. However, the use of a fluorescent probe to monitor pH was more sensitive, allowing for the use of sonicated unilamellar vesicles and shorter incubation times. The use of both an NMR and a fluorescent probe further demonstrated that the results were not dependent on the particular model system used.

Fluorescence probe pH measurements. The formation of pH gradients across sonicated unilamellar liposomes was monitored with the use of the fluorescent probe quinine (27). Sonicated unilamellar vesicles composed of soybean lipids (Sigma, phosphatidylcholine, type IV-S) were prepared at a concentration of 10 mg of lipid/ml in 20 mM sodium glutamate buffer, pH 6.0, containing 8.3 mM $ZnSO_4$. The use of chloride-containing salts or HCl was avoided as the chloride anion accelerates the dissipation of pH gradients (28). The extravesicular zinc was removed by passage of the sonicated preparation through a 1.5 × 20 cm column of Sephadex G-50, eluted with 32.3 mM sodium glutamate, pH 6.0. The vesicles were eluted in the void volume and were stored on ice until used for fluorescence measurements.

The assay was performed by adding 2 ml of 20 mM sodium citrate buffer, pH 6.0 to a 1-cm^2 fluorescence cuvette followed by 30 μl of the vesicle preparation with entrapped zinc and 20 μl of a quinine solution to give a final concentration of 0.5 μM quinine. The fluorescence spectrum was scanned over the wavelength range 360 to 487 nm using a Perkin-Elmer MPF-44 spectrofluorimeter in the ratio mode. The chelating agent to be tested was then added in a 20-μl volume to give the desired concentration (usually 0.1 mM), and the fluorescence spectrum was rescanned. In gradient dissipation experiments, a second reagent was added either simultaneously with the complexing agent or subsequently to the development of the pH gradient. A low emission value at 382 nm indicates a drop in pH inside the vesicles where more of the probe will partition (27). Finally, any pH gradient formed was dissipated with the addition of 3 ng/ml of the ionophore monensin and the resulting fluorescence spectrum again was monitored. The fluorescence spectra before the addition of chelating agent and after the addition of monensin were similar. The fluorescence of quinine at a given pH was altered by the presence of lipid. A standard curve was established by preparing vesicles in buffers of different pH and adding a small aliquot of them to the pH 6 buffer. This standard curve was used to calculate the extent of the perturbation of the intravesicular pH.

Solubilization of zinc-insulin complex. A suspension of zinc-insulin complex at pH 6.0 was prepared adding a 4-fold molar excess of $ZnSO_4$ to a 1 mM solution of insulin, both solutions having been adjusted initially to pH 3 with HCl. The final pH of the mixture was adjusted to 6.0 with KOH, whereupon the Zn-insulin complex precipitated. The complex was centrifuged for 10 min at 12,000 × *g*; the supernatant was decanted and replaced with distilled water to give a final concentration of 4 mM insulin. The insulin-zinc slurry was then mixed with the zinc-chelating agents. Because of the insolubility of some of the zinc-chelating agents, particularly dithizone, these agents were incorporated into lipid vesicles to distribute the chelating agents uniformly through the solution so as to attain equilibrium more rapidly. Egg phosphatidylcholine (27 mg/ml water) admixed with 5 mM chelating agent was vortexed and sonicated under a nitrogen atmosphere. The final pH was adjusted to 6.0 with 0.1 N KOH or 0.1 N H_2SO_4. The chelating agents plus lipid vesicles were mixed with the zinc-insulin complex to give a final concentration of 4 mM chelating agent and 1 mM Zn-insulin. The samples were then placed on a wrist action shaker for 1 hr at room temperature. Following this period of equilibration, the pH of the suspension was again monitored and the samples were centrifuged at 12,000 × *g* for 10 min at 4°. The concentration of insulin remaining in the supernatant was measured using the Bio-Rad dye-binding protein assay.

RESULTS

Effect of chelating agents in the permeability of zinc ions across phospholipid membranes. The abilities of chelating agents to accelerate the diffusion of zinc across the phospholipid bilayer of the vesicles are summarized by calculating the percentage of zinc which had escaped from the vesicles after 15 and 60 min (Table 1). For each vesicle preparation, the amount of zinc removed from the vesicle fraction in the presence of a chelating agent was corrected for the amount of zinc transport in the absence of chelating agent (control vesicles) after 15 and 60 min. This correction was always 2% or less of the total radioactivity associated with the vesicles. The presence of the water-soluble chelating agent EDTA did not enhance the rate of zinc transport. In the cases of DDC and dithizone, the presence of the chelating agent allowed more zinc to associate with the vesicles than occurred with the control. This was especially true for dithizone which has very limited water solubility even in the absence of zinc. DDC and dithizone may be poor ionophores because of the strong tendency of their zinc complexes to partition into the lipid bilayer.

The diabetogenic chelating agents oxine and 8-hydroxyquinaldine are the most potent zinc ionophores. Tolbutamide and chlortetracycline are relatively weak in promoting the transport of zinc. However, there is little correlation between the ability of these compounds to induce diabetes and their action as zinc ionophores. Thus, dithizone, which is diabetogenic (1, 2), is a poor zinc ionophore, while another diabetogenic agent, oxine (2), is a potent zinc ionophore. Thus, other factors must play an important role in determining the diabetogenicity of these chelating agents.

Acidification of the contents of zinc-containing vesicles by chelating agents. Using fluorine NMR, we observed

TABLE 1

Effect of chelating agents on the transport of Zn^{2+} from phospholipid vesicles

Release of intravesicular 10 mM labeled Zn^{2+} (as the chloride) from sonicated vesicles of egg lecithin:cholesterol (molar ratio, 4:1) at room temperature into 10 mM PIPES containing 15 mM KCl at pH 6.0.

Compound added (1.0 mM)	Transport[a]	
	15 min	60 min
	%	
8-Hydroxyquinoline (oxine)	90 ± 2	95 ± 1
8-Hydroxyquinaldine	92.6 ± 0.2	95 ± 2
8-Hydroxyquinoline-5-sulfonic acid	30.2 ± 0.9	70 ± 2
4,8-Dihydroxy-2-quinolinecarboxylic acid (xanthurenic acid)	15 ± 8	32 ± 3
Dithizone	−36.3 ± 0.2[b]	−22 ± 2[b]
DDC	−4.0 ± 0.2[b]	1.1 ± 0.8
Tolbutamide	5 ± 3	22 ± 5
Chlortetracycline	4.5 ± 0.3	12 ± 4
EDTA	0	0

[a] Mean ± SD (n = 4); corrected for release of the ^{65}Zn label in the absence of any chelating agent and expressed as per cent loss from zero time.

[b] Negative values indicate the presence of more ^{65}Zn in the vesicles containing the chelating agent than are found in control vesicles without chelating agent.

that the addition of oxine or of dithizone consistently gave rise to a shoulder on the resonance line for the $CF_3CH_2NH_2$ probe (Fig. 1). The position of this shoulder corresponded to an acidification of the intravesicular pH of ≥0.6 pH unit. Since the intravesicular volume is only about 5% compared with the total volume of the solution, the major peak corresponds to the extravesicular probe. The shoulder could be eliminated with the addition of excess Triton detergent. The appearance of the shoulder in the NMR spectra was not observed with vesicles not containing entrapped zinc ions. Unlike oxine or dithizone, the addition of EDTA did not lead to a shoulder on the NMR resonance line. The results support the hypothesis that diabetogenic chelating agents can acidify the contents of zinc-containing liposomes. In some cases, it was difficult to quantitate the extent of acidification of the vesicular contents because of the broadness of the signals. Among the factors contributing to the broadness of the peak is the instability of the pH gradient formed. This line-width enhancement was likely caused by a slow exchange of the $CF_3CH_2NH_2$ during the 10 min required to achieve a good signal to noise ratio. In addition, there may be heterogeneity of the intravesicular pH in the various compartments of the multilamellar vesicles. Cooling the suspension to 4° during the NMR experiments significantly reduced these problems but did not eliminate them completely. The fluorescence assay was used for more extensive studies.

The changes in quinine fluorescence after the addition of 0.1 mM chelating agent were dependent on the presence of Zn^{2+} entrapped in the vesicles. The diabetogenic zinc-chelating agent oxine, which liberates protons upon binding zinc, caused the acidification of vesicle contents (Table 2). The decrease in pH induced by oxine using this fluorescence assay was much greater than that observed with the NMR method. The two systems are difficult to compare directly, but some of the factors that may have contributed to the decreased pH shift in the NMR experiment are as follows. Multilamellar rather than single-walled vesicles were used for the NMR studies (to obtain sufficient signal strength) making it more difficult for the chelating agent to equilibrate with the inner core of the vesicles. The greater internal volume of the multilamellar vesicles requires a larger liberation of protons to affect the same pH change. The higher probe concentration for the NMR experiments and the longer time required to measure the response of the probe (about 15 min for signal averaging in the NMR experiment versus only 3–4 min to acquire the fluorescence spectra) potentiate the dissipation of any pH gradient that might form.

Using the fluorescence assay, we find that DDC, a chelating agent which does not liberate protons when it binds to zinc in aqueous solution, nevertheless strongly acidified the contents of zinc-containing vesicles (Table 2). The mechanism by which this occurs must have other contributions than zinc complex formation (see below). A more stable compound, structurally similar to DDC is ammonium dithiocarbamate (25).[2] This substance also acidified the contents of zinc-containing vesicles, albeit somewhat less than DDC (Table 2). Since DDC has been shown to protect rabbits against the induction of diabetes by oxine (13), we tested the ability of its analogue, ammonium dithiocarbamate, to decrease the extent of acidification of vesicle contents caused by oxine. No protective effect was observed in this *in vitro* system (Table 2). One of the products of the degradation of DDC, diethylamine, was able to reduce the extent of acidification of vesicle contents caused either by DDC or by oxine. This result suggests that diethylamine accelerates the dissipation of pH gradients as occurs in general with permeant weak bases. This was directly tested in our system either using an intravesicular pH of 2.5 induced by the addition of DDC (Table 3) or with a vesicle preparation with an internal pH of 3.0 suspended in a buffer at pH 6 (Table 4). It was found that ammonium sulfate and diethylamine can dissipate the pH gradient with a time course indicating a rapid release of protons from the vesicle within the first minute, followed by the release of protons at a rate at least an order of magnitude slower (Table 3). In the absence of added substance, there is a slow dissipation of the DDC-induced pH gradient (Table 3), possibly due to the formation of diethylamine from DDC breakdown or outward movement of DDC (see Discussion). The slower phase may be rate limited by ion transport required to maintain electrical neutrality. For subsequent measurements, the fluorescence readings were taken within 1 min after addition of the compound being tested, and the fractional change in the pH gradient was recorded. In the absence of added compound, or after addition of 100 μM sodium dithiocarbamate, there was no rapid dissipation of the pH gradient (Table 4). By contrast, very low concentrations of diethylamine as well as of ammonium sulfate rapidly dissipated the pH gradient (Table 4). This phenomenon may contribute to the protective effect of DDC.

Solubilization of insulin from zinc-insulin precipitates by zinc-chelating agents. We have simulated the major contents of insulin storage granules with zinc-insulin suspensions and have measured the ability of these chelating agents to solubilize the insulin from these suspensions. The solubility of the Zn-insulin precipitate was strongly dependent on pH with the minimum solubility occurring at about pH 6 and with markedly greater solubility below pH 5 or above pH 7. As expected, the diabetogenic chelating agents oxine and dithizone acidified the suspensions of zinc-insulin and solubilized a large proportion of the insulin (Table 5). EDTA also induced the dissolution of a significant quantity of insulin. This dissolution of insulin is not likely to be predominantly the result of removing zinc from the precipitate since we found that the solubility of zinc insulin was similar to zinc-free insulin, especially at more acidic pH values (data not shown). We suggest that the observed acidification accompanying solubilization is similar to events which could occur in the interior of insulin storage granules within pancreatic β-cells. Instead of acidification, DDC caused an increase in the pH of the zinc-insulin suspensions. Thus, the ability of this chelating agent to acidify the contents of zinc-containing vesicles is likely to occur by a mechanism different from

[2] R. M. Epand, A. R. Stafford, and E. Nieboer, unpublished results.

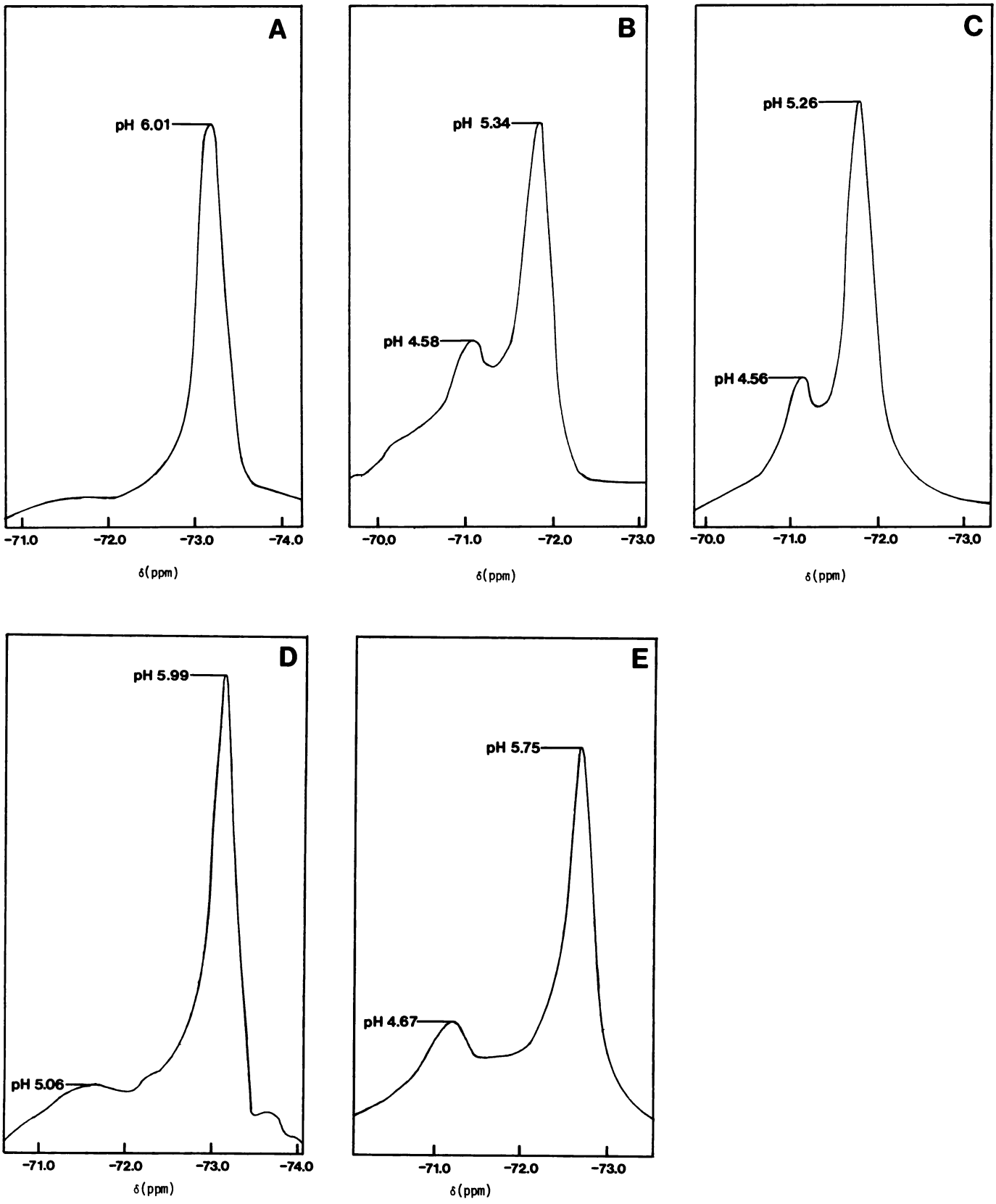

FIG. 1. *^{19}F NMR spectra of zinc-containing multilamellar vesicles*

Vesicles composed of lecithin, cholesterol, dicetylphosphate (7.5:2.0:0.5) were prepared (50 mg/ml) and chelating agents were added in the form of a vesicle suspension as described in Materials and Methods. The pH values shown were obtained from a pH chemical shift calibration curve for $CF_3CH_2NH_2$. Spectra (500 pulses) were recorded at 90 MHz and 4°. A, prior to addition of chelating agent; B, 5-min incubation with 1 mM oxine; C, 5-hr incubation with 1 mM oxine; D, 5-min incubation with 1 mM dithizone; E, 3-hr incubation with 1 mM dithizone.

TABLE 2

Fluorescent probe assay of acidification of the contents of zinc-containing vesicles

Intravesicular ΔpH was measured with quinine using sonicated vesicles of soybean lipids containing 8.3 mM Zn^{2+} (as sulfate) in 20 mM glutamate, pH 6.0. The extravesicular solution was 20 mM sodium citrate, pH 6.0. Values are mean ± SD (n = 2–10)

Compounds added (0.1 mM)[a]	Intravesicular ΔpH[b]
8-Hydroxyquinoline (oxine)	−3.2 ± 0.3
DDC	−3.5 ± 0.2
Ammonium dithiocarbamate	−1.8 ± 0.2
Ammonium dithiocarbamate + oxine[c]	−3.2 ± 0.2
Diethylamine + oxine[c]	−2.1 ± 0.2
Diethylamine + DDC[c]	−2.0 ± 0.1
Sodium dithiocarbamate	−1.5 ± 0.1
Sodium dithiocarbamate + oxine[c]	−3.5 ± 0.2
8-Hydroxyquinoline-5-sulfonic acid	0
Tolbutamide	0
EDTA	0

[a] The strong absorption of the zinc-dithizone complex in the UV/visible region of the spectrum prevented the use of dithizone in this experiment.

[b] Estimated from a standard curve comparing fluorescence change with ΔpH using preformed (known) gradients (see Materials and Methods).

[c] The two compounds were added together to give a final concentration of 0.1 mM each.

TABLE 3

Dissipation of preformed pH gradients with time in the presence of diethylamine or ammonium sulfate

A pH gradient between an external pH of 6 versus an internal pH of 2.5 was first generated with 0.1 mM DDC. Its dissipation with time was recorded after the addition of 0.1 mM ammonium sulfate or diethylamine. Other conditions as for Table 2. Values are mean ± SD (n = 3).

Time	Dissipation with no addition	Dissipation with ammonium sulfate	Dissipation with diethylamine
min	%	%	%
0[a]	0	28.3 ± 0.4	26 ± 3
1.0	4.4 ± 0.1	30.6 ± 0.2	33 ± 5
2.0	10 ± 1	33 ± 2	37 ± 5
5.0	21 ± 1	43 ± 2	46 ± 8

[a] T = 0 represents the minimum time (less than 1 min) after the addition of ammonium sulfate or diethylamine (0.1 mM).

that of the diabetogenic zinc-chelating agents. Preliminary data suggest that the accumulation of the DDC anion occurs by H^+ symport or OH^- antiport. The rise in pH occurs because DDC and ammonium dithiocarbamate are unstable at acidic pH, decomposing into carbon disulfide and a base (ammonia or diethylamine) with the consumption of 2 protons near neutral pH. Since insulin is more soluble above as well as below pH 6, DDC and ammonium dithiocarbamate cause some solubilization of insulin (Table 5). This decomposition could contribute to the protective effect that DDC gives against diabetogenic zinc-chelating agents by reducing the drop in pH caused by these agents. In addition, vesicular DDC efflux might have a similar ameliorating effect. Tolbutamide had very little effect in this system.

TABLE 4

Dissipation of preformed pH gradients

Conditions were as for Table 3.

Compound added	Concentration	Dissipation of pH gradient[a]
		%
Diethylamine	10 nM	25
	100 nM	37
	1 μM	47
	10 μM	50
	100 μM	63
	1 mM	75
Ammonium sulfate	100 μM	21
	300 μM	49
	500 μM	56
Sodium dithiocarbamate	100 μM	0
None		0

[a] Rapid dissipation of a preformed pH gradient (intravesicular pH 3, extravesicular pH 6) less than 1 min after the addition of the reagent.

TABLE 5

Effect of addition of chelating agents to zinc-insulin suspensions at pH 6.0 on the pH and on the extent of solubilization of insulin

This experiment was carried out in the presence of lipid vesicles (see Materials and Methods). Suspensions of insulin which had been precipitated with zinc at pH 6 were mixed with the indicated compound which had been previously sonicated with egg phosphatidylcholine (required to disperse the water-insoluble dithizone). The resulting change of pH and solubilization of insulin were measured. Values are mean ± SD (n = 3)

Compound added (4 mM)	ΔpH	Insulin Solubilized
		%
None	0.05 ± 0.05	7.4 ± 0.8
8-Hydroxyquinoline (oxine)	−1.08 ± 0.03	42 ± 3
Dithizone	−1.5 ± 0.1	97 ± 3
DDC	0.5 ± 0.1	26 ± 2
Ammonium dithiocarbamate	0.75 ± 0.09	39 ± 5
Tolbutamide	0.07 ± 0.03	9.4 ± 0.7
EDTA	−1.3 ± 0.4	31 ± 3

DISCUSSION

Zinc slows the release of insulin *in vivo* and probably contributes to stabilizing the storage form of insulin (5). Thus, one might anticipate that zinc ionophores would be diabetogenic as a result of their ability to remove zinc from pancreatic insulin storage granules. Oxine and 8-hydroxyquinaldine are cases in point as they readily transport Zn^{2+} from phospholipid vesicles, removing virtually all of the zinc in the first 15 min (Table 1). Further, the nondiabetogenic and hydrophilic chelating agents 8-hydroxyquinoline-5-sulfonic acid and xanthurenic acid are poorer ionophores, presumably because of their lower ability to partition into the bilayer. However, the diabetogenic ligand dithizone likely promotes the partitioning of the resulting zinc complex into the membrane, rather than acting as an ionophore (29). Chlortetracycline is thought to be a calcium ionophore (30) as is tolbutamide (31), although the ionophoretic ability of tolbutamide

has been questioned (32). Neither tolbutamide nor chlortetracycline transports zinc (Table 1). Therefore, our data suggest that diabetogenic zinc-chelating agents either accelerate the transport of zinc across phospholipid membranes or render it lipid soluble. It is concluded that the ability to cross lipid membranes and to complex zinc inside liposomes are attributes essential for biological activity related to the diabetogenicity of zinc-chelating agents.

The ability of chelating agents to acidify the interior of zinc-containing vesicles correlates most directly with diabetogenicity. The two potent diabetogenic zinc-chelating agents oxine and dithizone can create pH gradients across the bilayer of zinc-containing vesicles (Fig. 1 and Table 2). They are both lipophilic compounds and can therefore easily cross lipid membrane barriers. In general, the ability of a chelating agent to acidify the contents of zinc-containing vesicles will depend not solely on its permeability and the extent of proton release upon chelation of zinc in water. It will also be determined by the distribution of the zinc-chelating agent and its metal complex between aqueous and membrane phases and the effect of this partitioning on the proton ionization constants of the chelating agent (Table 6). Oxine, 8-hydroxyquinaldine, 8-hydroxyquinoline-5-sulfonic acid, and xanthurenic acid have a pK_a which is above 6. Thus, this group would be protonated at pH 6 leading to the liberation of a proton upon chelation of zinc. If the chelating agents partitioned into a nonpolar environment such as the phospholipid membrane, this pK_a would increase and a proton would still be liberated upon the chelation of zinc. In the cases of dithizone or DDC, pK_1 is below 6 and protons would not be liberated when zinc is chelated in an aqueous environment; however, this pK value is shifted above 6 in a nonpolar environment. Thus, in the presence of the typical lipid concentrations used, dithizone can release protons when partitioned in a membrane phase. This does not occur with DDC, EDTA, or insulin because they are less lipophilic.

Oxine and dithizone also promote the solubilization of zinc-insulin complexes at physiological pH values (Table 5). They would thus cause a marked increase in the osmotic pressure within insulin storage granules leading to their rupture, to their fusion with other organelles, or to exocytosis. By contrast, the nondiabetogenic (12) and more hydrophilic chelating agents 8-hydroxyquinoline-5-sulfonic acid and EDTA do not cause acidification of the vesicle contents (Table 2), presumably because they are not sufficiently permeable to the bilayer. EDTA does cause some solubilization of insulin-zinc complexes (Table 5), as expected from the release of protons accompanying complex formation at pH 6 (see Table 6). It appears that the water-soluble chelating agents are not diabetogenic because they are unable to enter the cell. Tolbutamide is not diabetogenic and it does not acidify the contents of zinc-entrapped vesicles (Table 2), nor does it solubilize zinc-insulin precipitates (Table 5). Thus, all of the diabetogenic chelating agents tested not only are lipophilic but they also release protons upon chelation of zinc as demonstrated by their ability to acidify the contents of zinc-containing vesicles. This reaction can take place with zinc-insulin as the source of Zn^{2+} ions because the diabetogenic chelating agents have a greater affinity for zinc than the weaker binding sites of insulin (compare apparent complex stabilities at pH 6 listed in Table 6). Inside granules, the concomitant acid-

TABLE 6

Proton dissociation constants and zinc-binding affinities of chelating agents

Compound	Ligand acidity[a]		Complex stability[a]		Fraction of ligand (α_L) present in unprotonated form at pH 6.0[b]	Apparent complex stability at pH 6.0[c], log β_n' (n)	Charge of metal complex ML_n, charge (n)	Protons liberated on complex formation of ML_n at pH 6.0[d]
	pK_{ai}	(i = 1,2, etc.)	log K_{ZnL}	log K_{ZnL_2}				
8-Hydroxyquinoline (oxine)	5.0 (1)	9.7 (2)	8.5	7.3	1.8×10^{-4}	8.3 (2)	0 (2)	2.2
8-Hydroxyquinaldine	5.6 (1)	10.0 (2)	7.8[e]	7.5[e]	7.2×10^{-5}	6.0 (2)	0 (2)	2.6
8-Hydroxyquinoline-5-sulfonic acid	3.9 (2)	8.4 (3)	7.5	6.8	4.0×10^{-3}	9.5 (2)	−2 (2)	2.0
Xanthurenic acid	3.9 (2)[e]	8.6 (3)[e]	6.4[e]	4.7[e]	2.5×10^{-3}	5.9 (2)	−2 (2)	2.0
Dithizone	4.5 (1)	10.0 (1)[f]	7.8	7.3	0.97, (10^{-4})[f]	15.1 (2), 7.1 (2)[f]	0 (2)	0, (2.0)[f] (2.0)[g]
DDC	3.4 (1)	7.0 (1)[f]	~6	~5	1.0, (9×10^{-2})[f]	11.0 (2), 8.9 (2)[f]	0 (2)	0, (1.8)[f] (0)[g]
EDTA	6.2 (3)	10.3 (4)	16.3		1.9×10^{-5}	11.6 (1)	−2 (1)	1.6
Insulin						5.3 (1)[h] 3.9 (1)[i]		

[a] Data are taken from current compilations of equilibrium constants; values refer to 25° and an ionic strength of 0.1 M.

[b] α_L was calculated as described in Laitinen and Harris (38).

[c] Apparent complex stability is equal to log $[\beta_n (\alpha_L)^n]$ with $\beta_n = K_{ML} \cdot K_{ML_2} \cdot \cdots \cdot K_{ML_n}$ (38).

[d] Calculated from $\alpha_{HL} + 2\alpha_{H_2L} + \cdots i\alpha_{H_iL}$ (38); for value of n, see previous column.

[e] Obtained by applying a correction factor to data determined in 50% dioxane-water solvent mixture.

[f] Corresponds to the equilibrium $\overline{HL} \rightleftarrows L^- + H^+$, with $\overline{HL}$ the reagent concentration in $CHCl_3$; L^- and H^+ refer to the aqueous phase. Equal volumes of organic and aqueous phases are assumed.

[g] This calculation takes into account that the lipid component constituted 2% of the total volume in the insulin solubilization experiments. Dithizone was present entirely as HL, dissolved in the lipid phase; DDC was largely present as the anion in the aqueous phase.

[h] Corresponds to "tightly" bound zinc ions (2 sites per hexamer at pH 6.0) (39).

[i] Corresponds to "weakly" bound zinc ions (10–12 sites per hexamer at pH 6–8) (39).

ification promotes the dissolution of insulin and the building up of osmotic pressure, and thus the postulated rupture of the granule.

A special case constitutes drugs which can hydrolyze or rearrange after they have entered the storage granule. A possible example of this is alloxan. This substance can readily be taken up by β-cells (33) but may not chelate zinc (10). However, it can readily rearrange to alloxanic acid (34). Alloxanic acid itself is not diabetogenic because it would probably be too polar to penetrate cell membranes. However, if alloxan were converted to alloxanic acid within the storage granules, the rearrangement itself would release protons and additional acidification might also take place upon chelation of zinc with the alloxanic acid. Alloxan is known to lower the pH within pancreatic islets, and this has been suggested to be of importance in its mechanism of action (35). Thus, alloxan cannot be excluded from our general mechanism of the diabetogenic action of zinc-chelating agents, although because of its reactivity other mechanisms may also be involved.

A more complicated example is DDC. This zinc-chelating agent has been found to protect rabbits against dithizone-induced diabetes, although, like the diabetogenic zinc-chelating agents, it has also been found to stimulate the release of insulin from the pancreas (36). A number of factors contribute to these effects. In our model system, DDC exhibits a property of the diabetogenic zinc-chelating agents by causing the acidification of zinc-containing vesicles. The mechanism by which DDC establishes a pH gradient is different from that of oxine or dithizone since DDC does not release protons upon chelation of zinc in aqueous media, nor is it expected to do so effectively in the presence of liposomes (see Table 6). This difference could account for the fact that DDC is not diabetogenic. We have clearly demonstrated that DDC is taken up by lipid vesicles at pH 6 (data not reported). Accumulation of DDC in the vesicle (both in the lipid phase and intravesicularly) requires protons. The process is driven by the chelation of zinc which is found only intravesicularly. Conversely, DDC efflux would result in an intravesicular pH increase, and this might contribute to the dissipation with time reported in Table 3 (no additions), and to the protective effect against dithizone reported for DDC in animal studies. It is also possible that the products of DDC degradation, diethylamine and carbon disulfide, pass through the membrane and then reform DDC inside the vesicle in the presence of zinc with the liberation of 2 protons. Any subsequent decomposition of the intravesicular DDC would consume protons. Thus, the diethylamine formed from the breakdown of DDC can function to dissipate pH gradients, both by acting as a base as well as by accelerating proton fluxes across the vesicle membrane. Inside the storage granule, the DDC anion can chelate zinc without the liberation of protons to form a hydrophobic complex which partitions into the membrane (29), perhaps serving as a reservoir of zinc ions which would be available for complexing with insulin. The ability of DDC to act as a radical scavenger (37) may also protect against the induction of diabetes.

We are currently testing the proposed osmotic mechanism using isolated β-cells and isolated insulin storage granules.

REFERENCES

1. Toroptsev, I. V., and V. A. Eshchenko. Toxic action of dithizone on insulin-producing cells. *Bull. Exp. Biol. Med.* **96**:1215–1217 (1983).
2. Kadota, I. Studies on experimental diabetes mellitus, as produced by organic reagents. *J. Lab. Clin. Med.* **35**:568–591 (1950).
3. Okamoto, H. Effect of quinaldic acid and its relatives on insulin-release from isolated Langerhans islets. *Acta Vitaminol. Enzymol.* **29**:227–231 (1975).
4. Kadota, I., and T. Abe. Chemical specificity of diabetogenic action of quinoline derivatives. *J. Lab. Clin. Med.* **43**:375–385 (1954).
5. Emdin, S. O., G. G. Dodson, J. M. Cutfield, and S. M. Cutfield. Role of zinc in insulin biosynthesis: some possible zinc-insulin interactions in the pancreatic β-cell. *Diabetologia* **19**:174–182 (1980).
6. Maske, H., H. Wolff, B. Stampfl, and F. Baumgarten. Beobachtungen uber den zinkstoffwechsel beim alloxan diabetes (ein beitrag zur alloxanwirkung). *Arch. Exp. Pathol. Pharmakol.* **216**:457–472 (1952).
7. Lazarow, A., and J. W. Patterson. Protection against alloxan diabetes with cobalt, zinc, and ferrous iron. *Anat. Record* **109**:377 (1951).
8. Tadros, W. M., R. Awadallah, H. Doss, and K. Khalifa. Protective effects of trace elements (Zn, Mn, Cr, Co) on alloxan-induced diabetes. *Indian J. Exp. Biol.* **20**:93–94 (1982).
9. Formby, B., F. Schmid-Formby, and G. M. Grodsky. Relationship between insulin release and ^{65}zinc efflux from rat pancreatic islets maintained in tissue culture. *Diabetes* **33**:229–234 (1984).
10. Resnik, R. A., and H. Cecil. The copper complex of alloxanic acid. *Arch. Biochem. Biophys.* **61**:179–185 (1956).
11. Kadota, I., and Y. Kawachi. Diabetic action of analogues of 8-hydroxyquinoline. *Proc. Soc. Exp. Biol. Med.* **101**:365–366 (1959).
12. Root, M. A., and K. K. Chen. Experimental diabetes produced by 8-hydroxyquinoline. *J. Pharmacol. Exp. Ther.* **104**:404–411 (1952).
13. Lazaris, J. A., and F. J. Babelskyi. Preventive effect of diethyldithiocarbamate on experimental diabetes produced by dithisone and 8-oxycholine. *Endocrinol. Exp.* **13**:39–51 (1979).
14. Greider, M. H., S. L. Howell, and P. E. Lacy. Isolation and properties of secretory granules from rat islets of Langerhans. II. Ultrastructure of the beta granule. *J. Cell Biol.* **41**:162–166 (1969).
15. Hutton, J. C., E. J. Penn, and M. Peshavaria. Low-molecular-weight constituents of isolated insulin-secretory granules: bivalent cations, adenine nucleotides and inorganic phosphate. *Biochem. J.* **210**:297–305 (1983).
16. Dean, P. M. Ultrastructural morphometry of the pancreatic beta-cell. *Diabetologia* **9**:115–119 (1973).
17. Pace, C. S., J. T. Tarvin, and J. S. Smith. The role of protons in glucose-induced stimulus-secretion coupling in pancreatic islet B cells, in *Intracellular pH: Its Measurement, Regulation, and Utilization in Cellular Functions* (R. Nuccitelli and D. W. Deamer, eds.). Alan R. Liss, Inc., New York, 483–512 (1982).
18. Matthews, E. K., D. B. McKay, M. D. L. O'Connor, and J. L. Borowitz. Biochemical and biophysical characterization of insulin granules isolated from rat pancreatic islets by an iso-osmotic gradient. *Biochim. Biophys. Acta* **715**:80–89 (1982).
19. Tanford, C., and J. Epstein. The physical chemistry of insulin. II. Hydrogen ion titration curve of crystalline zinc insulin; nature of its combination with zinc. *J. Am. Chem. Soc.* **76**:2170–2176 (1954).
20. Hutton, J. C. The internal pH and membrane potential of the insulin-secretory granule. *Biochem. J.* **204**:171–178 (1982).
21. Hutton, J. C., and M. Peshavaria. Proton-translocating Mg^{2+}-dependent ATPase activity in insulin-secretory granules. *Biochem. J.* **204**:161–170 (1982).
22. Howell, S. L., D. A. Young, and P. E. Lacy. Isolation and properties of secretory granules from rat islets of Langerhans. III. Studies of the stability of the isolated beta granules. *J. Cell Biol.* **41**:167–176 (1969).
23. Malaisse, W. J., A. Herchuelz, and A. Sener. The possible significance of intracellular pH in insulin release. *Life Sci.* **26**:1367–1371 (1980).
24. Smith, J. S., and C. S. Pace. Modification of glucose-induced insulin release by alteration of pH. *Diabetes* **32**:61–66 (1983).
25. Mathes, R. A. Ammonium dithiocarbamate. *Inorganic Synthesis* **3**:48–50 (1950).
26. Taylor, J. S., C. Deutsch, G. G. McDonald, and D. F. Wilson. Measurement of transmembrane pH gradients in human erythrocytes using ^{19}F NMR. *Anal. Biochem.* **114**:415–418 (1981).
27. Lee, H. C., and J. G. Forte. A novel method for measurement of intravesicular pH using fluorescent probes. *Biochim. Biophys. Acta* **601**:152–166 (1980).
28. Nozaki, Y., and C. Tanford. Proton and hydroxide ion permeability of phospholipid vesicles. *Proc. Natl. Acad. Sci. U. S. A.* **78**:4324–4328 (1981).
29. Nieboer, E., A. R. Stafford, S. L. Evans, and J. Dolovich. Cellular binding and/or uptake of nickel(II) ions, in *IARC Scientific Publication No. 53, Nickel in the Human Environment* (F. W. Sunderman, Jr., ed.). International Agency for Research on Cancer, Lyon, France, 321–331 (1984).
30. Schneider, A. S., R. Herz, and M. Sonenberg. Chlortetracycline as a probe of membrane-associated calcium and magnesium: interaction with red cell mem-

branes, phospholipids, and proteins monitored by fluorescence and circular dichroism. *Biochemistry* **22**:1680–1686 (1983).
31. Couturier, E., and W. J. Malaisse. Ionophoretic activity of hypoglycemic sulfonylureas. *Arch. Int. Pharmacodyn.* **245**:323–334 (1980).
32. Gylfe, E., B. Hellman, G. Arvidson, and J. Sandblom. Effect of hypoglycemic sulfonylureas on Ca^{2+} fluxes across lipid bilayers. *Biochem. Med.* **31**:246–253 (1984).
33. Gorus, F. K., W. J. Malaisse, and D. G. Pipeleers. Selective uptake of alloxan by pancreatic beta-cells. *Biochem. J.* **208**:513–515 (1982).
34. Seligson, D., and H. Seligson. The conversion of alloxan to alloxanic acid in plasma. *J. Biol. Chem.* **190**:647–657 (1951).
35. Boquist, L. A new hypothesis for alloxan diabetes. *Acta Pathol. Microbiol. Scand. Sect. A* **88**:201–209 (1980).
36. Kadota, I., and O. Midorikawa. Diabetogenic action of organic reagents: destructive lesions of islets of Langerhans caused by sodium diethyldithiocarbamate and potassium ethylxanthate. *J. Lab. Clin. Med.* **38**:671–688 (1951).
37. Evans, R. G., C. R. Engel, C. L. Wheatly, J. R. Nielsen, and L. J. Ciborowski. An *in vivo* study of the radioprotective effect of diethyldithiocarbamate (DDC). *Int. J. Radiat. Oncol. Biol. Phys.* **9**:1635–1640 (1983).
38. Laitinen, H. A., and Harris, H. E. *Chemical Analysis*, 2nd ed. McGraw-Hill, New York, 189 (1975).
39. Williamson, K. L., and R. J. P. Williams. Conformational analysis by nuclear magnetic resonance: insulin. *Biochemistry*. **18**:5966–5972 (1979).

Send reprint requests to: R. M. Epand, Department of Biochemistry, McMaster University Health Sciences Centre, Hamilton, Ontario L8N 3Z5 Canada.