ISOLATION AND PROPERTIES OF SECRETORY GRANULES FROM RAT ISLETS OF LANGERHANS

III. Studies of the Stability of the Isolated Beta Granules

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ABSTRACT

A partially purified secretory granule fraction, isolated from rat islets of Langerhans by differential centrifugation, was used for investigating the stability of the beta granules during incubation in various conditions. Effects of pH, temperature, and time were studied; the granules possessed optimal stability at 4° and pH 6.0, and could be solubilized at pH 4.0 or 8.5, or in the presence of sodium deoxycholate, but not by phospholipase *c*, ouabain, or alloxan. Incubation with glucose or some of its metabolites, or with tolbutamide, ATP, or cyclic 3′, 5′-AMP did not alter the stability of the beta granules Exogenous insulin-^{131}I was not bound by the isolated granules under the conditions used; no specific insulin-degrading activity could be detected in subcellular fractions of the islets. These findings indicate that intracellular solubilization of the granules with subsequent diffusion of the insulin into the extracellular space is not a likely mode of insulin secretion in vivo, and suggest that a crystalline zinc–insulin complex may exist in the matrix of the beta granules.

INTRODUCTION

The availability of a partially purified secretory granule fraction from rat islets of Langerhans, which contains 60–65% of the insulin content of the tissue, provides opportunities for an investigation of the properties of the isolated, unfixed beta granules. Such a study is of interest in providing data concerning the nature of the material within the granules. In addition, it is important to determine whether or not substances which might initiate insulin secretion from the beta cells in vivo may also alter the stability of their isolated secretory granules in vitro.

MATERIALS

Inorganic salts, glucose, and sucrose were Analar grade reagents obtained from Fisher Scientific Co., Fairlawn, New Jersey. All glucose intermediates, ATP, cyclic 3′,5′-AMP, alloxan, sodium deoxycholate, lecithinase c, and ouabain were obtained from Sigma Chemical Co., St. Louis. Two batches of tolbutamide were supplied by the Upjohn Co., Kalamazoo, Michigan. Pancreatic trypsin inhibitor was obtained from Worthington Ltd., Freehold, New Jersey. Beef insulin was supplied by Eli Lilly Ltd., Indianapolis. Insulin-^{131}I of specific activity

30–40 $\mu c/\mu g$ was obtained from Abbott Laboratories, Chicago.

METHODS

Isolation of a Secretory Granule Fraction

Islets of Langerhans were isolated from rat pancreas by the method of Lacy and Kostianovsky (1967): 70–100 islets were used for each experiment.

The islets were homogenized in 0.3 M sucrose buffered to pH 6.0 with 5 mM sodium phosphate under conditions already described (Howell, Fink, and Lacy, 1969). Nuclei, cell debris, and most of the mitochondria were removed in a common pellet by centrifugation at 5,500 *g* for 5 min. The pellet was resuspended in sucrose and again centrifuged under the same conditions, the supernatants being pooled and spun at 24,000 *g* for 10 min so as to provide a secretory granule fraction. The supernatant was rejected, and the granule fraction was resuspended in 0.4 ml of buffered 0.3 M sucrose by repeated aspiration of the solution into a finely drawn Pasteur pipette.

In most experiments, the incubation medium consisted of 160 mM KCl, 5 mM NaCl, 0.5 mM $MgCl_2$, 5 mM sodium phosphate, pH 6.0; the composition of the medium is discussed below. Test substances were added to the medium in concentrations indicated in the Results section.

Volumes of 0.02 ml of the resuspended granule fraction were transferred to polypropylene centrifuge tubes containing 0.5 ml of incubation medium and mixed thoroughly. They were incubated at 37° for 30 min and then centrifuged at 24,000 *g* (average) for 10 min at 4°C. The supernatants from this centrifugation were immediately removed and stored at 4°C for insulin assay at a later date. In each experiment, four tubes were not centrifuged at the end of the experiment but, instead, the granules were lysed by repeated freezing and thawing to −20°C, and the whole solution was retained for assay of its total insulin content. In most experiments, eight tubes contained control medium only, and eight additional tubes contained this medium together with added test substance.

Calculation of Results

In experiments in which the total insulin content of all 20 tubes was determined, it was found that a high degree of reproducibility of pipetting of the resuspended granule fraction could be obtained. Determinations of the total insulin content of each of 20 tubes in a typical experiment gave a mean of 2.02 milliUnits, with a standard error of the mean of 0.028 milliUnits. The total insulin content of the four tubes determined as described above, therefore, reliably represents the amount present in all the tubes. The insulin released into the supernatant during incubation was expressed as the mean (± standard error of the mean) percentage of the total insulin content of the tubes.

The significance of differences in percentage solubilization of insulin between the two sets of eight tubes in each experiment was assessed by the use of Student's *t* test.

Incubation with Insulin-^{131}I

In these experiments, subcellular fractions (obtained by the use of identical differential centrifugation conditions) of rat liver, adrenal medulla, and pituitary provided controls for the islet fractions. Comparable weights of tissue were used in each case.

Binding of Insulin-^{131}I by Subcellular Fractions

Nuclear, mitochondrial, and "granule" fractions were resuspended in the standard incubation medium described above. To this medium was added, in addition, 1 μg/ml beef insulin, together with a trace of beef insulin-^{131}I to give a final count rate of approximately 50,000 cpm/μg insulin. Volumes of 0.2 ml of this medium were incubated with each fraction, and with the supernatant from the 240,000 *g*-minute centrifugation, for 30 min at 37°C. The material was then repelleted by the use of the appropriate centrifugation conditions, and washed once by resuspension and recentrifugation, with incubation medium containing no added insulin. The supernatant and washings were retained, and the pellets were counted within the centrifuge tubes in a scintillation well counter (Picker Instruments Ltd., Cleveland) to a total of 10,000 accumulated counts. The pellets were subsequently dissolved in 1 N sodium hydroxide for determination of their protein content. The rate of insulin uptake or binding by the tissue fractions was expressed as μg insulin/100 mg protein/30 min.

Insulin Degradation by Subcellular Fractions

The protein content of the supernatant and washings from experiments in which binding of insulin-^{131}I by subcellular fractions was studied, was precipitated by the addition of an equal volume of 10% trichloroacetic acid (TCA), and the tubes were allowed to stand at 4°C for 2 hr. The precipitates were then spun down, washed once with 5% TCA, and the resulting pellets and final supernatants were counted in the manner already described. Results were expressed as the percentage of total counts which were present in the TCA supernatants.

RESULTS

Effect of pH

Fig. 1 shows the results of incubation of the secretory granules in KCl-rich medium whose pH was adjusted by the dropwise addition of 1 N NaOH or 1 N HCl to 4.0, 5.0, 6.0, 7.0, 7.4, or 8.5. The granules appeared to be stable in the pH range 5.0–7.0, with minimal degradation occurring at approximately pH 6.0. At pH 4.0 or 8.5, total solubilization of the insulin content of the granules was evident.

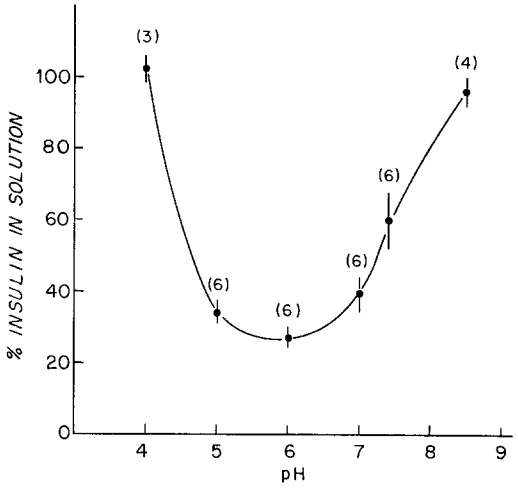

FIGURE 1 Effect of pH on the stability of the isolated beta granules during incubation at 37°C for 30 min. Means ± SEM of the number of observations in parentheses are shown.

Alteration of Ionic Composition of Incubation Medium

Results of experiments in which media of different composition were utilized are summarized in Fig. 2. The granules were clearly more stable in a potassium-rich medium than in a sodium-rich one, and it appeared that greater stability could be attained by the use of 220 mM potassium chloride (KCl). This concentration of KCl probably exceeds that found within the islet cells, and it was, therefore, not utilized in further experiments. The stability of the granules was not affected by presence or absence of calcium or magnesium, nor by alterations of phosphate concentration. A suitable medium, which approximated the intracellular environment and which provided reasonable stability of the granules, consisted of 160 mM KCl, 5 mM NaCl, 0.5 mM $MgCl_2$, 5 mM NaH_2PO_4, pH 6.0. This medium was used in all subsequent incubations.

Temperature and Duration of Incubation

The granules were more stable at low temperatures (4°) than at 25° or 37° (Fig. 3). Electron microscopic examination of the granule fraction after 30 min of incubation at 37° indicated that many of the granules had remained intact, and that they appeared similar to those present in the freshly isolated preparations (Fig. 4). The secre-

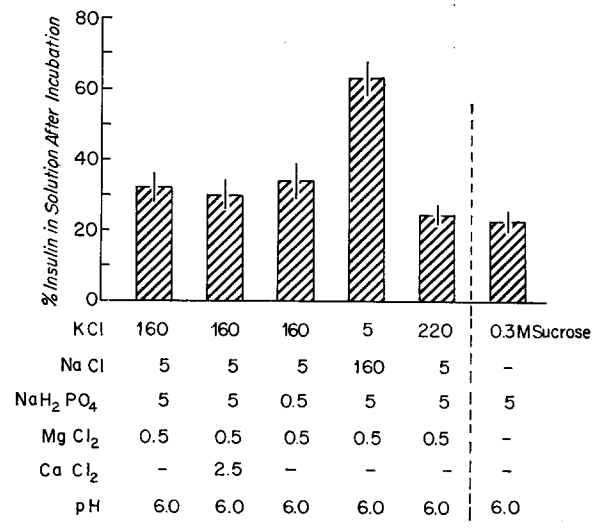

FIGURE 2 Effect of alteration of the ionic composition of the incubation medium on the stability of the isolated beta granules. Concentrations are shown in millimoles; incubation was at 37° for 30 min. Each result represents the mean ± SEM of four observations.

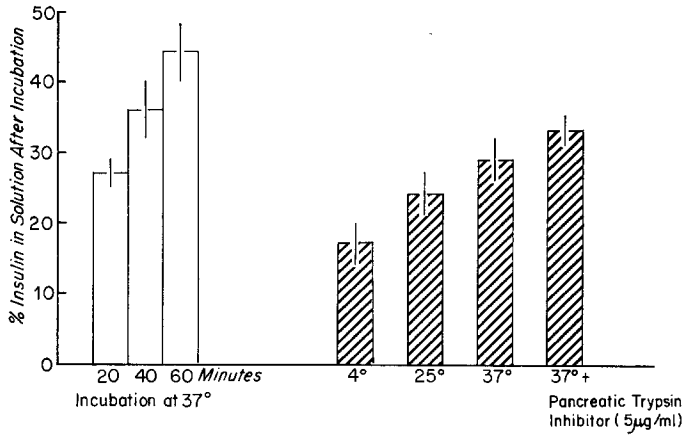

FIGURE 3 Effects of duration of incubation and of temperature on the stability of the isolated beta granules. 30 min of incubation was used in experiments designed for studying the effects of temperature. Results are given as mean ± SEM of four observations.

tory granule fraction prepared by differential centrifugation contained some mitochondrial and microsomal contamination. However, the time required to separate these components by density gradient centrifugation rendered additional purification of the granule fraction by this means impractical in the large number of experiments performed in this study. Activation of residual proteolytic enzymes in the fraction at the higher temperatures did not appear to be an important factor in eliciting breakdown of the granules, since addition of pancreatic trypsin inhibitor at a concentration of 5 μg/ml resulted in no increase in stabilization during incubation at 37°. Some part of the solubilization of the insulin content of the granules probably resulted from mechanical damage which occurred during resuspension of the pellet after centrifugation.

Effects of Glucose, Tolbutamide, Cyclic 3′,5′-AMP, ATP, and Arginine

Tolbutamide was used at a concentration of 0.2 mg/ml to investigate the effects of this agent on the isolated granules. Different incubation conditions, including the presence of 5 mM calcium chloride, were utilized, but the beta granules maintained their stability in each experiment (Table I): this finding confirmed the observation of Malaisse, Mayhew, and Wright (1967). Glucose (14 mM), cyclic 3′,5′-adenosine monophosphate (cyclic 3′,5′-AMP) at a concentration of 1.5×10^{-5} M, adenosine triphosphate (0.5 or 3.0 mM), and L-arginine (2.5 mM) were also without significant effects under these conditions (Table I).

Incubation in the Presence of Glucose Metabolites and Other Intermediates

Results of incubation of the isolated secretory granule fraction in the presence of various glucose metabolites and of other intermediates are shown in Table II. The concentrations used are shown in the Table and were comparable to those found within the intact islet tissue (These data were kindly provided by Dr. F. Matschinsky of Washington University). None of the agents used in this series of studies was effective in releasing insulin from within the granules.

Alloxan and Ouabain

These experiments were performed at 4°C in order to minimize the degradation of alloxan during the incubation period. At this temperature and pH 6.0, the half-life of this reagent appears to be at least 3 hr (Watkins, Cooperstein, and Lazarow, 1964). Alloxan at an initial concentration of 0.25 mM had no effect on the stability of the granules under these conditions (Table III).

Ouabain. A potent inhibitor of Na^+-K^+–activated adenosine triphosphatase activity in many tissues (Post, Merritt, Kinsolving, and Albright, 1960), ouabain (0.1 mM) was without action on the isolated beta granules (Table III).

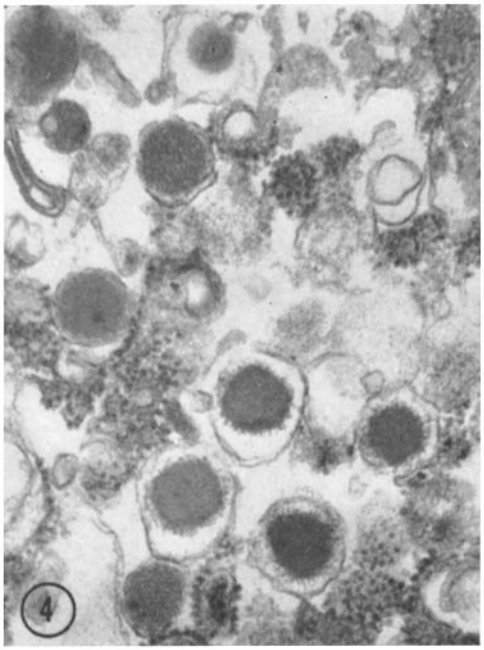

FIGURE 4 Appearance of granules in the isolated granule fraction after 30 min of incubation at 37°C in the KCl-rich medium utilized in these studies. The membrane of the granules remains intact, and their appearance remains similar to that of freshly isolated preparations. × 49,000.

Sodium Deoxycholate and Phospholipase c

In experiments designed to identify the material within the matrix of the beta granule, it was found that sodium deoxycholate completely solubilized the insulin content of the granules (Table III); these experiments were performed in medium of pH 7 in order to retain the deoxycholate in solution. Electron microscopic examination of the granule fraction after incubation with this agent indicated complete destruction of the organelles, none of which was still identifiable.

Phospholipase *c* (50 μg/ml) in the presence of 5 mM $CaCl_2$ had no significant effect on the stability of the isolated granules (Table III). The limiting membrane of the granules was still evident, by electron microscopy, after treatment with phospholipase *c*, and significant release of the insulin content of the granules was not observed after incubation of the granule fraction with this agent. Preliminary experiments with red cell suspension showed that, in the presence of 5 mM $CaCl_2$, phospholipase *c* (50 μg/ml) induced hemolysis during incubation of the red cells under conditions identical with those used during incubation of the isolated granules.

Binding of Insulin-^{131}I by Tissue Fractions

Results of experiments in which subcellular fractions of islet, liver, adrenal, and pituitary

TABLE I

Effects of Insulin Secretogogues on the Integrity of the Isolated Beta Granules

Substance	Concentration	Insulin content released from the granules*		No. of observations in each group
		Control	+ Added substance	
		%	%	
Glucose	14 mM	25 ± 4	24 ± 3	16
Tolbutamide	0.2 mg/ml	23 ± 3	26 ± 3	12
Tolbutamide + 5 mM $CaCl_2$	0.2 mg/ml	46 ± 4	49 ± 5	8
L-Arginine	2.5 mM	37 ± 6	34 ± 5	8
Cyclic 3′, 5′-AMP	1.5×10^{-5} M	39 ± 3	36 ± 5	14
Adenosine triphosphate	0.5 mM	26 ± 3	29 ± 5	8
	3.0 mM	21 ± 2	25 ± 3	8

* Results are expressed as Means ± SEM of the number of observations indicated in the final column.

TABLE II

Effects of Metabolic Intermediates on the Integrity of the Isolated Beta Granules

Intermediate	Concentration	Insulin content released from the granules* Control	+ Added intermediate	No. of observations in each group
	mM	%	%	
Glucose-6-phosphate	1.0	47 ± 7	44 ± 7	16
Fructose-6-phosphate	0.1	31 ± 3	36 ± 5	12
Fructose-1:6-diphosphate	0.1	34 ± 3	31 ± 3	12
Sodium citrate	3.0	29 ± 5	28 ± 4	10
6-Phosphogluconate	0.1	45 ± 6	37 ± 5	12
Ribulose-1:5-diphosphate	0.1	43 ± 3	37 ± 6	8
Uridine diphosphoglucose	1.0	41 ± 4	33 ± 5	10
Phosphocreatine	1.5	38 ± 7	43 ± 6	10

* Results are given as Means ± SEM of the number of observations shown in the final column.

TABLE III

Effect of Alloxan, Ouabain, Sodium Deoxycholate, and Phospholipase c on the Integrity of the Isolated Beta Granules

Substance	Concentration	Insulin content released from the granules* Control	+ Added substance	No. of observations in each group
		%	%	
Alloxan (4°C)	0.25 mM	23 ± 5	23 ± 3	15
Ouabain	0.1 mM	28 ± 5	34 ± 4	16
Sodium deoxycholate (pH 7.0)	0.16%	56 ± 4	103 ± 5	12
Phospholipase c + 5 mM $CaCl_2$	50 μg/ml	49 ± 5	58 ± 5	16

* Results are expressed as Mean ± SEM of the number of observations shown in the final column.

TABLE IV

*Binding of Insulin-^{131}I by Tissue Fractions**

	Nuclei	Mitochondria	"Granules"
Islet	23 ± 8	68 ± 12	61 ± 8
Liver	19 ± 3	51 ± 13	76 ± 12
Pituitary	30 ± 8	76 ± 19	68 ± 15
Adrenal medulla	41 ± 13	41 ± 5	82 ± 15

* Results are expressed as μg insulin bound/100 mg tissue protein/30 min. Means ± SEM of five determinations are shown.

TABLE V

*Insulin Degradation by Tissue Subcellular Fractions**

	Nuclei	Mitochondria	"Granules"	Supernatant
Islet	28 ± 4	32 ± 3	31 ± 1	34 ± 3
Liver	52 ± 4‡	36 ± 2	30 ± 4	67 ± 9‡
Pituitary	32 ± 4	34 ± 4	36 ± 7	30 ± 1
Adrenal medulla	34 ± 4	33 ± 2	31 ± 3	43 ± 6

* Results are expressed as Means ± SEM of the percentage radioactivity in the TCA supernatant (see text).

‡ $p < 0.05$ for differences from corresponding islet values.

tissues were incubated with 1 μg/ml insulin and a trace of insulin-^{131}I are shown in Table IV. The results have been expressed as μg insulin bound/100 mg of tissue protein/30 min. It is evident that every fraction of each tissue bound insulin-^{131}I to some extent, and that, on a weight basis, the degree of binding increased in fractions of smaller particle size (cell debris and nuclei < mitochondria < granules). This result probably reflects the nonspecific adsorption of small quantities of insulin to the surface of the organelles.

No evidence was obtained that the isolated beta granules were capable of specifically binding

large quantities of insulin-^{131}I under the incubation conditions used. Further experiments showed that no binding could be demonstrated in the presence of much lower concentrations (1 mμg/ml) of the iodinated hormone.

Degradation of Insulin-^{131}I by Tissue Fractions

Table V shows the percentage of insulin-^{131}I which could not be precipitated by 5% trichloroacetic acid after 30 min of incubation with subcellular fractions from four tissues. Estimation of the protein contents of the TCA precipitates confirmed that the weights of each tissue were comparable in the various fractions. Control experiments showed that approximately 25% of the insulin-^{131}I present in the incubation medium could not be precipitated by TCA after incubation in the absence of any tissue. In addition, each fraction possessed some slight insulin-degrading activity, but only the liver nuclear and supernatant fractions possessed activities significantly different from those seen in the islet subcellular fractions: these hepatic tissue fractions indicated the presence of significant insulin-degrading activity. An insulin-degrading enzyme or enzyme systems have already been extensively studied in the liver (Mirsky, Perisutti, and Dixon, 1955; Tomizawa and Halsey, 1959). It had been suggested that such an enzyme might play a role in insulin biosynthesis under some conditions (Kotoulos, Morrison, and Recant, 1965).

DISCUSSION

Incubation Procedure

It was shown in the previous paper (Howell, Fink, and Lacy, 1969) that 60–65% of the total insulin content of the islets is present in the secretory granule fraction obtained by differential centrifugation which was utilized in these experiments. It is assumed that the remaining 35% of the secretory granules possessed biochemical characteristics identical to those of the fraction studied.

Recoveries of the total insulin content of the incubated granules were shown to be highly reproducible (see Methods section), and were identical whether lysis was achieved by repeated freezing and thawing, by ultrasonic disruption, or with sodium deoxycholate.

The isolated granules were found to be stable in isotonic salt solutions as well as in 0.3 M sucrose. A potassium-rich medium appeared preferable to a sodium-rich one, although the reasons for this difference are not known. Greater stability could be achieved by the use of 220 mM KCl, possibly as a result of an increase in tonicity. Since this concentration probably exceeded the normal intracellular level of potassium, it was not utilized in subsequent studies. The stability of the granules appeared to be unaltered by changes of phosphate or calcium concentration.

Incubation at pH 6.0 and 37°C for 30 min. in the medium defined in the Methods section resulted in a 20–45% release of insulin from the granules. The results within each experiment were reproducible; however, the reasons for the day-to-day variation observed are not clear. The greater degree of solubilization seen at 37° as compared to 25° or 4° may reflect the greater solubility of the insulin within the granules with increasing temperature.

Preliminary experiments indicated that optimal stability of the isolated granules in 0.3 M sucrose could be achieved at pH 6.0, and this pH was utilized in the isolation and subsequently in the incubation media. The intracellular pH of the islet cells is unknown. The pH-solubility curve of the isolated granules in potassium-rich media closely resembles that of crystalline zinc-insulin incubated in saline (Fredericq and Neurath, 1950; Hallas-Moeller, Petersen, and Schlichtkrull, 1952).

Incubation with Glucose, Glucose Intermediates, Tolbutamide, Arginine, or Cyclic 3′5′-AMP

Elevation of blood glucose levels may provide the most important physiological stimulus to insulin secretion. The pathways of glucose metabolism which may lead to this stimulation are not known, although the activities of enzymes and the levels of intermediates within mouse islets have recently been studied (Matschinsky and Ellerman, 1968). There is some evidence that the pentose phosphate pathway may be important in regulating insulin secretion in the rat (Montague and Taylor, 1968).

In addition to glucose, a wide variety of other agents have been shown to influence rates of insulin secretion (Williams and Ensinck, 1966). Of these agents, tolbutamide exerts its effects specifically on the beta cells, while arginine may promote secretion of growth hormone and gluca-

gon in addition to insulin (Merimee, Lillicrap, and Rabinowitz, 1965; Floyd et al., 1965; Ohneda, Ketterer, Eisentraut, and Unger, 1967). Elevation of intracellular levels of cyclic 3′,5′-AMP may also promote insulin release, although the mechanism of its action in eliciting the effect is unknown (Malaisse, Malaisse-Lagae, and Mayhew, 1967).

Incubation of the isolated beta granules with glucose, with various intermediates of the glycolytic and pentose phosphate pathways, or with uridine diphosphoglucose resulted in no change in their stability. Furthermore, tolbutamide, either in the presence or in the absence of high calcium concentrations (Curry, Bennett, and Grodsky, 1968), arginine, and cyclic 3′,5′-AMP were also without effect. Arginine, cyclic 3′,5′-AMP, and tolbutamide may exert their effects on the process of secretion directly, without further metabolism to other products. Thus, in this study no evidence was obtained for a mode of secretion of insulin in which intracellular solubilization of the granules might occur, with subsequent diffusion of the hormone in solution out of the cells. It seems likely that the emiocytosis mechanism proposed by Lacy (1961), in which the secretory granule enclosed by its limiting membrane moves to the cell surface intact and liberates its insulin content after fusion of the granule sac with the plasma membrane, may provide the most important mode of insulin secretion in vivo. A possible mechanism for the translocation of the intact granules within the cell has recently been proposed (Lacy, Howell, Young, and Fink, 1968).

Incubation with ATP

Low concentrations of ATP (0.5 mM) have recently been shown to induce release of catecholamines from isolated secretory granules of the adrenal medulla, and of vasopressin from neurosecretory granules in vitro (Poisner and Douglas, 1968). However, even at concentrations of 3 mM, with an incubation medium similar to that used by those authors, no effect of ATP on release of insulin from the isolated beta granules could be detected.

Effects of Phospholipase c or Sodium Deoxycholate

Incubation with phospholipase *c* at a concentration of 50 μg/ml did not cause release of insulin from the isolated granules. Thus, a biochemically intact limiting membrane may not be essential to the survival of the granule in vitro, at least under some conditions. In addition, lecithin may be assumed not to constitute an essential part of the granule matrix, since it would have been removed by the enzyme with release of the insulin content of the granule into solution. However, sodium deoxycholate did cause complete disruption of the granules and release of their insulin content, as was also reported for secretory granules from goosefish islets by Lazarow, Bauer, and Lindall (1964). Electron microscopic examination of the granule fraction from rat islets after this treatment indicated that complete disruption of all organelles had occurred, and that the effects of deoxycholate could not be considered specific at the high concentrations (0.16%) used.

The Composition of the Beta Granule

Ultrastructural studies of the beta granule indicate that its matrix may consist of a crystalline material (Greider, Howell, and Lacy, 1969). This suggests the presence of an orderly molecular array within the granule matrix, either as a single protein or as a tightly bound complex of insulin together with a binding substance (or substances). Insulin bound in such a complex might be expected to exhibit physical properties markedly different from those of isolated and crystallized insulin.

It has already been noted above that the solubility characteristics of the insulin within the beta granules appear very similar to those of crystalline insulin. Furthermore, it is evident that the isolated granules show a lack of reactivity in the presence of external agents (such as ATP), which contrasts with the responses of the secretory granules of the adrenal medulla and posterior pituitary where binding materials have been shown to be present (Haselbach and Piguet, 1952; Banks and Helle, 1965). It seems possible that such a marked difference of behavior may reflect the absence of such binding materials from the beta granule matrix. Investigations are currently in progress to determine whether ATP or other small molecules may be present within the granules, and to further characterize the material obtained by extraction of the purified granule fraction.

It has long been known that the islet cells of the pancreas are rich in zinc (Okamoto, 1942),

and more recently a correlation between the granulation of the beta cells and their insulin content has been reported (Logothetopoulos, Kaneko, Wrenshall, and Best, 1964). Studies of the isolated beta granules have suggested that a pH close to 6 may be optimal for their survival within the cells. Thus, there may exist within the beta cells excellent conditions for the crystallization of insulin: a pH assumed to be close to the isoelectric point of rat insulins, and a high concentration of zinc. By contrast, when the granules reach the cell surface, the markedly different pH and zinc content of the lymph would induce immediate liberation of the insulin content of the granules into solution.

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