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Research Article

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Studies on the Secretion of Newly Synthesized Proinsulin

and Insulin from Isolated Rat Islets of Langerhans

HIROYUKI SANDO, JO BORG, and DONALD F. STEINER

From the Department of Biochemistry, University of Chicago, Pritzker School of Medicine, Chicago, Illinois 60637

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throughout the period of observation. With cycloheximide present, the concentration of proinsulin in the islets steadily declined while the specific activity of proinsulin remained high, indicating that the proinsulin pool is small and is turning over rapidly. In terms both of amount and radioactivity proinsulin amounted to 6–7% on a molar basis of the insulin in both the medium and the islets. Addition of dibutyryl cyclic 3',5'-adenosine monophosphate (DBCAMP) (0.002 M) with high glucose during the postlabeling period slightly increased the rate of insulin secretion (133% of control) but did not significantly alter the other parameters.

The results suggest that while newly synthesized insulin and proinsulin may be preferentially secreted to a slight degree, about 90% of the insulin released during 3 hr in response to glucose, or to glucose and DBCAMP, is derived from pre-existing granule stores. There were no indications of the existence of independent or nongranule pathways of insulin or proinsulin secretion.

INTRODUCTION

Studies on the biosynthesis of insulin in intact islets of Langerhans isolated from rat pancreas have shown that single chain proinsulin is synthesized in the rough endoplasmic reticulum, presumably on membrane-bound polyribosomes, and is subsequently transformed within the islet cells to insulin, the principal storage product of the beta granules (1–4). The subcellular site of transformation of proinsulin to insulin has been localized tentatively to the Golgi apparatus and/or the newly formed secretory granules (3, 5). The proteolytic conversion is a relatively slow process (half-time of conversion is approximately 1 hr), as shown by steady-state or pulse-chase labeling experiments (2). Previous studies have shown that newly synthesized insulin does not appear in appreciable amounts in the incubation medium until about 1 hr after the initiation of labeling; it is pre-

sumed that this delay represents the time required for the intracellular transfer of new secretory product from the ribosomes to the secretory granules (2), as has been amply documented in a variety of other secretory cells, such as pancreatic acinar cells and anterior pituitary cells (6, 7).

Recent studies of the insulin secretory response in isolated islets, perfused whole pancreas, or intact organisms have demonstrated a biphasic pattern characterized by a brief initial burst of secretion followed by a slower more sustained rise (8–10). It has been proposed that the rapid and quantitatively small first phase of secretion results from the release of stored material, while the second phase response is composed mainly of newly synthesized insulin as it is partially suppressed by inhibitors of protein synthesis (9).

In the experiments reported here, we have undertaken to determine the extent to which newly synthesized insulin and proinsulin are released from islets in response to glucose by comparing the specific activities of the proinsulin and insulin secreted into the medium with the specific activities of these proteins in the islets, in the presence and absence of cycloheximide. The results indicate that newly synthesized insulin (or proinsulin) does not comprise a major proportion of the secreted material during the initial 3 hr period after labeling the islets.

METHODS

Partially purified collagenase was obtained from the Worthington Biochemical Corp. (Freehold, N. J.). Hank's salt solution (11) was prepared as a stock concentrate ($\times$ 10) without bicarbonate and diluted before use and adjusted to pH 7.4 by appropriate gassing after addition of $NaHCO_3$. L-Leucine-4,5-^{3}H was supplied by the Radiochemical Centre, Amersham, England, at a specific activity of 22 Ci/mmole.

Two female Sprague-Dawley rats (180–250 g), that had been fed ad lib., were anesthetized by intraperitoneal injection of sodium pentobarbital (25 mg/animal). The excised pancreases were trimmed of fat, minced, and incubated with rapid stirring in a solution of collagenase (50–60 mg dissolved in 8 ml of Hank's salt solution) for about 25 min (12). Then, under a dissecting microscope, islets were picked up in a micropipette and transferred to a drop of medium maintained in an ice bath. About 400 islets were usually obtained from two rats. Residual exocrine cells were removed by repeated transfer of the islets. The islets were incubated in a small chamber made from a 7 mm length of vinyl tubing (10 mm diameter), with a bottom retainer made of fine mesh (5 μ) stainless steel screen (M.E.R. Chromatographic, Mountain View, Calif.; part No. 314).

In the first group of experiments, islets were incubated for 1 hr at 37°C (95% O_2/5% CO_2) in 200 μl of Hank's incubation medium (Ca^{++} — 2.4 mM, pH 7.4) containing 1 mg/ml of glucose, leucine-^{3}H (100 μCi/ml) and 200 μg/ml of bovine serum albumin (BSA,[1] Miles Research Div.,

[1] Abbreviations used in this paper: BSA, bovine serum albumin; DBCAMP, dibutyryl cyclic 3′,5′-adenosine monophosphate.

Miles Laboratories, Inc., Kankakee, Ill.) (pulse). Afterwards, the islets were washed 10 times with 0.5 ml of Hank's incubation medium containing 250 μg/ml of cold leucine to eliminate the radioactive leucine, and then incubated for 1 hr in medium containing 1 mg/ml of glucose, 250 μg/ml of unlabeled leucine, and 200 μg/ml of BSA (control period). After incubation, the medium was withdrawn and frozen, and about 10 islets were removed and homogenized in acid-ethanol (375 ml 95% ethanol: 7.5 ml concn. HCl:145 ml water) containing 1–2% BSA. The medium was replaced with fresh medium containing 3 mg/ml of glucose and unlabeled leucine as before. The medium was collected every 30 min and replaced with an equivalent volume of fresh medium containing the same components. About 10 islets were removed for extraction every 60 min. The total incubation period was 4 hr including the initial 1 hr labeling period (Fig. 1). In the second group of experiments, islets were chased in the presence of sufficient cycloheximide to inhibit protein synthesis in the islets by 95% or more (2). The medium used in the control period after labeling contained 0.5 mg/ml glucose, 100 μg/ml of cycloheximide, and 25 μg/ml of unlabeled leucine. This medium was replaced with medium containing 3 mg/ml of glucose and the same amount of cycloheximide and unlabeled leucine as in the control period. The medium and the islets were sampled in the manner described above. The total incubation period was 4 hr (Fig. 1).

20 μl of each medium sample was saved for measurement of secreted IRI. The medium and islets were extracted along with 1–2% BSA in acid-ethanol by a modification of the Davoren method (13). The ethanol-ether precipitate was dissolved in 3 M acetic acid and fractionated on a 1 $\times$ 50 cm column of Biogel P-30 (100–200 mesh, Bio-Rad Laboratories, Richmond, Calif.) equilibrated with 3 M acetic acid. Elution was at room temperature with the same solvent. 1 ml of each fraction, mixed with 15 ml of Triton-toluene scintillator (Triton X-100, 25 ml; toluene, 75 ml; 2,5-diphenyloxazole (PPO), 400 mg; 1,4-bis[2-(5-phenyloxazolyl)]benzene (POPOP), 5 mg), was counted in a Packard liquid scintillation spectrometer (Packard Instrument Co., Inc., Downers Grove, Ill.). Recovery of radioactive protein ranged from 95–100%. The remaining liquid in the fractions containing the peak radioactivity of either proinsulin or insulin was removed in vacuo after addition of 0.1 ml of 3% BSA. The dried material was dissolved in 2 ml of 0.1 M tris-HCl buffer (pH 7.65, 0.06 M
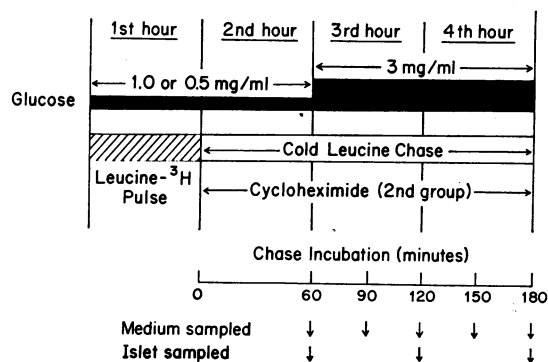

FIGURE 1 Experimental design (see methods for procedural details).

HCl, and 0.25% BSA). The proinsulin and insulin content were measured by a modification of the double antibody radioimmunoassay technique of Morgan and Lazarow (14, 15). The antiserum was prepared by standard procedures in guinea pigs with the use of porcine insulin as antigen (15). Rabbit anti-guinea pig globulin serum was obtained from the Sylvana Co. (Millburn, N. J.). The standards used were rat insulin and a mixture of rat proinsulin and intermediates (16). The specific activity of each fraction was calculated by dividing the radioactivity by the proinsulin or insulin content (Fig. 2). No correction for the difference in leucine content of rat proinsulin (10 leucines) and rat insulin (6 leucines) was necessary in these experiments since the connecting polypeptide (4 leucines) is quantitatively recovered in the insulin peak in this extraction and gel filtration method (16, 17). Five replicate experiments were carried out in each group.

RESULTS

Secretion of proinsulin and insulin. The cumulative secretion per 100 islets of both proinsulin (Fig. 3) and

Fractionation of Medium Extract
Biogel P-30: 3$\underline{M}$ HAc

$$SA \text{ of pro.} = \frac{Cp}{IRp}$$

$$SA \text{ of ins.} = \frac{\frac{Ci1}{IRi1} + \frac{Ci2}{IRi2}}{2}$$
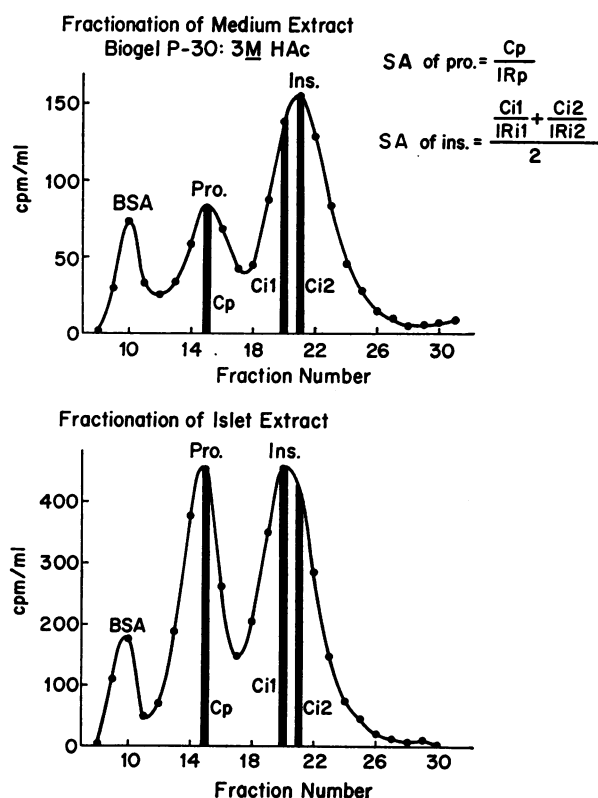
Fractionation of Islet Extract

FIGURE 2 Method for estimation of proinsulin and insulin-specific activity. After fractionation of the medium or islet extracts on columns of Biogel P-30 radioactivity was distributed as shown in these examples. One tube from the proinsulin peak and two from the insulin peak (black bars) were used for immunoassay of insulin and proinsulin (the distribution of immunoassayable and radioactive protein within each peak was uniform). Specific activities were calculated as shown (Cp, proinsulin cpm; IRp, immunoreactive proinsulin; Ci, insulin cpm; IRi, immunoreactive insulin).
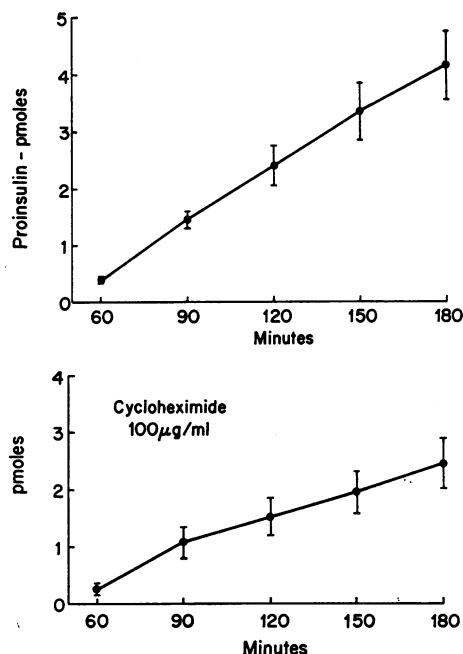

FIGURE 3 Cumulative immunocreative proinsulin secretion per 100 islets. Upper panel, without cycloheximide; lower panel, with cycloheximide. Brackets indicate SEM value of five experiments.

insulin (Fig. 4) increased in the high glucose medium (3 mg/ml) without or with cycloheximide. The total amount of proinsulin secreted was 4.2±0.6 pmole/100 islets without cycloheximide and 2.5±0.4 pmole/100 islets with cycloheximide during 3 hr. The rate of secretion of proinsulin was significantly reduced after the 2nd hr in the presence of cycloheximide ($P < 0.1$, 120–150 min; $P < 0.05$, 150–180 min). Although the initial values were somewhat lower, the rate of insulin secretion (slope) and the total amount of insulin secreted were not significantly different in the presence of cycloheximide (Fig. 4).

Ratio of proinsulin to insulin. The molar proportion of proinsulin amounted to about 6% of the amount of insulin both in the medium and the islets over the entire incubation period (Fig. 5, upper panel). The proinsulin content in the islets was almost the same as that measured in fresh whole pancreas from nonfasted rats (7%). In the experiments with cycloheximide, the proinsulin ratio progressively decreased in both the medium and the islets (Fig. 5, lower panel). The ratio in the medium fell from 7.6 to 4% during 3 hr incubation. The proportion of proinsulin in the islets incubated with cycloheximide was always significantly lower than that in the medium; it steadily declined from 3.9 to 1.3% of the insulin content during the 3 hr incubation period. These results indicate that without renewal by continuous synthesis the proinsulin pool rapidly becomes de-
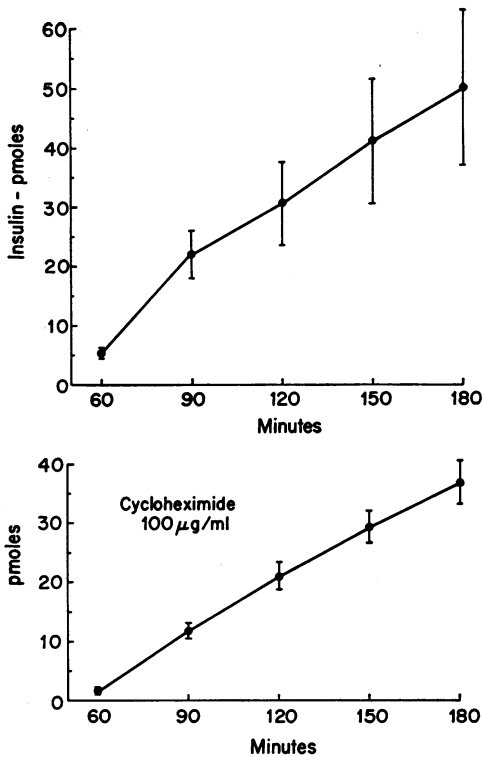
FIGURE 4 Cumulative immunoreactive insulin secretion per 100 islets. Upper panel, without cycloheximide; lower panel, with cycloheximide. Brackets indicate SEM value of five experiments.
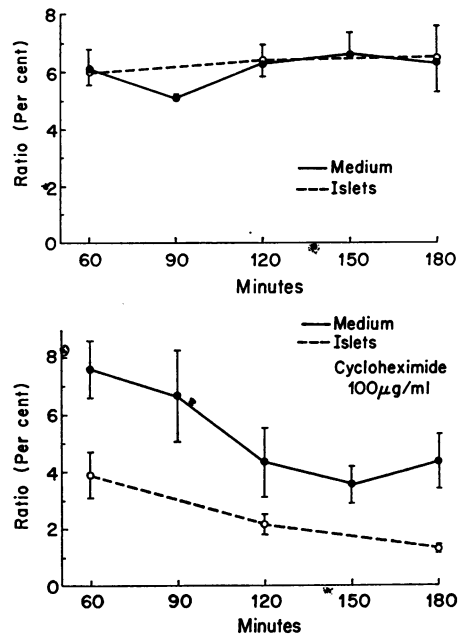

FIGURE 5 Molar ratio (in per cent) of proinsulin to insulin in the medium samples and in the islets. Upper panel, without cycloheximide; lower panel, with cycloheximide. Brackets indicate SEM value of five experiments.

pleted. The less marked and less rapid decline of proinsulin/insulin ratio in the medium as opposed to the islets suggests that proinsulin is not directly secreted from biosynthetic sites but instead is transferred to granules which subsequently undergo secretion.

Extent of conversion of proinsulin to insulin. The extent of conversion of proinsulin to insulin was calculated by dividing the radioactivity of the insulin by the sum of the radioactivities of proinsulin and insulin. In both sets of experiments, the extent of conversion increased during incubation (Fig. 6). Without cycloheximide, the ratio of radioactive insulin to proinsulin in the medium was higher than that in the islets by about 10%, indicating that proinsulin is not preferentially released.

Specific activity of proinsulin. The specific activity of the proinsulin in the islets decreased during incubation without cycloheximide (Fig. 7). Presumably the pool size of proinsulin in the islets is small and the continual dilution by newly synthesized nonlabeled proinsulin during the chase plays a role in this decline in the specific activity. The specific activity of proinsulin in the medium was the same as that in the islets, suggesting that preferential secretion of newly synthesized proinsulin does not take place in response to glucose stimulation. The
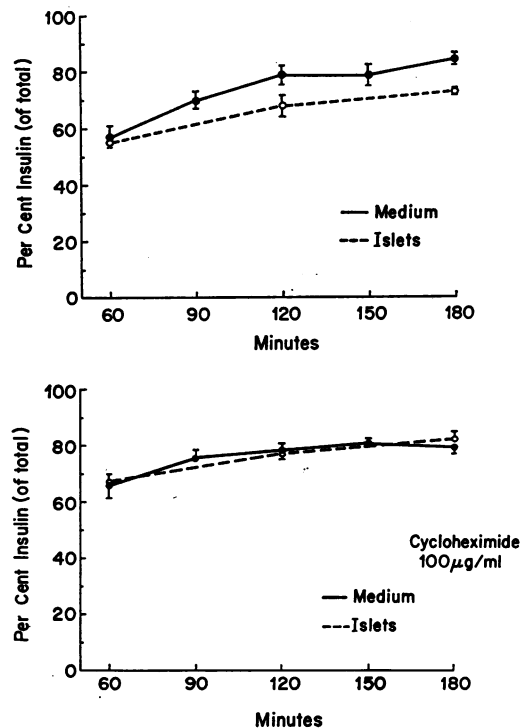

FIGURE 6 Extent of conversion of proinsulin to insulin in the medium samples and in the islets. Upper panel, without cycloheximide; lower panel with cycloheximide. Brackets indicate SEM value of five experiments.

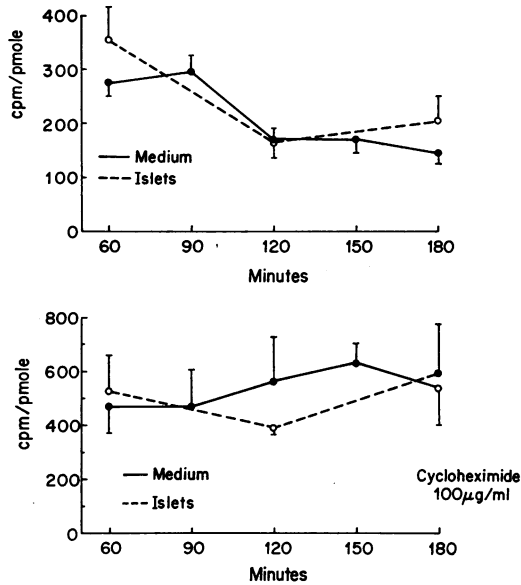*Secretion of Proinsulin and Insulin from Rat Islets* 1479

FIGURE 7 The specific activity of proinsulin in the medium samples and in the islets. Upper panel, without cycloheximide; lower panel, with cycloheximide. Brackets indicate SEM value of five experiments.

specific activity of the proinsulin in the medium and in the islets remained essentially constant during incubation with cycloheximide. The ratio of the specific activity of proinsulin in the medium to that in the islets remained about 1 during the entire incubation period, with or without cycloheximide (Fig. 8).

Specific activity of insulin. The specific activity of the insulin in the medium was always higher than that in the islets (Fig. 9). Without cycloheximide the ratio of the specific activity of the insulin in the medium to that in the islets showed a peak value of 2.4 at the 3rd hr after the beginning of labeling; it decreased to 1.4 by the
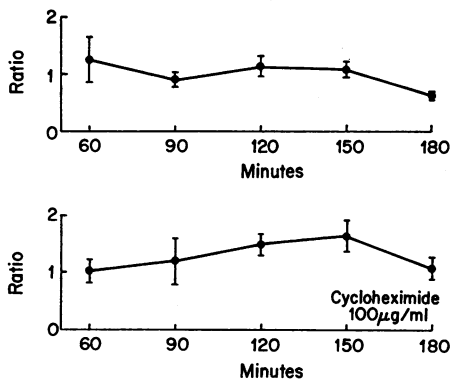

FIGURE 8 Ratio of the proinsulin specific activity in the medium samples to that in the islets. Upper panel, without cycloheximide; lower panel, with cycloheximide. Brackets indicate SEM value of five experiments.
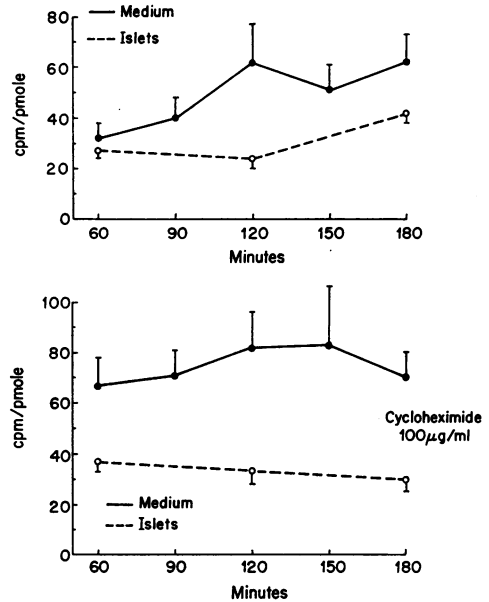

FIGURE 9 The specific activity of insulin in the medium samples and the islets. Upper panel, without cycloheximide; lower panel, with cycloheximide. Brackets indicate SEM value of five experiments.

4th hr (Table I). With cycloheximide the ratio of the specific activity of the insulin in the medium to that in the islets showed a peak value of 2.7 after 3.5 hr. There was no significant difference between the ratios of the specific activities of the insulin in the medium and the

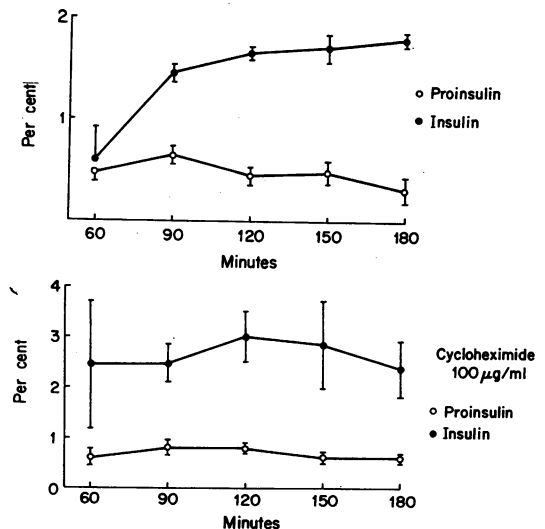FIGURE 10 Secretion of newly synthesized (³H-labeled) proinsulin and insulin expressed as a percentage of the total islet proinsulin and insulin radioactivity. Upper panel, without cycloheximide; lower panel, with cycloheximide. Brackets indicate SEM value of five experiments.

Minutes during chase	0–60	60–90	90–120	120–150	150–180
Without cycloheximide	1.18±0.15*	1.50±0.24	2.43±0.29	2.10±0.10	1.46±0.19
With cycloheximide, *100 µg/ml*	1.96±0.50	1.92±0.16	2.49±0.19	2.71±0.40	2.08±0.16
t value	1.1875	0.8603	0.0954	0.9115	1.2485
P value	NS	NS	NS	NS	NS

* SEM.

islets in the presence or absence of cycloheximide (Table I).

Percentages of secreted proinsulin and insulin. When expressed as a percentage of the total islet proinsulin/insulin radioactivity, less newly synthesized insulin was secreted into the incubation medium in the absence of cycloheximide (1–2%) than in its presence (2–3%) (Fig. 10). The corresponding values for total labeled insulin secreted into the medium in the absence and presence of cycloheximide were 6.7±1.0% and 12.9±2.4% respectively (Table II). This difference, however, was only marginally significant ($P < 0.1$). Total immunoreactive insulin secreted into the medium was not altered in the presence of cycloheximide (7.7±1.9% without cycloheximide; 6.1±1.0% with cycloheximide) (Table II).

When the quantity of newly synthesized proinsulin secreted into the medium was expressed as a percentage of the total islet proinsulin/insulin radioactivity, addition of cycloheximide did not significantly alter the results (2.9±0.5% without cycloheximide; 3.4±0.6% with cycloheximide) (Table II). The percentage of immunoreactive proinsulin secreted into the medium throughout the entire chase period was somewhat higher in the

presence of cycloheximide (13.6±3.4%) than in its absence (8.4±0.7%), but this difference was not statistically significant ($P < 0.2$) (Table II and Fig. 11). The percentage of the total immunoreactive proinsulin that was secreted (13.6±3.4%) was also somewhat higher than the percentage of the total immunoreactive insulin that was secreted (6.1±1.0%) when cycloheximide was present ($P < 0.1$). No significant difference between these values was observed in the absence of cycloheximide (Table II). Mean values for immunoreactive proinsulin, insulin, and total radioactivity per islet in the presence and absence of cycloheximide are shown in Table III. Only the proinsulin concentration in the islets was affected by the addition of cycloheximide. The amount of proinsulin decreased by about 50% during the 3 hr incubation.

TABLE II

Percentage of Secreted Proinsulin and Insulin

	Cycloheximide			
	(−)	(+)	*t* value	*P*
Total secreted radioactive proinsulin	2.9 ±0.5*	3.4 ±0.6	0.6	NS
Total secreted radioactive insulin	6.7 ±1.0	12.9 ±2.4	2.3	$P < 0.1$
Total secreted immunoreactive proinsulin	8.4 ±0.7	13.6 ±3.4‡	1.5	$P < 0.2$
Total secreted immunoreactive insulin	7.7 ±1.9	6.1 ±1.0‡	0.7	NS

* SEM.
‡ *t* = 2.13; *P* < 0.1 when compared with each other (total secreted immunoreactive proinsulin vs. insulin).
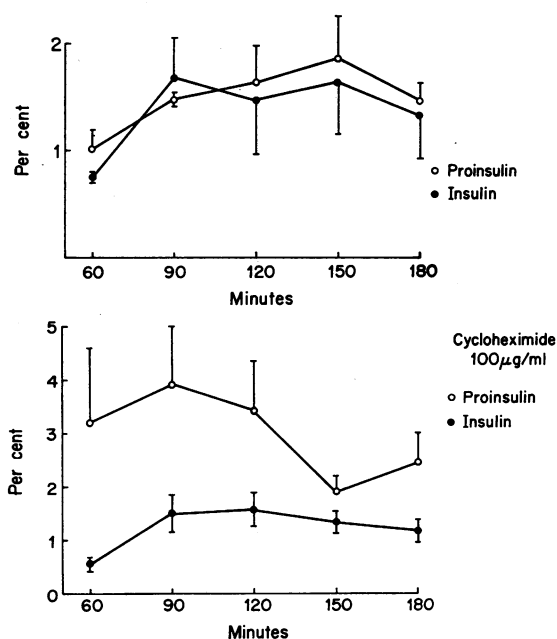

FIGURE 11 Secretion of immunoreactive proinsulin and insulin expressed as a percentage of the total islet proinsulin and insulin. Upper panel, without cycloheximide; lower panel, with cycloheximide. Brackets indicate SEM value of five experiments.

TABLE III

*Content per Islet of Proinsulin and Insulin, and Their Total Radioactivity**

| | Cycloheximide | | | |
	(−)	(+)	*t* value	*P*
Proinsulin, *ng/islet*	3.67± 0.43‡	1.95± 0.63	2.26	*P* < 0.1
Insulin, *mU/islet*	0.95± 0.08	0.90± 0.10	0.4	NS
Radioactivity, *cpm/islet*	301 ±30	295 ±47	0.10	NS

* Mean of values obtained throughout the 3 hr incubation.
‡ SEM.

DISCUSSION

Despite a growing list of substances that modify or stimulate insulin release from islet cells, the molecular details of the mechanism of insulin release are not yet understood. Thus in the case of glucose, the most important stimulus to insulin release under most physiological conditions, it is still unclear as to whether the glucose molecule per se (18) or one of its many metabolites derived along the various glycolytic pathways (19) is the initiating factor in the secretory response to this substance.

It has been questioned recently as to whether material secreted in response to glucose is derived exclusively from preformed secretory granules or, on the other hand, may be partly derived from the site of biosynthesis or other compartments within the cell. Although radioautographic studies on the biosynthesis and transfer of secretory products in a wide variety of cells strongly support the hypothesis that the secretory products are confined within the cell to the rough endoplasmic reticulum, Golgi apparatus and, finally, to the secretion granules (6, 7, 20–22), even more definitive proof of this strict compartmentation has been obtained in some instances by histochemical localization techniques (23). On the other hand, fractionation studies with disrupted cells have never succeeded in demonstrating this degree of sequestration (4, 24), presumably due to the fragility of some of the subcellular membranous structures, which release their contents during homogenization of the tissue. We might then first inquire as to whether evidence was obtained in these experiments for the existence of alternate or nongranule routes of insulin secretion from the islets. The extent of conversion of proinsulin to insulin in the medium sheds some light on this question since the rate of conversion of proinsulin to insulin is relatively slow and appears to begin only after a sufficiently long time interval has elapsed after biosynthesis to allow the newly labeled products to be transferred to the Golgi apparatus or to secretion granules (3). Moreover, the existence of an energy-dependent step before conversion has been demonstrated (3). This step is similar in its time relationships to an energy-dependent stage in the transfer of newly synthesized secretory protein from the rough endoplasmic reticulum to the peripheral Golgi tubules in pancreatic acinar cells (25). Thus, it seems highly probable that, if "direct" or nongranule secretion of insulin were to occur from islets, the secreted material should consist mainly if not entirely of proinsulin rather than insulin. This was not observed in these experiments, inasmuch as the ratio of proinsulin to insulin in the medium was always lower than that in the islets, indicating that newly synthesized insulin rather than proinsulin is released preferentially (Fig. 6). It is possible that the relatively small differences in extent of conversion between the islets and the medium observed here do not accurately reflect the degree of preferential insulin release from islets, since some of the radioactive protein in the incubation medium could have arisen from dissolution of cells near the periphery of the islets. It is difficult experimentally to exclude such a source. Further evidence against a nongranule route of insulin discharge is the almost total absence of secretion of newly synthesized proinsulin or insulin during the 1st hr (labeling period), as was also observed previously (3). Thus the long delay before secretion of newly synthesized materials begins to occur, as well as the slightly greater extent of conversion of the secreted materials both indicate that release is due largely, if not exclusively, to the discharge of preformed secretory granules.

A second question of considerable importance pertains to whether preferential release of newly synthesized, rather than stored protein occurs. This possibility has been raised by studies of the biphasic character of the insulin secretory response to glucose, particularly by the partial suppression of the second phase of the secretory response in the presence of inhibitors of protein synthesis such as cycloheximide and puromycin (9). The experiments reported here were designed in such a way that mainly the second phase of insulin secretion was studied. Addition of cycloheximide to the incubation medium during the 3 hr postlabeling period did not produce a statistically significant lowering of the rate

of release of insulin (Fig. 4). The inhibition reported by others working with perfused pancreas preparations (9) may be due to effects of these substances unrelated to inhibition of protein synthesis, since substantially higher concentrations were used in those experiments.

On the other hand, addition of cycloheximide did significantly lower the proinsulin content in the islets (Fig. 5) and the total amount of proinsulin released into the medium (Fig. 3), indicating that a major fraction of the proinsulin in the islets is rapidly turning over and is continually renewed by synthesis. This is reflected also in the high initial specific activity of the proinsulin in the islets (500 cpm/mole), which is about 10 times higher than that of the insulin, and by the steady decline of the proinsulin-specific activity when protein synthesis is not inhibited in the islets (Fig. 7). It seems likely, therefore, that the reduced secretion of proinsulin in the presence of cycloheximide is secondary to the decreased formation of proinsulin. The high specific activity of the proinsulin in the medium, similar to that in the islets (Fig. 7), suggests, moreover, that most of the proinsulin found in the medium is derived from more recently synthesized material, while this is not the case with the secreted insulin, which is derived in large part from a more stable pool contained in the older secretory granules. A corollary of this conclusion would necessarily be that the mature secretory granules contain a very low molar proportion of proinsulin, i.e. less than 2%, reflecting their much slower turnover time of many hours or even days. In order to explain the similar molar ratio of proinsulin to insulin in the medium to that in the islets in the absence of an inhibitor of protein synthesis, it must be concluded that a small proportion of the secretion granules released in response to glucose contain a very high proportion of proinsulin, i.e., that there is no preferential secertion of the "oldest" secretion granules before the release of newer ones.

If, on the other hand, mainly new granules, or newly synthesized insulin in some other particle or form, were preferentially released in response to glucose, we would expect to see a very large increase in the specific activity of the insulin released into the incubation medium as compared with that in the islets. However, we observed only a slight increase in the specific activity of the insulin in the medium compared with that in the islets throughout the entire chase incubation; the ratio of specific activities only increased by a factor of about 2 (Table I). The peak increase in specific activity occurred about 2 hr after the end of the labeling period in agreement with data reported earlier by Howell and Taylor (26). The delayed peak in specific activity probably is due to two factors; (a) the time required for conversion of newly synthesized proinsulin to insulin and (b) the time required for newly synthesized material to

be transported from the site of biosynthesis to the site of secretion. The slightly higher specific activity of the insulin in the medium could even be an artifact, due to uneven distribution of labeled material in the islets and/or the presence of a central core of nonviable islet tissue containing unlabeled insulin in some of the islets. We attempted to evaluate this possibility by radioautographic analysis of islets incubated for 1 hr with leucine-^{3}H under the same conditions as were used in these experiments. We indeed observed an uneven distribution in grain density from one islet to another and in many cases a heavier distribution of grains was found near the islet periphery.[3] These findings, although only tentative, seem to agree with observations of others that individuals islets isolated by the collagenase digestion technique vary in their insulin secretory response as well as in their insulin content (27).

Additional experiments were carried out in the same manner as those presented execpt that 2 mM dibutyrylcyclic 3',5'-AMP (DBCAMP), rather than cycloheximide, was added to the incubation medium during the 3 hr postlabeling incubation period. Although the results in these experiments were more variable, DBCAMP significantly increased the insulin secretory response to 3 mg/ml glucose (133% of control) (28). On the other hand, DBCAMP did not significantly alter the proportions of newly synthesized proinsulin or insulin secreted into the medium, or the maximum ratio of the specific activity of the insulin in the medium to that in islets (2.6:1). These results imply that DBCAMP elicits additional secretion of insulin by a similar mechanism to that for glucose, and from the same compartment in the beta cell; thus both glucose and DBCAMP release mainly preformed or stored hormone.

The initial specific activity of proinsulin in the islets provides a minimum estimate of the specific activity of newly labeled insulin derived from it, i.e., 500 cpm/pmole (Fig. 7, lower panel). The ratio of the observed specific activity of the secreted insulin (approximately 50 cpm/pmole, Fig. 9) to this value gives an estimate of the proportion of the total newly synthesized insulin that undergoes secretion. On this basis it is estimated that the maximum per cent of newly synthesized insulin released into the medium during the course of the 3 hr incubation is about 10% of the total insulin. The admixture in the medium of small amounts of newly synthesized insulin and proinsulin suggests that newly formed secretory granules mix with the pre-existing granule population and undergo release statistically rather than preferentially.

These findings bear on the interpretation of the observations in other laboratories that the insulin secretory response to glucose consists of two distinct components

[3]Dzoga, K., H. Sando, and D. Steiner, unpublished data.

(8–10). Rather than indicating the release of insulin from separate compartments within the cell, such as separate pools of stored and newly synthesized insulin, our results suggest that these changes in the kinetics of secretion must reflect the operation of complex regulatory circuits that control the rate of release of a mixture of old and new secretory granules. The brief initial rise of secretion followed by a sharp decline may indicate the rapid formation of a stimulating metabolite within the beta cells followed by the formation of an inhibitor which tends to counter its effect. Thus, the kinetic characteristics of the secretory response to glucose can be viewed as essentially cybernetic phenomena, rather than as manifestations of differences in ultrastructural organization or compartmentation within the cell.

Alternatively, functional compartmentation may exist in the cells such that the initial rapid phase of the secretory response represents the discharge of secretory granules that already are poised in close contact with the cell membrane so as to rapidly fuse with it when appropriate alterations in the properties of the plasma and/or secretion granule membrane occur. The slower second phase of release may be limited by two factors; (a) the rate of transport or diffusion of secretion granules towards the cell membrane and (b) further activation of the plasma or granule membranes for fusion. According to this view the total number of secretion granules available for discharge within a given beta cell might profoundly influence the observed response, both qualitatively and quantitatively. The magnitude of the first phase, in particular, might be greatly altered. Thus the diminished first phase of insulin secretion that occurs in many diabetic and prediabetic subjects (29), may reflect diminished insulin reserves or functional alterations in the regulatory mechanisms governing insulin secretion, rather than intrinsic changes in either pools or important components of the secretory mechanism (3). Our findings with DBCAMP support the hypothesis that DBCAMP (and presumably also cyclic 3',5'-adenosine monophosphate) acts within the beta cells by modulating the effect of glucose on the insulin release mechanism (30).

REFERENCES

1. Steiner, D. F., D. D. Cunningham, L. Spigelman, and B. Aten. 1967. Insulin biosynthesis: evidence for a precursor. *Science (Washington).* 157: 697.
2. Steiner, D. F. 1967. Evidence for a precursor in the biosynthesis of insulin. *Trans. N. Y. Acad. Sci.* 30 (No. 1): 60.
3. Steiner, D. F., J. L. Clark, C. Nolan, A. H. Rubenstein, E. Margoliash, F. Melani, and P. E. Oyer. 1970. The biosynthesis of insulin and some speculations regarding the pathogenesis of human diabetes. *In* The Pathogenesis of Diabetes Mellitus. Proceedings of the Thirteenth Nobel Symposium. E. Cerasi and R. Luft, editors. Almqvist & Wiksell, Publishers, Stockholm. 123.
4. Sorensen, R. L., M. W. Steffes, and A. W. Lindall. 1970. Subcellular localization of proinsulin to insulin conversion in isolated rat islets. *Endocrinology.* 86: 88.
5. Kemmler, W., and D. S. Steiner. 1970. Conversion of proinsulin to insulin in a subcellular fraction from rat islets. *Biochem. Biophys. Res. Commun.* 41: 1223.
6. Jamieson, J. D., and G. E. Palade. 1967. Intracellular transport of secretory proteins in pancreatic exocrine cell. I. Role of peripheral elements of Golgi complex. *J. Cell Biol.* 34: 577.
7. Mc Shan, W. H., and M. W. Hartley. 1965. Production, storage and release of anterior pituitary hormones. *Ergeb. Physiol. Biol. Chem. Exp. Pharmabol.* 56: 246.
8. Cerasi, E., and R. Luft. 1970. The pathogenesis of diabetes mellitus—a proposed concept. *In* The Pathogenesis of Diabetes Mellitus. Proceedings of the Thirteenth Nobel Symposium. E. Cerasi and R. Luft, editors. Almqvist & Wiksell, Publishers, Stockholm. 17.
9. Grodsky, G. 1970. Insulin and the pancreas. *Vitamins Hormones.* 28: 37.
10. Porte, D., Jr., and A. A. Pupo. 1969. Insulin responses to glucose; evidence for a two pool system in man. *J. Clin. Invest.* 48: 2309.
11. Hanks, J. H., and R. E. Wallace. 1949. Relationship of oxygen and temperature in the preservation of tissue by refrigeration. *Proc. Soc. Exp. Biol. Med.* 71: 196.
12. Lacy, P. E., and M. Kostianovsky. 1967. Method for the isolation of intact islets of Langerhans from the rat pancreas. *Diabetes.* 16: 35.
13. Davoren, P. R. 1962. The isolation of insulin from a single cat pancreas. *Biochim. Biophys. Acta.* 63: 150.
14. Morgan, C. R., and A. Lazarow. 1963. Immunoassay of insulin: two antibody system. *Diabetes.* 12: 115.
15. Rubenstein, A. H., D. F. Steiner, S. Cho, A. M. Lawrence, and L. Kirsteins. 1969. Immunological properties of bovine proinsulin and related fractions. *Diabetes.* 18: 598.
16. Clark, J. L., and D. F. Steiner. 1969. Insulin biosynthesis in the rat: demonstration of two proinsulins. *Proc. Nat. Acad. Sci. U. S. A.* 62: 278.
17. Rubenstein, A. H., J. L. Clark, F. Melani, and D. F. Steiner. 1969. Secretion of proinsulin C-peptide by pancreatic β cells and its circulation in blood. *Nature (London).* 224: 697.
18. Landgraf, R., J. Kotler-Brajtburg, and F. M. Matchinsky. 1971. Kinetics of insulin release from the perfused rat pancreas caused by glucose, glucosamine and galactose. *Proc. Nat. Acad. Sci. U. S. A.* 68: 536.
19. Taylor, K. W., and W. Montague. 1970. The synthesis, storage and release of insulin. *In* The Pathogenesis of Diabetes Mellitus. Proceedings of the Thirteenth Nobel Symposium. E. Cerasi and R. Luft, editors. Almqvist & Wiksell, Publishers, Stockholm. 123.
20. Schramm, M. 1967. Secretion of enzymes and other macromolecules. *Annu. Rev. Biochem.* 36: 307.
21. Howell, S. L., M. Kostianovsky, and P. E. Lacy. 1969. Beta granule formation in isolated islets of Langerhans: a study by electron microscopic radioautography. *J. Cell Biol.* 42: 695.
22. Orci, L., A. E. Lambert, Y. Kanazawa, M. Amherdt, C. Rouiller, and A. E. Renold. 1971. Morphological and biochemical studies of B cells of fetal rat endocrine pancrease in organ culture. Evidence for proinsulin biosynthesis. *J. Cell Biol.* 50: 565.

23. Bainton, D. F., and M. G. Farquhar. 1970. Segregation and packaging of granules enzymes in eosinophilic leukocytes. *J. Cell Biol.* **45**: 54.

24. Sorenson, R. L., A. W. Lindall, and A. Lazarow. 1969. Studies on the isolated goosefish insulin secretion granule. *Diabetes.* **18**: 129.

25. Jamieson, J. D., and G. E. Palade. 1968. Intracellular transport of secretory proteins in the pancreatic exocrine cell IV. Metabolic requirements. *J. Cell Biol.* **39**: 589.

26. Howell, S. L., and K. W. Taylor. 1967. The secretion of newly synthesized insulin *in vitro. Biochem. J.* **102**: 922.

27. Steinke, J., and T. Patel. 1971. Variable glucose response and extractable insulin content of single pancreatic rat islets. *Diabetes.* **20** (Suppl. 1) : 327. (Abstr.)

28. Tanese, T., N. R. Lazarus, S. Devrim, and L. Recant. 1970. Synthesis and release of proinsulin and insulin by isolated rat islets of Langerhans. *J. Clin. Invest.* **49**: 1394.

29. Cerasi, E., and R. Luft. 1969. The effect of an adenosine-3′,5′-monophosphate diesterase inhibitor (Aminophilline) on the insulin response to glucose infusion in prediabetic and diabetic subjects. *Horm. Metab. Res.* **1**: 162.

30. Montague, W., and J. R. Cook. 1971. The role of adenosine-3′,5′-cyclic monophosphate in the regulation of insulin release by isolated rat islets of Langerhans. *Biochem. J.* **122**: 115.