# Heterogeneity and Compartmental Properties of Insulin Storage and Secretion in Rat Islets

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ABSTRACT To investigate compartmental properties of insulin storage and secretion, isolated rat islets were used for pulse-labeling experiments, after which proinsulin and insulin were purified rigorously. Processing of proinsulin to insulin neared completion by 3 h without additional loss of either radioactive peptide by cellular or extracellular proteolysis. The amount of labeled hormone rapidly diminished in islets; it was secreted at a higher fractional rate than immunoreactive insulin, resulting in secreted insulin's having a higher specific activity than the average cellular insulin. Newly synthesized insulin, therefore, was secreted preferentially. Changes in the specific activity of secreted and cellular insulin with time were consistent with changes predicted for islets containing 33% of their total insulin in a glucose-labile compartment. Predictions were based on steady-state analysis of a simple storage-limited representation of B cell function. Islets from either the dorsal or ventral part of the pancreas also contained 33% of their total insulin in a glucose-labile compartment. The same compartment was mobilized by 20 mM glucose, 50 mM potassium + 2 mM glucose, or 20 mM glucose + 1 mM 3-isobutylmethylxanthine as indicated by the specific activity ratio of secreted vs. cellular insulin, even though average secretion rates with these stimuli differed by more than threefold. In the absence of calcium, the effectiveness of 20 mM glucose as a secretagogue declined markedly, and the older stored insulin was preferentially mobilized because secreted insulin had a lower rather than a higher specific activity than cellular insulin. Results provide insight into the mechanisms of nonrandom mobilization and secretion of insulin from the B cell.

## INTRODUCTION

Previous studies from this and other laboratories (1–10) using pulse-labeling techniques have suggested that insulin may be stored and secreted heterogeneously, as are several other proteins. Although most of these studies found that newly synthesized insulin is secreted selectively, little is known about how nonrandom secretion results from the integration of synthesis with the sequential cellular processing, storage, and mobilization of this protein. However, newly synthesized insulin appears to bypass the majority of the stored hormone entirely or to equilibrate with it very slowly. Hence, islets function as though they store a portion of their total insulin in a labile compartment that is both rich in newly synthesized hormone and preferentially mobilized for secretion in response to glucose. This compartment has been reported to diminish in size in islets isolated from older rats (8), but little else is known about its properties and its effect on B cell function. For example, what are the characteristics of newly synthesized proinsulin and insulin filling this compartment and their exchange with the remainder of the stored hormone? What is the size of this compartment, and is size fixed or does it vary with secretion rates or the type of secretagogue? Are newly synthesized proinsulin and insulin both secreted and degraded preferentially? Does heterogeneity of the cellular composition of islets from different regions of the pancreas, or heterogeneity of B cells, or heterogeneity of B granules produce nonrandom secretion? Finally, how do the kinetics of secretion of newly synthesized proinsulin and insulin compare with those of total immunoreactive insulin? Our experiments were designed to investigate these questions.

## METHODS

*Preparation of islets.* Male 300–350-g Long-Evans rats (Simmonson Company, Gilroy, Calif.) were fed ad lib. and at ~9 a.m. were anesthetized with sodium pentobarbital. Islets were prepared according to the methods of Lacy and Kostianovsky (11). Islets from different regions of four pancreases were obtained by excising *in situ* the duodenal division of each pancreatic head (ventral primordium) and a comparable amount of tissue from each pancreatic tail (dorsal primordium) (12). Digestion of pancreatic tissue was done

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at 37°C in 4.0 ml Hanks' balanced salt solution minus magnesium. Collagenase (CLS IV, Worthington Biochemical Corp., Freehold, N. J.; grade V, Sigma Chemical Co., St. Louis, Mo.) was used in a three-step procedure: the first period of hand agitation was with 16 mg, the second with 8 mg, and the third with 4 mg collagenase; each period was followed by two buffer washes in a clinical centrifuge. The final washed digest was centrifuged for 10 min at 1,500 *g* on an ice-cold, discontinuous gradient that consisted of 10 ml of 60% and 10 ml of 40% (vol/vol) Percoll (Pharmacia Fine Chemicals, Piscataway, N. J.). Islets banding at the interface between the 60 and 40% Percoll layers were withdrawn and manually selected under a dissecting microscope with a drawn glass pipette.

*Incubation and labeling of islets.* In each experiment, groups of 100–250 islets were put into 15 × 45-mm glass vials, which, like all glass- and plasticware, were siliconized with Prosil-28 (PCR Research Chemicals, Inc., Gainesville, Fla.). Buffer was removed, and 0.5 ml of Krebs-Ringer Bicarbonate (KRB)[1] + 25 mM *N*-2-hydroxyethyl-piperazine-*N*-2-ethane sulfonic acid, pH 7.3, was added. Incubations were at 37°C in an atmosphere of 95% $O_2$: 5% $CO_2$ using a Dubnoff metabolic shaker bath set for 50 strokes/min. After 45 min, buffer was removed, and fresh KRB + 20 mM glucose + 400 μCi/ml [$^3$H]leucine (TRK-170, Amersham Corp., Arlington Heights, Ill.) was introduced; for reference this time was called 0 min. 15 min later, radioactive incubation buffer was removed and KRB + 0.2 mM leucine + 20 mM glucose was added. All subsequent buffers also contained 0.2 mM leucine. Samples of secreted hormone were collected after incubation buffer was removed and islets were washed twice with a total of 1.0 ml of room-temperature KRB to eliminate all previously secreted hormones. Fresh KRB at 37°C was next introduced, and the islets were returned to incubation. A sample of hormones secreted during intervals specified in Results was removed and filtered through a plug of tightly packed glass wool. Secreted hormone and hormone remaining stored within the islets at the end of the sampling interval were extracted and purified separately. Purification was done at 4°C without the addition of carrier insulin as described below.

*Chemical extraction.* All samples were adjusted to 1.0 ml with fresh KRB and mixed with 4.28 ml of 356-ml ethanol:64.1 ml water:7.5 ml concentrated hydrochloric acid (13). The next morning 0.05 ml of 2 M ammonium acetate was added, and the pH was adjusted to 8.3 with ammonium hydroxide. After centrifugation, the resulting supernates were decanted, adjusted to pH 5.3 with 1.0 M hydrochloric acid, and 10 ml of ethanol and 20 ml of ether were added sequentially with mixing. The precipitate that formed after 2–4 h, was pelleted by centrifugation and air dried. The samples were dissolved and stored frozen in 4.0 ml of column buffer (40 mM sodium phosphate, pH 8.0, + 140 mM sodium chloride + 0.2% bovine serum albumin + 0.025% thimerosal). A 70% yield after the entire chemical extraction procedure was estimated by addition of a trace of porcine $^{125}$I-insulin to a sample of intact islets + 1.0 ml of incubation buffer.

*Affinity chromatography.* Antiinsulin-Sepharose was made from guinea pig antiinsulin serum (Miles Yeda, Israel) and Sepharose 4B (Pharmacia Fine Chemicals). A crude globulin fraction was prepared by dissolving lyophilized antiserum in 8 vol of water and, with stirring, adding saturated ammonium sulfate to a final concentration of 40% saturated. Precipitated protein was pelleted by centrifugation, redissolved, and then reprecipitated with the same procedure. The final precipitate was dissolved with and extensively dialyzed against water. Dialyzed, crude globulin from 3.0 ml of antiserum was covalently coupled to ~1.5 cm$^3$ (packed volume) of freshly prepared cyanogen bromide-activated Sepharose at pH 8.0 by stirring for 15 h at 4°C; excess reactive sites were blocked by stirring the beads for an additional 4 h in 1.0 M methylamine. Beads were extensively washed with acidic and basic buffers as described (14), and then packed into columns that bound in excess of 15 μg porcine insulin/1.0 cm$^3$ (packed volume).

Thawed experimental samples were applied to 1.0-cm$^3$ (packed volume) columns that were equilibrated with column buffer. 50 ml of column buffer was used to wash out unbound proteins, and elution of insulin and proinsulin was started with 50 ml of 1.0 N acetic acid. Insulin and proinsulin eluted with the first 10 ml of acetic acid and were precipitated from this sample with 10% (final) trichloroacetic acid. The precipitate was collected by centrifugation, washed three times with ether, then dissolved and frozen in 10 mM hydrochloric acid + 0.05% bovine serum albumin (Pentex Albumin fraction V, Miles Laboratories Inc., Elkhart, Ind.). A yield of 85% was estimated with a trace of porcine $^{125}$I-insulin applied to antiinsulin-Sepharose columns.

*Bio-gel chromatography.* Insulin and proinsulin were separated from each other on 1 × 110-cm columns of Bio-gel P-30 (Bio-Rad Laboratories, Richmond, Calif.) that were equilibrated and eluted with 3.0 N acetic acid + 0.05% bovine serum albumin at room temperature. Insulin (15) from the insulin peak was measured by radioimmunoassay with rat insulin (Novo Research Laboratories, Bagsvaerd, Denmark) as a standard, and radioactivity in all peaks was measured in Aquasol (New England Nuclear, Boston, Mass.) with a Packard Model 2425 Tri-Carb Liquid Scintillation Counter (Packard Instrument Co., Downers Grove, Ill.).

Fig. 1 is an example of an elution profile of a column of Biogel P-30 loaded with a sample of islets that were incubated for 95 min at 37°C, then extracted and the hormones purified as described above. There were only two peaks of radioactivity: the first was coincident with a small immunoreactive peak that marked the elution position of proinsulin; the second was coincident with the large peak of immunoreactive insulin. Insulin eluted with nearly constant specific activity (counts per minute/nanograms immunoreactive insulin) throughout its peak, indicating relative homogeneity. 100% of a trace of porcine $^{125}$I-insulin, but only 40% of the total immunoreactive insulin, was recovered in the insulin peak after day 2 of elution in 3.0 N acetic acid. For this reason the calculation of specific activity of insulin was done as follows: counts per minute eluted in the insulin region per nanograms immunoreactive insulin in sample before chromatography. Measurements of specific activity, therefore, included a small error (<10%) due to proinsulin in the original sample. Fractional secretion rates were calculated with 100% equal to the sum of the hormone secreted during the interval plus the hormone remaining stored in the islets at the end of the interval.

When required, the insulin (15) and glucagon (16) (Unger antiserum 04A) contents of islets were measured by radioimmunoassay of buffered dilutions of extracts that were obtained by mixing the islets for 2 h at 4°C in acid-ethanol + 50 mM benzamidine (17). All numbers are reported as the mean ± SE. Differences were assessed by Student's unpaired, two-tailed *t* test.

[1] *Abbreviations used in this paper:* IBMX, 3-isobutylmethylxanthine; KRB, Krebs-Ringer buffer.

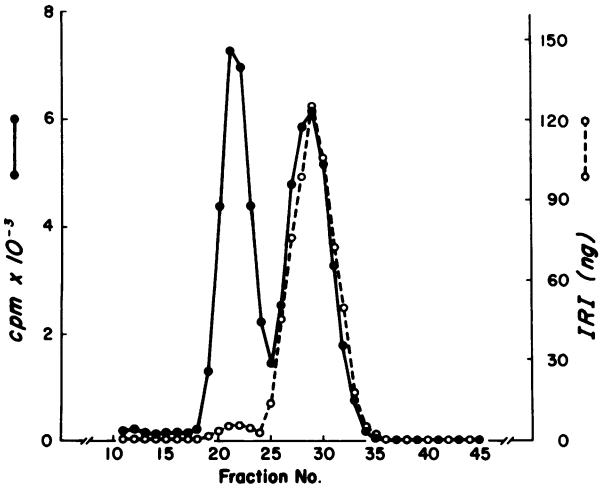


FIGURE 1 Profile of a typical sample eluted from a Biogel P-30 column. Islets were incubated continuously in KRB + 20 mM glucose beginning at −45 min, exposed to [$^3$H]leucine between 0 to 15 min, and extracted at 95 min with acidic ethanol. Proinsulin and insulin in these extracts were purified by antiinsulin-Sepharose chromatography. Purified samples were applied to a 1 × 110-cm column of Biogel P-30 and eluted at room temperature with 3 M acetic acid + 0.05% bovine serum albumin. Fractions are 2.0 ml each.

## RESULTS

*Conversion of proinsulin to insulin.* As shown in Fig. 2, and as others have shown (8, 18), conversion of newly synthesized proinsulin to insulin did not begin until ~30 min, then followed pseudo-first-order kinetics with a $t_{1/2}$ of ~50 min. In islets continuously stimulated with 20 mM glucose, almost all of the [$^3$H]leucine-containing proinsulin was converted by 3 h. Note that the radioactivity in insulin and proinsulin for this and all subsequent data was corrected for the leucine content of each protein because only 6 of the 11 leucines in a molecule of rat proinsulin (both forms) are retained in rat insulin (both forms ) (19). The other five are in the C peptide, which is lost during purification. Assuming that each leucyl residue has the same probability of being labeled, the radioactivity in a molecule of insulin can be compared directly with the radioactivity in a molecule of proinsulin after multiplying the former by 11/6.

*Efficiency of conversion and conservation of newly synthesized proinsulin and insulin.* Fig. 3 shows the recovery of radioactive proinsulin and insulin in both the islets plus incubation medium during the 15–135-min period. Radioactive data were corrected as explained above, which automatically corrected for the normal loss of radioactive C peptide during the conversion process. Although conversion of proinsulin was continuous and ~75% complete after 2 h, the radioactivity in proinsulin plus insulin obtained from combined samples of islets plus incubation buffer was not statistically different from that recovered just after the

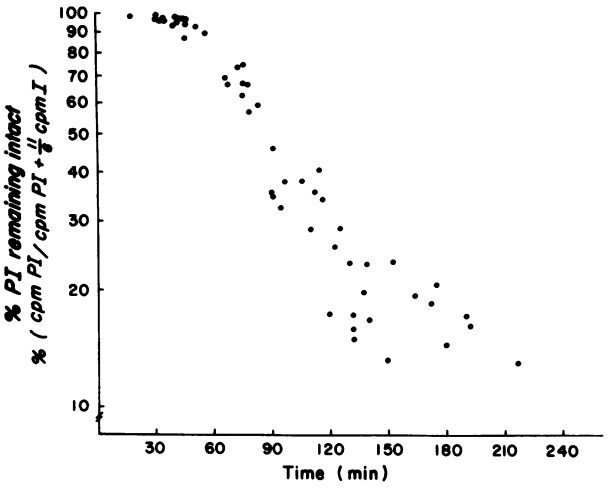


FIGURE 2 Effect of time on the conversion of newly synthesized proinsulin to insulin. Islets were incubated continuously in KRB + 20 mM glucose beginning at −45 min and exposed to [$^3$H]leucine between 0 to 15 min. Samples were obtained by extracting the islets and the accumulated incubation buffer together. The radioactivity eluting from columns of Biogel P-30 in the insulin peak was multiplied by 11/6 to correct for the difference in leucine content between insulin and proinsulin.

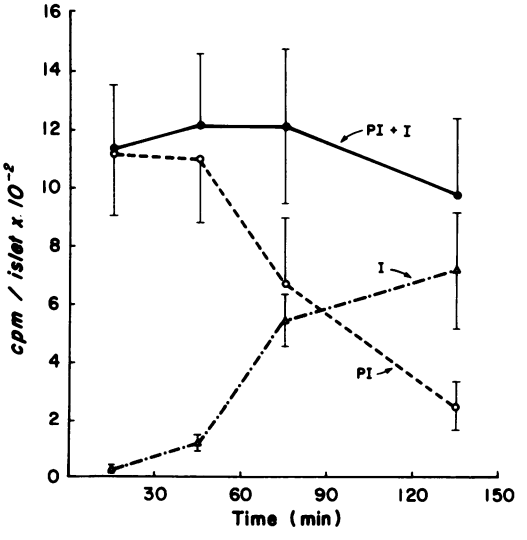


FIGURE 3 Effect of time on the recovery of proinsulin and insulin in the islets plus incubation buffer. Samples were obtained from islets, which were incubated continuously in KRB + 20 mM glucose beginning at −45 min and exposed to [$^3$H]leucine between 0 to 15 min. The radioactivity eluting from columns of Biogel P-30 in the insulin peak was multiplied by 11/6 to correct for the difference in leucine content between insulin and proinsulin. Points represent the mean ± SE from six experiments.

labeling period. Thus, newly synthesized proinsulin was either retained or quantitatively converted to insulin, indicating that conversion was highly efficient in glucose-stimulated islets. In addition, digestion of newly synthesized insulin in islets or in the incubation buffer was too slow a process to detect during a 2-h period.

*Preferential storage and release of hormone.* In contrast to the results above, a progressive and significant decrease of recovered radioactive insulin and proinsulin was evident when the islets alone were extracted. More than half of the newly synthesized hormone flowed through the B cells during these incubations (Fig. 4a). Note that the loss of total radioactive proinsulin plus insulin far exceeded the loss of immunoreactive insulin from these same islets (Fig. 4b).

Hormone released into the incubation buffer is considered in Fig. 5, which plots fractional secretion rates of immunoreactive insulin and of total radioactive

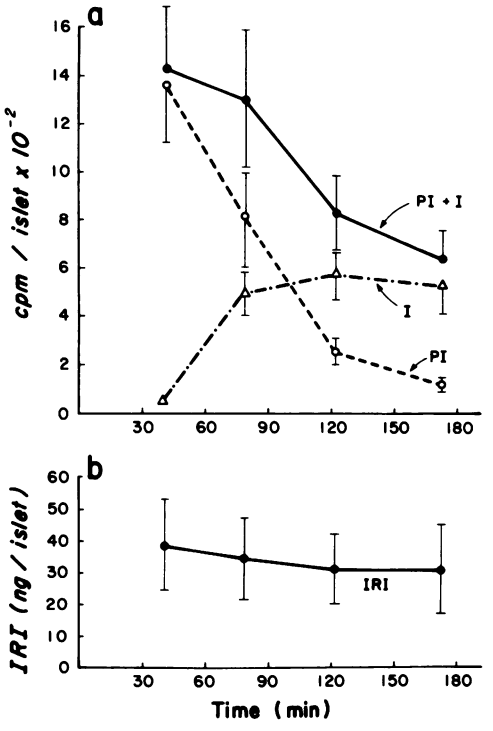


FIGURE 4 Effect of time on the recovery of proinsulin and insulin in islets. Samples were obtained from islets, which were incubated continuously in KRB + 20 mM glucose beginning at −45 min and exposed to [$^3$H] leucine between 0 to 15 min. In Fig. 4a, the radioactivity eluting from columns of Biogel P-30 in the insulin peak was multiplied by 11/6 to correct for the difference in leucine content between insulin and proinsulin. Points represent the mean ± SE from eight experiments. In Fig. 4b, the actual amount of immunoreactive insulin recovered after chemical extraction and affinity chromatography was plotted. Points represent the mean ± SE from eight experiments.

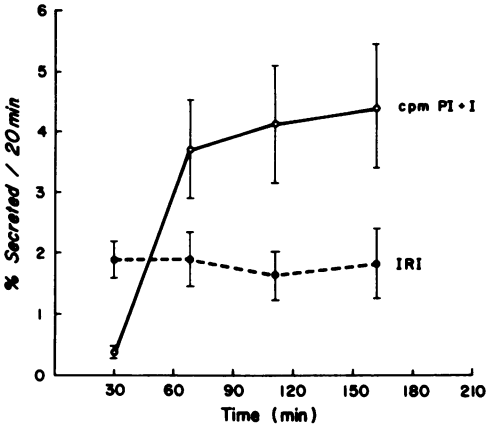


FIGURE 5 Effect of time on both the fractional secretory rate of radioactive proinsulin plus insulin and the fractional secretory rate of immunoreactive insulin. Islets were incubated continuously in KRB + 20 mM glucose beginning at −45 min and exposed to [$^3$H]leucine between 0 to 15 min. Islets were washed twice with KRB before a 20-min sample of secreted hormone was collected. The radioactivity eluting from columns of Biogel P-30 in the insulin peak was multiplied by 11/6 to correct for the difference in leucine content between insulin and proinsulin. Points were drawn at the center of the 20-min interval, and represent the mean ± SE from eight experiments.

hormone plus prohormone. Although fractional rates depend on equivalent yields of both secreted and cellular hormones for accuracy, fractional rather than actual rates were plotted because the isotope content of islets changed significantly with time (Fig. 4a). Rates were calculated from the quantity of hormone secreted during a 20-min interval; as an example, the rate at the 30-min time point was calculated from hormone secreted between 20 and 40 min. Immunoreactive insulin was secreted at an essentially constant fractional rate (~2%/20 min) throughout the experiments, indicating that the islets remained similarly responsive to glucose all through the period of study. The secretion pattern of radioactive hormone was markedly different: there was almost no secretion for the first 30 min; thereafter, fractional secretory rates increased rapidly, and, as reported (8), clearly exceeded the fractional secretion rate of immunoreactive insulin. At the 30-min point, newly synthesized proinsulin plus insulin was preferentially retained. After the first hour, however, newly synthesized hormone clearly was secreted preferentially.

The percentage of secreted radioactive hormone that eluted from columns of Biogel P-30 in the proinsulin peak changed with time and is listed in Table I for each of the time points in the figure above. During the earliest interval, secreted newly synthesized hormone was rich in prohormone and contained more radioactive proinsulin than insulin; note that it was not

quite as rich in newly synthesized proinsulin as the hormone in the islets (compare this percentage in Table I with those measured at the same time inside the cells of the islets in Fig. 2). However, during the period of preferential secretion of newly synthesized hormone that was observed after 1 h, secreted radioactive hormone generally mirrored the extent of completion of the cellular conversion of newly synthesized proinsulin to insulin.

*Size of labile compartment.* To predict how the size of a hypothetical, glucose-labile secretory compartment of insulin affects preferential secretion, a simplified mathematical expression was formulated, permitting integration of all the different time windows of sampling. It was based on the schematic representation of insulin biosynthesis coupled to heterogeneous storage and secretion shown in Fig. 6. Proinsulin and other constantly turning-over insulin precursors are represented by P, and insulin is represented by I. Lower case letters are rate constants. The continuous, upper, biosynthesis-to-secretion sequence represents the glucose-labile secretory compartment or "channel" of newly synthesized insulin; the lower box represents the remainder of the stored hormone. Both storage compartments contain proinsulin, which is secreted by the same mechanism as insulin. Proinsulin is represented in dynamic equilibrium with insulin in these compartments only as a mathematical convenience to describe a proteolytic reaction that does not go to completion.

Equations (not shown) were written for B cell synthesis and secretion of insulin at steady-state rates and were used to predict changes in the specific activity of cellular and secreted insulin with time. Hypothetical curves for different sizes of labile compartment were computer drawn in Fig. 7a. In No. 1, the labile compartment was 33%; in No. 2, it was 20%; and in

TABLE I
*Effect of Time on the Percentage of Radioactive Proinsulin in Secreted, Newly Synthesized Hormone*

| Average time of 20-min secreted sample | Percentage of counts per minute eluting in PI peak |
|---|---|
| *min* | *% cpm PI/cpm PI + I** |
| 30 | 71.3+7.5 |
| 68 | 62.6+6.2 |
| 111 | 32.3+4.2 |
| 162 | 15.6+2.1 |

* Mean±SE. The radioactivity eluting from columns of Biogel P-30 in the insulin peak was multiplied by 11/6 to correct for the difference in leucine content between insulin and proinsulin. Numbers represent the mean±SE from eight experiments and are an elaboration of data used for Fig. 5.

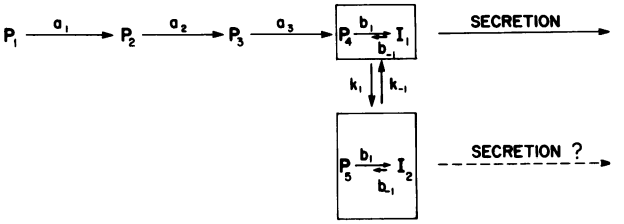


FIGURE 6 Schematic diagram of a storage-limited representation of B cell function. P represents proinsulin and other insulin precursors; I represents insulin; lowercase letters are rate constants.

No. 3, it was 11% of the total islet insulin. With only one homogeneous compartment, the specific activity of stored and secreted insulin would always be identical. With heterogeneous storage, as the size of the labile compartment gets smaller and smaller, the specific activity of secreted insulin would exceed that of cellular insulin more and more. The relative size of the labile compartment can be estimated visually because the approximate size of the compartment is inversely related to the ratio of maximal peak heights.

The observed changes with time of the specific activities of secreted and cellular insulin are shown in Fig. 7b. After the 15-min exposure to [$^3$H]leucine, the specific activity of cellular insulin continued to increase for 2 h, due to the slow cellular conversion of proinsulin to insulin. In agreement with previous reports (2, 5), after 1 h the specific activity of secreted insulin markedly exceeded that of the average cellular insulin. A comparison with the family of hypothetical curves in Fig. 7a indicates that this experimental datum most closely resembles that predicted for a glucose-labile compartment containing 33% of the total islet insulin.

*Sensitivity of labile compartment to various stimulatory conditions.* Secreted hormone was sampled in two sequential intervals between 90 and 132 min to test the effect of different secretagogues and secretion rates on mobilization of insulin storage compartments. In control experiments the secretagogue was 20 mM glucose in each interval. Both the secretion rates of immunoreactive insulin and the ratios of the specific activity of secreted to cellular insulin were identical in each interval (Fig. 8a). In Fig. 8b, sequential intervals were taken with KRB containing 20 mM glucose and KRB containing 2 mM glucose + 50 mM potassium. This elevated concentration of potassium induced insulin secretion by depolarizing the plasma membrane of the B cells (20). In Fig. 8c, sequential intervals were taken with KRB + 20 mM glucose and KRB + 20 mM glucose + 1 mM 3-isobutylmethylxanthine (IBMX). The interval with IBMX was shortened because preliminary experiments indicated

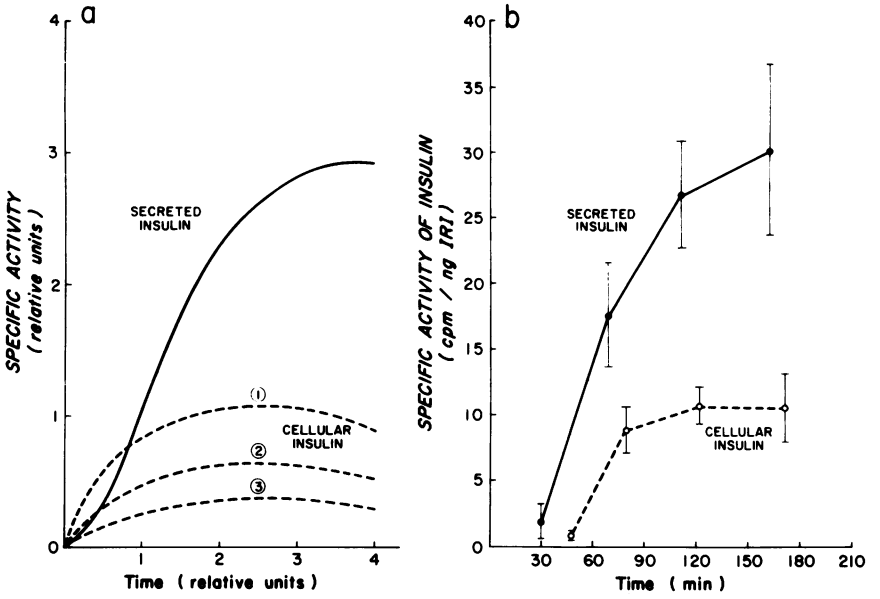


FIGURE 7 Effect of time on the predicted and experimentally determined specific activity of secreted and cellular insulin. In Fig. 7a steady-state equations were used to predict the relationships between the specific activity of secreted and cellular insulin if the percentage of total islet insulin contained in the glucose-labile compartment equals 33% in No. 1, 20% in No. 2, or 11% in No. 3. In Fig. 7b, experimental data were obtained from islets continuously incubated in KRB + 20 mM glucose beginning at −45 min and exposed to [$^3$H]leucine between 0 to 15 min. Islets were washed twice with KRB before a 20-min sample of secreted hormone was collected. Points representing secreted hormone are drawn at the center of the 20-min interval, and all points represent the mean ± SE from eight experiments.

that this compound elevated the secretion rate. With these very different stimuli, secretory rates differed by more than threefold (Fig. 8a–c). However, the specific activity ratios between secreted and average cellular insulin always remained the same, indicating that the same compartment was mobilized with each of these stimuli.

For the experiment in Fig. 8d, proteins were labeled as usual, then the islets were incubated in KRB made with 20 mM glucose but without added calcium, which is a cation required for secretion (21). The fractional secretory rate of insulin dropped dramatically without calcium in the buffer. Instead of a higher specific activity, as observed with the other stimuli, the secreted insulin had a lower specific activity than the average cellular insulin. Thus, older insulin was preferentially released from the islets under these conditions. Upon readdition of KRB containing calcium, the secretion rate and the specific activity ratio increased significantly, indicating that these islets remained viable and responsive to glucose.

*Role of geographic distribution of islets on compartment size.* Islets from the dorsal and ventral primordia of the pancreas were prepared in order to determine if preferential secretion of newly synthesized insulin would be comparable or different in islets with established differences in cellular composition (22). Islets from both regions contained identical amounts of insulin (Fig. 9), but those from the ventral primordia contained significantly less glucagon, as reported (23). After continuous incubation in 20 mM glucose, the fractional secretion rates of immunoreactive insulin from both groups of islets were identical. The specific activity ratios between secreted and cellular insulin also were identical, and the same as seen in experiments with total pancreatic islets (see Figs. 7b and 8a–c). Thus, regardless of the pancreatic location, glucose-labile compartments in islets were of the same relative size.

## DISCUSSION

*Experimental design.* Experiments reported here differ from previous pulse-labeling studies of islets because proinsulin and insulin were rigorously purified by a combination of chemical extraction, antiinsulin-Sepharose chromatography, and column chromatography. This resulted in elimination of radioactive contaminants at the void volume of the P-30 column and elimination of radioactive C peptide, which has been reported to elute in the insulin region on similar columns (2). Even in the earliest samples collected

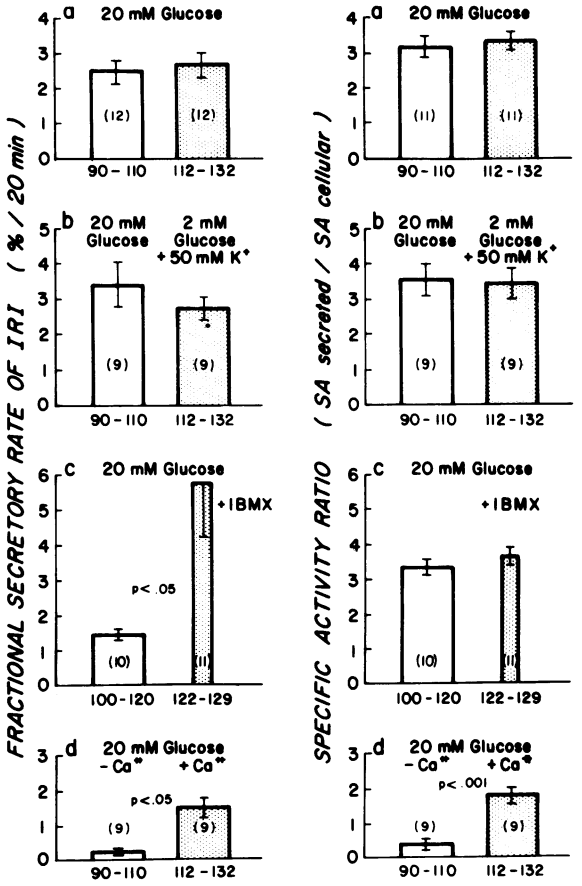


FIGURE 8 Effect of different stimuli on the rate of insulin secretion and the specific activity ratio between secreted and cellular insulin. Islets were continuously incubated in KRB + 20 mM glucose beginning at −45 min and exposed to [$^3$H]leucine between 0 to 15 min. The radioactivity eluting from columns of Biogel P-30 in the insulin peak was used for the calculation of the specific activity of insulin. In Fig. 8a, islets were washed twice with KRB + 20 mM glucose between 87 and 90 min and once between 110 and 112 min. Bars represent the mean ± SE from 11 or 12 experiments. In Fig. 8b, islets were washed twice with KRB + 20 mM glucose between 87 and 90 min and once with KRB + 2 mM glucose between 110 and 112 min. Bars represent the mean ± SE from nine experiments. In Fig. 8c, islets were washed twice with KRB + 20 mM glucose between 97 and 100 min and once between 120 and 122 min. A 7-min, rather than a 20-min sample of secreted hormone was collected in KRB + 1 mM IBMX in order to analyze equivalent amounts of secreted insulin in both intervals. Bars represent the mean ± SE from 10 or 11 experiments. In Fig. 8d, islets were washed twice each time with KRB + 20 mM glucose without added calcium—first at 15–18 min and again at 87–90 min. Islets were washed once with KRB + 20 mM glucose with calcium between 110 and 112 min. Bars represent the mean ± SE from nine experiments.

after the pulse, no distinct peak or shoulder attributable to preproinsulin was observed in the elution profiles; however, the fractions preceding the proinsulin peak always were above background. Thus, as reported (18), preproinsulin is transient and not observed in conventional pulse-labeling experiments.

Incubation of islets in 20 mM glucose before the labeling period was designed to establish constant near-maximal rates of insulin synthesis and secretion so that different time windows could be compared in comparably functioning islets. A secondary benefit was observed in six experiments, where islets previously incubated in 20 mM glucose incorporated 1,017±213 cpm/islet, whereas those incubated in 2 mM glucose incorporated only 248 ± 42. Thus, as reported (24), prior exposure of islets to elevated glucose concentrations significantly increases ($P < 0.01$) the subsequent rate of insulin synthesis.

*Conversion of proinsulin to insulin.* Although radioactive proinsulin was formed rapidly, ~30 min of incubation was required before conversion to insulin was detected. This 30-min lag probably marked the transit time for entry of proinsulin into the granule fraction of the B cell. A similar time has been reported

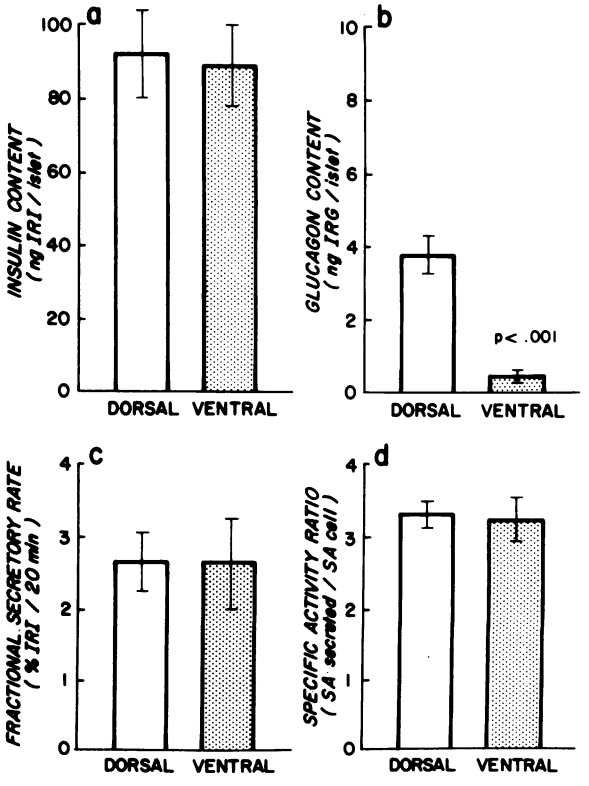


FIGURE 9 Effect of pancreatic region of origin on the insulin content, glucagon content, and insulin secretory rates of isolated islets. Islets were continuously incubated in KRB + 20 mM glucose beginning at −45 min and exposed to [$^3$H]leucine between 0 to 15 min. Islets were washed twice with KRB before a 20-min sample of secreted hormone was collected. Bars represent the mean ± SE from six experiments.

(25) for transport of radioactive grains from the endoplasmic reticulum (via the golgi) to granule profiles in islets of rat pancreas that also had been pulse labeled with [$^3$H]leucine. Enzymatic activities necessary for the processing of proinsulin to insulin also have been localized in a B granule-rich fraction prepared from homogenized islets (26). Thus, all lines of evidence indicate that the conversion process occurs in the B granules. From 30 min onward (presumably upon entering the B granule) proinsulin converts to insulin in our system in a pseudo-first-order process with a $t_{1/2}$ of 50 min, as reported (2, 8, 18).

*Efficiency of conversion and conservation of proinsulin and insulin.* We found that almost all of the radioactive proinsulin was recovered in the insulin fraction, indicating that all or most of the newly formed prohormone was exposed to the converting enzymes. Because the enzymatic activities necessary for the conversion are encapsulated within the membranes of isolated granules (26), it seems highly unlikely that, even under conditions of maximal glucose stimulation, any newly synthesized prohormone avoided being encapsulated in a secretory granule. The complete recovery of label in proinsulin plus insulin after 2 h indicates that conversion is a highly efficient process that occurs without unaccountable loss of proinsulin. Newly synthesized insulin also was not rapidly degraded. Thus, degradation of insulin in the glucose-stimulated B cell is a very slow process and occurs at a rate far below that of secretion. In this respect the B cell is unlike the parathyroid cell that degrades newly synthesized parathyroid hormone more rapidly than it secretes it (27). Because the autodigestion of secretable proteins is necessary to eliminate overproduction in cells with limited storage capacity, rapid autodigestion may be the rule for cells with a synthetic capacity in excess of their secretory capacity, and slow digestion, as observed in cultured islets (28) and here in freshly isolated islets, may be the rule where there is a lower synthetic than secretory capacity.

*Secretion of newly synthesized hormone.* Newly synthesized hormone was secreted concomitantly with the conversion process. Thus, immature storage components containing large amounts of unprocessed proinsulin were readily secreted. At 30 min, newly synthesized, secreted hormone was not quite as rich in proinsulin as was stored hormone, as observed (2, 5, 8). This could indicate either that B granules require partial maturation before secretion or that a large proportion of the radioactive proinsulin continues to reside in the lumen of the rough endoplasmic reticulum and golgi membranes rather than within the secretory granules at 30 min. The latter explanation seems most likely because the proportion of secreted, radioactive proinsulin to insulin generally mirrored the extent of the conversion process in the B cell after 1 h when the bulk of the newly synthesized proinsulin probably was in the granule fraction of the cells. These data suggest that newly formed secretory granules equilibrate with other granules within the labile compartment, and argue against a linear box-car arrangement of secretory granules in this compartment of the B cell.

*Preferential secretion of newly synthesized hormone.* Glucose-stimulated B cells, after 1 h in vitro, secreted newly synthesized proinsulin and insulin at a fractional rate that exceeded that of the older stored hormone. Also we observed that the amount of newly synthesized proinsulin and insulin in islets decreased more rapidly than could be explained by the fractional turnover of total immunoreactive insulin. The time lag before observation of preferential secretion of labeled hormone was comparable to the delay before the conversion of prohormone to hormone, and also may reflect the time required for transport of newly synthesized hormone into the secretory granules. Therefore, preferential secretion is probably a continuous phenomenon but becomes apparent in a pulse-labeling experiment only after 30 min when newly synthesized hormone enters the granules of the glucose-labile compartment.

*Size of the glucose-labile compartment.* As much as 33% of the islet insulin may be contained in a storage compartment that is both rich in newly synthesized hormone and preferentially mobilized for secretion. The remainder of the stored insulin is not mobilized as rapidly in response to glucose stimulation. This estimate of compartment size was based on a simple storage-limited scheme (29) and the use of steady-state equations to analyze heterogeneous insulin storage and transit through the B cell. The recovery study (Fig. 3) demonstrated that provision in this scheme for reutilization of proinsulin or degradation of insulin was unnecessary. Preferential secretion is not unique to insulin, and also has been observed for placental lactogen (30), prolactin (31), parathyroid hormone (32), pancreatic amylase (33), gonadotropin (34), vasopressin (35), thyroglobulin (36), and acetyl choline (37). For most of these secretory products, preferential secretion can be estimated to occur from a labile compartment not too dissimilar in size from the approximately 33% of the total storage compartment reported here. This schematic representation, therefore, also may be useful to describe secretion from several types of cell. However, a contrasting result, preferential retention of newly synthesized salivary amylase, has been observed in the parotid gland (38), indicating that preferential release does not occur in all secretory cells.

*Nature of the labile compartment.* The way that

insulin in the glucose-labile compartment is segregated from the remainder of the stored insulin remains unknown. There are several possible divisions in an islet: compartments could represent islets from different pancreatic regions, B cells in different environments, or different populations of B granules in each B cell. Islets from the dorsal and ventral regions of the pancreas were found to contain glucose-labile compartments of insulin that were of the same relative size as those found with islets from the total pancreas (compare the specific activity ratios in Fig. 9d with those for islets from the total pancreas in Figs. 8a–c, and those that can be calculated from the specific activities reported in Fig. 7b). Thus, it is unlikely that compartments from these different pancreatic regions represent the division. Previous studies also have shown that larger islets contain more insulin (39); however, large and small islets probably do not represent the compartmental division because we found no correlation between the amount of insulin recovered from islets and the ratio of specific activities between secreted and cellular insulin (data not shown). Although islet heterogeneity still must be considered as a plausible explanation for these data, it seems most likely, at the present time, that compartments represent either populations of B cells or of B granules.

*Sensitivity of the labile compartment to various stimulatory conditions.* Several secretory conditions were tested to see if the same or different compartments of islet insulin were mobilized in response to different secretagogues. In control experiments with 20 mM glucose, secretion rates and specific activities of insulin were identical in two consecutive intervals. Insulin secretion also was induced by depolarizing the plasma membrane of the B cell with 50 mM potassium in the absence of a stimulatory concentration of glucose and by increasing the cellular concentration of cyclic AMP with 1 mM IBMX, which inhibits phosphodiesterase and trebled the secretory rate produced by 20 mM glucose alone. With all these conditions, the specific activity of secreted insulin was the same. Therefore, it is likely that all these stimuli mobilized the same compartment of islet insulin. In addition, average fractional secretory rates ranged from 1.5 to 5.8%/20 min in the studies depicted in Figs. 8a–c, without a significant difference in the ratios of specific activities between secreted and cellular insulin. Therefore, the specific activity ratio of secreted to cellular insulin is not solely a function of secretory rates.

A different storage compartment, however, was mobilized by 20 mM glucose in the absence of calcium. Under conditions where the effectiveness of glucose as a secretagogue was blocked, islets secreted insulin with a specific activity that was lower than that of the average cellular insulin. Thus, there was preferential secretion of the older, stored insulin rather than the newly synthesized insulin. These islets were still responsive to glucose stimulation and quickly returned toward normal function after the readdition of calcium. No secretagogue yet tested preferentially mobilized the lower-specific-activity insulin and also increased the secretory rate above a basal level. Whether or not such an agent can be found, and if it will modify chronic B cell function in vivo, remain to be determined.

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## REFERENCES

1. Howell, S. L., D. G. Parry, and K. W. Taylor. 1965. Secretion of newly synthesized insulin in vitro. *Nature (Lond.).* **208:** 487.
2. Sando, H., J. Borg, and D. F. Steiner. 1972. Studies on the secretion of newly synthesized proinsulin and insulin from isolated rat islets of Langerhans. *J. Clin. Invest.* **51:** 1476–1485.
3. Creutzfeldt, C., N. S. Track, and W. Creutzfeldt. 1973. In vitro studies of the rate of proinsulin and insulin turnover in seven human insulinomas. *Eur. J. Clin. Invest.* **3:** 371–384.
4. Gutman, R. A., G. Fink, J. R. Shapiro, H. Selawry, and L. Recant. 1973. Proinsulin and insulin release with a human insulinoma and adjacent nonadenomatous pancreas. *J. Clin. Endocrinol. Metab.* **36:** 978–987.
5. Sando, H., and G. M. Grodsky. 1973. Dynamic synthesis and release of insulin and proinsulin from perifused islets. *Diabetes.* **22:** 354–360.
6. Schatz, H., C. Nierle, and E. F. Pfeiffer. 1975. (Pro-)insulin biosynthesis and release of newly synthesized (pro-)insulin from isolated islets of rat pancreas in the presence of amino acids and sulphonylureas. *Eur. J. Clin. Invest.* **5:** 477–485.
7. Track, N. S. 1977. Insulin biosynthesis. *In* Insulin and Metabolism. J. S. Bajaj, editor. Excerpta Medica, Amsterdam. 13–38.
8. Gold, G., G. M. Reaven, and E. P. Reaven. 1981. Effect of age on proinsulin and insulin secretory patterns in isolated rat islets. *Diabetes.* **30:** 77–82.
9. Gold, G., H. D. Landahl, J. R. Pettit, and G. M. Grodsky. 1981. Characteristics of a rapidly-labeled and rapidly-secreted compartment of islet insulin. *Clin. Res.* **29:** 94A.
10. Halban, P. 1981. Release of newly synthesized and of older, stored insulin from islets: a comparative study. *Diabetes.* **30**(Suppl. 1): 2A.
11. Lacy, P. E., and M. Kostianovsky. 1967. Method for the isolation of intact islets of Langerhans from the rat pancreas. *Diabetes.* **16:** 35–39.
12. Lambert, R. 1965. Surgery of the digestive system in the rat. Charles C Thomas, Publisher, Springfield, Ill. 90.
13. Davoren, P. R. 1962. The isolation of insulin from a single cat pancreas. *Biochim. Biophys. Acta.* **63:** 150–153.

14. Berne, C. 1975. Anti-insulin serum coupled to Sepharose 4B as a tool for the investigation of insulin biosynthesis in the B-cells of obese hyperglycemic mice. *Endocrinology.* **97:** 1241–1247.
15. Lundquist, I., R. Fanska, and G. M. Grodsky. 1976. Interaction of calcium and glucose on glucagon secretion. *Endocrinology.* **99:** 1304–1312.
16. Gerich, J. E., M. A. Charles, and G. M. Grodsky. 1974. Characterization of the effects of arginine and glucose on glucagon and insulin release from the perfused rat pancreas. *J. Clin. Invest.* **54:** 833–841.
17. Ensinck, J. W., C. Shepard, R. J. Dudl, and R. H. Williams. 1972. Use of benzamidine as a proteolytic inhibitor in the radioimmunoassay of glucagon in plasma. *J. Clin. Endocrinol. Metab.* **35:** 463–467.
18. Steiner, D. F., W. Kemmler, J. L. Clark, P. E. Oyer, and A. H. Rubenstein. 1972. The biosynthesis of insulin. *In* Handbook of Physiology, Endocrinology, Section 7 Vol. 1. D. F. Steiner and N. Freinkel, editors. Williams and Wilkins. Baltimore, Md. 175–198.
19. Lomedico, P., N. Rosenthal, A. Efstratiadis, W. Gilbert, R. Kolodner, and R. Tizard. 1979. The structure and evolution of two nonallelic rat preproinsulin genes. *Cell.* **18:** 545–558.
20. Epstein, G., R. Fanska, and G. M. Grodsky. 1978. The effect of potassium and valinomycin on insulin and glucagon secretion in the perfused rat pancreas. *Endocrinology.* **103:** 2207–2215.
21. Grodsky, G. M., and L. L. Bennett. 1966. Cation requirements for insulin secretion in the isolated perfused pancreas. *Diabetes.* **15:** 910–913.
22. Baetens, D., F. Malaisse-Lagae, A. Perrelet, and L. Orci. 1979. Endocrine pancreas: three-dimensional reconstruction shows two types of islets of Langerhans. *Science (Wash., D. C.).* **206:** 1323–1325.
23. Trimble, E. R., F. Malaisse-Lagae, and A. E. Renold. 1979. Insulin and somatostatin secretion by islets isolated from different parts of rat pancreas. *In* Proceedings of the Tenth Congress of the International Diabetes Federation, W. Waldhausl and K. G. M. M. Alberti, editors. Excerpta Medica (International Congr. Ser. 481). Amsterdam. 237.
24. Andersson, A., J. Westman, and C. Hellerstrom. 1974. Effects of glucose on the ultrastructure and insulin biosynthesis of isolated mouse pancreatic islets maintained in tissue culture. *Diabetologia.* **10:** 743–753.
25. 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 pancreas in organ culture: evidence for (pro)insulin biosynthesis. *J. Cell Biol.* **50:** 565–582.
26. Kemmler, W., D. F. Steiner, and J. Borg. 1973. Studies on the conversion of proinsulin to insulin. III. Studies in vitro with a crude secretion granule fraction isolated from rat islets of Langerhans. *J. Biol. Chem.* **248:** 4544–4551.
27. Morrissey, J. J., and D. V. Cohn. 1979. Secretion and degradation of parathormone as a function of intracellular maturation of hormone pools—modulation by calcium and dibutyryl cyclic AMP. *J. Cell Biol.* **83:** 521–528.
28. Halban, P A., and C. B. Wollheim. 1980. Intracellular degradation of insulin stores by rat pancreatic islets in vitro: an alternative pathway for homeostasis of pancreatic insulin content. *J. Biol. Chem.* **255:** 6003–6006.
29. O'Connor, M. D. L., H. D. Landahl, and G. M. Grodsky. 1980. Comparison of storage- and signal-limited models of pancreatic insulin secretion. *Am. J. Physiol.* **238:** R378–389.
30. Suwa, S., and H. Friesen. 1969. Biosynthesis of human placental proteins and human placental lactogen (HPL) in vitro. II. Dynamic studies of normal term placentas. *Endocrinology.* **85:** 1037–1045.
31. Swearingen, K. C. 1971. Heterogeneous turnover of adenohypophysial prolactin. *Endocrinology.* **89:** 1380–1388.
32. MacGregor, R. R., J. W. Hamilton, and D. V. Cohn. 1975. The by-pass of tissue hormone stores during the secretion of newly synthesized parathyroid hormone. *Endocrinology.* **97:** 178–188.
33. Slaby, F., and J. Bryan. 1976. High uptake of myo-inositol by rat pancreatic tissue in vitro stimulates secretion. *J. Biol. Chem.* **251:** 5078–5086.
34. Hoff, J. D., B. L. Lasley, C. F. Wang, and S. S. C. Yen. 1977. The two pools of pituitary gonadotropin: regulation during the menstrual cycle. *J. Clin. Endocrinol. Metab.* **44:** 302–312.
35. Sachs, H., P. Fawcett, Y. Takabatake, and R. Portanova. 1969. Biosynthesis and release of vasopressin and neurophysin. *Recent Prog. Horm. Res.* **25:** 447–491.
36. MacLeod, R. M., and A. Abad. 1968. On the control of prolactin and growth hormone synthesis in rat pituitary glands. *Endocrinology.* **83:** 799–806.
37. Collier, B. 1969. The preferential release of newly synthesized transmitter by a sympathetic ganglion. *J. Physiol. (Lond.).* **205:** 341–352.
38. Sharoni, Y., S. Eimerl, and M. Schramm. 1976. Secretion of old versus new exportable protein in rat parotid slices: control by neurotransmitters. *J. Cell Biol.* **71:** 107–122.
39. Reaven, E. P., G. Gold, and G. M. Reaven. 1979. Effect of age on glucose-stimulated insulin release by the B cell of the rat. *J. Clin. Invest.* **64:** 591–599.