# Effects of Tolbutamide Pretreatment on the Rate of Conversion of Newly Synthesized Proinsulin to Insulin and the Compartmental Characteristics of Insulin Storage in Isolated Rat Islets

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**SUMMARY**

**Tolbutamide (1 g/kg body wt) was administered to male rats for 3 days to determine the effects of this pretreatment on subsequent insulin biosynthesis and compartmental storage characteristics of freshly isolated islets. Islets were isolated 16 h after the last tolbutamide administration, at a time when fed plasma glucose concentrations were normal. Islet glucagon was unchanged but insulin content was significantly reduced (38 ± 1.2 ng IRI/islet from seven untreated rats versus 7.9 ± 1.2 ng IRI/islet from eight treated rats). After tolbutamide pretreatment, the rate of incorporation of $^3$H-leucine into islet proinsulin was unchanged, but the $t_{1/2}$ of labeled proinsulin-to-insulin conversion was significantly ($P < 0.001$) decreased from 36 to 20 min. After treatment, actual rates of glucose-stimulated insulin secretion were 50% lower, however, because due to the proportionately greater depletion of islet insulin content, the fractional rate of secretion was increased twofold. After treatment, there was evidence of compartmental, heterogeneous insulin storage, and glucose still marked newly synthesized insulin for preferential release; however, the differential release of new and old insulin converged rapidly with time. Mathematical integration of the data suggested dilution of the newly synthesized insulin compartment with unlabeled insulin during the chase period, but additionally indicated more rapid mixing of newly synthesized with previously stored, unlabeled insulin. Thus, tolbutamide-treated rats partially compensated for acute insulin depletion by (1) increasing the rate of proinsulin-to-insulin conversion, but not increasing the rate of proinsulin biosynthesis; (2) doubling the glucose-stimulated fractional secretory rate of the depleted cellular insulin storage compartment; and (3) retaining compartmental storage characteristics but mixing newly synthesized insulin more rapidly with the compartment of previously stored, unlabeled insulin.** DIABETES 1986; 35:6–12.

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In normal rat islets, a novel regulatory process, "marking," can partially couple insulin biosynthesis with secretion[1] and lead to the preferential secretion of newly synthesized insulin. Marking is regulated by the concentration of several secretagogues, including glucose, alpha-ketoisocaproic acid, and phorbol 12-myristate 13-acetate (TPA)[2], but occurs only at a critical time corresponding to when newly synthesized hormone is transiting the Golgi apparatus and new secretory granules are forming. Secretory rates and secretagogue concentrations before or after this critical time have little effect. Thus, insulin storage in the beta cell is heterogeneous and regulation by marking may represent a biochemical or cytostructural change that sorts secretory proteins during transport through the Golgi apparatus or during formation of new secretory granules.

However, marking may not only be characteristic of normal B-cells that continually alter synthesis and/or secretory rates with regulated responses to physiologic stimuli. For instance, with dispersed cells from a transplantable rat insulinoma, insulin was continuously secreted at a high fractional rate, which did not respond to changes in the glucose concentration.[3,4] In these cells, which store less insulin per gram tissue than islets,[4] both newly synthesized and stored insulins were secreted at identical elevated fractional rates as if both types of stored cellular insulin were highly marked for immediate release.[4]

To evaluate the role of diminished cellular insulin on insulin storage characteristics and marking regulation in glucose-responsive islets, normal rats were treated for 3 days with high doses of tolbutamide. Insulin-depleted islets from these animals were glucose responsive and retained both compartmental storage characteristics and marking regulation.



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Cells partially compensated for loss of stored insulin by increasing the rate of conversion of proinsulin to insulin and by also increasing the overall fractional rate for glucose-stimulated secretion of stored cellular insulin.

## MATERIALS AND METHODS

**Tolbutamide treatment.** Male Long-Evans rats weighing between 300 and 350 g (Simmonson Company, Gilroy, California) were housed with free access to food and water. Tolbutamide (Orinase; The Upjohn Company, Kalamazoo, Michigan) was orally administered as a 5% (wt/vol) solution in water. Each rat received 500 mg tolbutamide/kg body wt between 8 and 10 a.m. and again between 4 and 6 p.m. on three consecutive days.[5] Approximately 16 h after the last administration of tolbutamide, rats were anesthetized with sodium pentobarbital, tail blood was drawn, and fed plasma glucose concentrations were determined with a glucose analyzer (Beckman Instruments, Inc., Mountain View, California).

**Preparation of islets.** Pancreata were inflated via the bile duct with 20 ml Hanks' balanced salt solution minus magnesium, pH 7.3, plus 0.02% (wt/vol) bovine serum albumin (BSA) (Miles Scientific, Naperville, Illinois). Combined tissue from three excised pancreata was brought to 12 ml with Hanks' buffer, minced with scissors, and digested at 37°C according to the method of Lacy and Kostianovsky.[6] Three sequential incubations with collagenase (Worthington Biochemical Corporation, Freehold, New Jersey) were done with vortex mixing. Incubation times and collagenase concentrations were optimized for each batch of collagenase; typically, the first incubation was 11 min with 60 mg collagenase, the second was 5 min with 30 mg collagenase, and the third was 5 min with 15 mg collagenase. Both the second and third incubations contained a trace amount of DNAase I (Sigma Chemical Company, St. Louis, Missouri). Each incubation was followed by two buffer washes in a clinical centrifuge. The final washed tissue digest was layered onto an ice-cold gradient comprising 10 ml of 60% and 10 ml of 40% (vol/vol) Percoll (Pharmacia Fine Chemicals, Piscataway, New Jersey). After centrifugation for 10 min at 1500 × *g*, islets were withdrawn from the intraface between the Percoll layers and manually selected under a dissecting microscope with a drawn glass pipette.

**Incubation and labeling of islets.** Groups of 150–250 freshly isolated islets were gassed with 95% $O_2$:5% $CO_2$ and incubated at 37°C with 0.5 ml of Krebs-Ringer bicarbonate (KRB) plus 0.3% (wt/vol) BSA plus 25 mM N-2-hydroxyethylpiperazine-N′-2-ethane sulfonic acid (Hepes), pH 7.3. After 45 min in a Dubnoff metabolic shaker bath set for 50 strokes/min, incubation buffer was discarded and fresh KRB containing 400 μCi/ml $^3$H-leucine (Amersham Corporation, Arlington Heights, Illinois) was introduced for a 15-min pulse labeling; for reference, the labeling period was designated as 0–15 min. After labeling, buffer was discarded, islets were washed twice with KRB and then returned to incubation. All buffers after the labeling period contained 0.2 mM leucine.

The first experiments were time courses in which samples of incubation buffer and islets from untreated and tolbutamide-treated rats were collected at several times after the labeling period. Islets were continuously incubated with KRB containing 20 mM glucose and rates of proinsulin labeling, conversion of labeled proinsulin to insulin, and specific activities of both secreted and cellular insulins were determined. In a second set of experiments, paired groups of islets from each preparation were identically incubated and labeled as previously described. Between 15 and 90 min (the "marking" period, which is critical for regulating preferential secretion of newly synthesized insulin in islets from untreated rats), one group of labeled islets was incubated in KRB containing 20 mM glucose and the other group in KRB containing 2 mM glucose. At 90 min, both groups of islets were washed and returned to incubation with KRB containing 20 mM glucose. Total incubation buffers were collected at 110 min for the first 20-min test window; fresh KRB buffers were readded and then collected at 131 min for the second 20-min test window. The effects of glucose during the prior marking period on the specific activities of insulin in these secreted samples and in cellular insulin in islets collected at the end of each test period were assessed.

**Purification and assay of proinsulin, insulin, and glucose.** Proinsulin and insulin were purified at 4°C without carrier hormone from each secreted and cellular sample. After a 15-h extraction with ethanol containing 15% (vol/vol) water plus 1.75% (vol/vol) hydrochloric acid, 0.05 ml of 2 M ammonium acetate was added. Samples were then adjusted to pH 8.3 with ammonium hydroxide, centrifuged, and the supernatants were decanted and readjusted to pH 5.3.[7] A crude fraction containing insulin and proinsulin was precipitated with the addition of 10 ml ethanol plus 20 ml diethyl ether, collected by centrifugation, and dissolved in 4 ml phosphate buffer [40 mM $Na_2HPO_4$ plus 140 mM NaCl plus 0.2% (wt/vol) BSA plus 0.025% (wt/vol) thimerosal (ICN Nutritional Biochemicals, Cleveland, Ohio)]. Samples were then applied to anti-insulin columns, which consisted of 1.5 cm$^3$ of a support prepared using a cyanogen bromide procedure[8] to link a crude globulin precipitate (40% saturated ammonium sulfate) of 3–4 ml guinea pig anti-insulin serum (Miles Yeda, Rehovet, Israel, or Linco Research, Eureka, Missouri) to Sepharose 4B (Pharmacia). Excess reactive sites on the support were blocked with methylamine (Sigma). After washing bound samples with 60 ml phosphate buffer, proinsulin and insulin were co-eluted in 1 M acetic acid (10 ml), precipitated with 10% final (wt/vol) trichloroacetic acid (Mallinckrodt, Paris, Kentucky), pelleted by centrifugation for 10 min at 1500 × *g*, and washed three times with 4 ml ether. Nonspecific binding of label to anti-insulin columns was estimated at $<2.5\%$ by mixing one-half of a $^3$H-leucine-labeled rat islet sample with 5 mg "zinc-free" porcine insulin (Eli Lilly and Company, Indianapolis, Indiana) before application to the column. Immunopurified proinsulin and insulin were separated from each other by elution from 1 × 110-cm columns of Biogel P-30 (Bio-Rad Laboratories, Richmond, California) with 3 M acetic acid plus 0.05% (wt/vol) BSA. Recovery of IRI and label for these procedures was described previously.[9]

Radioactivity co-eluting from columns of Biogel P-30 with either proinsulin or insulin was determined by mixing with Hydroflour (National Diagnostics, Somerville, New Jersey) and counting on a Tri-Carb liquid scintillation counter (Packard Instrument Company, Downers Grove, Illinois). Total insulin was measured by radioimmunoassay[10] using a standard of rat insulin (Novo Research Laboratories, Bagsvaerd, Denmark).



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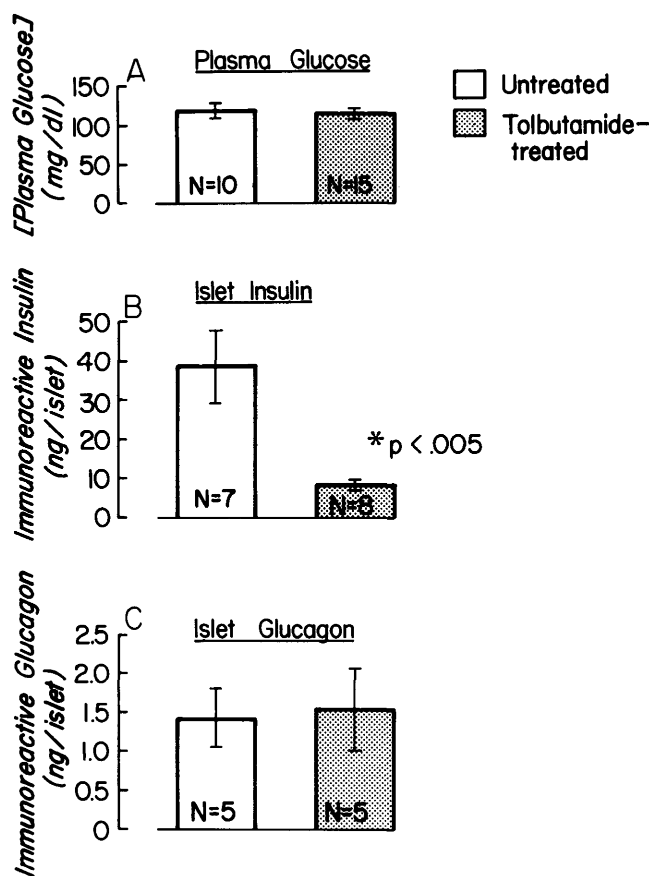


**FIGURE 1. (A) Effect of tolbutamide pretreatment on the concentration of plasma glucose. Tolbutamide-treated rats were administered tolbutamide orally (500 mg/kg body wt) twice daily for three consecutive days. Fed plasma glucose concentrations were measured on tail blood, which was drawn at 9 a.m. from anesthetized animals (approximately 16 h after the last tolbutamide administration). (B) Effect of tolbutamide pretreatment on the insulin content of isolated islets. Isolated islets were extracted at 4°C with acid-ethanol containing benzamidine. Insulin was measured by radioimmunoassay. Statistical differences were assessed with Student's two-tailed, unpaired *t*-test. (C) Effect of tolbutamide pretreatment on the glucagon content of isolated islets. Isolated islets were extracted at 4°C with acid-ethanol containing benzamidine. Glucagon was measured by radioimmunoassay.**

**Calculations.** Fractional rates of secretion were calculated by assigning the value of 100% to the sum of all islet and secreted hormone. Measurements of radioactivity were corrected for the loss of C-peptide during the purification procedures by multiplying radioactivity eluting in the insulin peak by 11/6.[9] Half-lives for the conversion of proinsulin to insulin were calculated by the least-squares method (logarithm of percent intact proinsulin versus time) for all points in the linear portion of the conversion curve. All numbers are reported as the mean ± SEM and differences were assessed with Student's unpaired, two-tailed *t*-test.

Computer modeling of insulin biosynthesis, compartmental storage, and fractional secretion was done on an IBM PC incorporating a flow pattern for labeled and unlabeled proinsulin and insulin (illustrated later in Figure 4 and to be published elsewhere in detail). This flow pattern features two cellular insulin storage compartments: one compartment (marked) represents marked granules that are rich in newly synthesized insulin, can be preferentially mobilized for secretion (high fractional rate), and contain a small fraction of the total cellular insulin. The second compartment (unmarked) contains the remainder of the total stored cellular insulin. The same rates for transport and conversion from proinsulin were used for labeled and unlabeled insulin. In fitting this pattern, rate of biosynthesis of labeled proinsulin, transport rate to secretory granules, fractional secretion of both labeled and unlabeled insulins, rate conversion of proinsulin to insulin, and islet content of labeled and unlabeled insulin were fixed by experimentally determined values taken from our previous study using identical conditions.[9]

## RESULTS

Approximately 16 h after the last tolbutamide administration, untreated and tolbutamide-treated rats had the same fed plasma glucose concentration (Figure 1A). Islets prepared at this time from treated rats appeared to be approximately the same average size as those from untreated rats, but were more translucent. Insulin was significantly depleted by >75% in these islets (Figure 1B), whereas glucagon content (Figure 1C) was the same as that from untreated rats. Thus, during the period of tolbutamide treatment, insulin release exceeded insulin production.

Effects of tolbutamide pretreatment on hormone biosynthesis were assessed with isolated islets pulse-labeled with $^{3}H$-leucine. As shown in Figure 2, islets from both untreated and treated rats incorporated $^{3}H$-leucine into proinsulin plus insulin at the same rate. As demonstrated in Figure 3, and as previously reported for islets from untreated rats,[9,11,12] conversion of labeled proinsulin to insulin normally began after an approximate 20-min delay associated with cellular transport of labeled proinsulin from site of synthesis (rough endoplasmic reticulum) to site of conversion (presumably the Golgi complex and secretory granules). Thereafter, conversion normally followed pseudo-first-order kinetics with a $t_{1/2}$ of 36 min. For islets from tolbutamide-treated rats, time of onset of conversion was unchanged. However, the $t_{1/2}$ of conversion significantly ($P < 0.001$) decreased to 20 min, indicating an accelerated rate for cellular maturation of hormone.

At several times after pulse-labeling of glucose-stimulated islets, specific activities of both cellular and secreted insulins were determined (Figure 4). An elevated specific activity of secreted versus cellular insulin indicates preferential release of newly synthesized insulin, whereas identical specific activities indicate random release. With islets from untreated rats (Figure 4C), the specific activity of cellular insulin rose slowly to approximately 10 cpm/ng IRI at 80 min and remained essentially unchanged for an additional 90 min. As shown by the fit of the data in the mathematical flow pattern (Figure 4A), this specific activity represents a dynamic balance between the secretion of labeled insulin from the marked compartment and the increasing production (after the pulse) of labeled insulin in this compartment, resulting from transport and the relatively slow conversion of the labeled proinsulin. As previously reported, at high glucose,[9] once labeled insulin was well established in this secretory compartment (60 min), the specific activity of secreted insulin exceeded that of the average cellular insulin by threefold (Figure 4C). With islets from tolbutamide-treated rats (Figure 4D), specific activities of islets and secreted insulin were 4–5 times greater than those of untreated islets, an expected

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result of (1) the normal synthetic rate into a diminished storage pool, and (2) a higher fractional secretion of unlabeled and labeled insulin (Figure 4B). The specific activity of secreted insulin also exceeded that of cellular insulin in the test periods between 90 and 131 min but with a slightly smaller ratio than that observed with islets from untreated rats. Note that, in this case, a plateau of specific activity of islet insulin was not achieved but specific activity transiently peaked at 80 min and then rapidly declined. A relatively close fit of the data was achieved with the flow diagram in Figure 4B if, in addition to the increased observed conversion and fractional secretion rates, a rapid change of marked to unmarked granules was assumed. Even though there are too many parameters to allow their independent determination, the diagram can account for the data.

A second series of pulse-labeling experiments was done to determine how treatment affected cellular storage characteristics or marking regulation (Figure 5). The results show that stimulated fractional secretion of total insulin from depleted islets during test windows was almost double that of normal islets. In both cases, elevated glucose in the marking period increased the secretion rate of insulin in test period

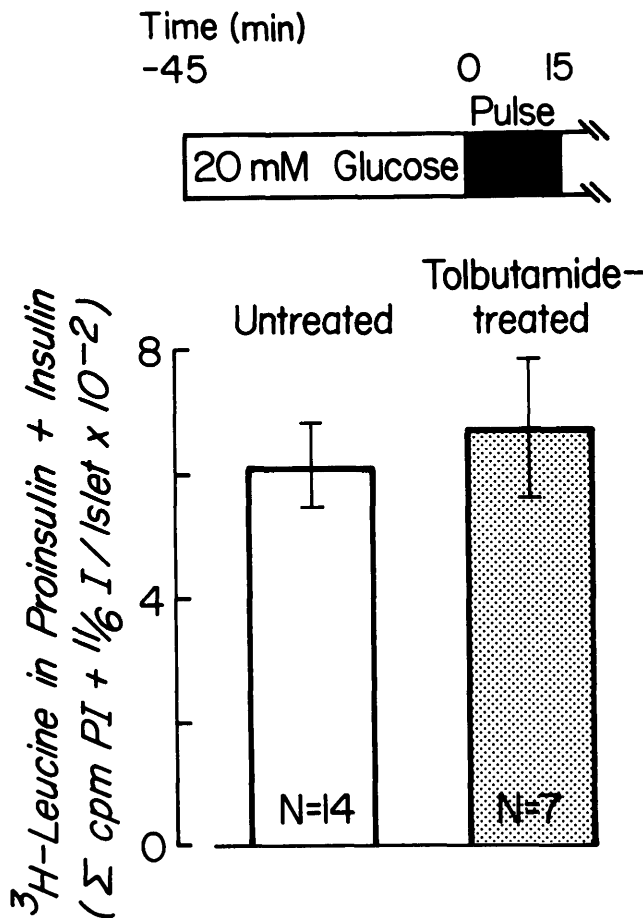


FIGURE 2. Effect of tolbutamide pretreatment on the incorporation of $^3$H-leucine into proinsulin + insulin in isolated islets. Values represent islets collected at later times (typically 131 min) plus all labeled proinsulin and insulin secreted from the time of the pulse to the time of islet collection. The radioactivity eluted 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.

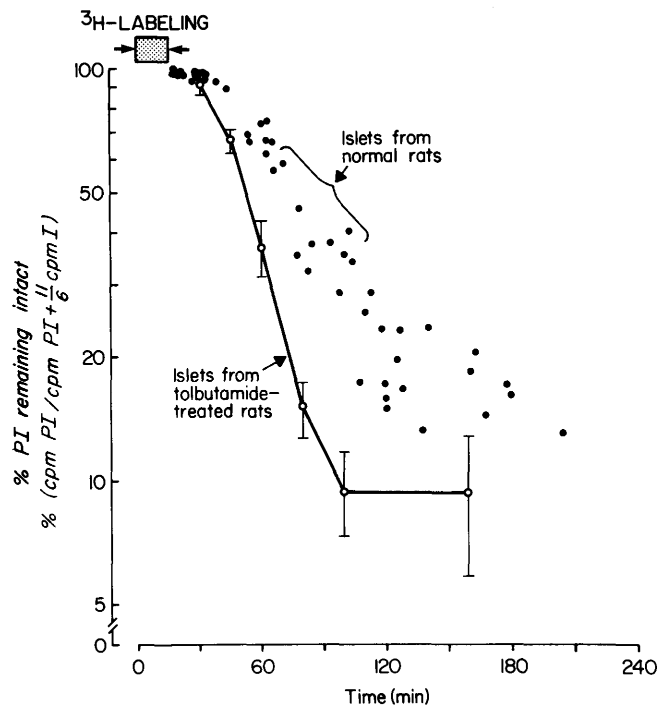


FIGURE 3. Effect of time on the conversion of labeled proinsulin to insulin in islets isolated from control and tolbutamide-treated rats. Radioactivity eluting from columns of Biogel P-30 in the insulin peak was multiplied by 11/6 to correct for the different leucine content between rat insulin and proinsulin. Closed circles represent experiments with islets from control rats (taken from ref. 9) and open circles represent the mean ± SEM of three experiments done on separate days with freshly isolated islets from tolbutamide-treated rats. Linear regression lines were calculated for each set of measurements using all values between 10% and 80% intact proinsulin: with normal rats, log (%PI remaining intact) = $-8.36 \pm 0.65 \times 10^{-3}$ min + 2.32 ($t_{1/2}$ = 36 min); with tolbutamide-treated rats, log (%PI remaining intact) = $-15.1 \pm 2.2 \times 10^{-3}$ min + 2.46 ($t_{1/2}$ = 20 min). Slopes of these two regression lines were significantly ($P < 0.001$) different.

A but not by test period B. This was previously observed[9] and was expanded, since glucose potentiates glucose-stimulated insulin release in a time-dependent manner.[2]

## DISCUSSION

Most published evidence suggests that tolbutamide potentiates the release of stored insulin while it causes either no change or a decrease (particularly at low concentations of glucose) of the rate of insulin biosynthesis.[13-19] These opposite effects can cause insulin secretion to outstrip production; after 3 days of this pharmacologic tolbutamide treatment, isolated islets were found to be significantly depleted of insulin but not glucagon. Fed plasma glucose concentrations were normal when islets were isolated (and probably were lower than normal during tolbutamide treatment). Comparable depletion of islet insulin was also reported in rats made hyperglycemic by glucose infusion.[20]

In pulse-labeling experiments, glucose-stimulated islets from tolbutamide-treated or untreated rats incorporated $^3$H-leucine at equal rates into proinsulin plus insulin. Therefore, amino acid transport and the size of intracellular leucine pools were unchanged with tolbutamide treatment,[21,22] and glucose-stimulated rates for the biosynthesis of proinsulin plus insulin probably were also equal. Also unchanged was the



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onset of conversion, which is thought to be associated with cellular transport of labeled secretory proteins from the rough endoplasmic reticulum across the Golgi into newly forming granules.[23,24] However, tolbutamide pretreatment hastened the production of mature hormone by reducing the half-time of conversion of labeled proinsulin to insulin from 36 to 20 min. Islets from rats made hyperglycemic by glucose infusion were also reported[20] to have accelerated rates of conversion of newly synthesized proinsulin to insulin (a $t_{1/2}$ of 24 min was estimated from reported data), suggesting that depletion of insulin stores, rather than tolbutamide treatment, glucose treatment, or altered serum glucose levels, is the stimulus for accelerated cellular maturation of hormone. Cellular insulin depletion may either stimulate the production or activity of the thiol protease(s) responsible for proinsulin-to-insulin conversion[25] or, alternatively, it may increase conversion by an alteration of the environment in secretory granules, where most of the conversion occurs.[26]

In normal islets, specific activity of stored and secreted insulin rose toward a maximum over 3 h (Figure 4 and ref. 9). This results from the slow transport of labeled proinsulin to secretory granules and its slow conversion to insulin. Despite very little change in the rate of proinsulin synthesis in pulse-labeling experiments with islets from tolbutamide-treated rats, the specific activity of islet and secreted insulin peaked at much higher levels than with islets from untreated rats; after treatment, newly synthesized insulin represented a larger percentage of the total stored insulin due both to a diminished insulin storage and to an accelerated rate of hormone maturation. Dropping the $t_{1/2}$ of conversion from 36 to 20 min after tolbutamide treatment could also diminish by as much as 24% (based on computer modeling) the amount of proinsulin released from these insulin-depleted cells during periods of stimulated secretion. In contrast, in identically performed experiments, dispersed cells from rat insulinomas also had a low hormone content but normal rates of proinsulin-to-insulin conversion and high, identical fractional secretion rates for both labeled and unlabeled insulins.[4] This state of highly marked granules, but with normal conversion rates, can explain the higher-than-normal secretion of proinsulin characteristic of many islet cell tumors.

During continuous glucose stimulation of islets from tolbutamide-treated rats, newly synthesized insulin was preferentially secreted as indicated by a ratio of specific activities of secreted versus cellular insulins that was elevated well above the 1.0 ratio of random release. Therefore, islets from tolbutamide-treated rats retained heterogeneous compartmental insulin storage characteristics during insulin depletion.

According to the model analysis of normal islets, during marking, all new granules enter a small pool of preexisting granules that had been similarly marked at some time before the pulse-chase experiments. At low glucose, they enter the more stable granular compartment (shift in rate from Golgi to the stable compartment depicted by the dashed line in flow system of Figure 4). The nature of the marking process is unknown, but observations that phorbol ester is a more effective marking agent than high glucose[2] suggests that phosphorylation of granule membrane proteins could be involved. The size of the initial marked compartment (1.1 ng/islet or 1.5% of total insulin content plus 3% as proinsulin) is calculated from the total unlabeled proinsulin synthesis and conversion to insulin during the in vitro exposure to glucose from −45 min. This value is estimated from total net insulin production/20 min in 24-h perifused islets (0.86 ng; unpublished observations) and should be considered approximate and minimum. A much larger synthetic rate, however, is unlikely since, according to the mathematical flow system for normal islets, this would dilute the marked compartment and cause a rapid decline of specific activity during the chase

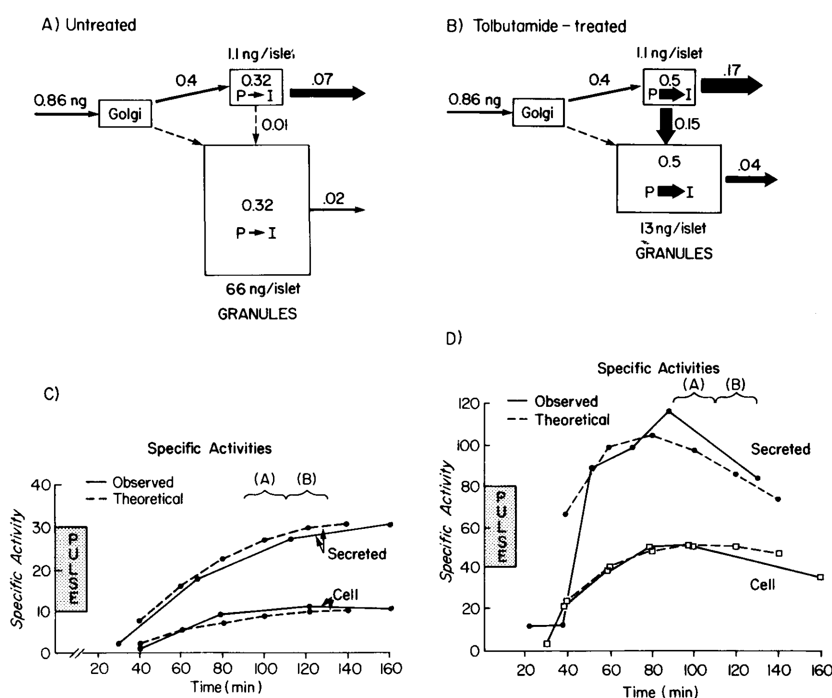


**FIGURE 4. (A and B) Flow diagrams for insulin biosynthesis, storage, and secretion in islets from untreated (A) and tolbutamide-treated (B) rats. All rate constants indicated by dashed or solid lines are fractional rates per 20 min. Best estimated values for the initial insulin content of each compartment are shown and relative differences (found in this study) between the insulin content of islets in normal and tolbutamide-treated rats have been maintained. (C and D) Effect of time on the specific activities (observed and theoretical) of secreted and cellular insulins in islets from untreated (C) and tolbutamide-treated (D) rats. Points represent mean ± SEM for three or more experiments. Data points are plotted at the center of the intervals, i.e., 15–45 min, in which incubation buffers were removed and replaced with fresh KRB. Observed specific activities for islets from tolbutamide-treated rats were normalized to the average incorporation of $^3$H-leucine shown in Figure 2. In these calculations, the minimal initial proinsulin and insulin values of the marked compartment were obtained by starting them at time zero and permitting the system to run for 1 h before introducing the pulse. To eliminate variability due to incorporation of $^3$H-leucine during the pulse, total radioactivity in proinsulin plus insulin at the end of the pulse was corrected to 6100 cpm/islet for individual controls and 6730 cpm/islet for individual tolbutamide-treated islets (data from Figure 2).**

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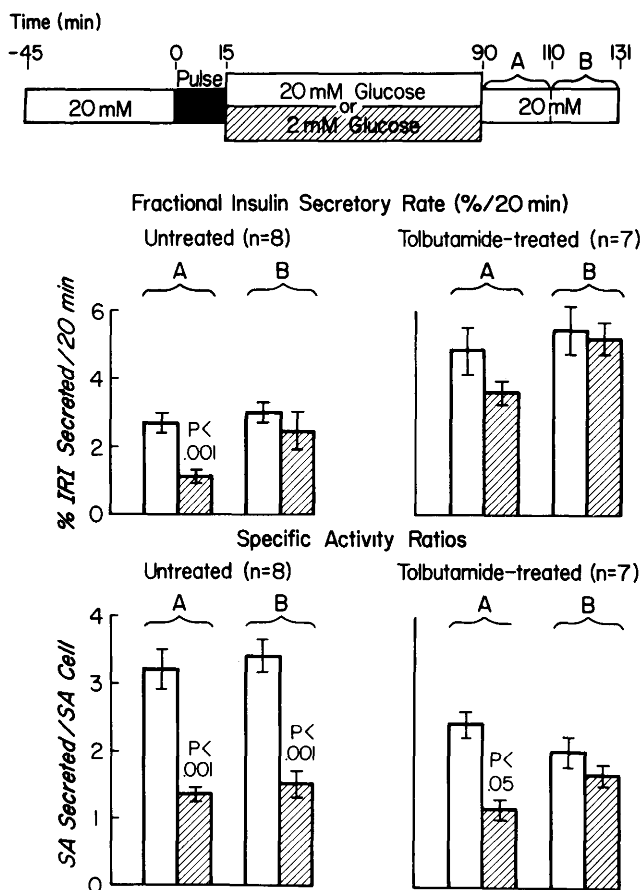


**FIGURE 5. Effect of glucose concentration on the marking for preferential release of newly synthesized insulin of islets from tolbutamide-treated rats. Fractional secretory rates were calculated with the sum of all secreted and islet insulin assigned the value of 100%. Bars represent the mean ± SEM from seven experiments.**

period—a phenomenon that did not occur during the 3 h of the experiment (Figure 4). Note that due to its small size, secretion from the marked compartment had little effect on the overall rate of insulin secretion in untreated islets.

In contrast, in the case of islets from tolbutamide-treated animals, the specific activity of secreted insulin reached a maximum and rapidly fell by 110–120 min during the chase periods. In part, the drop in specific activity could be explained by the more rapid fractional secretion of labeled (and unlabeled) insulin that occurred and its replacement by new unlabeled insulin (the insulin content of tolbutamide-treated islets decreased slightly between 30 and 160 min, 7.3 ± 0.9 and 6.6 ± 1.1 ng IRI/islet, respectively). However, the magnitude of the drop and the decrease in ratio of secreted to stored insulin (Figures 4 and 5) additionally requires rapid exchange from marked to the unmarked (or less-marked) compartment.

In pretreated islets, the marked, labile compartment represents a much higher proportion of total insulin than in normal islets. Furthermore, those granules rapidly converted to the less-marked compartment may still retain some of their labile characteristics. The combination of these phenomena could contribute to the increased fractional rates for total insulin, characteristic of these depleted islets (Figure 5).

In summary, insulin-depleted islets compensate by increasing the rate of conversion of proinsulin to insulin and not by increasing the glucose-stimulated rate of proinsulin biosynthesis, which already may be at maximal rate. Although exchange between marked to unmarked granules may be accelerated, cells retain their heterogeneous storage characteristics necessary for marked and preferential release of new hormone. The relatively larger marked compartment, in time, may contribute to the cell's increased fractional rate of insulin secretion.

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