STUDIES ON PROINSULIN AND PROGLUCAGON BIOSYNTHESIS AND CONVERSION AT THE SUBCELLULAR LEVEL

II. Distribution of Radioactive Peptide Hormones and Hormone Precursors in Subcellular Fractions after Pulse and Pulse-Chase Incubation of Islet Tissue

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ABSTRACT

Anglerfish proinsulin and insulin were selectively labeled with \[^{14}C\]isoleucine, while proglucagon, conversion intermediate(s), and glucagon were selectively labeled with \[^{3}H\]tryptophan. After various periods of continuous or pulse-chase incubation, islet tissue was subjected to subcellular fractionation. Fraction extracts were analyzed by gel filtration for their content of precursor, conversion intermediate(s), and product peptides. Of the seven subcellular fractions prepared after each incubation, only the microsome and secretory granule fractions yielded significant amounts of labeled insulin-related and glucagon-related peptides. After short-pulse incubations, levels of both \[^{14}C\]proinsulin and \[^{3}H\]proglucagon (mol wt \(\sim 12,000\)) were highest in the microsome fraction. This fraction is therefore identified as the site of synthesis. With increasing duration of continuous incubation or during chase incubation in the absence of isotopes, proinsulin, proglucagon, and conversion intermediate(s) are transported to secretory granules. Conversion of proinsulin to insulin and proglucagon to a \(\sim 4,900\) mol wt conversion intermediate and \(3,500\) mol wt glucagon occurs in the secretory granules. Converting activity also was observed in the microsome fraction. The recovery of most of the incorporated radioactivity in microsome and secretory granule fractions indicates that the newly synthesized islet peptides are relegated to a membrane-bound state soon after synthesis at the RER is completed. This finding supports the concept of intracisternal sequestration and intragranular maintenance of peptides synthesized for export from the cell of origin.

Results from recent studies designed to ascertain the mode of biosynthesis of glucagon indicate that a biosynthetic precursor is involved (8, 27–33, 47). Evidence from other studies performed over the last 10 yr indicates that many, if not all, peptide hormones are synthesized as peptides larger than the biologically active circulating hormone and are cleaved enzymatically before secre-
tion of their cell of origin (24, 25). These discoveries have led to increased interest in the manner in which intracellular proteolytic processing of these precursor molecules occurs. Studies on the mode of insulin biosynthesis at the subcellular level have been performed on islet tissue from fish (2, 6, 7), rat (10, 18, 35, 40, 42), and human isulomas (3, 19). Results from these studies, which employed both subcellular fractionation and electron microscope autoradiographic techniques, have indicated that proinsulin is synthesized on the rough endoplasmic reticulum. Sequential translocation of this molecule to the cis and trans cisternae of the endoplasmic reticulum, transfer to the Golgi complex, packaging in secretory granules, and exocytotic extrusion of insulin follow sequentially. Data from the studies of Sorenson et al. (40), Kemmler et al. (18), Creutzfeldt et al. (3), and Sun et al. (42) implicate the secretory granules and possibly components of the Golgi complex in the process of converting proinsulin to insulin.

Much less is known about the mechanisms of proglucagon biosynthesis and its proteolytic cleavage. We have recently reported results from studies on anglerfish islet tissue that provide evidence for sequential metabolic cleavage of a ~12,000 mol wt precursor for glucagon. The metabolic processing of this molecule by proteolysis appears to occur in three cleavage steps (27, 31).

Only two studies designed to elucidate the mechanisms of proglucagon biosynthesis and conversion at the subcellular level have appeared in the literature. In one preliminary report, anglerfish islet tissue that provide evidence for sequential metabolic cleavage of a ~12,000 mol wt precursor for glucagon. The metabolic processing of this molecule by proteolysis appears to occur in three cleavage steps (27, 31).

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**Materials and Methods**

**Incubation Procedure**

Anglerfish were obtained during the summer months at the Marine Biological Laboratory, Woods Hole, Mass. Islet tissue was removed, decapsulated, and sliced into pieces approximately 2 mm². Preincubation and incubation proceeded in Krebs-Ringer bicarbonate buffer (KRB) as previously described (31). During the incubation periods, the medium contained 20-70 µCi L-[¹⁴C]isoleucine (283 mCi/mmol) and 200-400 µCi L-[³⁵S]methionine (7.9 Ci/mmol). Isotopes were obtained from New England Nuclear, Boston, Mass. No other additions to the incubation medium were made except 40 µg/ml unlabeled L-isoleucine and L-tryptophan that were added to replace the isotopically labeled amino acids during chase incubations.

**Subcellular Fractionation and Extraction Procedures**

After continuous or pulse-chase incubation periods of varying length, the islet tissue was removed from the medium, homogenized in 0.25 M sucrose, and subjected to subcellular fractionation as described in the accompanying paper (28). All seven subcellular fractions obtained from each tissue homogenate were extracted by an acidified ethanol-trisodium citrate-methylene chloride technique as previously described (31). Equilibration in 1.5 ml and re-extraction in 1 ml of 2 M acetic acid followed removal of residual methylene chloride. The final volume of all extracts was 2.5 ml. A 250-µl aliquot for later use in protein and radioimmunoassay determinations was removed from all extracts before gel filtration. A modification of the Lowry procedure (22) was utilized to determine the protein content of the fraction extracts. The content of immunoreactive glucagon (IRG) in the fraction extracts was determined as previously described (31, 32), with the Unger 30 K antisera.

**Gel Filtration of Fraction Extracts**

The data shown in Fig. 1 were obtained by equilibration of whole tissue extracts in 2 M propionic acid and gel filtration in 1 M propionic acid. For convenience, all extracts of subcellular fractions were equilibrated in 2 M acetic acid and filtered in 1 M acetic acid. All gel filtration columns were packed with Bio-Gel P-100 (100-200 mesh; Bio-Rad Laboratories, Richmond, Calif.). Column calibration, collection of fractions, and continuous monitoring of column eluates for ultraviolet absorbance were performed as previously described (31). To each even-numbered eluate fraction, 7 ml of Preblend 3a70B liquid scintillation cocktail (Research Products International Corp., Elk Grove Village, Ill.), was added, and radioactivity was assayed in a Beckman liquid scintillation spectrometer (Beckman Instruments Inc., Palo Alto, Calif.). All glassware that came into contact with tissues, tissue extracts, extracts of subcellular frac-
Data Computation

The raw data from scintillation counting were corrected for background counts and spill of 4C-radioactivity into the 4H-channel. The data obtained were then converted to dpm per unit tissue weight, milligram protein or microgram immunoadsorbable glucagon in the fraction extracts. These computations and plotting of the data on a Tektronix graphics terminal (Tektronix Inc., Beaverton, Ore.) were facilitated by use of computer programs designed for these purposes. A print-out of the derived values used in plotting each data point was also obtained. The data in Figs. 2-6 and Table I were obtained by summation of the total dpm attributable to molecules of varying size. The 4C-radioactivity attributable to proinsulin and insulin in gel filtration eluates is easily computed as the sum of all dpm in the appropriate peak. For example, in Fig. 1 peaks 1 are radioactive proinsulin and peaks 2 are radioactive insulin. The 4H-radioactivity attributable to glucagon and its precursor is not so readily separated. For convenience, the total [4H]dpm in peaks a and b in eluates from gel filtration of fraction extracts are expressed together as radioactivity in the "precursor region," and the total dpm in peaks c and d are expressed together as radioactivity in the "glucagon peak" in Figs. 4-6. The rationale for using this method of computation is given below.

RESULTS

Distribution of Radioactivity in Proteins Found in Whole Tissue Extracts after Continuous Incubation

Anglerfish proinsulin contains two residues of isoleucine as components of the insulin A chain but has no tryptophan (26, 48). Glucagon and a larger glucagon-related peptide from anglerfish islet contain no isoleucine but have one tryptophan residue (46). This information, our previous analyses of incorporation of [4H]tryptophan and [4C]isoleucine into anglerfish islet proteins (27, 29, 31), and unpublished observations from ion exchange separations of labeled islet proteins all indicate that (a) tryptophan becomes incorporated selectively into glucagon, its precursor, and metabolic cleavage intermediates; and (b) isoleucine becomes incorporated only into insulin-related peptides.

In our previous studies, gel filtration was performed in 1 M acetic acid. The data shown in Fig. 1 were obtained by gel filtration of extracts of tissue incubated in the presence of labeled isoleucine and tryptophan for 2 h (A), 4 h (B), and 5 h (C) in 1 M propionic acid. Filtration in propionic acid appears to provide better resolution of the various peptides in the extract than filtration in acetic acid (31). Peaks 1 and 2 in Fig. 1 are proinsulin and insulin, respectively (27, 29, 31). Peaks a in Fig. 1 have been identified as proglucagon (mol wt ~12,000), and peaks b identified as a possible metabolic conversion intermediate in cleavage of proglucagon to glucagon (31). Peak d is the elution position of 3,500 mol wt anglerfish glucagon, and peak c is formed by the presence of a ~4,900-mol wt metabolic conversion intermediate for anglerfish glucagon (27). This latter peptide is quite basic and is probably similar to the fragment of bovine/porcine glucagon characterized by Tager and Steiner (43). In previous work in which whole islet tissue was subjected to pulse-chase incubation, it has been established that metabolic transfer of 4H-radioactivity from peaks a and/or b to peaks c and d occurs during chase incubation (30). Because peak a is not completely separated from peak b and, similarly, peak c is not completely separated from peak d by gel filtration, isotope incorporation into glucagon-related peptides extracted from subcellular fractions in Figs. 4-6 and Table I is expressed as total "precursor region" dpm (peak a plus peak b) and total "glucagon" peak dpm (peak c plus peak d).

Determination of the Effectiveness of the Fractionation Procedure When Applied to Incubated Tissue

Because subcellular fractionation of tissue that

| Table I |

| Percentage of Labeled Precursors and Products in the Cell Supernate (F-IV) |

| Incubation period | [4C]Isoleucine-labeled proinsulin | [4C]Isoleucine-labeled insulin | [4H]Tryptophan-labeled proinsulin | [4H]Tryptophan-labeled glucagon |
|-------------------|---------------------------------|-----------------------------|---------------------------------|---------------------------------|
| min               | %                               | %                           | %                               | %                               |
| Continuous incubation |
| 20                | 31.2                            | 32.1                        | 15.1                            | 18.4                            |
| 60                | 10.6                            | 21.7                        | 10.9                            | 10.7                            |
| 180               | 6.6                             | 9.3                         | 4.2                             | 7.8                             |
| Pulse-chase       |
| 20 + 0            | –                               | –                          | 9.9                             | 3.9                             |
| 60 + 60           | –                               | –                          | 6.0                             | 3.2                             |
| 60 + 180          | –                               | –                          | 1.9                             | 1.7                             |

Data were obtained by summation of total radioactivity (dpm/100 mg protein) attributable to precursor or product in extracts of each of the seven subcellular fractions at each time interval. The values in this table are the proportions of the total dpm in all fractions present in F-IV.
FIGURE 1 Continuous incubation of 370 mg of anglerfish islet for 2 h (A), 232 mg for 4 h (B), and 300 mg for 5 h (C). Incubations proceeded in the presence of 10 μCi L-[^14]C]isoleucine (313 μCi/mmol) and 100 μCi L-[^3H]tryptophan (1.15 Ci/mmol). After extraction, each sample was subjected to gel filtration on a 1.6 × 90-cm column of Bio-Gel P-10 (100-200-mesh) in 1 M propionic acid. Fractions of 1.5 ml were collected. Peaks 1 and 2 represent ^14C-labeled proinsulin and insulin, respectively. Peaks a–d are glucagon-related peptides labeled with ^3H]tryptophan.

has been subjected to in vitro incubation may yield fractions having more cross-contamination than fractions isolated from fresh tissue, several control experiments were performed. The modified subcellular fractionation procedure described in the accompanying paper (28) was applied to islet tissue subjected to 20- and 180-min in vitro incubation. The fraction contents were prepared for and subjected to ultrastructural examination as described for fractions isolated from fresh islet tissue (28). No differences in fraction content or integrity were observed between fractions prepared from fresh or incubated tissue.

Another potential artifact in determination of precursor and product peptide content in subcellular fractions from incubated islet tissue is the possibility that conversion occurs during the fractionation procedure. To test this possibility, islet tissue was incubated for 20 min in the presence of [^14]C]isoleucine and [^3H]tryptophan. Previous determinations have shown that the only labeled peptides in tissue extracts are the precursor peptides after 20 min of incubation (31). The incubated tissue was homogenized in 0.25 M sucrose, and one-half of the homogenate was immediately extracted as described in Methods. The remainder of the homogenate was allowed to incubate at 4°C for 4 h which is the period of time required to complete the extraction procedure. The extracts of both portions of the homogenate were subjected to gel filtration at different times on the same Bio-Gel P-10 column. There was no difference in the pattern of elution of radioactivity between the two extracts, indicating that maintenance of tissue components at 4°C in unbuffered sucrose solutions prevents precursor-to-product cleavage during execution of the fractionation procedure.

A third consideration is the possibility that data obtained from tissue incubated in a medium devoid of energy-yielding substrate may not accurately reflect the rates of synthesis, transport, and cleavage that occur in vivo. If one computes the total radioactivity incorporated into insulin, glucagon, and their precursors from the data in Fig. 1, the accumulation of both ^3H- and ^14C-labeled peptides is found to be linear through 5 h of continuous incubation. Although incorporation may proceed more rapidly in vivo, this observation suggests that an adequate endogenous energy supply is available to support the short-term incubations employed to obtain the data presented in Figs. 2–6.

Proinsulin and Insulin Content of Subcellular Fractions Isolated after Continuous Incubation

Islet tissue was subjected to 20 min, 1 h, and 3 h
of continuous incubation in the presence of \([1^{14}C]\)isoleucine and \([3^{3}H]\)tryptophan before subcellular fractionation. Several experiments of this nature were performed. Because the data from all experiments were comparable, only the results of the most complete experiment are presented here. After gel filtration of the extracts from the subcellular fractions, the total \([14^{C}]\)-radioactivity attributable to proinsulin and insulin was ascertained as described in Materials and Methods. The results are shown in graphic form in Fig. 2. After 20 min of incubation, proinsulin in the microsome fraction (F-II) possessed the highest level of radioactivity. This implicates the microsomes as the site of synthesis. The amount of labeled proinsulin extracted from the microsome fraction increased in a linear manner with increasing incubation time. Levels of \([14^{C}]\)proinsulin found in the secretory granule fractions (F-IIb and F-IIIc) after 20 min of incubation were quite low but increased markedly with increase in duration of incubation which is possibly an indication of transfer of proinsulin into these fractions. It should also be noted that the levels of proinsulin-associated radioactivity found in the F-I, F-IIc, and F-IVb fractions were very low at all time periods. This suggests that the components of these fractions play an insignificant role, if any, in synthesis and processing of proinsulin. The only fraction other than the microsome and secretory granule fractions in which significant levels of \([14^{C}]\)proinsulin were found was the cell supernate (F-IV). However, if one compares the proportion of the total \([14^{C}]\)proinsulin that can be extracted from F-IV with that from all the other fractions at each time period studied, it can be seen that the proportion in F-IV diminishes markedly with increasing incubation time (Table I).

The data in Fig. 2B show the levels of \([14^{C}]\)insulin extracted from each of the subcellular fractions after 20, 60, and 180 min of continuous incubation. Insulin levels were highest in the microsome fraction after 20 min of incubation and increased in this fraction with increase in duration of the incubation. The data in Fig. 2B also indicate that very little \([14^{C}]\)insulin was present in the secretory granules after 20 min of incubation but accumulated in these fractions with increasing incubation time. As in the case for proinsulin (Fig. 2A), the levels of \([14^{C}]\)insulin found in the F-I, F-IIc, and F-IIb fractions are insignificant in comparison to the amounts observed in the microsome and secretory granule fractions. Although the proportion of \([14^{C}]\)insulin extracted from the cell supernatant fraction is high after the 20-min incubation period, that proportion decreases markedly in the F-IV fractions from tissue incubated 60 and 180 min (Table I).

Fig. 3 shows the data from microsome and secretory granule fractions in Fig. 2A and B in composite form. Data from the fractions in which insignificant amounts of proinsulin and insulin were found are omitted, and the total proinsulin or insulin radioactivity in the F-IIb and F-IIIc fractions is expressed as a sum (proinsulin in granule fractions or insulin in granule fractions, Fig. 3). Inasmuch as neither beta nor alpha cell secretory granules predominate in either of the two secretory granule fractions (28), expression as total accumulation in both granule fractions is perhaps a more accurate assessment of accumulation of proinsulin and insulin in secretory granules with increasing incubation time. With the data expressed in this manner, the relationships between the accumulation of proinsulin and that of insulin in the microsome and secretory granule fractions are more readily discerned. Both peptides accumulate in the secretory granule fraction, with the accumulation of insulin occurring later and more slowly than that of proinsulin.

Proglucagon, Conversion Intermediate(s), and Glucagon Content of Subcelluar Fractions Isolated after Continuous Incubation

The data in Figs. 4 and 5 show the results from analysis of \([3^{3}H]\)tryptophan incorporation into proglucagon and other glucagon-related peptides in the various subcellular fractions after the same 20-, 60-, and 180-min continuous incubations from which the data for proinsulin and insulin accumulation were derived. The data in Fig. 4A indicate that the level of \([3^{3}H]\)tryptophan radioactivity found in the "precursor region" of the gel filtration eluate is highest in the microsome fraction after 20 min of incubation. This result suggests that the microsome fraction is the site of proglucagon biosynthesis. Both proglucagon and the putative first conversion intermediate (peaks a and b, Fig. 1) were present in extracts of fractions from all time periods, but the proportion of radioactivity attributable to the intermediate peak was found to increase with increasing incubation time. It should also be noted in Fig. 4A that total accumulation of \([3^{3}H]\)-radioactivity in the precursor region was linear with increasing incubation time.
A

\[14C\] Radioactivity
Proinsulin Peak

B

\[14C\] Radioactivity
Insulin Peak

Incubation Time (min)
The levels of radioactive precursor and first intermediate in the secretory granule fractions were low after 20 min of incubation but accumulated rapidly in these fractions with increase in duration of incubation. This suggests that transfer of these peptides from the microsomes to the secretory granules occurs after their synthesis at the rough endoplasmic reticulum.

The data in Fig. 4 B indicate that $^3$H-radioactivity attributable to glucagon-sized peptides showed very little accumulation in any of the fractions after 20 min of incubation. The amounts observed in the microsome fraction accumulated most rapidly with increasing incubation time. The greatest proportion of this $[^3]$H]tryptophan radioactivity eluting after anglerfish insulin on gel filtration of the extracts of microsomes was associated with the ~4,900 mol wt component (i.e., peak c in Fig. 1). The $^3$H-radioactivity attributable to glucagon-sized peptides also accumulated in the secretory granule fractions with increasing duration of incubation but at a lower rate than that observed in the microsome fraction. The data in Fig. 4 A and B show that accumulation of glucagon-related peptides in the F-I, F-II, and F-IIW fractions is insignificant. Some accumulation of $[^3]$H]tryptophan-labeled glucagon-related peptides was observed in the cell supernatant fraction (F-IV) with increase in incubation time. However, as shown in Table I, the proportion of the total $^3$H-radioactivity in F-IV was not only fairly low but decreased markedly with increasing duration of incubation.

Fig. 5 is a composite of the data from Figs. 4 A and B, with the total radioactivity in both secre-
Figure 4 Levels of [3H]tryptophan-labeled ~12,000 mol wt proglucagon and ~9,000 mol wt intermediate I (Precursor Region, A) compared with [3H]tryptophan-labeled ~4,900 mol wt intermediate II, and 3,500 mol wt glucagon (Glucagon Peak, B) in subcellular fractions prepared after 20, 60, and 180 min of continuous incubation. Anglerfish islet (146 mg) was incubated in the presence of 400 μCi 1-[3H]tryptophan. Tissue removal, extraction, and gel filtration were performed as described in Fig. 2.
Figure 5 Combined data from the microsome and secretory granule fractions in Fig. 4 expressed in terms of specific radioactivity (dpm/microgram immunoassayable glucagon in fraction extracts). The data confirm the microsome fraction as the site of proglucagon synthesis. Metabolic processing of the larger peptides to the ~4,900 mol wt peptide and glucagon apparently occurs in both the microsomes and secretory granules.

The data from fractions that appear to be insignificant in the biosynthesis and processing of proglucagon are omitted. In addition, these data are expressed as dpm per microgram immunoas-
sayable glucagon (IRG) in the fraction extracts. The IRG determinations were made on aliquots taken from extracts of fractions before gel filtration. Thus, the IRG measured represents the sum of the reactivities of all glucagon immunoreactive components present (e.g., proglucagon, intermediates, and glucagon). Expression of the data as dpm/microgram IRG when the IRG value is de-

erived from the presence of more than one glucagon immunoreactive component is only an esti-

mate of specific radioactivity. With the antisera employed, the amount of cross-reactivity per mole of glucagon-related peptide diminishes with in-

creasing molecular size (27, 29, 31 and unpublished data). This observation is similar to the finding that antibodies specific for an antigenic determinant on the insulin molecule cross-react less well with proinsulin. At least small amounts of the ~4,900 mol wt second conversion intermediate and 3,500 mol wt glucagon are apparently present in the microsome fraction (Fig. 4 B). Because these peptides are more highly immunoreac-

tive than proglucagon on a molar basis, the actual specific radioactivity of the larger [H]-labeled mol-

eules in the precursor region is probably underestimated.

Even though specific radioactivity in the "pre-

cursor" region is artifically low, the data ex-

pressed in this manner still yield useful informa-

tion. The "specific radioactivity" in proglucagon and the first conversion intermediate were highest
in the microsome fraction at the termination of the 20-min incubation. This finding, added to the observation that the total 3H-radioactivity attributable to precursor region molecules is also highest in F-III after 20 min (Fig. 4 A), confirms the microsomes as the site of proglucagon biosynthesis. With increasing incubation time, the specific radioactivity of precursor peptides in the microsomes increases in a linear manner. The levels of proglucagon and first conversion intermediate in the secretory granule fractions are quite low after the 20-min pulse incubation (1,830 dpm/µg IRG) but increase rapidly to reach 340,930 dpm/µg IRG at the end of the 3-h incubation period. The dpm/microgram IRG attributable to the second conversion intermediate and glucagon increased in a nearly linear manner in F-III throughout the incubation period, suggesting that some conversion may occur in this fraction. The specific radioactivity of these peptides in the secretory granules is very low after only 20 min of incubation and increases only very slowly with increasing incubation time. This relatively slow accumulation is probably due to secretion of some of the newly synthesized product molecules during the incubation period and to the possibility that the final conversion steps may occur much more slowly than the initial steps. (See Discussion.)

FIGURE 6 Distribution of [3H]tryptophan-labeled glucagon-related precursor and product peptides in subcellular fractions after pulse-chase incubation. After a 20-min pulse incubation of 200 mg anglerfish islet in the presence of 200 µCi L-[3H]tryptophan (7.9 Ci/mmol), one-third of the tissue was removed for subcellular fractionation. The incubation was then continued for an additional 40 min. One-half of the remaining tissue was removed for fractionation after 60 and 180 min of chase incubation in the absence of isotope. Although all seven subcellular fractions from each time period were extracted and subjected to gel filtration as described in Fig. 2, only the data from microsome and combined secretory granule fractions are shown. The data are expressed as dpm/microgram protein in fraction extracts.

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Content of Glucagon-Related Peptides in Subcellular Fractions Isolated after Pulse-Chase Incubations

The data in Fig. 6 show the results from determination of the distribution of "precursor" and "product" peptides in subcellular fractions prepared after pulse-chase incubation. One-third of the incubation tissue was removed for fractionation after 20 min of incubation. The remainder was allowed to incubate for an additional 40 min to allow sufficient accumulation of labeled peptides for recovery after chase incubation, and then was washed free of isotope for chase incubation in the absence of isotope. Equivalent portions of tissue were removed for fractionation after 60 and 180 min of chase. Only the data from the microsome and secretory granule fractions are shown because the proportion of the total labeled glucagon-related peptides recovered in F-IV was quite low (Table I) and the content in the other fractions was even lower. The data are expressed as dpm/microgram protein in the fraction extract. Because extracts of the microsome fraction usually possess approximately 15% of the total extractable protein, and the combined secretory granule fractions possess 38-40% (28), the dpm/microgram protein values for precursor and glucagon in the microsome fraction in Fig. 6 are elevated correspondingly by this mode of representation of data.

Expressed in this manner, these data re-emphasize that after a 20-min pulse incubation, the levels of proglucagon and first conversion intermediate are highest in the microsome fraction. With increasing duration of chase incubation, the amounts of these peptides are progressively depleted in the microsome fraction. This indicates that these large glucagon-related peptides are cleaved to smaller peptides in F-III, or that they are transported out of F-III to another fraction or fractions, or that a combination of both of these processes occurs. The remainder of the data in Fig. 6 support the latter possibility. The precursor peptides accumulated in the secretory granule fractions with increasing duration of chase incubation. This suggests transport of these peptides from F-III to the secretory granules. The amounts of the second conversion intermediate and glucagon in the microsome fraction ("Glucagon in F-III" in Fig. 6), although low after a 20-min pulse, accumulated with increasing duration of chase incubation. This suggests that some component of the microsome fraction may participate in cleavage of precursor to product.

The level of glucagon-sized [3H]tryptophan-labeled peptides found in the secretory granule fractions ("Glucagon in granule fractions" in Fig. 6) was very low after the 20-min pulse incubation. Although the level of these peptides increased threefold over the 20-min value at the 60-min pulse—60-min chase time period, accumulation did not occur in the secretory granules between the 60- and 180-min chase periods. This finding is consistent with the data in Figs. 4 B and 5, which indicate that product peptides accumulated very slowly in the secretory granules, even during a continuous incubation.

DISCUSSION

The results from several other studies have a bearing on the interpretation of the data presented here. That insulin and glucagon are the secretory products of the pancreatic islet beta and alpha cells, respectively, has long been accepted. Recent immunohistochemical determinations indicate that somatostatin is a product of the pancreatic islet delta cell in several species (1, 9, 23, 34). In recent studies, we have been able to localize insulin, glucagon, and somatostatin in separate cells of anglerfish islet by immunohistochemical techniques (16). Because somatostatin-containing cells appear to comprise nearly 30% of all anglerfish islet cells, we have, within the past year, completed a number of experiments designed to determine whether anglerfish islets synthesize somatostatin. During the course of these studies, it became evident that acid ethanol-methylene chloride-acetic acid extraction of TCA-precipitated islet tissue homogenates yielded poor recovery of immunoreactive somatostatin. Direct homogenization and extraction in 2 M acetic acid resulted in good recovery. When acetic acid extracts were subjected to gel filtration on Bio-Gel P-10, the predominant peak of immunoreactive somatostatin was found to elute just before the salt volume of the column. This elution position is completely separate from the insulin- and glucagon-containing portion of the eluate. No isoleucine incorporation was observed, but radioactive tryptophan does become incorporated into anglerfish somatostatin during incorporation experiments.1 However, be-

1 B. D. Noe, G. C. Weir, and G. E. Bauer. 1976. Biol. Bull. 151:422-423a. (Abstr.); full manuscript in preparation.
cause somatostatin is recovered only sparingly in acid ethanol extracts of TCA-precipitated material and because its elution position on gel filtration totally separates it from insulin and glucagon, the \(^3\)H-radioactivity observed in gel filtration eluates of the present study is that which has become incorporated into proglucagon, cleavage intermediates, and glucagon. The contribution of \(^3\)H-radioactivity incorporated into prosomatostatin to the levels observed in the precursor region is negligible when TCA precipitation-acid ethanol extraction is used.

In addition to use of an improved fractionation procedure, another aspect of the experimental protocol employed in the present study should be emphasized as an improvement over procedures followed in other studies. In most previous work designed to ascertain the subcellular sites of protein synthesis, transfer, and conversion, whether by using electron microscope autoradiography or subcellular fractionation techniques, radioactively labeled leucine was the isotope incorporated (2, 3, 12, 18, 35, 40, 42). In one such study, labeled phenylalanine was used (7). It should be emphasized that leucine and phenylalanine are components of insulin and glucagon. Therefore, one would expect some incorporation of radioactivity into glucagon and glucagon-related peptides as well as proinsulin and insulin, even under conditions in which high concentrations of glucose were present in the incubation medium. In the present study, such overlap in labeling is not possible. Proinsulin and insulin are labeled selectively with isoleucine, whereas proglucagon, conversion intermediates, and glucagon are labeled selectively with tryptophan.

Although certain aspects of the data presented in this paper must be considered preliminary in nature, several salient features of the experimental results should be emphasized. Our findings that the intracellular site of proinsulin biosynthesis is the microsomes and that transfer to the secretory granules occurs (Figs. 2 and 3) correlate well with the results from other studies in which the sites of synthesis and transfer have been determined (2, 7, 12, 17, 35, 40–42). The data in Figs. 4–6 provide new evidence that proglucagon is also initially synthesized at the rough endoplasmic reticulum and transported subsequently to the secretory granules. This information confirms the results from an earlier report in which electron microscope autoradiography was used to assess transfer of \(^3\)H-leucine and \(^3\)H-tryptophan through the

guinea pig alpha cell (11). The data from the present study, however, identify the specific peptides involved and further characterize the dynamics of the process. Both proinsulin (Fig. 3) and proglucagon (Fig. 5) accumulate in the microsome fraction during continuous incubation. The levels of these precursors in secretory granules are initially low but increase rapidly with increasing duration of incubation. This is interpreted as an indication that transport of precursors to the granules occurs more rapidly than the accumulation of these peptides in the microsomes. Thus, the levels of proinsulin and proglucagon in the secretory granules are much higher than the content of the same peptides in the microsomes after 180 min of incubation (Figs. 3 and 5).

That beta cell secretory granules participate in conversion of proinsulin to insulin has been demonstrated clearly in numerous studies (3, 7, 18, 40, 42). Results from the present study confirm this finding and indicate that conversion of proglucagon and intermediate(s) also occurs in secretory granules. The data in Figs. 5 and 6 indicate that the rate of accumulation of glucagon-sized peptides in secretory granules is relatively slow. It has been demonstrated in previous studies that \(^3\)H-tryptophan-labeled glucagon-sized peptides as well as immunoreactive glucagon accumulate in the incubation medium during incubation (31). This suggests that secretion of both stored and newly labeled glucagon occurs. Therefore, the low levels of labeled glucagon-sized peptides observed in the secretion granules after 180 min of continuous (Fig. 5) and chase incubation (Fig. 6) may be due in part to secretion. However, the major factor contributing to slow product accumulation in secretory granules is probably that there is a slow rate of conversion of proglucagon to glucagon in this fraction. This slow conversion rate is reflected in the rapid accumulation of precursor in secretory granules (Figs. 5 and 6).

Precursor-to-product conversion also appears to occur in the microsome fraction. The amounts of both labeled insulin and labeled glucagon-sized peptides were found to increase appreciably in the microsomes with increase in duration of incubation (Figs. 2–6). Inasmuch as cross-contamination of fractions prepared from tissue subjected to incubation and cleavage of precursors during the fractionation procedure have been essentially ruled out as sources of error by control experiments, the observed accumulation is apparently a result of cleavage in the microsomes. This finding
differs from the results of several previous studies. Creutzfeldt et al. (3), Sorenson et al. (40), and Sun et al. (42) have reported studies in which both microsomes and secretory granules isolated from islet tissue were analyzed specifically to assess activity in converting proinsulin to insulin. Only limited converting activity was observed in microsome fractions.

There are, however, several important differences in the fractionation procedure used in the present study and those employed previously. In this study, a centrifugation of $6.0 \times 10^4$ g-min was utilized for preparation of a crude fraction containing mitochondria and secretory granules. Subfractionation of the crude F-II yields an uncontaminated secretory granule fraction having granules that possess a range in mean diameter of 220–310 nm (F-IIb). It is important to note that not all of the secretory granules in the homogenates are isolated in the F-IIb subfraction. The secretory granules that remain in the supernate of the crude F-II preparation are removed from the microsomes by ultracentrifugation over 1.4 M sucrose (28). These granules, which comprise the F-IIc subfraction, possess a range in mean diameter of 150–200 nm. The observation that these secretory granules are physically smaller than those in the F-IIb and that they reside in the crude F-III preparation (supernate of the crude F-II) after the $6.0 \times 10^4$ g-min centrifugation suggests that they have a lower sedimentation velocity in 0.25 M sucrose.

Components of the Golgi apparatus, such as microsomal components derived from Golgi flattened saccules, would be expected to possess an even lower sedimentation velocity than the secretory granules of the F-IIIc. These Golgi components, then, probably comprise a portion of the purified F-IIIc. Ultrastructural examination of this fraction confirms the presence of many smooth microsomal vesicles (28).

Other investigators have routinely assumed that Golgi components sediment with the secretory granules (3, 6, 7, 18, 40, 42). Without exception, where comparable fractionation procedures were employed to fractionate islet tissue, a more rigorous centrifugation than $6.0 \times 10^4$ g-min was used to prepare the crude F-II fraction. The procedures followed utilized centrifugations ranging from $13.0 \times 10^4$ g-min (6) to $3.0 \times 10^5$ g-min (42). It is thus probable that, in these procedures, condensing vacuoles and other smooth membranous components of the Golgi apparatus sediment with the secretory granules at this step in the fractionation procedure. This contention is supported by data on proinsulin conversion. If one considers estimates of transport time and findings from experiments in which transport was blocked, the Golgi apparatus is implicated as the site of initiation of cleavage of proinsulin to insulin (17, 40, 41). Yet, when the converting activity of various subcellular fractions is assessed, nearly all conversion of proinsulin to insulin is found to occur in secretory granules (3, 40, 42).

In light of these considerations, the most reasonable explanation for the converting activity observed in the microsome fraction in the present study is that the fractionation procedure employed results in isolation of Golgi apparatus components with the microsomes. Confirmation of this proposal requires further investigation. Assays for Golgi enzyme markers or procedures such as those of Dallner et al. (4, 5) or Tartakoff and Jamieson (44) which separate smooth from rough-surfaced components of endoplasmic reticulum should be employed. Use of such techniques should further clarify the role of the Golgi apparatus in the conversion process and the ultimate disposition of product peptides that are apparently generated in this structure.

In addition to establishing the efficacy of the modified fractionation procedure for studying proinsulin and proglucagon biosynthesis and conversion in anglerfish islet tissue, the data in this paper provide support for intracisternal processing and packaging of newly synthesized (pro)insulin and (pro)glucagon. The greatest portion of the total insulin (>84%) and glucagon (>93%) immunoreactivity found in all fractions was present in the microsome and secretory granule fractions. The data in Figs. 2, 4, and Table I suggest that newly synthesized insulin- and glucagon-related peptides are relegated to a membrane-bound state soon after their synthesis at the rough endoplasmic reticulum. The data in Table I also show clearly that, with increase in incubation time, there is a progressive decline in the proportion of selectively labeled insulin- and glucagon-related peptides in the cell supernate. This pattern was found to be consistent during both continuous and pulse-chase incubations. When considered along with the finding that the nuclear and mitochondrial fractions possess exceedingly low amounts of these labeled peptides at all time periods tested (Figs. 2 and 4), the progressive decline in the levels of labeled peptides in F-IV supports the concept of intracisternal and intragranular sequestration of peptides.
synthesized for export from the cell.

Jamieson and Palade (15) have performed subcellular fractionation studies with guinea pig exocrine pancreas. They reported that the proportion of radioactively labeled proteins and pancreatic amylase recovered in their postmicrosomal supernatant fractions is usually in the range of 15–17%. It was also stated that this proportion could be increased appreciably if homogenization is prolonged. The data in Table I show that although the proportions of 14C-radioactivity in proinsulin and insulin recovered in F-IV after a 20-min pulse are higher than 17%, this value decreases markedly to below 10% for both proinsulin and insulin with increasing duration of incubation. The proportions of 3H-labeled glucagon precursor and glucagon-sized 3H-labeled peptides recovered in F-IV after a 20-min pulse were found to be in the 15–19% range and to subsequently diminish markedly during prolongation of continuous or chase incubation. The initially higher proportion of both 14C- and 3H-radioactivity found in the cell supernate after the 20-min pulse periods can be attributed to very low incorporation during this short pulse (especially for [14C]isoleucine). In addition, after a short incubation, the proportion of newly synthesized peptides that have been transported into the endoplasmic reticulum (ER) cisternae compared to labeled nascent peptides is low. With increasing duration of incubation, the proportion of labeled peptides found in subcellular fractions that contain primarily vesicular components increases rapidly (Figs. 2-6 and Table I).

It is suggested that these data on proinsulin and proglucagon synthesis and transport are more consistent with the intracisternal transport and packaging theories proposed by Jamieson and Palade and their co-workers for exocrine pancreatic cells (13-15, 39, 45) than with the theories of free interchange between cellular compartments postulated for the same system by Rothman and his co-workers (20, 21, 36-38). However, direct comparisons between the exocrine and endocrine pancreatic systems should be made with caution. Each of the exocrine pancreatic cells synthesizes numerous different peptides for export, whereas most of the evidence available to date indicates that each of the islet hormones is synthesized and secreted from a single islet cell type. Nevertheless, the parallels between the data reported here and the data that support intracisternal transport and processing of newly synthesized peptides in exocrine pancreatic cells are striking.

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