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Detection and kinetic behavior of preproinsulin in pancreatic islets

(secretory prepeptides/precursor cleavage/proinsulin/insulinoma/polyacrylamide gel electrophoresis–fluorography)

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ABSTRACT **Newly synthesized rat islet proteins have been analyzed by polyacrylamide slab gel electrophoresis and fluorography. A minor component having an apparent molecular weight of 11,100 was identified as preproinsulin by the sensitivity of its synthesis to glucose, the pattern of NH_2-terminal leucine residues, and the rapidity of its appearance and disappearance during incubation of islets or islet cell tumors. A small amount of labeled peptide material which may represent the excised NH_2-terminal extension of preproinsulin or its fragment was also detected. The kinetics of formation and processing of the preproinsulin fraction were complex, consisting of a rapidly turning over component having a half-life of about 1 min and a slower minor fraction that may have bypassed the normal cleavage process. The electrophoretic resolution of the preproinsulin and proinsulin fractions into two bands each is consistent with the presence of two closely related gene products in rat islets rather than intermediate stages in the processing of these peptides.**

Many recent studies have shown that the *in vitro* translation products of the mRNAs for various secretory proteins carry a 20- to 30-residue NH_2-terminal extension that contains a high proportion of hydrophobic amino acids (1–4). This region is believed to assist in the formation of the ribosome–membrane junction leading to the vectorial discharge and segregation of the nascent polypeptides (1, 2). Several studies with reconstituted systems have provided additional evidence that this signal sequence is rapidly cleaved from the nascent secretory product before polypeptide chain completion by a protease(s) associated with the rough microsomes (5, 6), thus accounting for the apparent difficulty in detecting these presecretory forms in intact tissues (7, 8). In the present studies, intact rat islets of Langerhans or insulinoma cells were incubated for brief periods with labeled amino acids and then analyzed by sodium dodecyl sulfate ($NaDodSO_4$)/acrylamide slab gel electrophoresis and fluorography in an attempt to detect preproinsulin (3, 9, 10) and to assess its kinetic behavior as a precursor of proinsulin.

METHODS AND MATERIALS

Tissue Sources. Islets were isolated from Sprague–Dawley rats as described (11). Lobules of exocrine pancreatic tissue were collected during this isolation procedure. Insulin-producing tumors were either maintained from an x-ray-induced insulinoma in NEDH inbred rats (12) or induced by injection of streptozotocin and nicotinamide in Holtzman rats (13). Samples of 25–100 islets or equivalent amounts of exocrine tissue, or of tumor cells, were incubated in 50 μl of Hanks' buffer supplemented with 2.5 or 25 mM glucose, amino acids according to Eagle's minimal essential medium (omitting leucine), and 0.5 μCi of [^{3}H]leucine per islet, at 37° ([3,4,5-^{3}H(N)] leucine, specific activity, ~110 Ci/mmol, obtained from New England Nuclear Corp., Boston).

Electrophoresis and Fluorography. Islets, or cells, were washed briefly, after incubation, with excess buffer containing 1 mM unlabeled leucine, collected by centrifugation, boiled for 3 min in 12.5 μl of 125 mM Tris·HCl, pH 6.8/2% $NaDodSO_4$/5% 2-mercaptoethanol/250 mM sucrose/0.01% bromphenol blue, and then electrophoresed on 12 × 12 × 0.15 cm polyacrylamide slabs at 200 V according to Laemmli (14) modified as follows: the separation gel contained 16% acrylamide and 0.25% bisacrylamide, and the running buffer was diluted 1:5. Gels were fixed and stained as described by Weber and Osborn (15) and treated for fluorography by soaking in dimethyl sulfoxide, 2,5-diphenyloxazole in dimethyl sulfoxide, and water (16). The frozen gels were dried overnight on a plate of porous plastic in an evacuated rubber bag at room temperature (16). To increase sensitivity, x-ray films (X-Omat R, Eastman Kodak) were activated prior to exposure (17).

Characterization of Protein Bands. Unstained protein bands, localized on dried gels by alignment with the corresponding fluorograph, were excised, the adherent filter paper was removed, and the gel bits were homogenized in performic acid at 0° with an all-glass homogenizer. After incubation for 12 hr at 0°, homogenates were extracted twice with performic acid and the combined supernates were lyophilized. Samples were then dissolved either in 1.0 ml of 50% acetic acid with 4 mg of bovine serum albumin as carrier for automated sequence determination in a Beckman 890 C sequenator or in 1.0 ml of 0.1 M $(NH_4)_2CO_3$/2 mM $CaCl_2$ for digestion with 1 μg of trypsin (L-1-tosylamide-2-phenylethyl chloromethyl ketone-treated, Worthington Biochemicals) at 37° for 24 hr. After drying, samples were taken up in 50 μl of 30% formic acid, spotted on cellulose thin-layer plates (Eastman no. 6064), and electrophoresed in 30% formic acid at 100 V for 4 hr. Fluorographs of these peptide maps were obtained by coating the plates with 2,5-diphenyloxazole (18) and exposing them on activated x-ray films at −70°.

Quantitative Analysis of Fluorographs. For quantitation, protein bands cut from fluorographed gels were dissolved in 200 μl of 30% H_2O_2 at 60° overnight and assayed for radioactivity in 15 ml of Bray's solution. Similarly, fluorographed tryptic peptides were collected from thin-layer plates, eluted, and assayed in 10 ml of Aquasol (New England Nuclear Corp.). Densitometric scanning of fluorographs was performed on a Schoeffel densitometer (Westwood, NY).

RESULTS

Electrophoretic Separation of Newly Synthesized Islet Proteins. The pattern of newly synthesized rat islet proteins as separated by polyacrylamide electorphoresis and visualized by

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Abbreviation: $NaDodSO_4$, sodium dodecyl sulfate.

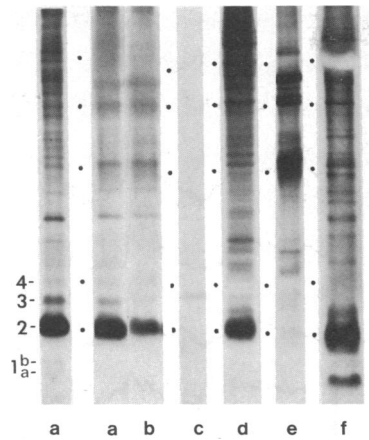

FIG. 1. Electrophoretograms of newly synthesized proteins in pancreatic islets and related tissues. Lanes: a, 50 islets incubated for 5 min in 25 mM glucose; b, 50 islets incubated for 5 min in 2.5 mM glucose; c, *in vitro* translation product of partially purified insulinoma mRNA (19); d, x-ray induced insulinoma cells incubated for 5 min in 25 mM glucose; e, exocrine pancreatic tissue incubated for 5 min in 25 mM glucose; f, lyophilyzed medium from 50 islets incubated for 3 hr in 25 mM glucose. Dots indicate (from top to bottom) the position of the following marker proteins: bovine serum albumin (65,000), ovalbumin (43,000), chymotrypsinogen A (25,700), cytochrome *c* (12,400), bovine proinsulin (8700).

fluorography is shown in Fig. 1, lane a. The experimental conditions were optimized for the detection of a short-lived precursor of proinsulin by stimulating insulin biosynthesis maximally with glucose and by optimizing the resolution of the system in the range of 9000 to 13,000 daltons (3). To avoid overlap of labeled protein bands on the fluorograph, the pulse incubation of islets with [^{3}H]leucine was limited to 5 min. Even after this short incubation, proinsulin (band 2, Fig. 1, lane a) was found to be the most prominent fraction. Several slower migrating fractions could also be resolved, among which band 3 (estimated molecular weight, 11,100) showed the highest leucine incorporation. Above band 3, another band was consistently observed (band 4; estimated molecular weight, 12,400). The electrophoretic pattern varied somewhat from one experiment to another, occasionally resulting in a splitting of bands 2 and 3 (designated a and b). Material of molecular weight lower than that of proinsulin was consistently found after 5 min of incubation and could also occasionally be separated into two bands (bands 1a and 1b). Band 3 corresponded most closely in molecular size to the 11,500-dalton translation product of mRNA from normal islets and from the x-ray-induced insulinoma (Fig. 1 lane c) (3). The formation of band 3 (as well as of band 2) was clearly stimulated by glucose (Fig. 1, lanes a and b). Furthermore, this peptide also was present in the electrophoretogram of the beta cell tumor cells (Fig. 1, lane d) but was not seen in exocrine pancreatic tissue (Fig. 1, lane e) or in the pattern of proteins secreted by islets during a 3-hr incubation with [^{3}H]leucine (Fig. 1, lane f). Band 3 was therefore tentatively identified as preproinsulin (3, 9, 10).

NH_2-Terminal Sequence Determinations. To corroborate this assumption, the enumerated protein bands were eluted from slab gels after electrophoresis of the lysate of 1200 islets incubated for 5 min in 25 mM glucose and [^{3}H]leucine. The eluates were then subjected to automated sequential Edman degradation to determine the position of [^{3}H]leucine residues in the NH_2-terminal sequence of 25–30 amino acids. These results are compiled in Fig. 2 which compares the resulting radiosequencing patterns to the known sequence of rat preproinsulin (3) or proinsulin. Band 2 was resolved in this experiment into two bands (designated a and b), both of which clearly showed the sequence of the leucine residues of proinsulin—i.e., of the insulin B chain. Despite a rather high background of radioactivity, all of the slower migrating fractions, (i.e., 3a, 3b, and 4) were similar to preproinsulin in their NH_2-terminal sequences, showing peaks at positions 2, 7, 9, and 15. The groups of doubled leucine residues between positions 9 and 15, however, were less clearly resolved. Stronger deviations appeared in the profile of band 3a, which may be explained in part by some contamination from the adjacent proinsulin band (band 2b). The large peak at position 26 may be an artifact or may represent contamination by an unknown small exocrine pancreatic protein (see Fig. 1, lane e). The ma-

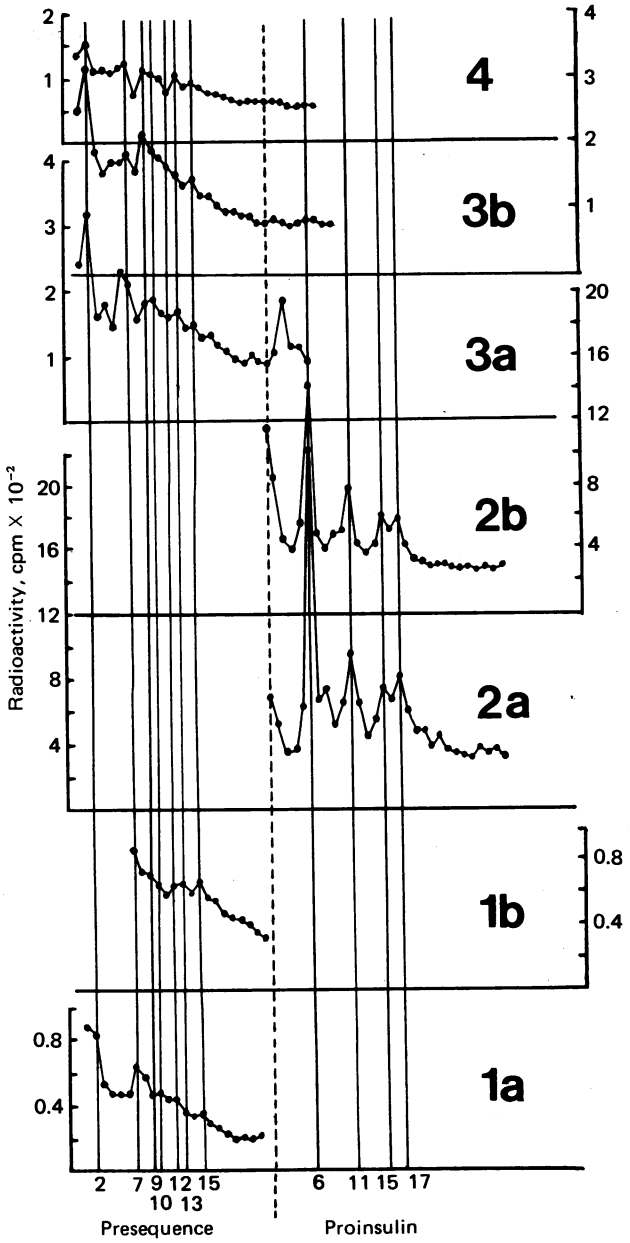

FIG. 2. Determination of the position of leucine residues in the NH_2-terminal sequence of various protein bands eluted from slab electrophoretograms. Thiazolinone intermediates from the sequenator were assayed for radioactivity in a toluene-based scintillation cocktail. Vertical lines indicate the position of leucine residues as determined in the *in vitro* translation product of islet RNA (3) and of the insulin B chain, respectively. Fractions numbered as in Fig. 1.

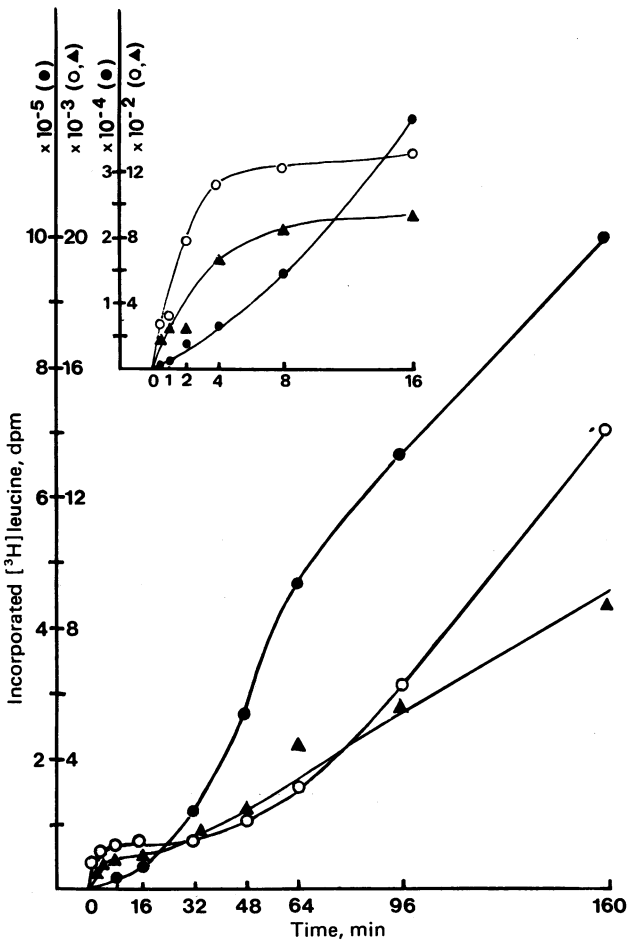

FIG. 3. Time course of [^{3}H]leucine incorporation into bands 2 (●), 3 (○), and 4 (▲) of Fig. 1. Each point represents the radioactivity found in the respective band after electrophoresis of the lysate of 50 islets after incubation in 25 mM glucose at 37° for the indicated time. (*Inset*) Early phase in more detail.

terial corresponding to bands 1a and 1b yielded only low amounts of radioactivity. Their sequence patterns were tentatively interpreted as being the NH_2-terminal extension of preproinsulin (1a) or a part of it (1b), starting at position 7 and being of unknown length (no residues from the B chain appeared in the COOH-terminal portion of either pattern).

Tryptic Peptides. For further characterization of eluted proteins of bands 1–4, their tryptic peptides were analyzed by one-dimensional peptide mapping. Because, under the conditions of electrophoresis used, the oxidized B chain, the C peptide, and the NH_2-terminal peptide(s) of preproinsulin are all positively charged (3, 20) and not well resolved, only the oxidized A chain, which is strongly negatively charged, could be unambiguously identified. Bands 2a and 2b and bands 3a and 3b gave the expected ratios of A chain to total radioactivity for proinsulin and preproinsulin, respectively (data not shown).

Time Course of Leucine Incorporation. Fig. 3 shows the time course of the incorporation of [^{3}H]leucine into bands 2, 3, and 4. Bands 2 and 3 were not well resolved into their subfractions a and b in the electrophoretograms. The kinetics of incorporation of [^{3}H]leucine were different for each of the bands. Band 2 showed a sigmoidal curve that reached its inflection at about 48 min. The curve for band 3 seemed to be composed of two parts: an early phase consisting of a rapid increase in incorporation which leveled off after about 8 to 16 min, and a later phase of almost linear increase that did not

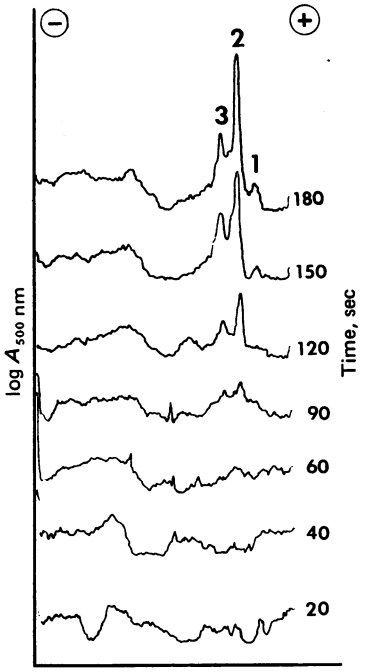

FIG. 4. Time course of [^{3}H]leucine incorporation into fractions 1, 2, and 3 at lower temperature (25°) as evaluated by densitometry. Incubation periods are indicated at the right.

reach saturation within the experimental period. The characteristics of band 4 were similar to those of band 3. The *inset* shows the early phase in more detail: in contrast to the steep parabolic increase of incorporation into band 2, incorporation into bands 3 and 4 reached plateaus within 16 min.

In order to study the early phase of incorporation in greater detail, the incubation temperature was reduced to 25° (Fig. 4). The peaks for bands 2 and 3 were about equal in size up to 90–120 sec; thereafter, band 2 predominated, in agreement with the results of the experiment shown in Fig. 3. Note the appearance of band 1 components after 150-sec incubation.

Pulse-Chase Experiments. The fluorographs of a set of pulse-chase experiments performed with isolated islets and cell suspensions of streptozotocin and x-ray-induced insulinomas are presented in Fig. 5. In each case, band 3 was conspicuous after the 2-min pulse. A chase period of 3 min resulted in the almost complete disappearance of this band. Band 4 could be detected with certainty only in the islet material and not in the tumor material, and it seemed to persist for a longer time. The fluorographs in Fig. 5 also show the conversion of proinsulin (band 2), but this processing was delayed in the insulinoma material. Band 1 material, which was present after the pulse period in some experiments, tended to disappear rapidly.

From a separate fluorograph of an islet pulse-chase experiment in which bands 2 and 3 were separated into two portions (a and b), the material was collected from the gel and quantitated by scintillation counting (Fig. 6). Bands 3a and 3b disappeared almost completely within chase periods of 5–10 min. Band 4, however, showed a further increase of leucine incorporation during the chase phase. Band 2b showed a sharp decline within 10–30 min but did not vanish completely during the experimental period of 2 hr. In contrast, band 2a, although less prominent immediately after the pulse, increased during the chase period and reached a maximum at 10 min. Thereafter, it remained the dominant component. Again, bands 1a and 1b could not be resolved but formed a rapidly disappearing peak when quantitated by densitometry (data not shown).

Studies on the Conversion of Preproinsulin to Proinsulin.

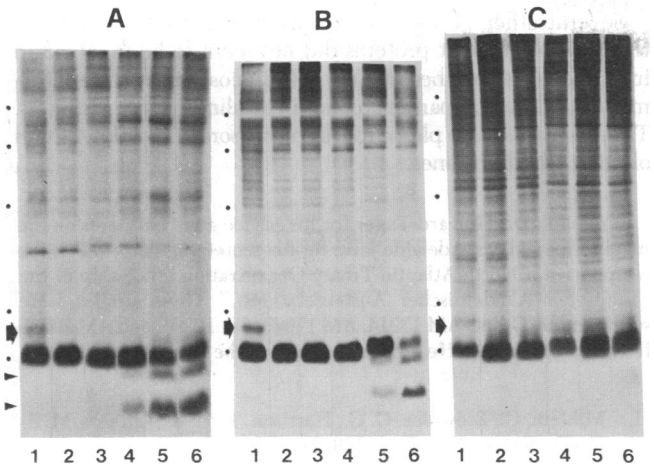

FIG. 5. Pulse-chase experiments with isolated islets (*A*) and insulinoma cells induced by streptozotocin (*B*) or x-rays (*C*). Samples of 50 islets or approximately equivalent amounts of tumor cells were incubated for 2 min in [^{3}H]leucine and 25 mM glucose. Thereafter, 5 ml of buffer containing 1 mM unlabeled leucine was added. Total incubation periods (including the pulse period) were: lane 1, 2 min; lane 2, 5 min; lane 3, 10 min; lane 4, 30 min, lane 5, 60 min; lane 6, 120 min. Big arrows point to band 3, and dots indicate the position of marker proteins as described in the legend of Fig. 1. Note accumulation of proinsulin conversion products after 30 min incubation of islets (arrowheads).

Table 1 summarizes the effects of various inhibitors—i.e., of proteases [TLCK (7), TPCK (8), iodoacetate], of binding of ribosomes to endoplasmic reticulum membranes [ATA (21), FDA (22)], and of the secretory pathway [cyproheptadine (23), Colchicine, (24), DNP (25), lack of Ca^{2+} (26)]—on the conversion of preproinsulin (band 3) to proinsulin (band 2). In addition, the effect of islet incubation at lowered temperature (25°) was studied. A "normal" ratio of preproinsulin to proinsulin of approximately 1:10 was found after 5 min of incubation. Most of the listed agents and conditions led to a decrease in protein synthesis accompanied by an increase in the ratio of "pre" to "pro" such as to suggest the existence of an approximately linear correlation between these two effects.

Table 1. Influence of various drugs and incubation conditions on the ratio of preproinsulin to proinsulin in pancreatic islets

Addition or condition*	[^{3}H]Leucine incorporation, dpm			
	"Pre" (band 3)	"Pro" (band 2)	"Pre" + "pro"	"Pre"/"pro"
Control	9312	103,739	113,051	0.09
TLCK	6516	55,003	61,519	0.12
TPCK	3781	35,576	39,357	0.11
FDA	4798	39,921	44,719	0.12
CHD	2504	13,516	16,020	0.19
No Ca^{2+}	3684	34,119	37,803	0.11
Control	9719	64,100	73,819	0.15
25°	9831	45,017	54,848	0.22
Colchicine	6735	39,287	46,022	0.17
ATA	9051	67,709	76,760	0.13
Iodoacetate	8699	59,473	68,172	0.15
DNP	2535	8,568	11,103	0.30

Fifty islets were preincubated under the indicated conditions for 20 min at 37°. Concentration of added drugs was 0.1 mM. The reaction was started by addition of 25 μCi of [^{3}H]leucine and incubation was continued for 5 min. Islets were then washed, lysed, and electrophoresed; the respective bands of the fluorographed gel were cut out and assayed. Data represent means of duplicates.

* TLCK, *N*-α-*p*-tosyl-L-lysine chloromethyl ketone; TPCK, L-1-tosylamido-2-phenylethyl chloromethyl ketone; FDA, fluorescein diacetate; CHD, cyproheptadine; ATA, aurintricarboxylic acid; DNP, 2,4-dinitrophenol.

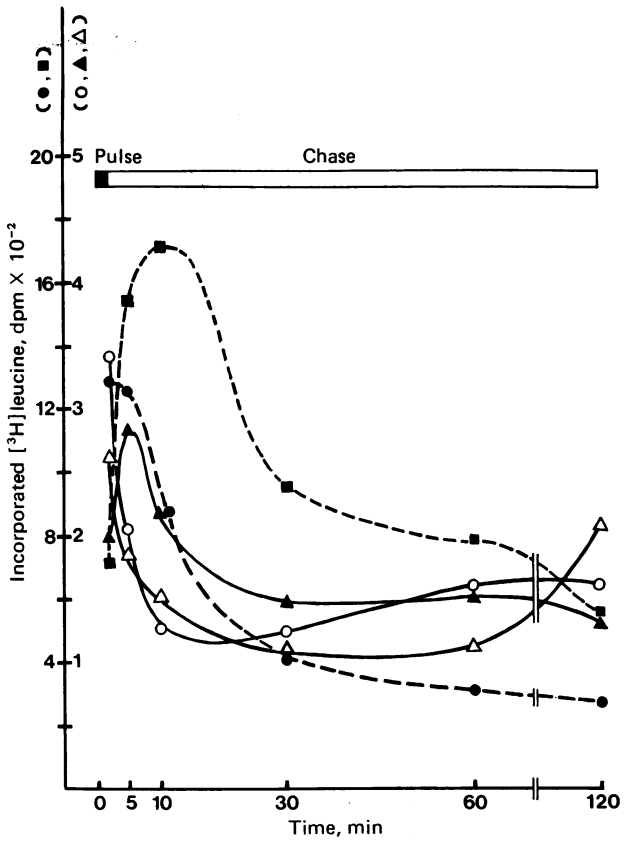

FIG. 6. Pulse-chase experiment with isolated islets as quantitated by scintillation counting. Experimental conditions as in the legend to Fig. 5. Data represent means of duplicates. ■, Band 2a; ●, band 2b; △, band 3a; ○, band 3b; ▲, band 4.

DISCUSSION

These results provide insight into the very early events of insulin biosynthesis. As an essential prerequisite for our conclusions, however, it was necessary to identify appropriately the protein fractions detected during the labeling of islets. Proinsulin was readily identified as band 2 on the basis of the following criteria: (*i*) apparent molecular weight, (*ii*) stimulation of its formation by glucose, (*iii*) position of leucine residues in the NH_2-terminal sequence, (*iv*) time course of its formation and processing, and (*v*) immunoreactivity—i.e., reactivity with anti-insulin serum either in islet homogenates (followed by electrophoresis of the immunoprecipitate) or reactivity of band 2 material after elution from gel slabs (data not shown). Because bands 2a and 2b both show an intact NH_2 terminus (B chain, Fig. 2) as well as the proinsulin COOH terminus (A chain), these two fractions therefore likely represent the two rat proinsulins, despite their somewhat different rates of turnover (Fig. 6). Proinsulin is thus the predominant product of islet protein biosynthesis.

By using similar criteria, band 3 was identified as preproinsulin by (*i*) molecular weight, (*ii*) sensitivity to glucose, (*iii*) NH_2-terminal pattern of leucine residues, and (*iv*) rapid appearance and disappearance in pulse-chase experiments. Our failure to immunoprecipitate preproinsulin from islet homogenates could be due to its rapid breakdown or turnover.

Moreover, because preproinsulin reacts poorly with anti-insulin serum unless folded correctly (20), the lack of immunoreactivity of band 3 does not contradict its identification as preproinsulin. Because the occasionally resolved bands 3a and 3b show so many similarities, we suspect that these bands represent the two rat preproinsulins.

Band 4 also seemed to fulfill some of the above criteria. However, its higher molecular weight, slower formation and disappearance in pulse-chase experiments, and insensitivity to glucose stimulation indicate that this protein is not related to preproinsulin. It may, in fact, represent a precursor of glucagon, somatostatin, or pancreatic polypeptide.

Finally, bands 1a and 1b show features expected for the NH_2-terminal extension (or parts of it) after its removal from preproinsulin. Its rapid disappearance during the chase period and its sequence of leucine residues show that this fraction does not represent early products of the processing of proinsulin itself. Because proinsulin is the major secretory product being synthesized, the contribution of other prepeptides to this fraction is presumably quite small. It is of interest that these fragments never accumulated label in amounts commensurate with their leucine content, thus indicating the rapidity of their degradation in the intact system.

Taken together, these data show that preproinsulin is formed in pancreatic islets. Its low proportion compared to proinsulin and its rapid turnover rates are consistent with the predictions made from *in vitro* studies on other similar peptide precursors (2, 6). In cell-free or reconstituted systems, the cleavage of the nascent peptide chain of a protein precursor has been estimated to occur after the polymerization of at least 70–80 amino acid residues (2, 6). Preproinsulin (109 residues) and preproparathyroid hormone (115 residues) are both only slightly longer than this minimum length. It is thus possible that some completed chains of these peptides escape immediate cleavage—i.e., removal of the NH_2-terminal extension—thereby resulting in a tiny accumulation of the intact precursor molecule (27). Furthermore, such an interpretation would explain the failure of others to detect precursors of considerably larger secretory proteins in intact cells under normal conditions (7, 8).

The kinetics of formation and processing of preproinsulin were complex in that a major part of this fraction was formed within 1–2 min and disappeared within a 3-min chase period, whereas a minor portion remained for several hours (Fig. 6). As a tentative interpretation, these two components may represent preproinsulin accumulating in different compartments: an intracisternal component, which is undergoing rapid processing and which is represented by the transient saturation in the curve of leucine-incorporation in Fig. 3 (0–16 min), and a more stable cytoplasmic component, which may result from heterotopic translation (Fig. 3, 16–160 min).

The data in Table 1 indicate, rather unexpectedly, that any reduction in protein synthesis may lead to a relative increase in the ratio of preproinsulin to proinsulin, although this phenomenon cannot readily be explained. The inhibitory effect of the protease inhibitors on protein synthesis shows that these agents are taken up by the islets. However, it remains unclear as to whether they also enter the intracisternal space, where the protease(s) involved in the conversion of "pre" to "pro" is presumably localized. The correlation between degree of inhibition of protein synthesis and of conversion was poorest in the case of islets incubated at lowered temperature, suggesting that the converting protease(s) has a temperature-sensitivity different from that of protein synthesis.

Several other peptides that appeared consistently on the fluorographs of islet proteins did not seem to be involved in insulin biosynthesis because of their glucose-independent formation and their apparent absence in insulin-producing tumors. They may therefore play a role as precursors in the biosynthesis of other islet hormones.

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1. Milstein, C., Brownlee, G. G., Harrison, T. M. & Mathews, M. B. (1972) *Nature New Biol.* **239**, 117–120.
2. Blobel, G. & Dobberstein, B. (1975) *J. Cell Biol.* **67**, 835–851.
3. Chan, S. J., Keim, P. & Steiner, D. F. (1976) *Proc. Natl. Acad. Sci. USA* **73**, 1964–1968.
4. Kemper, B., Habener, J. F., Mulligan, R. C., Potts, J. T., Jr. & Rich, A. (1974) *Proc. Natl. Acad. Sci. USA* **71**, 3731–3735.
5. Blobel, G. & Dobberstein, B. (1975) *J. Cell Biol.* **67**, 852–862.
6. Boime, I., Szczesna, E. & Smith, D. (1977) *Eur. J. Biochem.* **73**, 515–520.
7. Schmeckpeper, B. J., Adams, J. M. & Harris, A. W. (1975) *FEBS Lett.* **53**, 95–98.
8. Sussman, P. M., Tushinski, R. J. & Bancroft, F. C. (1976) *Proc. Natl. Acad. Sci. USA* **73**, 29–33.
9. Permutt, M. A., Biesbroeck, J., Chyn, R., Boime, I., Szczesna, E. & McWilliams, D. (1976) in *Polypeptide Hormones: Molecular and Cellular Aspects*, Ciba Foundation Symposium no. 41, eds. Porter, R. & Fitzsimons, D. W. (Elsevier, Amsterdam), pp. 97–116.
10. Lomedico, P. T. & Saunders, G. F. (1976) *Nucleic Acids Res.* **3**, 381–386.
11. Lernmark, Å., Nathans, A. & Steiner, D. F. (1976) *J. Cell Biol.* **71**, 606–623.
12. Chick, W. L., Warren, S., Chute, R. N., Like, A. A., Lauris, V. & Kitchen, K. C. (1977) *Proc. Natl. Acad. Sci. USA* **74**, 628–632.
13. Rakieten, N., Gordon, B. S., Beaty, A., Cooney, D. A., Davis, R. A. & Schein, P. S. (1971) *Proc. Soc. Exp. Biol. Med.* **137**, 280–283.
14. Laemmli, U. K. (1970) *Nature New Biol.* **227**, 680–685.
15. Weber, K. & Osborn, M. (1969) *J. Biol. Chem.* **244**, 4406–4412.
16. Bonner, W. M. & Laskey, R. A. (1974) *Eur. J. Biochem.* **46**, 83–88.
17. Laskey, R. A. & Mills, A. D. (1975) *Eur. J. Biochem.* **56**, 335–341.
18. Randerath, K. (1969) *Anal. Chem.* **41**, 991–992.
19. Duguid, J. R., Steiner, D. F. & Chick, W. L. (1976) *Proc. Natl. Acad. Sci. USA* **73**, 3539–3543.
20. Lomedico, P. T., Chan, S. J., Steiner, D. F. & Saunders, G. F. (1977) *J. Biol. Chem.*, **252**, 7971–7978.
21. Borgese, N., Mok, W., Kreibich, G. & Sabatini, D. D. (1974) *J. Mol. Biol.* **88**, 559–580.
22. Grollman, A. P. & Huang, M. T. (1973) *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **32**, 1673–1678.
23. Wold, J. S., Longnecker, D. S. & Fischer, L. J. (1971) *Toxicol. Appl. Pharmacol.* **19**, 188–201.
24. Malaisse, W. J., Malaisse-Lagae, F., Walker, M. O. & Lacy, P. E. (1971) *Diabetes* **20**, 257–265.
25. Jamieson, J. D. & Palade, G. E. (1968) *J. Cell Biol.* **39**, 589–603.
26. Grodsky, G. M. & Bennett, L. L. (1966) *Diabetes* **15**, 910–913.
27. Habener, J. F., Potts, J. T., Jr. & Rich, A. (1976) *J. Biol. Chem.* **251**, 3893–3899.