Insulin Biosynthesis: Studies of Islet Polyribosomes*
(nascent peptides/sucrose gradient analysis/gel filtration)
M. A. PERMUTT AND D. M. KIPNIS
Department of Medicine, Washington University School of Medicine, St. Louis, Missouri 63110
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ABSTRACT A method is described for separation of polyribosomes from as few as 25 isolated islets of Langerhans, representing about 250 μg of pancreatic tissue. Islets are labeled with [3H]leucine and polysomes are isolated with liver polyribosomes, which serve as carrier and inhibitor of ribonuclease activity. Islets incubated at 37°C for 46 min in 15.5 mM glucose, then pulsed with [3H]leucine, incorporated about 2-3 times more label into nascent peptides on islet polysomes than islets incubated in 2.8 mM glucose. Sucrose gradient analysis of the labeled polysomes indicated that raising the glucose concentration preferentially stimulated synthesis of peptides on trisomes and larger polyribosomes. Islets incubated with [3H]leucine for 15 min incorporated two-thirds of the label into proteins on membrane-bound polysomes. At least 85% of the proinsulin synthesis during this time occurs on membrane-bound polysomes.

Glucose stimulates insulin synthesis in isolated islets of Langerhans of the pancreas (1-5). Evidence has been presented that the glucose effect occurs at both the transcriptional and post-transcriptional levels during a 2-hr incubation in vitro (1). To further define these processes, it became necessary to separate polyribosomes from pancreatic islets. Since the aggregate weight of islets isolated from the pancreas of a rat by collagenase digestion is of the order of a milligram (1), the usual procedures for isolating polyribosomes were not adequate. Furthermore, polyribosomes are very susceptible to breakdown during isolation; the problem was further compounded by the presence of significant ribonuclease activity in preparations of isolated islets. Islet RNA extracted in the presence of phenol, heparin, and bentonite consistently yielded degraded material. These difficulties have been overcome by a modification of the technique of Scherr and Uhr (6) for the study of immunoglobulin synthesis by myeloma cells. The procedure is based on the use of liver polyribosomes as a carrier for isolation of labeled polysomes from small quantities of tissue. Liver was used as the polysome donor because of the presence of a potent ribonuclease inhibitor in liver cytosol, and the ease with which sufficient quantities of intact liver polysomes could be isolated for use as a carrier to provide readily visible pellets on centrifugation. With this technique, intact polysomes have been isolated from as little as 25 islets, representing less than 250 μg of tissue.

MATERIALS AND METHODS
Islets of Langerhan were isolated from pancreases of adult Sprague-Dawley rats (300-450 g) that were allowed free access to Purina Chow diet. The islets were prepared by collagenase digestion of pancreatic tissue, as described by Lacy and Kostianovsky (7). [3H]Leucine (55 Ci/mmol) was obtained from New England Nuclear Corp., Boston, Mass.; puromycin from Nutritional Biochem; sodium deoxycholate and bovine insulin from Sigma. All other chemicals were reagent grade from Fisher.

Preparation of Polyribosomes. Isolated rat islets (30-100) were incubated in 500 μl of Krebs–Ringer bicarbonate buffer (pH 7.4), continually gassed with 95% O2-5% CO2, at 37°C for 45 min, then pulsed with [3H]leucine as indicated. All further procedures were performed at 0-4°C. The islets were quickly mixed with 4 ml of liver post-mitochondrial supernatant fraction that was prepared immediately before use according to the method of Blobel and Potter (8, 9). The islets were homogenized in a Dounce homogenizer with 15 strokes of the tight-fitting (B) pestle and centrifuged at 17,000 × g for 10 min. The supernate was mixed with fresh 10% sodium deoxycholate, to a final concentration of 1%, in order to release membrane-bound polysomes, then layered over a 2-layer discontinuous sucrose gradient containing ribonuclease inhibitor; polysomes were pelleted as described by Blobel and Potter (8). The polysome pellets were either digested in Soluene (1 ml) and counted in Instagel (5 ml), or analyzed on sucrose gradients as described below. Where indicated, polysomes were separated into membrane-bound and free polysomes by the procedure of Scherr and Uhr (6), as outlined in Fig. 1.

Sucrose Gradient Analysis. Polysome pellets were suspended in 0.5 ml of 0.25 M sucrose–TKM [0.05 M Tris·HCl (pH 7.5 at 20°C)–25 mM KCl–5 mM MgCl2]–S8 solution (8), layered over a 12-ml linear 10-40% sucrose–TKM gradient, and centrifuged for 90 min at 25,000 rpm in a Beckman model L ultracentrifuge (SW-28 rotor, stopped without braking). The gradients were then aspirated from the top with a Buchler Auto-Densiflow, pumped through a Gilford spectrophotometer (2-mm cell) recording at 260 nm, and collected into a fraction collector. Fractions were mixed with an equal volume of cold 10% trichloroacetic acid; the precipitates were collected by centrifugation, washed with 5% trichloroacetic acid, with ethanol–ether 2:1, digested in Soluene (1 ml), and counted in Instagel (5 ml) on a Packard liquid scintillation counter at 30% efficiency.

Sephadex Chromatography. Subcellular fractions of islets of Langerhan were extracted with acid–alcohol; insulin was partially purified by the procedure of Davoren (10) through the ethanol–ether precipitation step; 1 mg of bovine insulin per 10 ml was added as carrier. The partially purified insulin

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precipitate was dissolved in 1 ml of 1 M acetic acid and chromatographed on Sephadex G-50 (1 x 100 cm) as described (1). The fractions were analyzed for absorption at 280 nm, then mixed with 5 ml of Instagel for liquid scintillation counting.

**RESULTS**

**Time course and characteristics of nascent peptide labeling**

The time course of labeling of nascent peptide on islet polysomes is shown in Fig. 2A. Isolated islets were incubated for 45 min and then pulsed with [3H]leucine for the times indicated. Incorporation of [3H]leucine into the polysomes was extremely rapid; half-maximal labeling occurred within the first 2 min and maximal labeling was reached within 5 min. Islets were incubated for 45 min, “pulsed” for 5 min with [3H]leucine, and then “chased” with unlabeled 1 mM leucine (Fig. 2B). Only about half of the label was removed in 10 min, but about 90% of this removal occurred within 5 min.

Sucrose gradient analysis of the [3H]leucine-labeled polysomes (Fig. 3) demonstrated a characteristic polysome profile identified as monosomes through pentasomes and larger polysome aggregates. [3H]Leucine was incorporated mainly into the rapidly sedimenting polyribosome region. RNase treatment of the polysomes before sucrose gradient analysis resulted in the ultraviolet light-absorbing and radioactive material sedimenting in the monosome region.

Further evidence that [3H]leucine was incorporated into nascent islet polypeptide rather than bound nonspecifically to carrier liver polysomes was obtained in the following manner. Islets were pulsed with [3H]leucine for 5 min and then divided into two aliquots—one was incubated with puromycin for an

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**Fig. 1.** Preparation of bound and free islet polyribosomes.

**Fig. 2.** Incorporation of [3H]leucine into islet polysomes. (A) Islets (30) in each of four separate tubes were incubated at 37°C in Kreb’s buffer (pH 7.4) containing 15.5 mM glucose for 45 min, then [3H]leucine was added (200 μCi/ml) and the islets were homogenized at the times indicated; the polysomes were prepared and counted as described in Methods. (B) Islets (30) in each of four tubes were incubated at 37°C for 45 min as above, pulsed with [3H]leucine (200 μCi/ml) for 5 min, then 1 mM [3H] leucine was added to each tube and the islets were homogenized; polysomes were prepared as in (A), at the indicated times.

**Fig. 3.** Sedimentation profiles of islet polysomes identified by [3H]leucine-labeled nascent peptides, and carrier liver polysomes identified by absorption at 260 nm. Islets (100) were incubated at 37°C for 45 min, pulsed with [3H]leucine (200 μCi/ml) for 5 min; polysomes were prepared and analyzed on sucrose gradients as described in Methods. The solid lines (———) represent the absorbance at 260 nm, cpm (O—–O). In the lower panel the polysomes were treated with RNase (0.25 μg/ml, incubated at 37°C for 5 min) before gradient analysis.
and then determine whether proinsulin is synthesized on free or bound polysomes, islets were pulsed with [³H]leucine (200 
μC/ml) for 15 min, then fractionated into subcellular fractions
(6) as outlined in Fig. 1. The distribution of labeled protein in
the subcellular fractions is shown in Table 1. Labeled proteins in the
postmicrosomal supernatant comprised 7.7% of the total, and are presumed to be products of the free

Effect of glucose on nascent peptide formation
Islets were incubated for 45 min in either 2.8 or 15.5 mM
glucose, and then pulsed with [³H]leucine for various periods (Fig. 5).
The time course for the labeling of nascent peptides in islets incubated in 2.8 mM glucose was similar to that of
islets incubated in 15.5 mM glucose, but the absolute amount of labeled nascent peptide was reduced about 50–60%.

The effect of glucose on the distribution of [³H]leucine in
polysome nascent peptides was determined by sucrose gradient analysis (Fig. 6). Glucose markedly increased the
incorporation of [³H]leucine into nascent peptides associated with large polysomes. Of particular interest is the abrupt
increase of labeled nascent peptides in the trisome region.

Distribution and nature of labeled peptides from
bound and free polyribosomes
In several mammalian cells, secretory proteins have been
shown to be synthesized on membrane-bound polysomes and
to be released into the cisternae of the endoplasmic reticulum.
Nonsecretory proteins, on the other hand, may be syn-
thesized on free polysomes (not attached to membranes) and then released directly into cell cytosol (6, 11, 26–27).
To determine whether proinsulin is synthesized on free

Fig. 4. Effect of puromycin “chase” on labeled nascent peptides of islet polysomes. The solid line represents the absorbance
at 260 nm; the hatched bars represent cpm. Lower panel: islets were pulsed with [³H]leucine for 5 min, then polysomes were
prepared and analyzed on a sucrose gradient as described in Methods. Upper panel: same as lower panel except that the 5-min
[³H]leucine pulse was followed by 5-min incubation with puromycin (5 X 10⁻⁴ M).

Fig. 5. Effect of glucose on labeling of nascent peptides of islet polysomes. Islets were incubated at 37°C for 45 min in 2.8
(ΟΟΟΟΟ) or 15.5 mM (ΟΟΟΟΟΟ) glucose, then pulsed with [³H]-
leucine for the indicated times; labeled polysomes were prepared and counted as described in Methods.

Fig. 6. Effect of glucose on sedimentation profiles of islet polyribosomes. Islets (50) were incubated at 37°C in Kreb's
buffer (pH 7.4) with 2.8 or 15.5 mM glucose (2 groups at each concentration) for 45 min; nascent peptides were labeled for
5 min with [³H]leucine; the polysomes were prepared and analyzed as described in Methods. Solid line represents the absorbance
at 260 nm of carrier liver polysomes; bars represent the cpm in nascent peptide of islets that were incubated at 37°C in 15.5
mM (open bars), or in 2.8 mM (hatched bars) glucose (each bar is the mean of two determinations).
incubations with [3H]amino acid would predictably label ribosomal proteins (15), as well as nascent peptide. We have observed that 15-min pulses with [3H]leucine yield label in polysomes that cannot be stripped by subsequent puromycin treatment (Permutt and Kipnis, unpublished). Evidence that [3H]leucine is incorporated into nascent peptide on islet polysomes, rather than nonspecifically bound to carrier liver polysomes, includes (a) islets mixed with [3H]leucine and immediately homogenized contained about 10% of the radioactivity of the islets that were incubated 10 min (Fig. 5), (b) the labeled amino acid is preferentially associated with polysomes rather than monosomes (Fig. 3), and (c) puromycin removed 90% of the polysome-associated radioactivity (Fig. 4).

An increase of the glucose concentration in the medium from 2.8 to 15.5 mM over 45 min nearly doubled the polyribosome activity. This correlates well with the observed doubling of total islet protein synthesis in medium with high glucose content (1). Nutritional effects on mammalian protein synthesis have been reported to occur at several control points (16-19). Van Venrooij et al. (20) found that protein synthesis in Ehrlich ascites tumor cells diminished with glucose depletion of the incubation medium and increased when glucose was restored. This was accompanied by an increase in monosomes aggregating to form polysomes without immediate change in the rate of peptide chain elongation, and implied that initiation of peptide synthesis was stimulated by glucose. They also noted (20) that glucose stimulated peptide synthesis per unit of polysome-associated ribosome, i.e., an accelerated rate of ribosome translation along the mRNA strand.

At the present time, it is not known how glucose controls the activities of islet polysomes. Glucose could affect polysome activity by increasing the amount and/or activity of the macromolecular components. For example, islets incubated for 1 hr in a medium with high glucose concentration incorporated twice as much [3H]uridine into a soluble RNA and polysome-associated RNA fraction (presumed to be mRNA) as did islets incubated in a medium with low glucose concentration (1). It is conceivable that mRNA production is rate-limiting to glucose-stimulated activity of islet polyribosomes.

**DISCUSSION**

Modification of a method has been described that permits the separation of free and membrane-bound polysomes from as little as 25 isolated rat islets. Since only microgram quantities of RNA are obtained from the few milligrams of islet tissue that can be feasibly isolated from 2–3 rat islets, islet polysomes are identified by labeling nascent peptides with [3H]amino acids. The kinetics of labeling of nascent peptides on islet polysomes during short pulses with [3H]leucine follows the same pattern observed in other mammalian systems (13, 14). Longer

**Table 1. Distribution of [3H]leucine-labeled protein in subcellular fractions of isolated rat islets**

<table>
<thead>
<tr>
<th>Subcellular fraction</th>
<th>Percent of total radioactivity*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Postnuclear supernatant</td>
<td>100</td>
</tr>
<tr>
<td>Postmicrosomal supernatant</td>
<td>7.7 ± 1.1</td>
</tr>
<tr>
<td>2M Sucrose I</td>
<td>9.2 ± 2.0</td>
</tr>
<tr>
<td>Free polyribosomes</td>
<td>12.0 ± 3.8</td>
</tr>
<tr>
<td>Postmicrosomal supernatant</td>
<td>61.4 ± 5.8</td>
</tr>
<tr>
<td>2M Sucrose II</td>
<td>5.6 ± 1.2</td>
</tr>
<tr>
<td>Bound polyribosomes</td>
<td>1.0 ± .4</td>
</tr>
<tr>
<td>% Recovered</td>
<td>96.9 ± 3.8</td>
</tr>
</tbody>
</table>

* Mean ± SE for eight experiments. Islets (100) were labeled for 15 min with [3H]leucine (200 μCi/ml) in Krebs buffer (pH 7.4) containing 15.5 mM glucose, then homogenized; subcellular fractions were prepared as in Fig. 1. Aliquots of the fraction were precipitated with CH2COOH and counted as described in Methods.
The effect of glucose on islet protein synthesis is specific for insulin, for when islets were incubated in a medium with low glucose concentration (2.8 mM) for 90 min, about 6% of the newly synthesized protein was proinsulin and insulin, whereas an increase of the glucose concentration in the medium to 15.5 mM increased the proportion of proinsulin and insulin to 22% (1). In the present studies, the effect of glucose on the distribution of [3H]leucine in nascent peptides of islet polysomes indicated a preferential stimulation of peptides synthesized on trisomes and larger polysome aggregates. If we assume that rat proinsulin mRNA is monocistronic and has only three nucleotides coding for each of the 80-84 amino acids in proinsulin (22), it should have about 250 nucleotides and should be able to accommodate at most three ribosomes (21, 22). It is interesting to note that islets incubated in 2.8 mM glucose for 45 min synthesize about 7% of their nascent peptides on trisomes (Fig. 6). Islets incubated in 15.5 mM glucose for 45 min, on the other hand, synthesized about 12% of their nascent peptide on trisomes.

Fractionation of islets incubated for 15 min in [3H]leucine revealed that over two-thirds of islet protein synthesis occurred on membrane-bound polysomes. It is estimated that at least 85% of the proinsulin synthesized by the islet is synthesized on membrane-bound polysomes. This agrees closely with the estimates of Kemmler and Steiner (29), who found that 82% of the proinsulin in rat islets associated with cytoplasmic membrane particles. At the present time, it is impossible to say whether the proinsulin recovered in the cell cytosol represents protein that is actually synthesized on free polysomes or that is simply released from broken cytoplasmic membrane particles. Lisowska-Bernstein et al. (27) have demonstrated the synthesis of immunoglobulins on free polysomes. In the unstimulated state, about 5-10% of the insulin released by islets is in the form of proinsulin (28). Since conversion of proinsulin to insulin occurs in cytoplasmic-membrane particles (29), it is possible that the proinsulin that is released from the beta cell is primarily that which is synthesized on free polysomes.

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