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Research Article

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Synthesis and Release of Proinsulin and Insulin by Isolated Rat Islets of Langerhans

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ABSTRACT Isolated rat islets of Langerhans were incubated for 60, 120, and 180 min and the incorporation of leucine-^{3}H into proinsulin and insulin moieties was followed. Synthesis and release of these hormones could be followed by separate extractions of islets and incubation media.

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INTRODUCTION

The existence of proinsulin as a precursor molecule for insulin is now established (1–3). Recent reports have indicated that proinsulin may be found in the serum of human subjects suggesting that under certain circumstances proinsulin release from pancreatic islets occurs (4, 5). Conditions conducive to this in vivo release have been partially defined following a glucose stimulus. Clark and Steiner (6) demonstrated that in vitro incubation of rat islets of Langerhans, in increasing concentrations of glucose ranging from 2 to 6 mg/ml resulted in the release of an increasing proportion of newly synthesized proinsulin relative to insulin. The present study was undertaken to define in greater detail the conditions leading to proinsulin release in vitro utilizing physiologic and pharmacologic agents known to affect release of pancreatic insulin.

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METHODS

Preparation of islets. Islets of Langerhans were isolated from the pancreas of fed male Sprague-Dawley rats (250–270 g). The technique of Lacy and Kostianovsky was utilized (7) with minor modifications. Preparations of collagenase were obtained from Worthington Biochemical Corp., Calbiochem, General Biochemicals, and Pierce Chemical Co. Preparations varied in their digestive abilities and each required screening in order to determine optimal incubation times. Collagenase (12 mg/ml) in 5 ml Hanks' solution, pH 7.4, was added to minced pancreas (2–2.5 g wet weight) and incubated with continuous stirring at 37°C for various periods of time. Digestion was monitored throughout the incubation by use of a dissecting microscope. At the appropriate time, digestion was stopped by the addition of 20 ml of Hanks' solutions (4°C). The washing procedure, microdissection, and fishing of islets were as described (7). Careful choice of intact islets was made so that only 20–40 islets could be harvested per pancreas. Often several pancreas preparations were pooled.

Incubation procedure. 10–20 islets were incubated in 0.5 ml Krebs-Hanseleit-bicarbonate buffer pH 7.4 containing all amino acids (5 μg/0.5 ml of each), soy bean trypsin inhibitor (0.5 μg/0.5 ml), and bovine serum albumin (1 mg/0.5 ml). Either 5.3 mM or 16 mM glucose was present with or without the following agents: 5 mM DAMP[1]; 5 mM theophylline; 0.04–0.4 mM tolbutamide; or 5 mM 5′-AMP. In all incubations, leucine-^{3}H (5.0 Ci/mmole, 100 μCi/ml; New England Nuclear) was substituted for leucine. Incubations were carried out in a Dubnoff shaker for periods from 60 to 180 min and gassed with 95% O_2–5% CO_2. The procedure followed closely that described by Steiner and Oyer (8).

Extraction procedure. The extraction of proinsulin and insulin was carried out separately on the tissue and on the 0.5 ml incubation medium. The extraction procedure of Davoren (9) was carried through the final 15% NaCl precipitation. The following modifications were made. All work was performed at 4°C. Specimens were centrifuged at 12,000 *g* for 60 min. 1 mg of crystalline beef insulin was

[1] DAMP, N^6O^2-dibutyryl cyclic adenosine-3′5′-monophosphate.

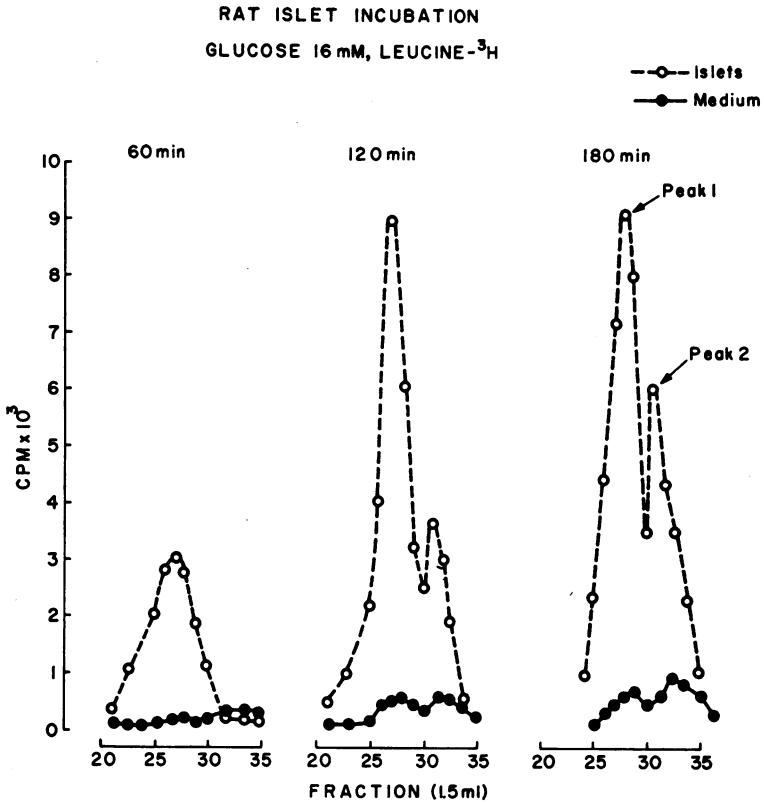


FIGURE 1 Sephadex G-50 gel filtration of acid alcohol extracts of islets and media incubated for 60, 120, and 180 min. See text for details.

routinely added to the acid alcohol extract of both the islets and the medium. This insulin served as an internal control to test the efficacy of the extraction procedure. Only those extractions that showed similar recoveries of insulin as measured by optical density at 275 mμ were used. Variations as a result of faulty extraction procedures were thus eliminated. In those experiments where material, immunoassayable as insulin, was to be followed, albumin was added as the carrier protein. The final precipitates of the extraction procedure were taken up in 1 ml of 1 M acetic acid.

Gel filtration procedures. Filtration was carried out on Sephadex G-50 medium columns (1.2 × 50 cm), flow rate 1 drop per 20 sec, and fraction volumes of 1.5 ml. Elution was with 1 M acetic acid. 05 ml of each fraction was added to 10 ml of Aquafluor (New England Nuclear) and counted in a scintillation counter. Optical densities at 275 mμ were recorded on each fraction. All peaks were rechromatographed on Sephadex after pooling and lyophilization. Selected peaks from islet tissue and media were subjected to further identification procedures. The elution position of crystalline porcine insulin and pure porcine proinsulin (Eli Lilly & Co., and Elanco Products Div., a subsidiary of Eli Lilly, respectively) were determined. Porcine proinsulin was iodinated with ^{131}I according to the method of Greenwood and Hunter (10) and counted in a gamma counter.

Identification procedures. Polyacrylamide-gel electrophoresis pH 8.6 as described by Davis (11) was performed utilizing a 15% gel. Further definition of the radioactive peaks obtained on alkaline gels was carried out utilizing gel electrophoresis at pH 4.4 (15% gel). Stock solutions were degassed before usage to prevent bubble formation within the gels. Separating gel columns were 7 × 0.5 cm. Samples were run at 5 mamp/tube for 4 hr. Gels were removed intact from the tubes by cracking the tubes. Staining procedures in both gels were as described by Davis (11).

The gels were cut in 1.5-mm segments and digested as described by Clark and Steiner (6). Leucine-^{3}H was counted as before and the radioactive patterns determined. Rat insulin (courtesy of Dr. K. Becker) and porcine proinsulin were utilized as standards.

Trypsin treatment of tentatively identified radioactive proinsulin peaks was carried out in 0.125 M borate buffer pH 9.4 (4 mg trypsin per ml). 1 mg of unlabeled insulin was added. Digestion varied from 8 to 30 min and was terminated by the addition of 1 M acetic acid just before further gel filtration on Sephadex G-50. Immunoassay for insulin was performed by the method of Morgan and Lazarow (12).

RESULTS

Incubation of islets in 16 mM glucose. Islets were incubated for 60, 120, and 180 min in 16 mM glucose. Acid alcohol extracts of islets and media were subjected to gel filtration on Sephadex. Fig. 1 shows the sequential patterns obtained. It should be noted that at 60 min only a single peak is seen in the islets, while

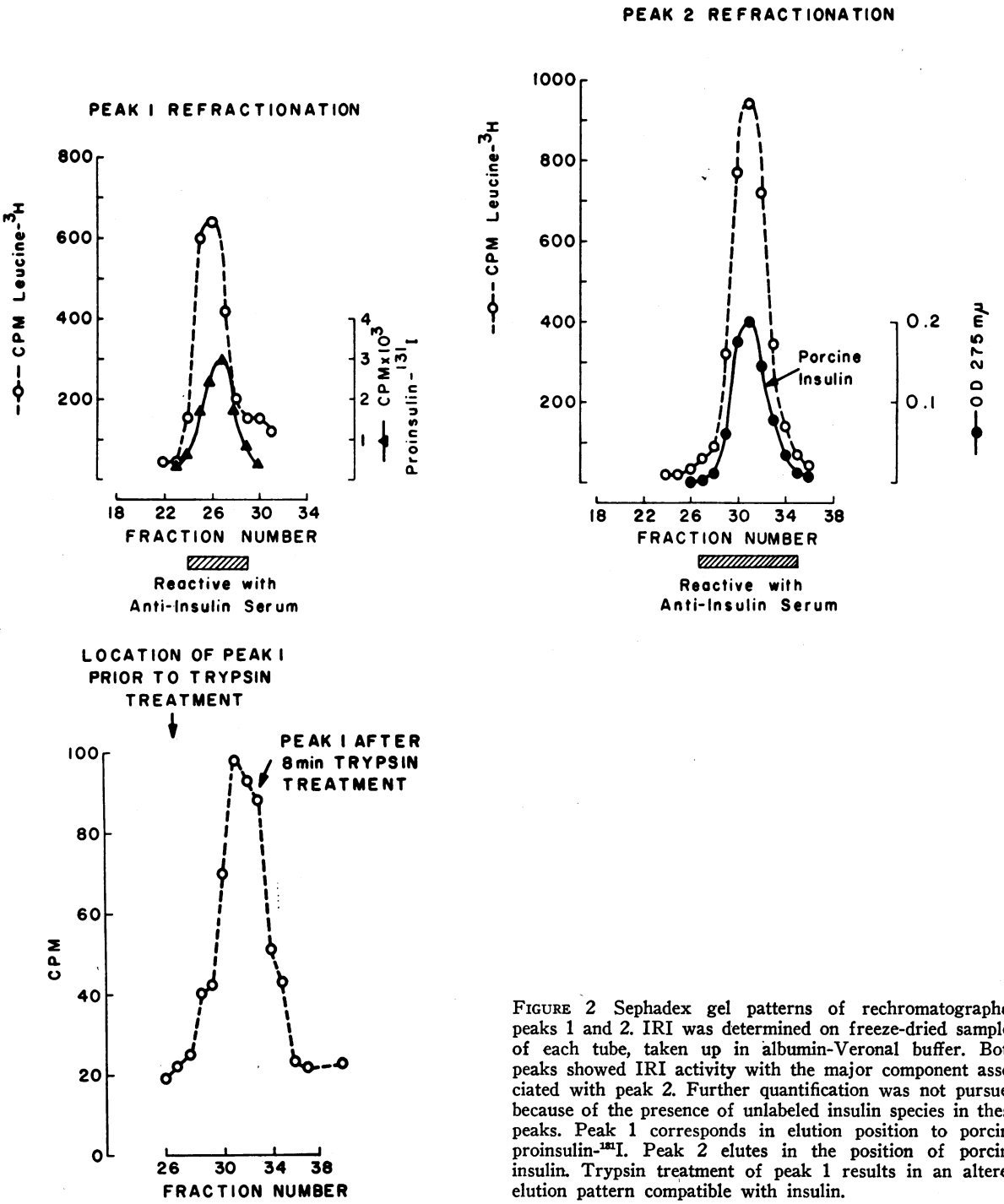


FIGURE 2 Sephadex gel patterns of rechromatographed peaks 1 and 2. IRI was determined on freeze-dried samples of each tube, taken up in albumin-Veronal buffer. Both peaks showed IRI activity with the major component associated with peak 2. Further quantification was not pursued because of the presence of unlabeled insulin species in these peaks. Peak 1 corresponds in elution position to porcine proinsulin-^{131}I. Peak 2 elutes in the position of porcine insulin. Trypsin treatment of peak 1 results in an altered elution pattern compatible with insulin.

at 120 and 180 min, two peaks are defined. The total counts in the islets increase with time. At 180 min, no further increase in peak 1 is seen, while peak 2 is becoming more prominent. Radioactivity in the medium also increases with time. At 60 min only few counts are noted, and no peaks are seen. At 180 min, two peaks are noted.

Fig. 2 shows the Sephadex gel patterns obtained after rechromatography of peaks 1 and 2. Peak 1 occupies a similar elution position to porcine proinsulin-^{131}I.

Peak 2 elutes in the same position as crystalline porcine insulin. Immunoassay of peaks 1 and 2 indicate that while both react with insulin antibody, peak 2 is more reactive than peak 1.

In an effort to identify the radioactive peaks more precisely, trypsin digestion of peak 1 was carried out. Fig. 2 shows the conversion by trypsin of peak 1 radioactivity to material migrating in the position of insulin. This behavior is consistent with the probability that peak 1 contains proinsulin.

Clark and Steiner (6), using polyacrylamide-gel procedures, emphasized the existence of two rat insulins (I_1 and I_2) as well as two proinsulins and possibly several proinsulin intermediates. Proinsulin is an intact single chain while the intermediates are forms in which cleavage has occurred to produce two-chain derivatives. For this reason, rechromatographed peaks from islets and medium were subjected to alkaline polyacrylamide-gel electrophoresis. Fig. 3 shows the results utilizing peaks obtained from Sephadex after 180 min of incubation. Varying amounts of peak sample were applied to the gels. Rat insulin was used to determine the position of the two insulin moieties. Because of the unavailability of rat proinsulin, porcine proinsulin was used as a standard. Examination of the first Sephadex peak, both from islets and medium, reveals the presence of two major components, one coinciding in position with proinsulin and the second running just before rat insulin. The second Sephadex peak has three major components in both islets and medium. Two appear compatible with insulins 1 and 2. The third peak position coincides with proinsulin.

In order to obtain further identification of these polyacrylamide-gel peaks, acid polyacrylamide-gel electrophoresis was performed using peaks 1 and 2 from Sephadex. Fig. 4 compares the alkaline and acid gels of Sephadex peak 1 obtained from islets after 60 min of incubation. The findings suggest that the major peak is located in the position of proinsulin (and or intermediates) while a smaller amount of insulin is also present. Comparison of alkaline and acid gels obtained from Sephadex peak 2 after 180 min of islet incubation are also shown in Fig. 4 In this case, the major components appear to be insulins 1 and 2. A minor component is either proinsulin or intermediates.

The per cent of proinsulin, intermediates, or insulin in each peak obtained after Sephadex chromatography was calculated from the data obtained after polyacrylamide electrophoresis (Table I, Figs. 3 and 4). These data suggest the following. (*a*) Both proinsulin and insulin are already present in the islets after 60 min of incubation, though no significant release into the medium occurs until a later time. (*b*) At 180 min, proinsulin and insulin are found in the medium. The ratio

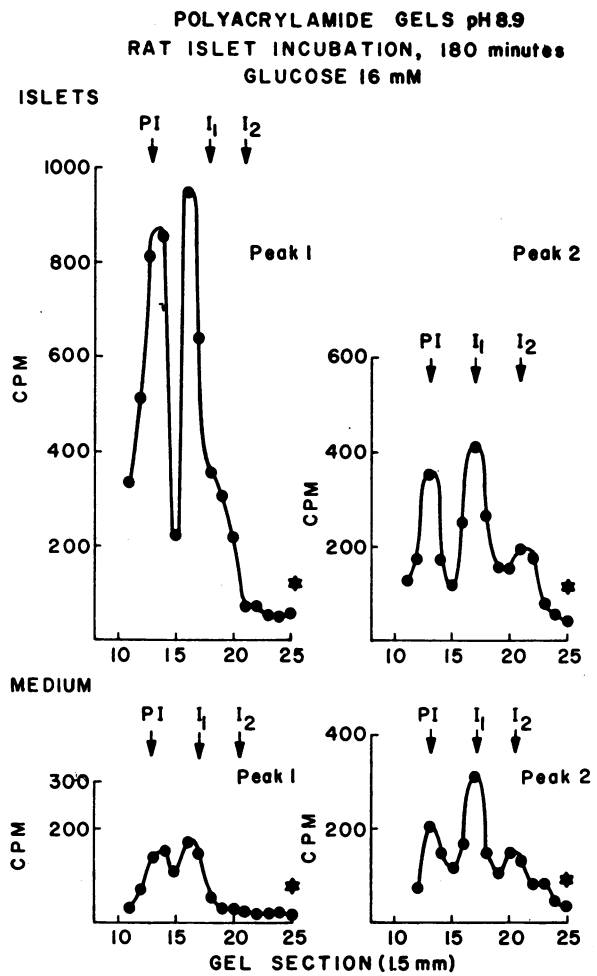
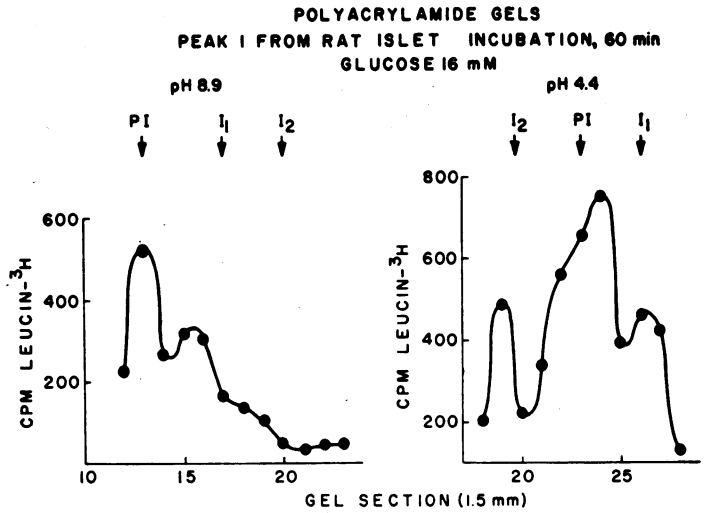


FIGURE 3 Alkaline polyacrylamide-gel electrophoresis of leucine-^{3}H Sephadex peaks obtained from islets and media. The positive pole is to the right of the figure. The arrows mark the position of PI (porcine proinsulin) and I_1 and I_2 (rat insulins 1 and 2). The asterisk indicates the position of tracker dye. It should be noted that it is difficult to differentiate intermediate forms from insulin (I_1) under these conditions.

of proinsulin in islets/proinsulin in the medium is 13.8/1, while the ratio of insulin in islets/medium is only 8.5/1. (*c*) The ratio of proinsulin to insulin in the medium can be calculated to be 0.46, assuming that 40% of the radioactivity in proinsulin is derived from C peptide (13). And finally, (*d*) because no radioactivity was found on the gels in the position of the tracker dye, is was concluded that Sephadex insulin peaks were not contaminated with radioactivity due to the presence of the C peptide.

Incubation with 5 mM DAMP in the presence of 16 mM glucose. Fig. 5 shows the Sephadex elution pattern obtained from islets incubated with 5 mM *DAMP*



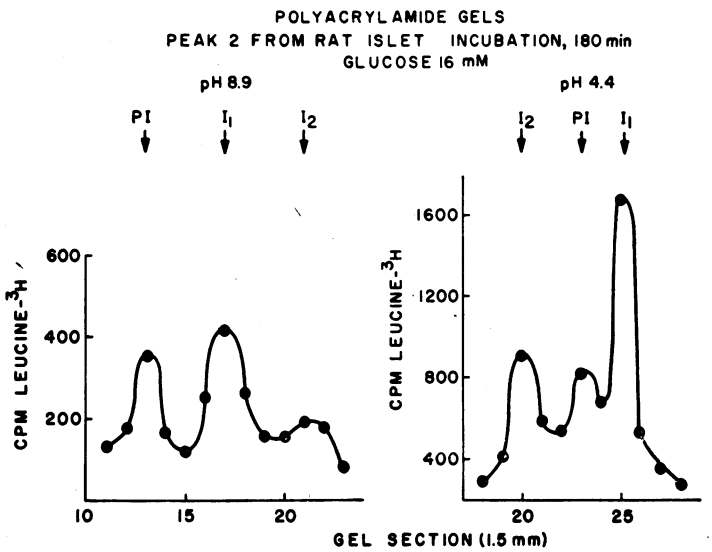


FIGURE 4 Comparison of alkaline and acid polyacrylamide-gel patterns of Sephadex peak 1 (60 min incubation) and peak 2 (180 min incubation). Acid gels were utilized to determine the amounts of insulin 1 and 2 present in the alkaline gels. These figures were then utilized to determine the amounts of proinsulin and intermediates on the alkaline gels. Peak 1 is similar to the alkaline gel pattern obtained after 180 min (Fig. 3), except that the insulin component is less marked. This is confirmed on acid gel. Peak 2 contains insulins 1 and 2 as well as proinsulin(s) (intermediates) in both gels. The major component is insulin.

and 16 mM glucose for 180 min. In contrast with the glucose control (Fig. 1) two features are noteworthy: (*a*) incorporation of radioactivity in the islets is increased 1.4-fold, and (*b*) radioactivity in the medium is increased 5-fold. Fig. 6 shows the patterns of the rechromatographed Sephadex peaks on alkaline polyacrylamide gels. These patterns are qualitatively similar to those of the glucose control (Fig. 3). Table II summarizes the measurements of radioactivity in the islet and medium proinsulin, intermediates and insulin. The ratio of islet/medium proinsulin is 3.9/1 while islet/medium insulin is 2.5/1 The ratio of proinsulin to

TABLE I

180 min Incubation in 16 mM Glucose

	Islets						Medium					
Sephadex peak.....	1			2			1			2		
Fraction*.....	PI	Int.	I	PI	Int.	I	PI	Int .	I	PI	Int.	I
Total counts in sephadex peak	44,192			26,543			2200			3800		
% of total counts on alkaline gel§	28.4	22.8	4.7	20.0	—	43.0	31.2	26.3	7.8	18.0	—	45.0
Adjusted % based on acid gel§	28.4	11.4	16.1	20.0	—	43.0	31.2	13.2	21.0	18.0	—	45.0
Total counts in peaks 1 + 2:												
PI + intermediates			22,896						1660			
I			18,528						2172			

* PI = proinsulin; Int. = proinsulin intermediates; I = insulins (1 + 2).

‡ 50–60% of counts placed on polyacrylamide gels are recoverable in the proinsulin, intermediates, and insulin positions. The residual counts probably represent noninsulin protein.

§ Comparison of acid and alkaline gels shows that the intermediate position on alkaline gels may represent 50% insulin contamination. This correction was taken into account when calculating the total number of counts in proinsulin, intermediates, and insulin.

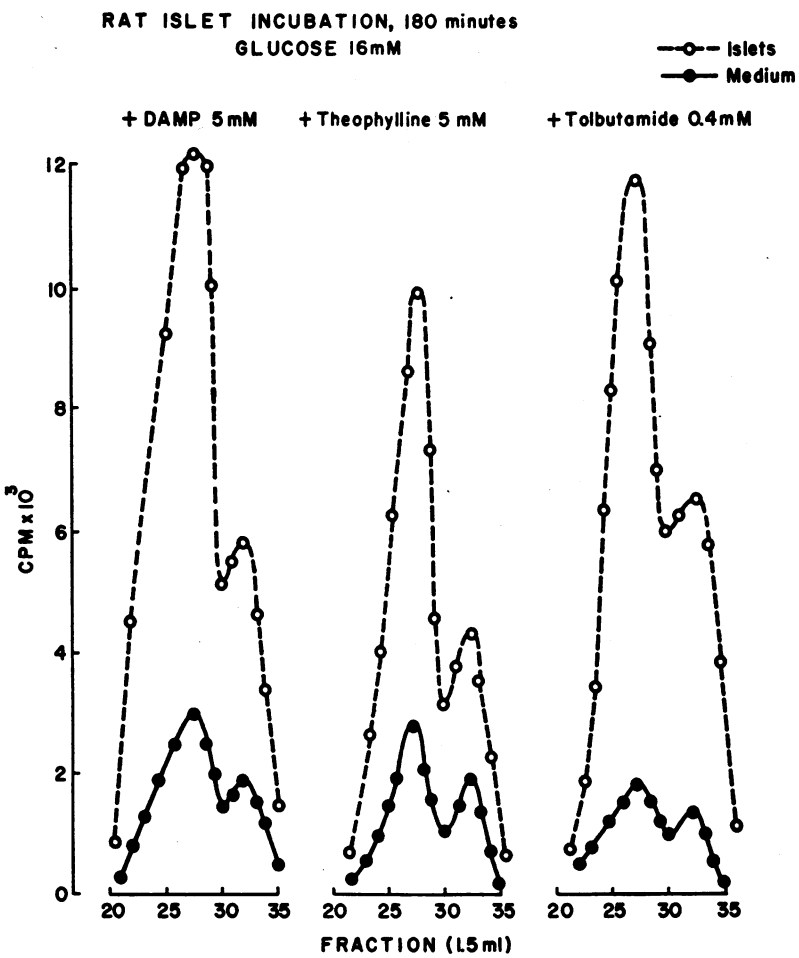


FIGURE 5 Sephadex G-50 gel filtration of acid alcohol extracts of islets and media incubated for 180 min in the presence of 16 mM glucose plus DAMP, theophylline, or tolbutamide.

TABLE II

180 min Incubation in 5 mM DAMP + 16 mM Glucose

	Islets						Medium					
Sephadex peak.....	1			2			1			2		
Fraction*.....	PI	Int.	I	PI	Int.	I	PI	Int.	I	PI	Int.	I
Total counts in Sephadex peak	61,667			32,366			14,314			15,586		
% of total counts on alkaline gel‡	42.0	20.6	2.5	24.0	—	49.0	24.0	34.4	1.3	27.0	—	42.0
Adjusted % based on acid gels§	42.0	10.3	12.8	24.0	—	49.0	24.0	17.2	18.5	27.0	—	42.0
Total counts in peaks 1 + 2:												
PI + Intermediates	40,019						10,105					
I	23,752						9,194					

* PI = proinsulin; Int. = proinsulin intermediates; I = insulins (1 + 2).

‡ 50–60% of counts placed on polyacrylamide gels are recoverable in the proinsulin, intermediates, and insulin positions. The residual counts probably represent noninsulin protein.

§ Comparison of acid and alkaline gels shows that the intermediate position on alkaline gels may represent 50% insulin contamination. This correction was taken into account when calculating the total number of counts in proinsulin, intermediates, and insulin.

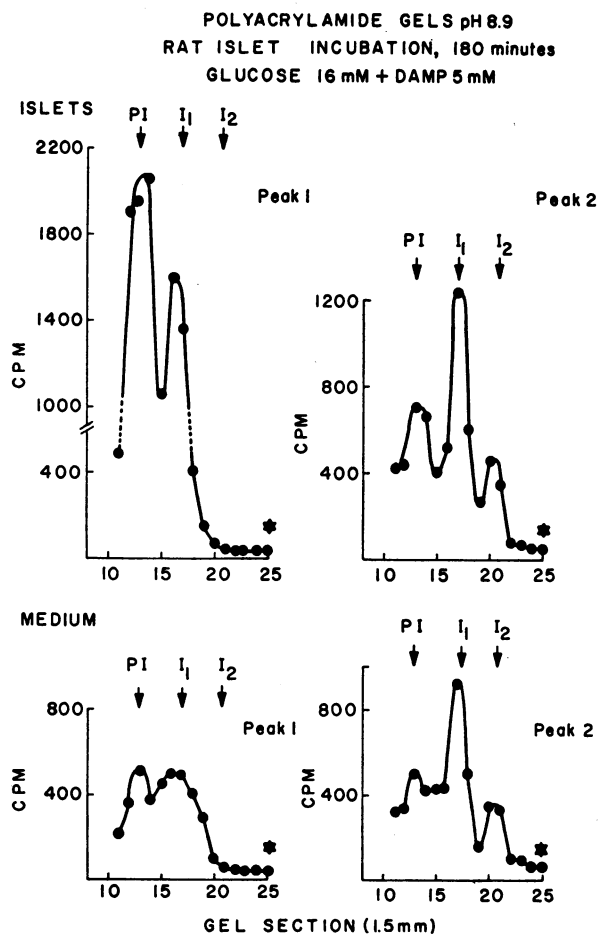


FIGURE 6 Alkaline polyacrylamide-gel patterns of Sephadex peaks obtained from islets and media incubated with 5 mM DAMP, 16 mM glucose, and leucine-^{3}H for 180 min. See Fig. 3 and text for details.

insulin in the medium (40% of PI counts assumed to be C peptide) is 0.66.

Incubation with 5 mM theophylline, 0.4 mM tolbutamide, or 5 mM 5'AMP. After incubation in 16 mM glucose for 180 min, the Sephadex elution peaks obtained from islets and media incubated with theophylline or tolbutamide were indistinguishable from the pattern obtained with DAMP (Fig. 5). Increased incorporation of leucine in both islet peaks and marked release of radioactivity in the medium were noted. 5'AMP produced a pattern similar to those of the controls. Incubation with DAMP, theophylline, or tolbutamide for *60 min* (Fig. 7), increased the incorporation of leucine into islet protein by a factor of 1.5–2 compared with the glucose control (Fig. 1). Despite this, medium radioactivity was not increased over the control.

Incubation at 5.3 mM glucose. Incubation of islets for 180 min at a glucose concentration of 5.3 mmoles/liter results in the Sephadex pattern observed in Fig. 8. In contrast with 16 mM glucose, the total number of counts in the islet peaks is 50% less and essentially *no radioactivity appears in the medium.* Theophylline in the presence of 5.3 mM glucose causes a small increase both in the total number of counts incorporated into the Sephadex peaks and in the quantity of radioactivity released. DAMP with 5.3 mM glucose increased incorporation and release to levels observed with 16 mM glucose alone.

Tolbutamide (0.4 mmole/liter) completely inhibited synthesis of both peaks and no radioactivity appeared in the medium. This inhibition of synthesis by tolbutamide could be overcome by either raising the glucose concentration to 16 mmoles/liter (Fig. 5) or lowering the tolbutamide concentration to 0.04 mmole/liter (Fig. 9).

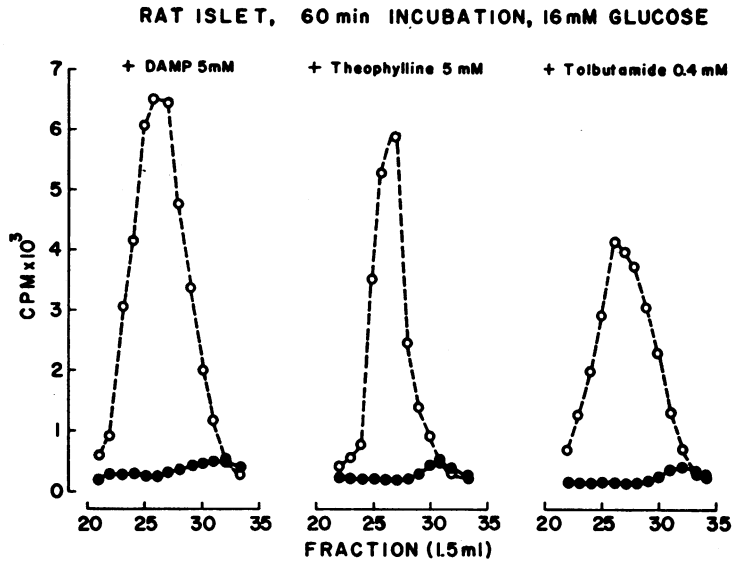


FIGURE 7 Sephadex G-50 gel filtration of leucine-^{3}H acid alcohol extracts of islets and medium incubated for 60 min with 16 mM glucose plus DAMP, theophylline, or tolbutamide. Note increase in incorporation of leucine-^{3}H into islet peaks (open circles) without significant release into the medium (closed circles).

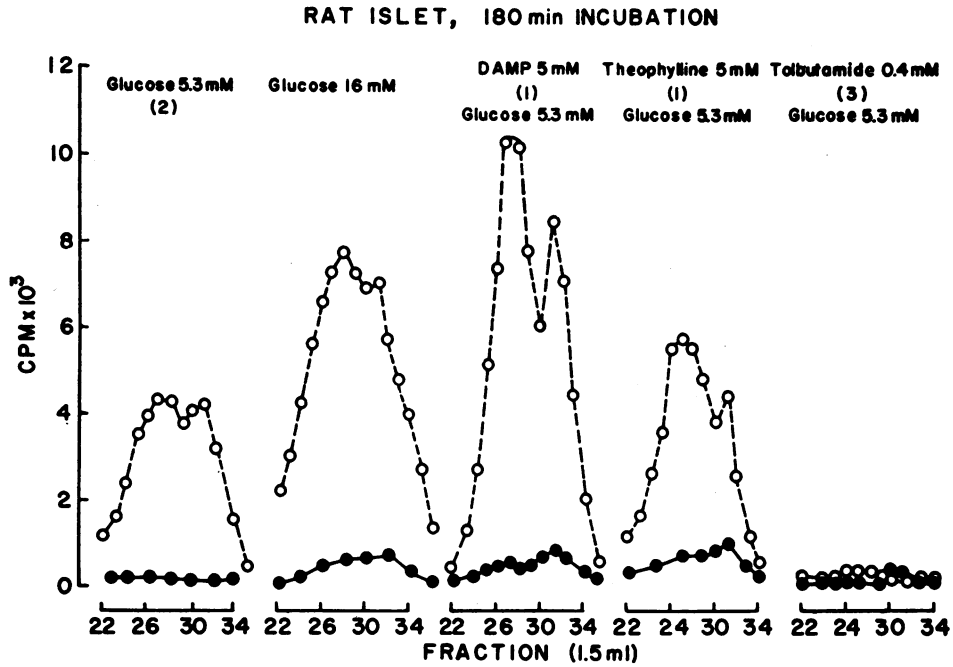


FIGURE 8 Sephadex G-50 gel filtration patterns of leucine-^{3}H acid alcohol extracts of islets (open circles) and media (closed circles) incubated for 180 min. Numbers in parentheses represent number of experiments. Note stimulatory effect of 16 mM glucose, and of DAMP and theophylline on synthesis and release of insulin moieties and inhibitory effect of tolbutamide.

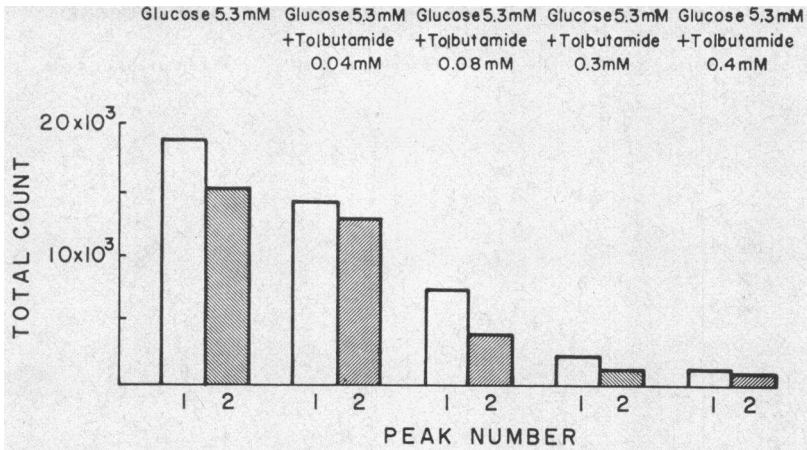


FIGURE 9 Effect of tolbutamide on incorporation of leucine-^{3}H in rat islet. Concentration dependence of the inhibitory effect of tolbutamide on incorporation of leucine-^{3}H into acid alcohol extracts of islets at 5.3 mM glucose.

DISCUSSION

Isolated rat islets of Langerhans remain viable for 3 or more hr of incubation (13) as evidenced by the sequential incorporation of radioactive leucine into acid-soluble protein in the islets. During 180 min incubation, reproducible, time-related increases in islet radioactivity occur. Sephadex patterns evolve from a single radioactive peak at 60 min to two peaks at 120 and 180 min with an increase in the second peak relative to the first after 120 min. These observations confirm the original report of Steiner and Oyer (8).

It is important to ascertain that the radioactivity appearing in the medium represents *release* and not *leakage* of insulin moieties from damaged islets. To obviate this possibility, only those islets which showed intact smooth contours after collagenase treatment were chosen. The following experimental results confirm that release and not leakage of radioactivity occurred. After 60 min of incubation in 16 mM glucose, only a minimal amount of radioactivity was present in the medium despite considerable accumulation of radioactive acid-soluble protein in the islets. Incubation for 60 min with 5 mM DAMP, theophylline, or tolbutamide while resulting in marked increases in islet radioactivity compared with glucose alone, did not increase medium radioactivity. Incubation with these agents for 180 min resulted not only in increases in islet radioactivity ($\sim 1.5 \times$ the control), but more marked increases in medium radioactivity ($\sim 5 \times$ the control medium). Thus leakage of radioactivity does not occur with 60 min of incubation. Incubation for 3 hr in 16 mM glucose resulted in sequential, reproducible increases in radioactive acid-soluble protein in the medium. If the islets progressively deteriorated, then one might anticipate leakage at the later time periods. However, experiments in which islets were incubated with 5.3 mM glucose for 180 min, clearly demonstrated that although significant incorporation of radioactivity (with two well defined Sephadex peaks) occurred in the islets, essentially *no radioactivity could be found in the medium.* Addition of DAMP or theophylline in the presence of 5.3 mM glucose, resulted in increased release of radioactivity similar to the 16 mM glucose control, but less than that observed with 16 mM glucose and these agents.

It seems clear, therefore, that specific biochemical requirements exist for radioactivity to appear in the medium: namely, (*a*) the need for longer than 60 min of incubation, (*b*) the need for a higher than 5.3 mM glucose concentration, or (*c*) the action of insulin releasing agents (DAMP, theophylline, and tolbutamide) potentiated by concentrations of glucose higher than 5.3 mmoles/liter.

The identification of the radioactive (leucine-^{3}H) peaks in rat islets and medium is complicated by the fact that rat islets synthesize two proinsulins and two insulins under glucose incubation. This was first demonstrated by Clark and Steiner (6) who further indicated that in the conversion of the proinsulins to insulins, several intermediate proinsulin forms and free C peptide (which contains 40% of the leucine residues in proinsulin) are also produced. Since Sephadex gel chromatography is unable to discriminate adequately amongst these various forms, more precise definition of the two radioactive peaks obtained on Sephadex was necessary. For this reason the Sephadex peaks from *islets and media* were analyzed by alkaline and acid polyacrylamide-gel electrophoresis.

Clark and Steiner (6) used 7.5% polyacrylamide gels, which on a molecular weight basis discriminate mole-

cules of 100,000 to 1,000,000. Since the molecular species to be separated ranged from 3000 to 10,000 molecular weight, it seemed appropriate to use a 15% gel which is recommended for molecular weights of 10,000 or less. Rat insulin and porcine proinsulin served as standards. On 15% alkaline gels, the rat insulins ran as reported on 7.5% gels, and porcine proinsulin migrated in a position similar to that of rat proinsulins. The 15% alkaline gels differed from the 7.5% gels in that an additional peak assumed to be proinsulin intermediate migrated more slowly than rat insulin I. The C peptide was not seen on these gels. However, human C peptide runs close to the tracker dye (14) as previously shown with 7.5% gels (15). The absence of C peptide here can be ascribed to the 15% NaCl precipitation step used in the insulin extraction which probably eliminates this component.[3] Since complete separation of intermediate forms from insulin I does not take place in either 7.5% or 15% alkaline gels, acid gels were utilized to separate the insulins. The rat insulin and porcine proinsulin patterns on the 15% acid gels were similar to the 7.5% gels reported (6). Separation of insulin I and II from proinsulin was obtained.

On the basis of the gel patterns, confidence can be placed on the identification of proinsulin and insulin, but the designation of the proinsulin intermediate remains a logical assumption. It should be stated that without molecular identification of the gel peaks obtained from rat islets and media, absolute identification of the species is not possible. Unfortunately the small amounts of hormone synthesized precluded this possibility. Recognizing these limitations of the methods, certain conclusions and calculations (Tables I and II) regarding the relationship between total proinsulin and insulin in islets and media are, however, warranted.

Several features emerge from the data presented.

The role of glucose. 5.3 mM glucose stimulates proinsulin and insulin synthesis in the islets, but no release into medium occurs. Release is observed only at higher glucose concentrations (16 mmoles/liter) where synthesis is approximately twice that at 5.3 mM glucose. Release of insulin appears to be preferentially accomplished relative to release of proinsulin and intermediate forms. The ratio of islet/medium insulin is 8.5/1 while that of islet/medium proinsulin is 13.8/1. The proportion of insulin/proinsulin in the medium is of the order of 2.2.

The role of cyclic Amp and tolbutamide. At 16 mM glucose, DAMP, theophylline, or tolbutamide significantly increased synthesis of all insulin moieties. However, their enhancing effect upon release was even more striking as can be seen by the islet/medium ratios for proinsulin (3.9/1) and for insulin (2.5/1) when compared with the effect of glucose alone. Again insulin appeared to be preferentially released although the medium contained roughly equal amounts of insulin and proinsulin. This may well reflect the failure of the islet-converting enzyme to keep pace with the markedly increased rate of proinsulin synthesis. It is also of note that some enhancement of synthesis and release was produced by these agents at lower concentration of glucose. The mechanism of action of tolbutamide in this system is not known. Presumably DAMP and theophylline exerted similar biochemical actions, namely provision of cyclic Amp. Cyclic AMP has been shown to induce enzyme synthesis in fetal liver explants (16) and to increase protein synthesis in parotid slices (17). It is, therefore, likely in this system to be exerting an action on protein synthesis per se.

Of particular note are the experiments with tolbutamide indicating a dose-related inhibition of insulin synthesis at low incubation concentrations of glucose. We have no explanation for this action of tolbutamide, though the observations are compatible with the reports of Malaisse, Malaisse-Lagae, Mayhew, and Wright (18) and Creutzfeld, Frerichs, and Creutzfeldt (19) who showed impairment of pancreas insulin responses to glucose in vitro and in vivo after tolbutamide.

Transit time of insulin moieties. Although proinsulin and insulin synthesis could be demonstrated after 60 min of incubation, release could not be induced even with cyclic AMP until a later time. This time course is compatible with the data of Caro and Palade (20) and that of Howell, Parry, and Taylor (21). The latter using rabbit pancreas pieces concluded that newly synthesized insulin was released only in small quantity before 2½ hr of incubation. Caro and Palade, studying acinar pancreas, demonstrated that 1 hr elapses between synthesis of enzyme protein and the arrival of the zymogen granules at the membrane. One might postulate that 16 mM glucose, DAMP, theophylline, and tolbutamide affect release only when newly synthesized proinsulin and insulin are strategically located with respect to the plasma membrane and that the time delay represents intracellular transit time. The preferential release of insulin over proinsulin may represent the preferential release of granule contents over nongranular hormone. Evidence reported by Tung and Yip (22) and Howell, Kostianovsky, and Lacy (23) suggests that only insulin and double-chain proinsulin intermediate forms are present in granules.

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[3] Personal communication from the laboratory of Dr. D. F. Steiner.

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