Regulation of proinsulin synthesis in pancreatic islets and a new aspect to insulin-dependent diabetes*

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I. Introduction

The biosynthesis of insulin in *B*-cells of pancreatic islets of *Langerhans* involves the transcription of proinsulin mRNA from the proinsulin gene, translation of the proinsulin mRNA by ribosomes

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associated with the rough endoplasmic reticulum, conversion of proinsulin to insulin in the Golgi aparatus, formation of *B*-granules, and secretion of the insulin from the *B*-granule. With the recent development of systems for the cell-free translation of messenger RNA (mRNA), it has become possible to examine directly the initial polypeptide product, preproinsulin, encoded in mRNA extracted from islets (1) or islet *B*-cell tumors (2).

Although it is well known that the synthesis of insulin in pancreatic islets is mainly regulated by glucose, the regulation mechanism of insulin biosynthesis has not been well understood. Recently proinsulin mRNA was purified from rat *B*-cell tumors induced with streptozotocin and nicotinamide, and complementary DNA (cDNA) was synthesized using purified proinsulin mRNA as a template. These advances as well as recent improvements in the method for islet isolation made it possible to study the regulatory mechanism of insulin biosynthesis, and diabetes itself is being examined at the molecular level. In this article, some results of our recent experiments are described in the following order.

II. Isolation of *Langerhans* islets from rat pancreas

The collagenase digestion method for isolation of pancreatic islets was originally developed by Lacy & Kostianovsky (3). However, the possibility that some functions of islets are impaired during isolation may not be totally ignored. Nevertheless, little attention has been fixed on this possibility. Therefore, we at first examined the possible impairment of islet functions by determining the insulin secreting ability since if any damage occurred to islets during the isolation process, it would be reflected first in the insulin secreting ability rather than the insulin synthesizing function.

When an excess amount of albumin or a protease inhibitor, aprotinin, was added to the medium for collagenase-treatment of rat pancreas, islets with considerable secreting ability were obtained (4). It was further observed that reduction of the incubation period with collagenase elevated that capability. As shown in Fig. 1, compared to the islets obtained after 25 min treatment, those obtained after 10 min treatment exhibited a steeper response of insulin secretion to glucose stimulation. In the

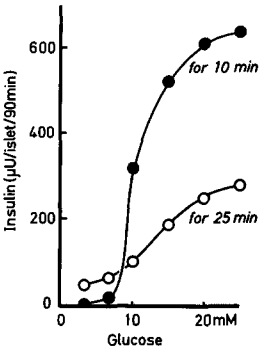

Fig. 1. Insulin release from isolated pancreatic islets. Rat pancreatic islets were isolated by the method of Okamoto *et al.* (4). The collagenase treatment was carried out for 10 min (●) or 25 min (○). The insulin release from isolated islets was examined as described previously (38–41).

presence of 20 mM glucose, more than 600 μU of insulin were secreted from one islet during 90 min of incubation. This value is 2 to 3-fold greater than that reported by Lacy & Kostianovsky (3). The islets obtained after relatively brief treatment with collagenase also displayed a distinct susceptibility to somatostatin, which was no longer observed in islets obtained after relatively long treatment. Somatostatin (10 ng/ml) inhibited insulin secretion by approximately 50% from the islets obtained after 10 min collagenase treatment, whereas the insulin secretion from islets obtained after 25 min treatment was not affected at all by somatostatin (4). Fig. 2 shows the effect of varing periods of collagenase treatment on the amount of insulin

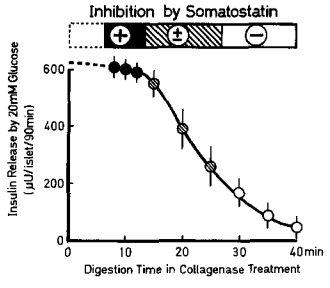

Fig. 2. Effect of varing periods of collagenase treatment on islet insulin release and on its susceptibility to somatostatin. Results represent the mean ± S.E. in μU of insulin released in 4 incubation flasks (4). A flask contained 6 isolated islets.
+: The insulin release was inhibited by somatostatin.
−: The insulin release was not inhibited by somatostatin.
±: Inhibition by somatostatin of insulin release was not prominent.

secreted in the presence of 20 mM glucose and on the susceptibility to somatostatin. As shown in this figure, treatment with collagenase for 20 min or more, drastically diminished both the amount of insulin secreted and the susceptibility to somatostatin. The amount of insulin secreted reached approximately 600 μU/islet/90 min as the treatment period decreased. We are thus led to conclude that the value which is obtained by extrapolation of the treatment period to 0 min gives the amount of insulin secreted from an 'intact' islet. Therefore, that almost 'intact' pancreatic islets were obtained was confirmed by examining the amount of insulin secreted from an isolated islet, the response curve of insulin secreted corresponding to change in glucose concentration, and the sensitivity to somatostatin.

III. Purification and properties of proinsulin mRNA

In order to elucidate the detailed processes of proinsulin biosynthesis and its regulation at the molecular level, it is necessary to purify and characterize proinsulin mRNA. Several investigators have tried to purify proinsulin mRNA from the pancreatic islets of rats (1), carp (5), or catfish (6). However, they could not obtain pure mRNA because of the difficulty of getting large amounts of fresh pancreatic islets. Duguid *et al.* (7) used an X-ray-induced transplantable *B*-cell tumor as the material for mRNA purification. The tumor seemed to be a promising material because it is large enough and transplantable. The proinsulin mRNA content, however, was too low to be purified (8). Recently, Itoh *et al.* (9, 10) succeeded in the purification of mRNA from rat *B*-cell tumor induced by both streptozotocin and nicotinamide. The tumor is not transplantable and not very large (3 to 8 mm), but it contains almost the same amount of proinsulin mRNA as the pancreatic islet of normal rats do. The mRNA was purified from the total nucleic acid fraction by LiCl precipitation, oligo (dT) cellulose column chromatography and sucrose gradient centrifugation (Table 1). A high degree of purity of the final preparation was confirmed by analysis using sucrose gradient centrifugation and polyacrylamide gel electrophoresis in the presence of formamide (9, 10), and by examination of the kinetics of hybridization with complementary DNA (9).

The translation product of the purified proinsulin mRNA in a cell-free protein-synthesizing system of wheat germ was analyzed by SDS-polyacrylamide gel electrophoresis (Fig. 3). The [^{3}H]leucine-labeled trichloroacetic acid-insoluble translation product gave a single peak that was clearly larger than that of proinsulin. The molecular weight of the product was estimated to be approximately 11 500 on the gel. The [^{3}H]leucine-labeled immunoprecipitated translation product was also analyzed by SDS-polyacrylamide gel electrophoresis. This translation product gave a single peak which corresponded to that of the trichloroacetic acid-insoluble translation product (Fig. 3).

To characterize the cell-free product further, the [^{3}H]leucine-labeled immunoprecipitated translation product was digested with Tos-Phe-CH_2Cl-

Table 1. Purification of proinsulin mRNA from streptozotocin-nicotinamide induced rat *B*-cell tumor (9).

Fraction	Nucleic acid	Proinsulin mRNA activity counts min^{-1}		Yield
	μg	μg RNA^{-1}	-fold	%
Total nucleic acid	8560	1450	(1.0)	100
LiCl-precipitable RNA	4880	1940	(1.3)	76
Poly(A)-containing RNA	55.5	120 000	(82.8)	54
1st sucrose gradient	10.2	507 000	(350)	42
2nd sucrose gradient	5.6	525 000	(362)	24

Proinsulin mRNA activity is given as the [^{3}H]leucine incorporated into preproinsulin synthesized in the wheat germ system. Numbers in parentheses give the degree of purification.

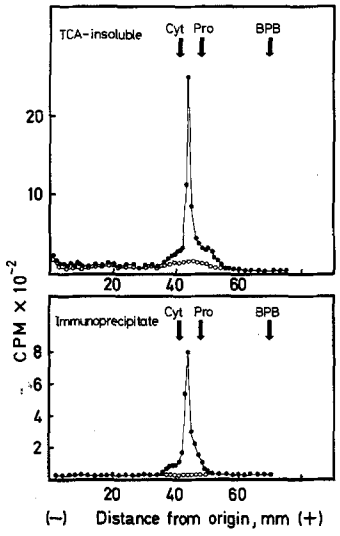

Fig. 3. SDS-polyacrylamide gel electrophoresis of the cell-free translation product of purified proinsulin mRNA (9). ●: assay with proinsulin mRNA; ○: assay without proinsulin mRNA. Arrows indicate relative migration of: Cyt, horse heart cytochrome c (Mr 13 400); Pro, rat proinsulin (Mr 9000); and BPB, bromophenol blue, run on parallel gels.

treated trypsin and the tryptic peptide fragments were characterized by SDS-polyacrylamide gel electrophoresis, and compared with the tryptic peptide fragments from [^{3}H]leucine-labeled rat proinsulin (9). Digestion of rat proinsulin with trypsin yielded two major peptide fragments, which were similar in electrophoretic mobility to rat insulin and C peptide respectively. The two major fragments were also obtained from the trypsin-digested material of the cell-free translation product, but there was one additional peak which was faster in electrophoretic mobility than C peptide. The peak was expected to be a tryptic peptide from the prepeptide portion of preproinsulin. The trypsin-digested cell-free translation product was also analyzed by paper electrophoresis (in 30% formic acid). The two major peaks corresponding to rat insulin and C peptide were also detected.

We therefore deduced that the cell-free translation product corresponded to 'preproinsulin' which was the predominant product from nucleic acid fractions of rat pancreatic islets (1).

It is well known that rat pancreatic islets contain two types of proinsulin, proinsulin I and proinsulin II. The four amino acid residues are different from each other, and it has been reported that although proinsulin I has no methionine residue, proinsulin II has a metionine residue at position 29 in the *B*-chain (11). By the experiment of cyanogen bromide with the cell-free translation product (preproinsulin), preproinsulin was found to consist of two types of preproinsulins, I and II, in approximately equal amounts. This result indicated that the purified proinsulin mRNA consisted of proinsulin I mRNA and proinsulin II mRNA in approximately equal amounts (9).

The molecular weight of proinsulin mRNA was determined to be approximately 200 000, which corresponds to a length of approximately 600 nucleotides (9, 10). As the translatable sequences coding for preproinsulin are 330 nucleotides, proinsulin mRNA is assumed to have approximately 45% of untranslatable sequences (Fig. 4). Recent studies have demonstrated that most eukaryotic mRNAs have unique terminal structures, a capped structure at the 5′ terminus and a poly(A) sequence at the 3′ terminus (12). As shown in Fig. 4, proinsulin mRNA also had the same unique terminal structures (9).

The structures of the proinsulin gene of rats, human and chickens have recently been determined (13–17). In rats, there were two nonallelic proinsulin genes (I and II). The proinsulin II gene contained two introns: a 499 base pair intron interrupting the region encoding the C-peptide, and a 119 base pair intron interrupting the segment encoding the 5′ noncoding region of the mRNA. The proinsulin I gene contained only the smaller intron of a 119 base pair (13, 14). Although the nucleotide sequences of rat proinsulin mRNA have

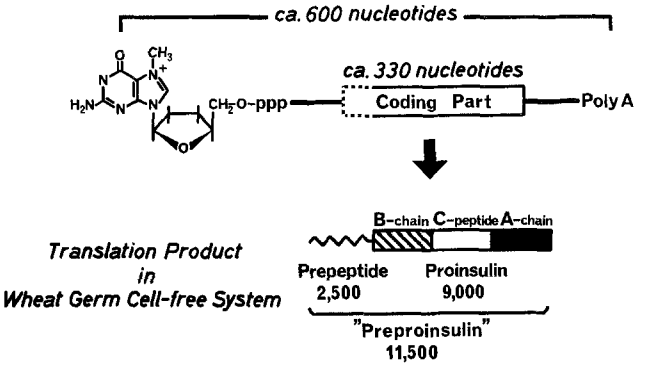

Fig. 4. Schematic structure of proinsulin mRNA and preproinsulin.

already been determined from the poly (A) site to the 47 base-upstream site from the translation initiation site with cloned proinsulin cDNA (8, 18, 19), the 5′ terminal nucleotide sequences of the mRNA have not yet been determined. Therefore, it is still uncertain where the cap site (the putative transcription initiation site) is located and whether or not any other than the above two introns exists in the 5′ noncoding region of the mRNA.

To resolve these questions, we have recently determined the 5′ terminal sequences of rat proinsulin mRNA (20). After removing the cap (m^7G^5ppp-) from the proinsulin mRNA, the 5′ end of the mRNA was labeled with ^{32}P using γ-^{32}P-ATP and T_4 polynucleotide kinase. The 5′ terminal nucleotide sequences were determined by base specific RNase digestions followed by gel electrophoresis, and the 5′ end nucleotide was identified by nuclease P1 digestion followed by thin layer chromatography. The 5′ terminal nucleotide sequences determined were:

$m^7G^5ppp^5m^6AmAxxCUxAGUGA$ ..... proinsulin I mRNA
$m^7G^5ppp^5m^6AmGxxCUxAGUGA$ ..... proinsulin II mRNA

x: not determined; m^6Am: N^6,2′-o-dimethyladenosine

From the 5′ terminal nucleotide sequences, we identified the cap site of the two proinsulin genes as 'A' located in the 176 base pair-upstream site from the translation initiation site of the genes, and concluded that the proinsulin genes contained no intron other than the above two intron (20).

IV. Determination of proinsulin mRNA in pancreatic islets

Isolation of pancreatic islets from the pancreas made it possible to examine the *in vitro* biosynthesis of proinsulin and its conversion to insulin. However, until recently, the synthesis of proinsulin mRNA itself was not studied at all.

Our recent success in the purification and characterization of proinsulin mRNA made possible an approach to the study of insulin biosynthesis at the transcription and translation levels by quantifying proinsulin mRNA in pancreatic islets. In the following section, methods for the determination of proinsulin mRNA are described.

1. Quantification of proinsulin mRNA by using a cell-free protein synthesizing system (10, 21)

It is possible to determine proinsulin mRNA by assaying preproinsulin synthesized in a cell-free protein synthesizing system of wheat germ or reticulocyte. When nucleic acid extracted from islets was incubated at 25 °C in the cell-free system, [^{3}H]leucine was incorporated into protein at a linear rate with the incubation time up to 90 min (2). SDS-polyacrylamide gel electrophoresis of the [^{3}H]-labeled product revealed that the cell-free translation product consisted of preproinsulin (10, 21). The amount of radioactivity incorporated into the preproinsulin fraction was increased proportionally to the amount of nucleic acid added to the cell-free system (10, 21). Furthermore, as mixing of purified proinsulin mRNA of rat *B*-cell tumor with islet nucleic acid resulted in the additive formation of [^{3}H]preproinsulin by the cell-free system, it was confirmed that a trace amount of proinsulin mRNA in nucleic acid extracted from islets was quantitatively translated in the system (10, 21).

2. Quantification of proinsulin mRNA by hybridization with complementary DNA (22, 23)

It is also possible to determine the mRNA in the nucleic acid fraction by hybridization with complementary DNA (cDNA) which is synthesized from purified proinsulin mRNA. The determination method using cDNA is 100 to 1 000 times as sensitive as that using the cell-free protein synthesizing system. As shown in Fig. 5a, trace

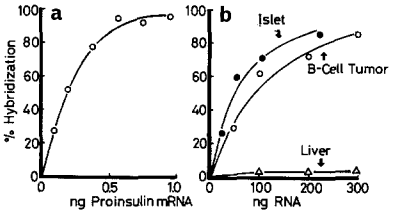

Fig. 5. Quantification of proinsulin mRNA by hybridization with proinsulin cDNA (22, 23).
a: Hybridization of purified proinsulin mRNA with [^{3}H]proinsulin cDNA.
b: Hybridization of the total RNA from rat pancreatic islets (●), rat B-cell tumors induced with streptozotocin and nicotinamide (○) and rat liver (△) with [^{3}H]proinsulin cDNA.

amounts of proinsulin mRNA (0.05–0.5 ng) could be measured by hybridization with proinsulin cDNA. As it is difficult to prepare many islets from rat pancreas, this sensitive hybridization assay is of particular value. To examine the specificity of the hybridization reaction, we prepared total RNA from rat pancreatic islets, rat *B*-cell tumors and rat liver, and examined the hybridization of proinsulin cDNA with total RNA from these tissues (Fig. 5b). From the extent of the hybridization, the proinsulin mRNA content of total RNA from islets and that from *B*-cell tumors were determined to be 0.38% and 0.25%, respectively. Total RNA of rat liver did not hybridize with proinsulin cDNA. These results suggest that the hybridization reaction was highly specific.

3. Preparation of a recombinant DNA containing proinsulin cDNA sequences (24, 25)

A large amount of proinsulin cDNA is necessary for determining newly synthesized proinsulin mRNA in islets. We prepared a recombinant DNA containing proinsulin cDNA sequences. Double-stranded cDNA prepared with the purified proinsulin mRNA as a template was inserted into the *Pst* I site of the plasmid pBR322 by the G-C tailing technique, and the recombinant DNA was used to transform *E. coli* strain χ1776. Transformants containing putative proinsulin cDNA sequences were identified by the colony hybridization method with proinsulin cDNA as a probe. The recombinant plasmid DNA from one positive colony was designated as pBR322 IN. The digestion of pBR322 IN with *Pst* I generated two DNA fragments. One was a large fragment that corresponded to a linear pBR322 and the other was a 340-nucleotide fragment that represented the inserted DNA. Assuming a 20 base-pair-long G-C tail at either end of the double-stranded cDNA, the length of the cloned proinsulin DNA sequences was estimated to be 300 nucleotides. As the size of proinsulin mRNA was estimated to be 600 nucleotides (9, 10), the pBR322 IN appeared to contain approximately 50% of proinsulin mRNA sequences.

The ability of pBR322 IN to hybridize selectively to proinsulin mRNA was examined by a hybridization-translation procedure. The pBR322 IN was linearized with *Eco* R1 and immobilized on a nitrocellulose filter. Total RNA of the *B*-cell tumor was hybridized to the filter-bound pBR322 IN. After unhybridized RNA was washed, hybridized RNA was eluted from the filter and translated in a wheat germ cell-free system, and the translation product was analyzed by an immunoprecipitation procedure with antiserum to bovine insulin. The result indicated that the pBR322 IN selectively hybridized to proinsulin mRNA (24, 25).

V. Regulatory mechanism of proinsulin biosynthesis by glucose

Glucose is known to stimulate proinsulin synthesis in pancreatic islets of rats as well as of many other animals, and the examination of the glucose effect has been one of the main subjects of the study of insulin biosynthesis under physiological and pathological conditions. As described above, recent success in the purification of proinsulin mRNA and its application to the determination of proinsulin mRNA in the islets made it possible to study on the mechanisms of the stimulation effect of glucose on insulin biosynthesis at the molecular level.

1. Translational control – glucose stimulates proinsulin synthesis in pancreatic islets without increasing the amount of proinsulin mRNA

The synthetic processes of proinsulin consist of at least two parts; one is transcription of proinsulin mRNA from the proinsulin gene and the other is the translation of proinsulin mRNA on ribosomes. Which is the target of the glucose effect? If the transcription is affected by glucose, *de novo* synthesis of proinsulin mRNA is induced by glucose, resulting in the accumulation of proinsulin mRNA and the stimulation of proinsulin synthesis. On the other hand, it is also possible the proinsulin induction is regulated at the translation level. In this case, the rate of proinsulin synthesis may not be correlated with the amount of proinsulin mRNA.

In order to clarify this problem, we examined the effect of glucose on the synthesis of proinsulin and on the amount of proinsulin mRNA in isolated islets of rats (10, 21, 22, 26, 27).

As shown in Fig. 6, when the concentration of glucose in the incubation medium was increased from 2.8 mM to 25 mM, the proinsulin synthesis was greatly stimulated (~10-fold) and proinsulin

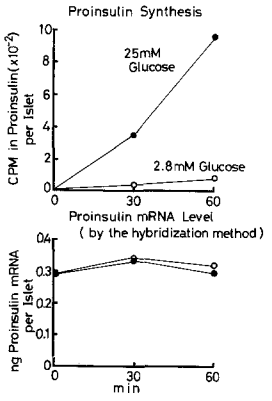

Fig. 6. Induction of proinsulin synthesis by glucose in pancreatic islets and proinsulin mRNA level during the induction (22). Rat pancreatic islets were incubated in the presence of 2.8 mM (○) or 25 mM (●) glucose at 37 °C for the indicated time.

Table 2. Subcellular distribution of proinsulin mRNA in pancreatic islets (22).

Glucose	Membrane-bound polysome	Free polysome	Post-polysomal supernatant
Expt 1			
2.8 mM	0.099 (39.4)	0.033 (13.1)	0.119 (47.5)
25 mM	0.136 (57.9)	0.028 (11.9)	0.071 (30.2)
Expt 2			
2.8 mM	0.109 (41.4)	0.031 (11.8)	0.123 (46.8)
25 mM	0.145 (58.4)	0.036 (14.5)	0.067 (27.1)

One hundred and fifty islets were incubated at 37 °C for 60 min in the presence of 2.8 mM or 25 mM glucose. After addition of 0.1 g wet weight of rat liver as carrier, the incubated islets were homogenized and membrane-bound polysome, free polysome and post-polysomal supernatant were prepared from the post-nuclear supernatant. After extraction of nucleic acid from subcellular fractions, aliquots of the nucleic acids were used for quantification of proinsulin mRNA by hybridization with proinsulin cDNA. Values are expressed as ng proinsulin mRNA per islet. The numbers in parentheses give the percentage of the post-nuclear supernatant.

synthesis in the glucose-stimulated islets comprised 53% of the total protein synthesis. In contrast, the non-proinsulin protein synthesis was only slightly stimulated (~1.3-fold), suggesting that the glucose-stimulation effect on protein synthesis in islets is highly specific to proinsulin synthesis.

When quantitated by the hybridization method, the amount of proinsulin mRNA in the glucose-stimulated (25 mM glucose) or -unstimulated (2.8 mM glucose) islets remained unchanged during the period of proinsulin induction for up to 60 min (Fig. 6). The amount of proinsulin mRNA in islets as determined by a wheat germ cell-free translation assay remained also unchanged during the period of proinsulin induction by glucose (10, 21). Thus, glucose seems to regulate proinsulin synthesis at the translation level (translational control). Proinsulin mRNA, and mRNAs coding for other secretory proteins, have been shown to be translated on the membrane-bound polysome (28). As shown in Table 2, in the examination of the subcellular localization of proinsulin mRNA in the glucose-stimulated or -unstimulated islets, only a small difference between the amount of proinsulin mRNA in both membrane-bound polysome fractions was observed (58% and 40% in the stimulated and unstimulated islets, respectively). These results indicate that proinsulin synthesis is mainly regulated by enhancing the translation efficiency of proinsulin mRNA on the membrane-bound polysome.

However, a significant amount of proinsulin mRNA was also detected in the post-polysomal supernatant fraction; this was more marked in the unstimulated islets. In some eucaryotic cells, some mRNAs have been shown to be present in a post-polysomal supernatant fraction as messenger ribonucleoprotein (mRNP; actin mRNP (29), histone mRNP (30), globin mRNP (31), albumin mRNP (32)). As it has been suggested that the mRNP may be involved in the regulation of the mRNA translation (29, 32), it would be interesting to determine whether proinsulin mRNA is present in the post-polysomal supernatant fraction complexed with protein to form mRNP. To characterize the form of proinsulin mRNA present in the supernatant fraction, the supernatant was fractionated by sucrose gradient centrifugation, and proinsulin mRNA sequence in each gradient fraction were quantified with proinsulin cDNA. The sedimentation profile of proinsulin mRNA sequences in the supernatant fraction exhibited essentially a single major peak in the region of approximately 9 S, which corresponds to the sedimentation value of intact proinsulin mRNA free from protein (22). As the proinsulin mRNA content in total RNA of islets determined by the hybridization method was consistent with that determined by a wheat germ cell-free translation assay, it is reasonable to assume that proinsulin

mRNA detected in the post-polysomal supernatant exists as an intact and translatable form.

As can be seen in Table 2, the amount of proinsulin mRNA in the supernatant fraction was decreased by glucose stimulation. This decrease in proinsulin mRNA in the supernatant fraction was closely correlated with an increase in proinsulin mRNA in the membrane-bound polysome fraction. Therefore, the regulation by glucose of proinsulin synthesis in pancreatic islets is assumed to be achieved also in part by the transfer of proinsulin mRNA in the supernatant to the membrane-bound polysome.

2. Determination of the number of proinsulin mRNA molecules in a B-cell of pancreatic islets

The number of molecules of proinsulin mRNA per rat pancreatic *B*-cell, an insulin producing cell in the islets, was estimated to be approximately 2.7×10^5 from the following additional evidence: i) The molecular weight of rat proinsulin mRNA was estimated to be approximately 2×10^5 (9). ii) The amount of DNA in rat pancreas was shown to be 7.4 ng per cell (33) and that in rat pancreatic islets was found to be 20 ng per islet (unpublished data). iii) The *B*-cells comprised 82% of the total cell populations of rat pancreatic islets (34).

Several investigators have estimated the number of specific mRNA molecules in certain types of cells. For example, the numbers of globin mRNA molecules in a reticulocyte (35) and of ovalbumin mRNA molecules in an oviduct cell (36) were reported to be 1.4 to 1.5×10^5 mRNA per cell, and that figure has been thought to be the highest mRNA content of eucaryotic cells. The number of proinsulin mRNA molecules per rat pancreatic *B*-cell (2.7×10^5 mRNA per cell) estimated in the present paper was comparable to those of globin mRNA in a reticulocyte and of ovalbumin mRNA in an oviduct cell.

3. Effect of glucose on proinsulin gene transcription in pancreatic islets

As described above, the proinsulin synthesis was mainly regulated at the translation level. However, the transcriptional regulation of the proinsulin gene expression by glucose in pancreatic islets has been completely unknown, although the structure of the proinsulin gene has recently been determined (13–17).

Recently, we determined the transcriptional activity of the proinsulin gene in pancreatic islets using a recombinant DNA containing proinsulin cDNA sequences as a probe. Islets were incubated at 37 °C for 20, 40 and 60 min in a medium containing 2.8 mM or 25 mM glucose and [^{3}H]uridine. [^{3}H]Uridine was incorporated at a linear rate into total RNA during the period of incubation up to 60 min (24, 25). The total RNA synthesis was only slightly stimulated by glucose (1.3 ~ 1.4-fold). Newly synthesized proinsulin mRNA sequences in the islets incubated for 60 min were determined by a filter hybridization method with a pBR322 IN bound filter. In contrast to the total RNA synthesis, the proinsulin mRNA sequences synthesis was stimulated 1.9-fold by glucose (Table 3). This result indicated that the glucose-stimulation effect on the RNA synthesis in islets was slightly but significantly specific to the proinsulin mRNA sequences synthesis. The effect of α-amanitin, an RNA polymerase II inhibitor, on the total RNA and proinsulin mRNA sequences synthesis was also examined. Although the total RNA synthesis was only slightly inhibited by α-amanitin, the proinsulin mRNA sequences synthesis was strongly inhibited.

Table 3. Effect of glucose on synthesis of total RNA and proinsulin mRNA sequences in pancreatic islets (24, 25).

Glucose	Total RNA synthesis	Proinsulin mRNA sequences synthesis
	^{3}H d.p.m. per islet	
2.8 mM	3.57×10^4	24.4
2.8 mM (+ 40 μg ml^{-1} α-amanitin)	3.08×10^4	4.9
25 mM	5.14×10^4	46.5
25 mM (+ 40 μg ml^{-1} α-amanitin)	4.49×10^4	11.4

One hundred and sixty islets were incubated at 37 °C for 60 min in 100 μl of Dulbecco's modified Eagle's medium containing 2.8 mM glucose, no or 4 μg of α-amanitin and 20% rat serum, and incubated further at 37 °C for 60 min by adding 10 μl of Dulbecco's modified Eagle's medium containing 200 μCi ^{3}H-uridine and 2.8 mM or 248 mM glucose (the final concentration was 25 mM) glucose. After the incubation, nucleic acid was prepared from the incubated islets, and aliquots of the nucleic acid were used for quantification of proinsulin mRNA sequences by a filter hybridization method with a pBR 322 IN-filter (25).

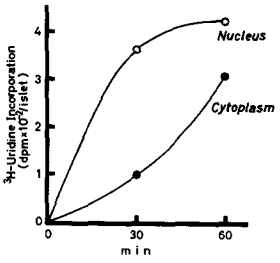

Fig. 7. Kinetics of newly synthesized proinsulin mRNA sequences accumulation in the nucleus and cytoplasm of pancreatic islets (24, 25).

The accumulation kinetics of newly synthesized proinsulin mRNA sequences in the nucleus and cytoplasm of pancreatic islets were also examined as shown in Fig. 7. After islets were incubated at 37 °C for 30 min and 60 min in a medium containing 25 mM glucose and [^{3}H]uridine, the islets were homogenized, and the nucleus and cytoplasm were prepared. RNA extracted from the subcellular fractions was used for determination of newly synthesized proinsulin mRNA sequences by the filter hybridization method. A significant amount of newly synthesized proinsulin mRNA sequences was detected in the cytoplasm of islets incubated for 30 min. This result indicated that proinsulin mRNA sequences synthesized in the nucleus were transported into the cytoplasm within 30 min. In the cytoplasm of islets incubated for 60 min, nearly half of the newly synthesized proinsulin mRNA sequences were recovered.

4. Biological significance of translational control of proinsulin synthesis by glucose

As summarized in Fig. 8, the glucose induction of proinsulin synthesis in pancreatic islets was mainly achieved by enhancement of the translation efficiency of proinsulin mRNA on the membrane-bound polysome and in part by the transfer of proinsulin mRNA in the cytoplasm to the membrane-bound polysome.

As glucose only slightly stimulated total RNA synthesis in pancreatic islets, the great increase of translation efficiency of proinsulin mRNA by glucose seemed not to be achieved by the stimulation of rRNA or tRNA synthesis in the islets. Since the glucose-stimulation effect on protein synthesis in the islets was highly specific to the proinsulin

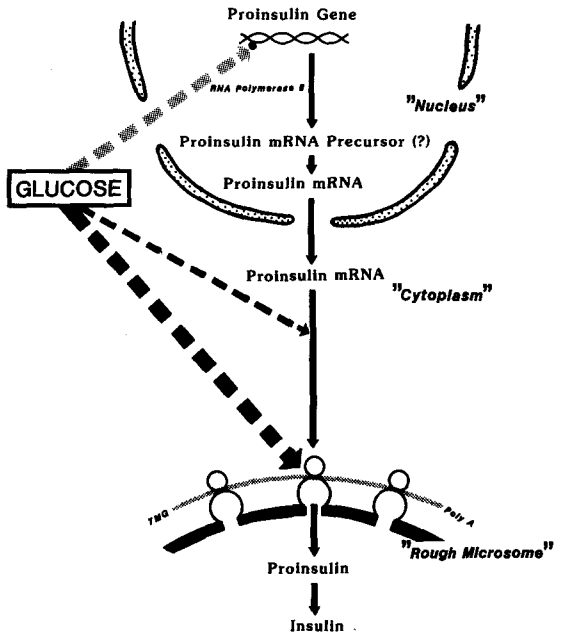

Fig. 8. Schematic representation of the glucose effect on proinsulin synthesis in pancreatic islet B-cells.

Wide dashed line: Enhancement of the translation efficiency of proinsulin mRNA on the membrane-bound polysome.

Narrow dashed line: Transfer of proinsulin mRNA in the cytoplasm to the membrane-bound polysome.

Shaded dashed line: Slight stimulation of the proinsulin gene transcription.

synthesis, it may be expected that there is some initiation factor specific to the proinsulin mRNA translation.

Although the proinsulin mRNA content in the islets was reduced in both spontaneously diabetic and fasted rats (37), the proinsulin synthesizing ability *in vitro* of islets isolated from these rats was 2- to 3-fold higher than that of the control (37). This indicates that even under the abnormal conditions in the diabetic or fasted rats, *B*-cells of pancreatic islets may maintain or elevate their ability to synthesize proinsulin by increasing the efficiency of translation of proinsulin mRNA. It is therefore suggested that the translational control of proinsulin biosynthesis, which was originally observed in pancreatic islets of normal rats, may play a significant role in pathological conditions.

Although the proinsulin synthesis was stimulated 10-fold, the synthesis of proinsulin mRNA sequences was stimulated only 1.9-fold. As the

proinsulin mRNA synthesis is strongly inhibited by α-amanitin, the proinsulin gene seemed to be transcribed by RNA polymerase II in pancreatic islets. Although glucose stimulated the synthesis of proinsulin mRNA sequences, and although the newly synthesized proinsulin mRNA in the nucleus was rapidly transported to the cytoplasm, the proinsulin mRNA level in pancreatic islets remained unchanged during the period of glucose stimulation. The role of glucose stimulation at the transcription level is still uncertain, but one possible explanation may be that the stimulation at the transcription level plays a part in the maintenance of the proinsulin mRNA level in pancreatic islets.

The proinsulin gene expression, therefore, was regulated at both the translation level and the transcription level by glucose, although the major regulation site was at the translation level. As the regulation of gene expressions in eucaryotic cells as well as in procaryotic cells has been attributed mainly to regulation at the transcription level, the regulation of proinsulin gene expression by glucose in pancreatic islets described here seems to be a unique regulation of gene expression in eucaryotic cells. As insulin regulates the glucose level in the blood, the rates of both insulin secretion and proinsulin synthesis are required to change rapidly according to the change in the blood glucose level. This regulation ('Translational Control') of proinsulin gene expression may be a means of ensuring rapid regulation of proinsulin synthesis by glucose.

Insulin is known to be only one hypoglycemic factor in animals whereas there are many hyperglycemic factors such as glucagon, catecholamines, ACTH, glucocorticoids, growth hormone, and thyroid hormone. Therefore, insulin competes with many kinds of hyperglycemic factors to maintain the blood sugar level. From this point of view, it seems reasonable to speculate that the translational control of insulin biosynthesis together with the high content of proinsulin mRNA in pancreatic *B*-cells may contribute a great deal towards the homeostasis of the blood glucose level, which is so vital to higher animals.

VI. Regulation of insulin biosynthesis by kynurenine metabolites

As described above, glucose is the primary factor which regulates proinsulin synthesis in pancreatic islets. However, in addition to glucose, there may be other factors such as hormones and various biogenic metabolites which also have some regulatory effect on proinsulin synthesis.

We have already shown that kynurenine metabolites belonging to quinoline derivatives, such as quinaldic acid, 8-hydroxyquinaldic acid, xanthurenic acid and kynurenic acid, cause insulin release from the isolated pancreatic islets but inhibit glucose-induced insulin release (38–41). In the following section, a possible regulation of proinsulin synthesis by kynurenine metabolites is discussed.

1. Effect of kynurenine metabolites on proinsulin synthesis

During the past decade, there have been many papers reporting an increased level of kynurenine metabolites, such as kynurenic acid and xanthurenic acid, in urine of steroid-treated patients, women using contraceptive steroid, and pregnant women (42–45). It has also been reported that in such cases a diabetic state is generally observed (46–48). In animal experiments, xanthurenic acid was reported to be diabetogenic (49). However, there has been no available information concerning the effect of kynurenine metabolites on insulin release and/or synthesis in pancreatic islets.

As a continuation of our study on insulin release (38–41), we have investigated the effect of various kynurenine metabolites on insulin biosynthesis in isolated pancreatic islets of rats (50–52). When islets were incubated with [^{3}H]leucine for 60 min, most of the radioactivity appeared as a peak which corresponded in its elution position to that for

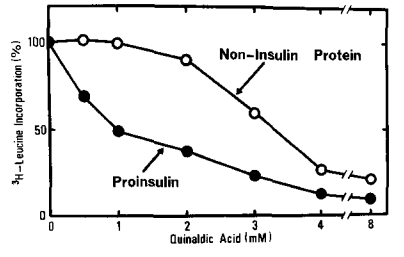

Fig. 9. Effect of quinaldic acid on [^{3}H]leucine incorporation into proinsulin and non-insulin proteins (52). Results are expressed in % of the corresponding total radioactivity of the control without quinaldic acid.

authentic rat proinsulin (52). Therefore, in order to determine the effect of quinaldic acid on proinsulin synthesis, islets were incubated for 60 min in 20 mM glucose in the presence of various concentrations of quinaldic acid. It may be seen in Fig. 9 that addition of 0.5, 0.1 and 2.0 mM quinaldic acid to the incubation medium decreased the rate of [^{3}H]leucine incorporation into the proinsulin fraction to 70, 50 and 38%, respectively, of the control value. In contrast, at the same concentrations of quinaldic acid, the incorporation into non-insulin proteins was almost unaffected. Although higher concentrations of quinaldic acid (3–8 mM) were inhibitory on both proinsulin and non-insulin protein synthesis, some difference between the sensitivity of proinsulin synthesis and non-insulin protein synthesis to the compound was still observed.

Effects of various tryptophan metabolites on proinsulin synthesis are summarized in Table 4. 8-Hydroxyquinaldic acid was also found to inhibit proinsulin synthesis significantly. Xanthurenic acid and kynurenic acid, though far less effective than quinaldic acid, inhibited proinsulin synthesis without affecting the synthesis of non-insulin proteins. L-Tryptophan and L-kynurenine slightly inhibited proinsulin synthesis as well as the synthesis of non-insulin proteins. 3-Hydroxyanthranilic acid and quinolinic acid had no effect on protein synthesis in the islets.

Table 4. Effects of tryptophan metabolites on proinsulin synthesis.

Additions	[^{3}H]leucine incorporation (%)	
	Proinsulin	Non-insulin proteins
20 mM glucose	100	100
+ quinaldic acid	42	97
+ 8-hydroxyquinaldic acid	31	90
+ xanthurenic acid	75	98
+ kynurenic acid	79	102
+ L-tryptophan	88	89
+ L-kynurenine	90	92
+ 3-hydroxyanthranilic acid	106	102
+ quinolinic acid	101	98

Islets were incubated with [^{3}H]leucine for 60 min in the presence of 20 mM glucose and 1 mM tryptophan metabolite as indicated. Total radioactivity of proinsulin fraction and of non-insulin proteins was measured (52). Results are expressed in % of the corresponding total radioactivity of the control without tryptophan metabolite.

2. Effect of kynurenine metabolites on the conversion of proinsulin to insulin

In order to examine the effect of quinaldic acid on the conversion of proinsulin to insulin, an alternative approach was chosen (52). Islets were pre-labeled with [^{3}H]leucine in 20 mM glucose for 60 min and after removing free [^{3}H]leucine by washing, they were incubated further. After incubation for 60 or 120 min at 37°C, the proinsulin which had been labeled with [^{3}H]leucine was converted to insulin. The half-time for the conversion was approximately 1 h, as previously reported by Steiner *et al.* (53). Addition of 2 mM quinaldic acid to the incubation system preincubated with [^{3}H]leucine caused no change in the conversion pattern of proinsulin to insulin. None of the kynurenine metabolites examined was found to exert any discernible effect upon the conversion of proinsulin to insulin (52).

Further evidence for the conversion of proinsulin to insulin under the condition of reduced proinsulin synthesis was obtained from steady-state experiments in which islets were incubated in 20 mM glucose for 4 h in the presence of various concentrations of quinaldic acid. Increasing concentrations of quinaldic acid significantly reduced the [^{3}H]leucine incorporation into both proinsulin and insulin, and the ratio of the total count incorporated in the insulin fraction to that in the proinsulin fraction remained close to the ratio of the control without quinaldic acid (52).

3. Pathological significance of regulation of proinsulin synthesis by kynurenine metabolites

On the basis of experimental results obtained *in vitro* on both insulin release (38–41) and proinsulin synthesis (50–52), it is suggested that kynurenine metabolites with a quinoline ring, such as quinaldic acid, 8-hydroxyquinaldic acid, kynurenic acid and xanthurenic acid, have dual functions against *B*-cells of pancreatic islets (Fig. 10): 1) by inducing insulin release from preformed B-granules, and 2) by inhibiting the glucose-induced proinsulin synthesis. These *in vitro* effects were observed in *in vivo* experiments (54). Therefore it is reasonable to assume that when an excess amount of kynurenine metabolites is produced, the insulin level in the *B*-cell is decreased after a transient hypersecretion

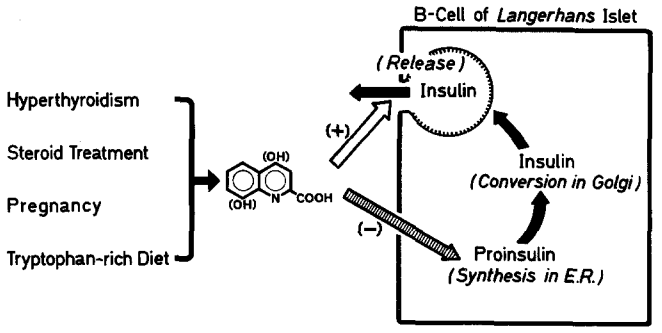

Fig. 10. Effects of kynurenine metabolites with a quinoline ring on the B-cell of pancreatic islets.

(+): The kynurenine metabolites induce insulin release from preformed B-granules.

(–): The kynurenine metabolites inhibit proinsulin synthesis.

of insulin. As shown in Fig. 10, it is further suggested that secondary diabetes observed in patients of hyperthyroidism, in steroid-treated patients, or in pregnant women may be ascribed to the hyperproduction of kynurenine metabolites.

Quinaldic acid and 8-hydroxyquinaldic acid have been shown to be metabolites of kynurenic acid and xanthurenic acid, respectively (55–57). It has been shown that kynurenic acid and xanthurenic acid are increased in urine of steroid-treated patients, women using contraceptive steroid, pregnant women (42–45), and hyperthyroid rats (58). Patients receiving combined progestogen-estrogen preparations excreted 1 000–3 600 μmol of xanthurenic acid in 8 h following an oral load of tryptophan (43). Khattab *et al.* (59) reported an approximately 3-fold increase in the excretion of kynurenic acid and xanthurenic acid in diabetic patients. Normal human subjects excreted only about 5 μmol of quinaldic acid / day, but ingestion of kynurenic acid by man led to the excretion of quinaldic acid sufficient to account for as much as 30% of the ingested kynurenic acid (55). Although no report on the concentrations of quinaldic acid and 8-hydroxyquinaldic acid in plasma or in *B*-cells of pancreatic islets has been presented, it appears reasonable to assume that an increased formation of quinaldic acid and 8-hydroxyquinaldic acid occurs in the above mentioned pathological conditions.

VII. A new aspect to experimental diabetes - Streptozotocin and alloxan induce diabetes through a common mechanism

Approximately half of NAD in mammalian cells is synthesized from tryptophan via kynurenine (40). When kynurenine is metabolized to compounds with a quinoline ring such as quinaldic acid and 8-hydroxyquinaldic acid, NAD synthesis is decreased. As described in the preceding section, it is suggested that excessive formation of the quinoline compounds due to a disorder in tryptphan metabolism is involved in the pathogenesis of a certain diabetic state.

On the other hand, it is known that streptozotocin, a typical diabetogenic agent, depresses NAD content in pancreatic islets and inhibits proinsulin synthesis (60, 61). As nicotinamide prevents these effects of streptozotocin (62–64), it has been so far considered that streptozotocin impairs NAD synthesis in islets to cause diabetes (65, 66). However, Hinz *et al.* suggested that the streptozotocin-induced depression of mouse islet NAD content may be ascribed to increased NAD degradation (67). More recently, we found that streptozotocin as well as alloxan, another typical diabetogenic agent, depress the islet NAD level by increasing the activity of islet nuclear poly(ADP-ribose) synthetase, an NAD degradating enzyme (68, 69). This finding may provide an important clue in understanding the mechanism of action on the islet *B*-cell of diabetogenic substances.

1. Both streptozotocin and alloxan increase islet nuclear poly(ADP-ribose) synthetase activity and depress islet NAD content

Alloxan and streptozotocin exhibit the most potent diabetogenicity and have been most widely used for induction of experimental diabetes. Although considerable research has been carried out on the diabetogenicity of alloxan and streptozotocin, the mechanism of action on the islet *B*-cell of the two substances is still not understood.

Quite recently, both alloxan and streptozotocin have been found to increase the islet nuclear poly(ADP-ribose) synthetase activity (68, 69). Poly(ADP-ribose) synthetase is tightly associated with chromatin and catalyzes the synthesis of acceptor-bound poly(ADP-ribose) from the ADP-

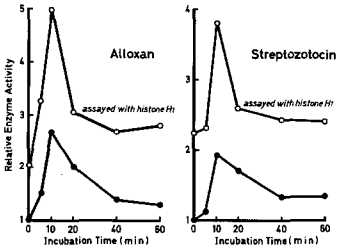

Fig. 11. Effect of alloxan or streptozotocin on islet poly(ADP-ribose) synthetase activity (69). Batches of 300 islets were incubated in the presence of 1 mM alloxan or 2 mM streptozotocin at 37 °C for the indicated time. After incubation, nuclear fractions were prepared and the poly(ADP-ribose) synthetase was assayed in the presence (○) or absence (●) of calf thymus histone H1 (0.2 μg/μl) as described previously (68). All activities were related to that assayed without histone H1 at 0 min (11 pmoles poly(ADP-ribose) synthesized/10 min/μg of islet nuclear DNA).

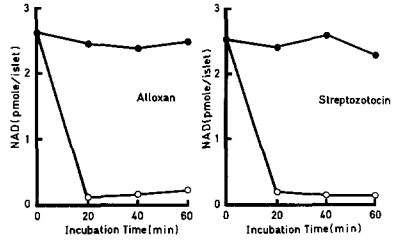

Fig. 12. Effect of alloxan or streptozotocin on islet NAD level (69). Batches of 100 islets were incubated in the presence (○) or absence (●) of 1 mM alloxan or 2 mM streptozotocin at 37 °C for the indicated time. The NAD content of incubated islets was assayed as previously described (68).

ribosyl moiety of NAD (70, 71). It has been highly purified from rat liver (72) and calf thymus (73–75). The biological significance of poly ADP-ribosylation is not clear but poly(ADP-ribose) synthetase has been suggested to exert a major role in NAD degradation in many mammalian cells (76, 77). The presence of poly(ADP-ribose) synthetase has been clearly demonstrated in rat pancreatic islet nuclei (68). Pancreatic islets of rats were incubated with alloxan or streptozotocin for 0–60 min, and the nuclear poly(ADP-ribose) synthetase activity was measured. As shown in Fig. 11, streptozotocin induced about a 2-fold increase in the enzyme activity with a peak at 10 min. A quite similar time course for the enzyme activity was observed in the nuclear fraction of alloxantreated islets. Addition of histone H1, a main acceptor protein of poly(ADP-ribose) (78), to the reaction mixture for assaying poly(ADP-ribose) synthetase resulted in a significant amplification of the streptozotocin- or alloxan-induced increase in the enzyme activity. These results show that both alloxan and streptozotocin significantly increase islet poly(ADP-ribose) synthetase activity with a quite similar kinetics.

It has been found that NAD levels of liver and islets are decreased with streptozotocin, while the liver NAD was not changed following alloxan treatment (79). Since liver NAD was not altered by alloxan it has been suggested that islet NAD also may not be changed (80). We examined the effect of alloxan on the NAD level in isolated pancreatic islets of rats. It was found that the islet NAD content is lowered by alloxan in a dose-dependent manner. Moreover, a striking temporal correlation is found between the time-course of increase in poly(ADP-ribose) synthetase activity and that of decrease in islet NAD level after treatment with either alloxan or streptozotocin (Fig. 12), indicating that rapid islet NAD reduction induced by the substances may be ascribed to enhanced poly ADP-ribosylation mediated by rapid increase in the poly(ADP-ribose) synthetase activity.

2. Inhibitors of islet poly(ADP-ribose) synthetase protect against islet NAD depletion as well as depression of proinsulin synthesis induced by either streptozotocin or alloxan

Nicotinamide as well as picolinamide (2-pyridine-carboxylic acid amide) was found to be a strong inhibitor of poly(ADP-ribose) synthetase of nuclei from rat pancreatic islet cells, and the inhibitors protected against streptozotocin-induced depression of proinsulin synthesis as well as against streptozotocin-induced reduction of NAD content (68). As summarized in Table 5 and 6, the alloxan-induced NAD depletion was found to be completely abolished by the inhibitors and the decrease in proinsulin synthesis of alloxan-treated islets was reversed by the coexistence of either nicotinamide or picolinamide. These results suggest that alloxan as well as streptozotocin acts on the islet *B*-cell to impair proinsulin synthesis, a major function of the *B*-cell, through a common mechanism mediated by poly ADP-ribosylation.

Table 5. Effect of nicotinamide or picolinamide on streptozotocin- or alloxan-induced depletion of islet NAD content (69).

Addition	Islet NAD content pmole/islet	(%)
none	2.84	(100)
1 mM alloxan	0.52	(18)
1 mM alloxan & 2 mM nicotinamide	2.94	(104)
1 mM alloxan & 2 mM picolinamide	2.81	(99)
2 mM streptozotocin	0.34	(12)
2 mM streptozotocin & 2 mM nicotinamide	2.91	(102)
2 mM streptozotocin & 2 mM picolinamide	2.93	(103)
2 mM nicotinamide	3.19	(112)
2 mM picolinamide	3.09	(109)

Batches of 100 islets were incubated at 37 °C for 20 min under the condition indicated, and the NAD content of incubated islets was assayed as previously described (68). The numbers in parentheses give the percentage of the control without diabetogenic agents.

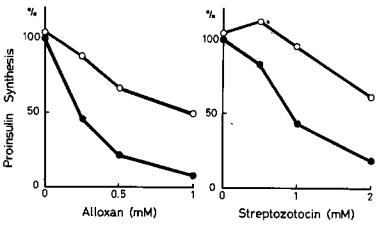

Fig. 13. Effect of glucose on alloxan-induced or streptozotocin-induced inhibition of proinsulin synthesis. Islets were exposed to 0–1 mM alloxan or 0–2 mM streptozotocin for 5 min in the presence of 2.8 mM glucose (●) or 20 mM glucose (○), and then incubated for 60 min in the presence of [^{3}H]leucine and 20 mM glucose. Proinsulin synthesis was determined as described in Table 6. Test values are expressed as the percentage of the control incubated for 5 min in the presence of 2.8 mM glucose and then with [^{3}H]leucine and 20 mM glucose for 60 min ([^{3}H]proinsulin synthesized 3428 cpm/60 min/ islet). (Yamamoto, H. & Okamoto, H., in preparation).

Table 6. Effect of nicotinamide or picolinamide on streptozotocin-or alloxan-induced depression of proinsulin synthesis (69).

Addition	^{3}H-Proinsulin synthesized cpm/60 min/islet	(%)
[Expt 1]		
none	3119	(100)
0.5 mM alloxan	1466	(47)
0.5 mM alloxan & 2 mM nicotinamide	3084	(99)
0.5 mM alloxan & 2 mM picolinamide	2277	(73)
[Expt 2]		
none	3410	(100)
1 mM alloxan	307	(9)
1 mM alloxan & 2 mM nicotinamide	1194	(35)
1 mM alloxan & 2 mM picolinamide	921	(27)
[Expt 3]		
none	3302	(100)
1 mM streptozotocin	2041	(62)
1 mM streptozotocin & 2 mM nicotinamide	2995	(91)
1 mM streptozotocin & 2 mM picolinamide	2942	(89)
2 mM streptozotocin	1155	(35)
2 mM streptozotocin & 2 mM nicotinamide	2760	(84)
2 mMstreptozotocin & 2 mM picolinamide	2486	(71)

Batches of 30 islets were incubated at 37 °C for 60 min in the presence of ^{3}H-leucine under the condition indicated, and the amount of proinsulin synthesized was determined as previously described (22, 68). The numbers in parentheses give the percentage of the control without diabetogenic agents.

3. High glucose protects against inhibition of proinsulin synthesis by both streptozotocin and alloxan

A protecting effect of glucose against the action of alloxan has been shown both *in vivo* (81) and *in vitro* (82), whereas the action of streptozotocin has been described not to be affected by glucose (61, 62). However, our recent study also revealed that the inhibitory effects on proinsulin synthesis not only of alloxan but also of streptozotocin are significantly lessened by glucose. As shown in Fig. 13, proinsulin synthesis in pancreatic islets was inhibited in a dose-dependent manner by either alloxan or streptozotocin. When the glucose concentration in the medium in which islets were exposed to the chemicals was raised from 2.8 mM to 20 mM, proinsulin synthesis was found to be less inhibited by either alloxan or streptozotocin at every concentration of the agent added. Further, it was shown that 0.23 millimoles of alloxan and 0.94 millimoles of streptozotocin are about equivalent with regard to inhibition of proinsulin synthesis in rat islets. The molar equivalence was compatible with those estimated in the studies using whole animals (83).

4. Some aspects of the basic mechanisms of development of insulin-dependent diabetes and B-cell tumors

Since the two highly *B*-cytotoxic compounds, alloxan and streptozotocin, are structually different compounds, their mechanisms of action on islet *B*-cells are of special importance in understanding the pathogenesis of insulin-dependent diabetes

which is thought to be caused by many different factors. From the evidence available it has been considered that the two substances do not act in an identical way, though some observations suggest great similarities between particular pathogenic reactions (80, 84). Such similarities are the protecting effect of nicotinamide against the induction of diabetes and the chromatin clumping in *B*-cells by either drug (80).

Our recent study using isolated pancreatic islets of rats have demonstrated that both alloxan and streptozotocin increase the poly(ADP-ribose) synthetase activity to reduce the NAD level and inhibit proinsulin synthesis. Inhibitors of islet nuclear poly(ADP-ribose) synthetase were found to prevent the reduction of NAD level and the inhibition of proinsulin synthesis. From these findings, we propose that the actions of alloxan and streptozotocin converge into a common pathway to stimulate poly(ADP-ribose) synthetase, to depress NAD level, and to inhibit proinsulin synthesis in pancreatic islets (Fig. 14). Therefore, it is reasonable to assume that alloxan and streptozotocin cause an increased flux from NAD through poly(ADP-ribose) by increasing poly(ADP-ribose) synthetase activity to depress islet NAD level and inhibit islet cell functions, including proinsulin synthesis. Inhibitors of poly(ADP-ribose) synthetase may prevent the diabetogenic action of alloxan and streptozotocin by maintaining the intracellular NAD level. In this respect, it should now be added that not only nicotinamide and picolinamide but also other species of poly(ADP-ribose) synthetase inhibitors, such as 3-aminobenzamide, 3-nitrobenzamide, 3-methoxybenzamide and pyrazinamide, are found to protect against streptozotocin-induced depression of proinsulin synthesis (Yamamoto, H. & Okamoto, H., unpublished data). The protection against alloxan action on islets by methylxanthines (85) may also belong to this category, because methylxanthines also have been reported to be poly(ADP-ribose) synthetase inhibitors (86, 87).

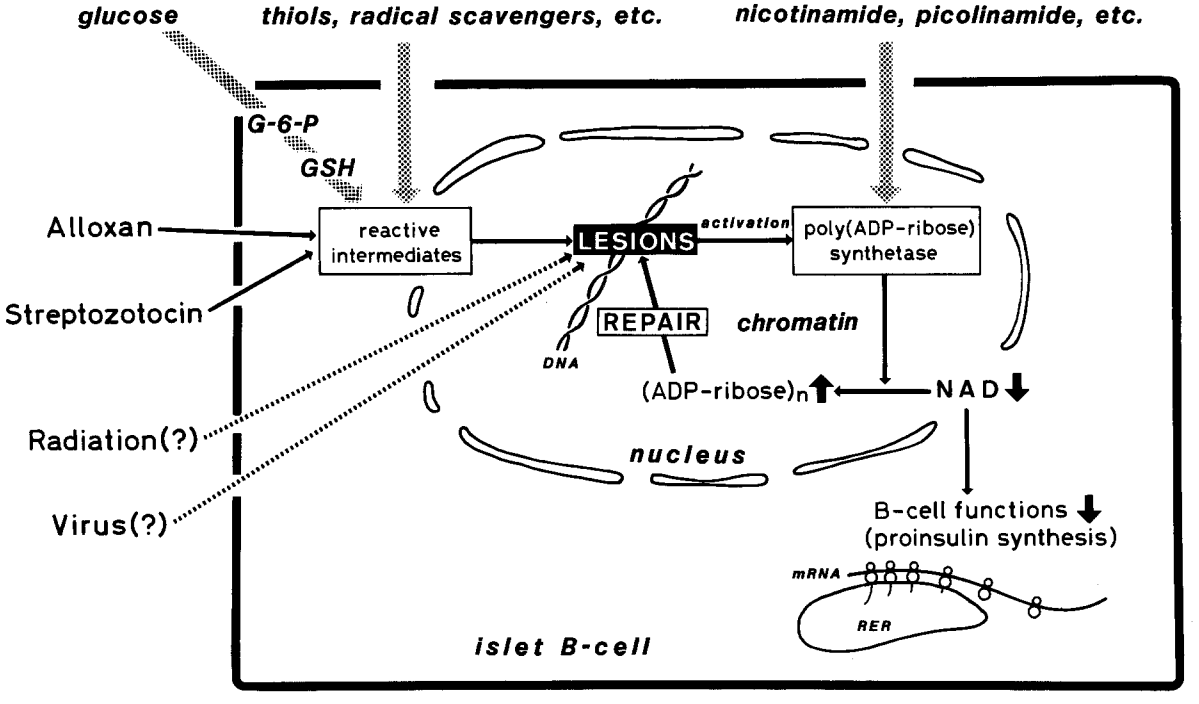

Fig. 14. Proposed mechanisms of action of streptozotocin and alloxan on pancreatic B-cells. As indicated by the shaded lines, the protection by poly(ADP-ribose) synthetase inhibitors such as nicotinamide and picolinamide against the alloxan- or streptozotocin-induced depression of NAD level and of proinsulin synthesis may be due to the blockage of the activated enzyme activity, and thiols and radical scavengers may protect against the diabetogenic acition of alloxan and streptozotocin by inactivating reactive intermediates generated from the diabetogenic agents, and high glucose may prevent the action of the agents by increasing the level of reduced glutathione (GSH), which is generated via glucose 6-phosphate(G-6-p) and NADPH.

The effect on the poly(ADP-ribose) synthetase activity and on the NAD level suggest that the common step occurs rapidly after alloxan and streptozotocin interact with the *B*-cell. In fact, damage to the nuclei of *B*-cells evidenced by chromatin clumping has been observed within 15 min after treatment (80, 88). The activity of poly(ADP-ribose) synthetase is known to be increased under the conditions that cause DNA damages. These conditions include exposure to chemical carcinogens (89–91), nuclease treatment (92, 93) and UV- or γ-irradiation (91, 94). Ohgushi *et al.* recently described the direct evidence that poly(ADP-ribose) synthetase purified from bovine thymus is activated only when the enzyme is bound to nicked or fragmented DNA (95). These observations lead to the proposal that alloxan- or streptozotocin-induced increase in activity of islet nuclear poly(ADP-ribose) synthetase may be triggered by DNA lesions caused by treatment with these chemicals (Fig. 14). More recently, we found that alloxan and streptozotocin cause DNA strand breaks in pancreatic islet cells (Yamamoto, H. & Okamoto, H., in preparation).

Thiols and radical scavengers are known to compose another family of chemical substances that can protect against the diabetogenic action of alloxan (96–98). Holmgren and Lyckeborg suggested that hydroxyl radicals generated from alloxan may mediate the diabetogenic action of alloxan (99). Independently, Robbins *et al.* recently found that the diabetogenic action of streptozotocin is also abolished by superoxide dismutase, a potent radical scavenger (100). Therefore, as shown in Fig. 14, it may be assumable that alloxan and streptozotocin are metabolized in islet *B*-cells to yield free radicals or electrophilic intermediates, and that such reactive metabolites, instead of the agents themselves, damage islet cell DNA to activate the chromatin-bound poly(ADP-ribose) synthetase.

The present study also demonstrated that the high concentration of glucose protects against depression of proinsulin synthesis induced by both alloxan and streptozotocin. It has already been reported that the ratio of reduced glutathione/oxidized glutathione in the islets is increased by the administration of glucose (101). Therefore, glucose seems to prevent the action of the agents by increasing the endogenous thiol level, though some ideas that have been proposed concerning the mechanism of the protection by glucose against alloxan action are not negligible (80, 102).

The increase in islet poly(ADP-ribose) synthetase activity induced by streptozotocin or alloxan was shown to result in a significant depletion of islet NAD. Decrease in intracellular NAD content to such an unphysiological level may severely affect islet cell functions including proinsulin synthesis. That NAD metabolism may play an essential role in proinsulin synthesis is also shown by another of our experiments using rats maintained on a tryptophan-niacin deficient diet. Islets isolated from these rats contained less NAD than those from the control and exhibited the diminished proinsulin synthetic ability (Yamamoto, H. & Okamoto, H., unpublished data).

We quantitated proinsulin mRNA sequences in islets treated with streptozotocin in order to test which step of the proinsulin biosynthetic processes is mainly involved in the streptozotocin-induced inhibition of proinsulin synthesis. Incubation of islets in the presence of streptozotocin did not cause any change in the islet proinsulin mRNA level, while proinsulin synthesis of the islets was markedly depressed (Yamamoto, H. & Okamoto, H., unpublished data). Therefore, it is suggested that the translational process of proinsulin synthesis may be mainly affected in islets whose NAD levels are decreased by treatment with streptozotocin or alloxan.

Regardless of the specific details, the recognition that the two highly B-cytotoxic compounds, alloxan and streptozotocin, act at least in part through a specific biochemical pathway is a prerequisite to understanding the mechanism of action of the diabetogenic compounds and the pathogenesis of *Diabetes Mellitus*. Thus, as summarized in Fig. 14, not only the chemical compounds but also physical factors like irradiation or certain viruses, which are known to stimulate poly(ADP-ribose) synthetase (91, 94, 103), may be expected to be etiological factors of insulin-dependent diabetes.

The present study has dealt with the mechanism of the diabetogenic action of alloxan and streptozotocin. The available evidence, however, might also explain the biochemical basis of *B*-cell tumor development. It is well known that a single injection of streptozotocin successfully induces pancreatic *B*-cell tumors when combined with nicotinamide

(104). As above described, streptozotocin may cause damage of pancreatic *B*-cell DNA, and poly(ADP-ribose) synthetase inhibitors may serve for the persistence of such DNA lesions by blocking the poly ADP-ribosylation which is suggested to be closely coupled with DNA repair (91, 105, 106). In the coexistence of poly(ADP-ribose) synthetase inhibitors, islet *B*-cells are protected against the diabetogenic action of streptozotocin and may survive with such damage within their own genetic apparatus. *B*-cell tumors presumably develop from some of them. This idea proposes a possible *B*-cell oncogenicity of alloxan because the mode of action of alloxan is found to be identical to that of streptozotocin in our present study. In fact, Kazumi *et al.* recently reported islet cell tumors of the pancreas found in rats given alloxan and nicotinamide (107).

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