Diabetologia 12, 471–481 (1976)

Diabetologia

Effects of Streptozotocin in vitro on Proinsulin Biosynthesis, Insulin Release and ATP Content of Isolated Rat Islets of Langerhans*

A. Maldonato**, P.A. Trueheart***, A.E. Renold and G.W.G. Sharp

Institut de Biochimie Clinique, Université de Genève, Genève, Switzerland

Summary. Proinsulin synthesis, insulin release and intracellular ATP concentrations were measured in isolated rat islets of Langerhans under control conditions of in vitro incubation and after treatment with several concentrations of streptozotocin for different periods of time. It was found that streptozotocin inhibited proinsulin synthesis, as well as insulin release, in a time and concentration dependent manner. The characteristics of the inhibition of these two processes were similar in general terms, but one dissimilarity was noted, i.e. after 60 min exposure to a high concentration of streptozotocin, proinsulin synthesis was inhibited more than insulin release. ATP content was reduced by high concentrations of streptozotocin, but it was found that proinsulin synthesis and insulin release could be inhibited without any effect on ATP content by a low (0.22 mM) concentration of streptozotocin. The effect of streptozotocin on proinsulin synthesis was judged to be the result of a target specificity for the B-cell rather than a specific effect on proinsulin relative to total protein synthesis.

Key words: Streptozotocin, proinsulin synthesis, insulin release, rat islets, isolated islets of Langerhans, ATP content, proinsulin content, nicotinamide, methyl-nicotinamide.

Streptozotocin, an antibacterial agent [1] produced by Streptomyces achromogenes [2] and subsequently shown to exhibit antitumour [3] and diabetogenic [4] activity, has been structurally characterized as 2-deoxy-2-(3′-methyl-3′-nitrosoureido)-D-glucopyranose [5]. The compound has been used extensively for the induction of experimental diabetes in laboratory animals and may, because of the reproducibility of its effects, be the agent of choice for this purpose [6–16].

The mechanism by which streptozotocin selectively destroys the B-cells of the islets of Langerhans is not known, although lowering of intracellular nicotinamide adenine dinucleotide (NAD) levels may be involved. Certainly, the administration of nicotinamide, a precursor of NAD in many tissues, protects against the diabetogenic effects of subsequently administered streptozotocin [17–25]. This protection may, however, unmask a long term oncogenic effect of the drug [26, 27].

Studies of the effects of streptozotocin on islets of Langerhans have shown a correlation between the extent of B-cell destruction and the decrease in islet NAD [25]; decreased oxidation of glucose by islets isolated after in vivo administration of a diabetogenic dose of streptozotocin [28]; and a dose-related inhibition of glucose-induced insulin secretion during two hours of incubation, when the drug was added in vitro to isolated islets [29]. The purpose of the present study was that of investigating the effects of streptozotocin on proinsulin synthesis, while simultaneously measuring its effects upon insulin release. Only one preliminary report dealing with proinsulin synthesis and streptozotocin has appeared [30].

* This work was supported by the Swiss National Scientific Foundation (Grant No. 3106073), Berne, Switzerland, and by a grant-in-aid from Hoechst Pharmaceuticals, Frankfurt-Hoechst, Germany

Preliminary data from this work were presented at the European Symposium on Hypoglycemia, Rome, April 5–6, 1974

** Current address: Cattedra di Endocrinologia, c/o II Clinica Medica, Università di Roma, Rome, Italy

*** Current address: Department of Medicine, Washington University School of Medicine, St. Louis, Missouri, U.S.A.

Materials and Methods

Islet Isolation, Incubation and Protein Extraction

Male Wistar rats fed ad libitum and weighing 180–200 g were killed by decapitation and islets isolated by the collagenase digestion method of Lacy and Kostianovsky [31]. The Hank's solution used during the isolation was supplemented with 5 mM D-(+)-glucose obtained from Merck, Darmstadt, Germany. Collagenase was obtained from Worthington Biochemical Corporation, Freehold, New Jersey. For each experiment 400–800 islets from four pooled pancreata were used.

Incubation was in a Krebs-Ringer bicarbonate buffer supplemented with a mixture of the following eleven amino-acids: L-arginine, L-cystine, L-histidine, L-isoleucine, L-lysine, L-methionine, L-phenylalanine, L-threonine, L-tryptophane, L-tyrosine, L-valine (all obtained from Merck, Darmstadt, Germany) in the concentrations used for Eagle's culture medium [32]; 0.5% bovine serum albumin (BSA) (Behringwerke A.G., Marburg, Germany) and 16.7 mM glucose were also added. This mixture is referred to in the text as KRB-medium. The buffer was gassed continuously with 95% O_2, 5% CO_2, the pH being adjusted to 7.4.

All islets were preincubated for 60 min in the presence or absence of different concentrations of streptozotocin before the start, at time zero, of a 30 min incubation in the presence of ^{3}H-leucine. For the studies on the effect of different exposure times to streptozotocin, the drug was also added at $-30'$ or $-5'$. After isolation, the islets were aspirated into glass micropipettes and transferred to 12 incubation vials, each containing 1 ml ice-cold KRB-medium. In order to randomize the size of islets in each vial, the islets were transferred in groups of five to all twelve vials sequentially, with random numbering of the vials only after all islets had been transferred. Each vial contained between 20–30 islets, the number being constant in any one experiment. The medium was then removed by aspiration under microscopic observation, and 1 ml fresh, cold KRB-medium added to each vial, with or without 10 µl concentrated streptozotocin solution (lot No. 9681-GGS-118Fl-U-988 g kindly supplied by Dr. William Dulin, Upjohn Co., Kalamazoo, Michigan, U.S.A.) made up in 1 mM citric acid and 0.9% NaCl. The final concentrations of streptozotocin tested ranged from 0.02 to 11.0 mM. The control vials received only streptozotocin vehicle. The final concentration of citric acid in the incubation medium was 10 µM for all vials. When streptozotocin was added at times other than the beginning of the preincubation ($-60'$), that is, at $-30'$ or $-5'$, the appropriate control was included.

Every experimental condition, including the appropriate controls, was examined in triplicate. Accordingly, the number of variables or conditions usually examined in any one experiment was limited to four.

After the 60 min preincubation, the islets were washed three times with warm oxygenated KRB-medium and incubation was started by the addition of 1 ml KRB medium supplemented with L-leucine-4, 5-^{3}H (The Radiochemical Centre, Amersham, Bukkinghamshire, U.K.; specific activity 55 Ci/mmol) to the final concentration of 100 µCi/ml or 1.82×10^{-6}M leucine. After 30 min, the incubation was stopped by immersion of the vials in ice-cold water, the medium was aspirated and the islets washed three times with 1 ml cold KRB-medium. As the three washes between the preincubation and the incubation periods took less than 5 min per two vials, the preincubations were started and stopped at 5 min intervals between pairs of vials. To minimize the effect of differing time periods in cold buffer, before the beginning of the preincubation, the sequence of conditions was changed for each set of experiments.

After incubation and washing, 2 ml 0.2 M glycine buffer, pH 8.8, containing 0.25% human serum albumin (referred to as GB in the text) was added; the islets were broken by 40 sec sonication using the Sonifier B-12 (Branson Sonic Power Company, Danbury, Connecticut, U.S.A.) in position 4 of the output control. In control experiments, recovery of immunoreactive insulin (IRI) from sonicated isolated islets was similar to that after acid-ethanol extraction according to Davoren [33], as already shown by others [34, 35]. In particular, the heat produced during 40 sec sonication was without effect since sonication of rat insulin standard for periods well in excess of 40 sec gave rise to standard curves superimposable on those obtained from the standard solutions not having undergone sonication. Sonication was preferable to acid-ethanol extraction since it could be performed in the same buffer that was used for both chromatography and radioimmunoassay, thereby avoiding the cumbersome process of drying each fraction after acid-ethanol extraction before redissolving it in buffer.

Chromatographic Separation

1 ml aliquots of sonicated islet homogenates were chromatographed on 1 × 120 cm columns of Sephadex G50 fine (Pharmacia, Uppsala, Sweden) previously equilibrated in GB and saturated with 2 ml human serum containing 2 µg/ml rat insulin. They were calibrated with ^{125}I-albumin, ^{125}I-proinsulin, ^{125}I-insulin, ^{125}I-glucagon, ^{3}H-leucine, ^{125}I. Six col-

umns were used simultaneously and 1 ml fractions of the effluent were collected in Ultro Rac 7000 fraction collectors (LKB Produkter AB, Bromma, Sweden), the flow rate being approximately 20 ml/h.

Radioimmunoassay and Radioactivity Measurements

Immunoreactive insulin (IRI) was assayed in the incubation media, in the whole islet homogenates, and in each ml fraction of eluates of islet homogenates. Radioimmunoassays were performed using rat insulin as standard (kindly supplied by Dr. J. Schlichtkrull, Novo Research Institute, Bagsvaerd, Denmark), an antiinsulin serum (AIS) to porcine insulin in guinea pigs, kindly supplied by Dr. P.H. Wright of Indianapolis, Indiana, U.S.A., ^{125}I-porcine insulin and a charcoal-dextran separation technique [36]. ^{125}I-insulin was prepared by the method of Greenwood et al. [37] by incubating pork insulin in phosphate buffer with Na^{125}I and chloramine T for 30 sec. The reaction was stopped by the addition of sodium metabisulphite and potassium iodide. The ^{125}I-insulin was purified on 1 × 120 cm column of Sephadex G-50 by elution with phosphate buffer containing human serum albumin. Sensitivity of the assay was such, that a significant displacement of labelled insulin was obtained with a concentration of 0.125 ng/ml pork proinsulin. Rat insulin and pork proinsulin standard curves were parallel up to 3 ng/ml proinsulin, the affinity for pork proinsulin being 65% of that for rat insulin. Therefore, IRI values measured in the proinsulin region as rat insulin equivalents should be multiplied by 1.54 in order to express them as pork proinsulin equivalents.

To measure ^{3}H incorporation, 0.5 ml of each fraction to be assayed was mixed with 6 ml of Instagel (Packard Instrument Company, Warrenville, Illinois, U.S.A.) in plastic vials and counted in a liquid scintillation spectrometer (LS-330, Beckman Instruments, Fullerton, Ca., U.S.A.). The efficiency of ^{3}H counting was 30% and constant in all experiments.

Calculations and Expression of Results

Insulin release was estimated from the IRI present in the incubation media at the end of the incubations. Proinsulin content (expressed as IRI) was derived by integration of the area under the proinsulin peak (Fig. 1). Ten fractions were taken into consideration, namely the peak fraction plus the five preceding and the four following fractions.

Incorporation of ^{3}H-leucine into proinsulin and into total proteins was quantified by integrating the corresponding areas of the radioactivity elution profiles (Fig. 2). To minimize contamination of proinsulin with other peptides, only the right half of the proinsulin region (peak fraction + four following fractions) was taken into consideration and multiplied by two. With this procedure contamination with peptides other than proinsulin was found to represent a constant 40% of the total, even when protein synthesis was strongly inhibited, as in Figure 2 with 2.2 mM streptozotocin. This was estimated by incubation of the pooled fractions of the proinsulin region with excess AIS followed by gel-filtration on a column of Sephadex G75, 1 × 24 cm. Two peaks of radioactivity were obtained, the first one due to the antibody-bound peptides and considered to represent proinsulin, the second one due to non-antibody-bound peptides.

About 2% of the counts, distributed to the insulin region, were lost when integrating the first portion of the elution profiles for total proteins (Fig. 2). All results are calculated per islet.

In order better to compare different experiments and different variables, the results were finally expressed as percent of the mean of the triplicate controls for each experiment. Each experiment also resulted in triplicate values for each experimental condition. For statistical analysis the mean of the triplicates was the result used.

Measurement of ATP

A separate series of experiments were performed in order to evaluate the effect of streptozotocin on islet ATP content. The assay used was the luciferase assay of Strehler et al. [38] as modified by Wettermark et al. [39] and Ashcroft et al. [40]. Fire-fly extract was obtained from Worthington Biochemical Corporation. The experimental design was the same as previously described. Islets were incubated in 0.4 ml KRB medium, and ATP was measured at the beginning (min 0) and at the end (min 30) of incubation. The incubation was stopped by adding 0.2 ml ice-cold 2% $HClO_3$ to each vial and the islets were sonicated and centrifuged. 0.1 ml of the supernatant was then added to 5 ml fire-fly enzyme solution, the samples were gently shaken and read for 0.1 min – after a constant interval of 14 sec – in the Beckman liquid scintillation spectrometer set out-of-coincidence. Standard ATP (a gift of Dr. F.H. Schmidt, Boehringer Mannheim GmbH, Germany) was also dissolved in 2/3 KRB and 1/3 $HClO_3$ and a standard curve obtained after elimination of the precipitate. These standard curves were reproducibly linear up to 500 pmoles ATP per 0.1 ml sample.

Results

The results of an experiment in which isolated islets of Langerhans were incubated for 30, 120, or 180 min

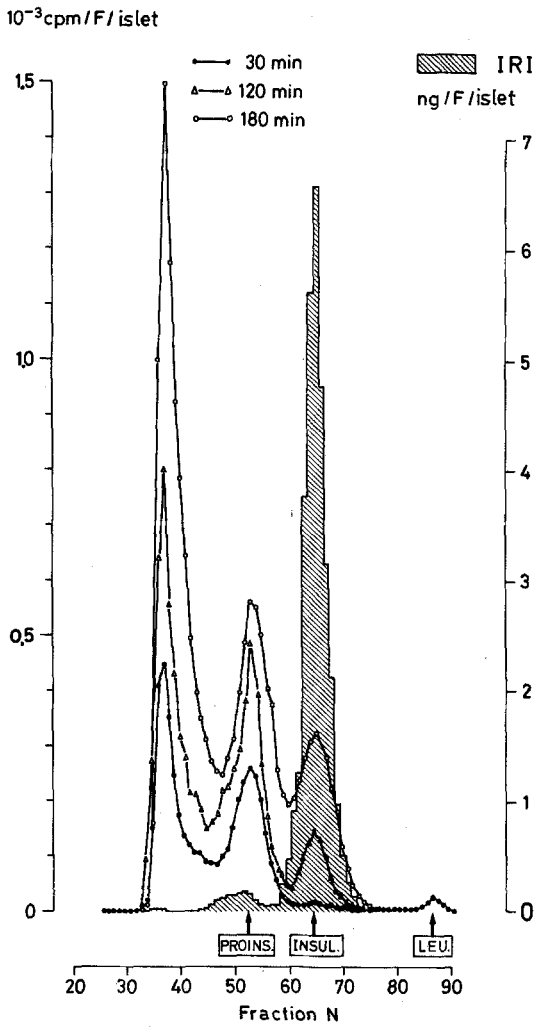

Fig. 1. Radioactivity profiles resulting from the chromatography of extracts from isolated islets incubated in presence of 16.7 mM glucose and ^{3}H-leucine for 30 min (•——•), 120 min (△——△) or 180 min (○——○). The shaded area represents the insulin immunoreactivity profile expressed as ng/fraction (F)/islet

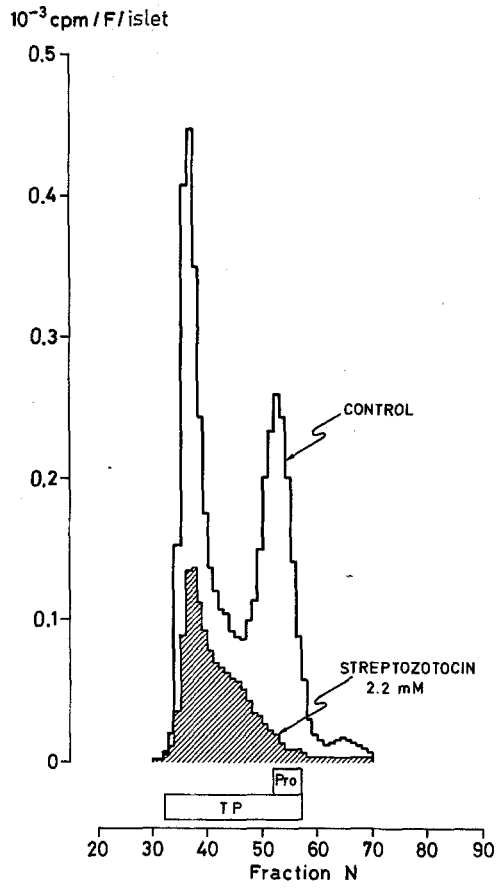

Fig. 2. Radioactivity profiles resulting from the chromatography of extracts from homogenized isolated islets preincubated 60 min in presence and absence of 2.2 mM streptozotocin. The rectangles below the elution profiles indicate which fractions were taken to represent the incorporation into total proteins (TP) or into half of the proinsulin (Pro)

with ^{3}H-leucine in the presence of 16.7 mM glucose are shown in Figure 1. At the end of each incubation period the islets were sonicated in 2 ml GB, of which 1 ml was chromatographed on Sephadex G-50. Four peaks of radioactivity were detected. First, the large protein peak at around fraction 37, secondly the proinsulin peak at fraction 53, thirdly the insulin peak at fraction 65 and finally the small free leucine peak at fraction 87. Proinsulin and insulin were identified by their immunoreactivity and were distinctly separate from each other although not, of course, from other similarly sized peptides.

After incubation for 30 min, radioactivity was present in the large protein and in the proinsulin peaks, only barely in the insulin peak. After 120 min, there was more radioactivity in the large protein and in the proinsulin peak and radioactivity now appeared clearly in the insulin peak. A further increase of incorporation occurred in all three peaks after 180 min. It is clear from these results that the rate of proinsulin synthesis is most conveniently studied when using an incubation period of 30 min with ^{3}H-leucine, identifying the proinsulin fraction and measuring ^{3}H-leucine incorporation in that fraction. Under these conditions, little or no radioactivity is detected in insulin and thus none can be lost by insulin release.

While this simple method of determining ^{3}H-leucine incorporation into proinsulin is satisfactory for control or stimulated conditions, it becomes less favourable when studying the *inhibition* of proinsulin synthesis, i. e. when the ^{3}H-leucine incorporation is low. Thus, in the experiment shown in Figure 2, islets of Langerhans were incubated with 2.2 mM streptozotocin for 60 min prior to incubation with ^{3}H-leucine for 30 min. The incorporation of

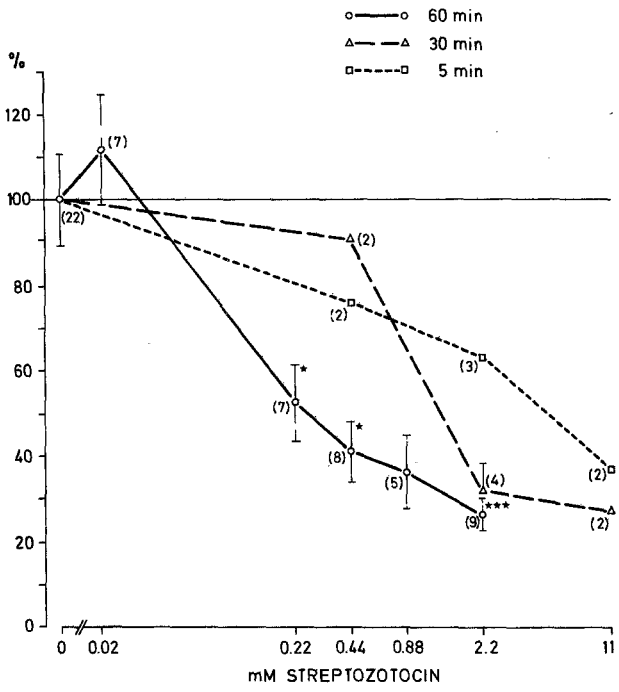

Fig. 3. Insulin release into the incubation medium during the 30 min incubation as a function of the concentration (abcissa) and of the time of previous exposure to streptozotocin: 60 min (o——o), 30 min (△----△), 5 min (□----□). The average release from the three control batches of islets in each experiment was taken as 100%. The release from all other batches of the same experiment was expressed as percent of the control average. 100% corresponds to 2.92 ± 0.35 ng IRI/islet. The number of experiments performed (in triplicate) is indicated in brackets. The stars show the statistical significance of the difference between test and control values, calculated with the paired Student's "t" test: * = $P < 0.05$, ** = $P < 0.01$, *** = $P < 0.001$

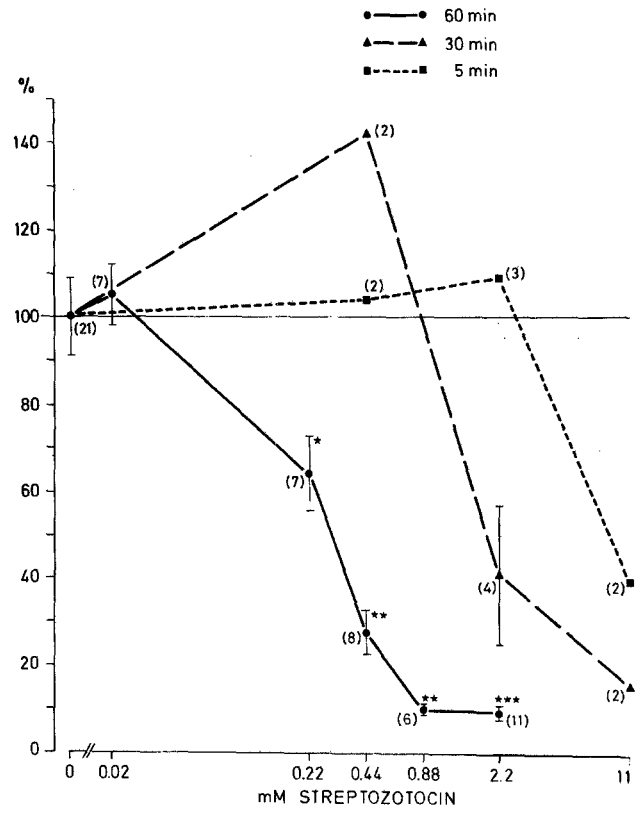

Fig. 4. Incorporation of ^{3}H-leucine into proinsulin during the 30 min incubation following exposure to streptozotocin for 60 (●——●), 30 (▲----▲) and 5 (■----■) min. Test values are expressed as percent of the control average in each experiment. 100% coresonds to 2022 ± 179 cpm/islet

Table 1. Total IRI content (ng/islet), proinsulin content (ng/islet) and $\frac{\text{proinsulin}}{\text{total IRI}} \times 100$ (ng/ng) at the end of the 30 min incubation following exposure to streptozotocin for 60 min. Proinsulin is expressed as rat insulin equivalents. The concentration of streptozotocin used is indicated in the column "mM streptozotocin", and the corresponding values are in the column "streptozotocin". △ = difference between test and control values. n = number of experiments (all performed in triplicate). "p" values were calculated with the Student's "t" test for paired data

	mM Streptozotocin	Control	Streptozotocin	△	n	p <
IRI (ng/islet) (± SEM	0.02	47.8 ± 12	56.0 ± 16	8.2 ± 5	7	0.20
	0.22	47.8 ± 12	52.8 ± 13	5.0 ±4	7	0.30
	0.44	51.7 ± 9	55.2 ± 9	3.4 ± 4	8	0.50
	0.88	53.8 ± 12	64.1 ± 15	10.3 ± 5	6	0.20
	2.2	39.4 ± 7	46.2 ± 9	6.8 ± 3	12	0.10
Proinsulin (ng/islet) (± SEM)	0.02	1.35 ± 0.2	1.64 ± 0.3	0.29 ± 0.2	7	0.20
	0.22	1.35 ± 0.2	1.32 ± 0.2	−0.03 ± 0.2	7	1.0
	0.44	1.06 ± 0.2	0.92 ± 0.2	−0.14 ± 0.2	8	0.50
	0.88	1.14 ± 0.3	0.91 ± 0.2	−0.23 ± 0.2	6	0.30
	2.2	1.22 ± 0.2	0.88 ± 0.1	−0.34 ± 0.1	12	0.05
$\frac{\text{Proinsulin}}{\text{Total IRI}} \times 100$	0.02	3.65 ± 0.9	4.0 ± 0.9	0.35 ± 0.2	7	0.10
	0.22	3.65 ± 0.9	3.17 ± 0.7	−0.48 ± 0.4	7	0.30
	0.44	1.97 ± 0.1	1.40 ± 0.8	−0.57 ± 0.1	8	0.05
	0.88	2.04 ± 0.2	1.20 ± 0.1	−0.84 ± 0.2	6	0.01
	2.2	3.91 ± 0.7	2.34 ± 0.3	−1.57 ± 0.4	12	0.005

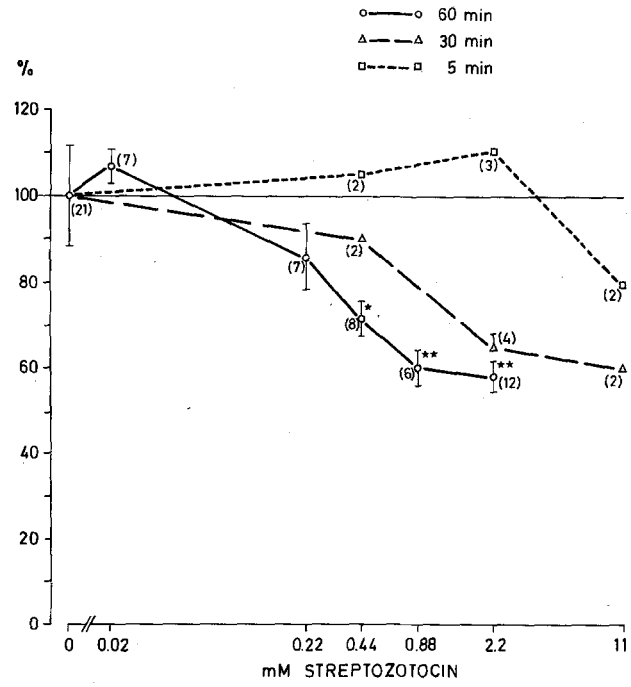

Fig. 5. Islet proinsulin content at the end of incubation expressed as percent of total IRI content. The 100% control value for proinsulin content was 3.68 ± 0.44% of insulin content

^{3}H-leucine was markedly reduced. Whereas the radioactivity profile from the control islets exhibited the two usual and distinct peaks – large proteins and proinsulin – that from the islets inhibited by streptozotocin exhibited only one peak of radioactivity, albeit a skewed one. The ^{3}H-leucine incorporation into proinsulin was strongly inhibited, and reduced to a slight shoulder on the descending slope of the large protein peak. While this is an extreme example, it draws attention to the need for a suitable quantitation technique. Figure 2 exemplifies the method used for all of the studies reported here, and defines the fractions taken to represent total proteins and those included in the estimation of half the proinsulin peak.

The Effects of Streptozotocin Concentration and Time of Exposure on Insulin Release and Proinsulin Synthesis

The inhibition of insulin release obtained after exposure of isolated islets to concentrations of streptozotocin varying from 0.02 to 11 mM, is shown in Figure 3. Exposure to the drug was for 5, 30 or 60 min. The results are expressed as percent release relative to untreated control islets in each experiment. The extent of the inhibition was related both to time and to concentration. Thus, when the exposure was 60 min, 0.22 mM streptozotocin resulted in 48% inhibition of

Table 2. Effects of 60 min exposure to 2.2 mM streptozotocin (5), 10 mM nicotinamide (N), 4 mM methyl-nicotinamide (MN) and of N and MN in combination with streptozotocin on the incorporation of ^{3}H-leucine into proinsulin and on IRI release. Numbers represent means ± SEM for triplicate determinations

	^{3}H-leucine incorporation into proinsulin (cpm/islet ± SEM)	IRI release (ng/islet ± SEM)
Control	2135 ± 106	2.47 ± 0.13
S	457 ± 51	0.28 ± 0.09
N	2103 ± 44	3.05 ± 0.05
S + N	1103 ± 104	1.82 ± 0.16
MN	2132 ± 126	2.65 ± 0.29
S + MN	1263 ± 162	2.00 ± 0.24

Table 3. Effects of 60 min exposure to 0.44 mM streptozotocin on the incorporation of ^{3}H-leucine into proinsulin and on IRI release in the absence of glucose and in the presence of 16.7 and 33.4 mM glucose. Numbers represent means ± SEM for triplicate determinations

Glucose (mM)	Streptozotocin (mM)	^{3}H-leucine incorporation into proinsulin (cpm/islet ± SEM)	IRI release (ng/islet ± SEM)
0	0	609 ± 33	0.18 ± 0.05
16.7	0	1721 ± 167	2.43 ± 0.14
33.4	0	1701 ± 58	2.61 ± 0.15
0	0.44	229 ± 64	0.50 ± 0.16
16.7	0.44	755 ± 68	0.73 ± 0.12
33.4	0.44	855 ± 70	0.76 ± 0.10

insulin release; increasing the concentration to 2.2 mM increased the inhibition to 73%. With 30 min of exposure, inhibition was marked at 2.2 mM streptozotocin, and even with only 5 min exposure, 2.2 mM caused 37% inhibition and 11 mM 63% inhibition.

These inhibitory effects of streptozotocin added in vitro on insulin release were somewhat similar to the inhibitory effects on proinsulin synthesis, as shown in Figure 4. Exposure for 60 min to 0.22 mM streptozotocin caused 36% inhibition of proinsulin synthesis, whereas inhibition at 0.88 and 2.2 mM was 90%. With a 30 min exposure period, inhibition of proinsulin biosynthesis was not apparent at concentrations of 0.44 mM and below. When the exposure was 5 min, no inhibition was noted except with 11 mM streptozotocin. Thus, the effects of streptozotocin on insulin release and proinsulin synthesis were similar in general terms. However, it appeared that after 60 min exposure to 0.88 mM and 2.2 mM streptozotocin, proinsulin synthesis was more inhibited than insulin release.

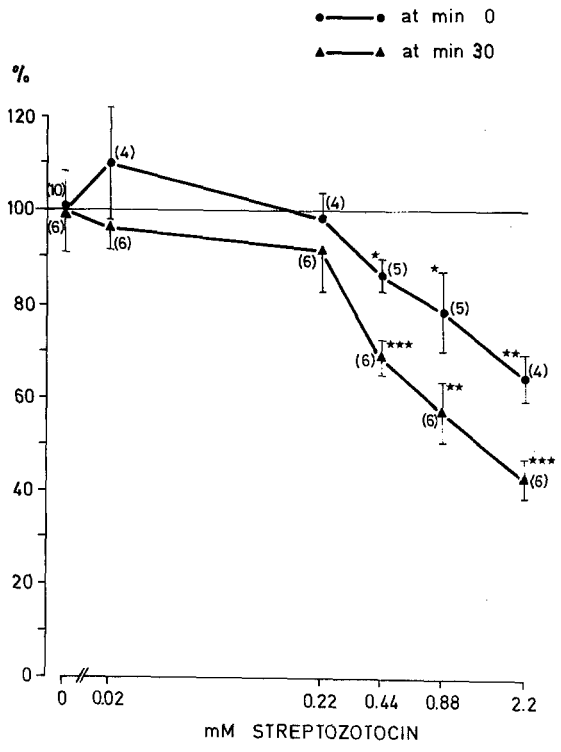

Fig. 6. Islet ATP content at the beginning (min 0, ●——●) and at the end (min 30, ▲——▲) of a 30 min incubation following 60 min exposure to different concentrations of streptozotocin (abscissa). The 100% control value corresponds to 12.0 ± 1.07 pmol ATP/islet at min 0 and to 11.6 ± 1.05 pmol ATP/islet at min 30

The inhibitory effect of streptozotocin on proinsulin synthesis was also detected by measuring proinsulin content and insulin content, both expressed as IRI, at the end of the exposure and incubation periods. These results are shown in Table 1. While significant decreases were observed in proinsulin content due to streptozotocin treatment, no significant changes in IRI content were detected. However, in all cases the IRI content of islets treated with streptozotocin were non-significantly higher than their paired controls because of the higher IRI release in the control islets. In Figure 5, where the content of proinsulin is expressed as a ratio of the IRI content and normalized to 100% (the control ratio), is can be seen that the content of proinsulin decreased as a function of the concentration of streptozotocin and the exposure time.

In characterizing the effects of streptozotocin, it was of interest to know whether nicotinamide or methylnicotinamide could protect against the streptozotocin-induced inhibition of proinsulin and total protein synthesis, just as they have been reported to protect the insulin release capability [17–24]. In one experiment, 10 mM nicotinamide or 4 mM methyl nicotinamide were included in the incubation medium with or without 2.2 mM streptozotocin. It is clear from the results shown in Table 2 that both nicotinamide and methyl-nicotinamide partially protected the islets against the effects of streptozotocin, with respect both to insulin release and to proinsulin synthesis. Thus 2.2 mM streptozotocin inhibited proinsulin synthesis by 79%. However, in the presence of 10 mM nicotinamide and 4 mM methylnicotinamide the inhibition was reduced to 48% and 41% respectively.

One further characterization study was performed. As streptozotocin appears to exert specific effects on the B-cell, the possibility existed that the glucose moiety of the molecule is responsible for the specificity, perhaps by virtue of a glucose transport system or glucose receptor targeting. Therefore, evidence for a protective effect of glucose against streptozotocin was sought, and the effects of streptozotocin were studied in the presence of different concentrations of glucose, i.e. 0, 16.7 and 33.4 mM. Only a slight effect of glucose against streptozotocin was observed on proinsulin synthesis and no effect on insulin release. The results are shown in Table 3.

Effect of Streptozotocin on ATP Content of Isolated Islets

In an attempt to correlate the inhibitory effects of streptozotocin on insulin synthesis and release with the likely presence of general deterioration of cell function and metabolism, measurements were made of the ATP content of islets 0 or 30 min after treatment for 60 min with different concentrations of streptozotocin. As shown in Figure 6, the ATP content of islets decreased as a function of both concentration and time from exposure. At time 0′ after exposure, a significant depression of ATP levels occurred at 0.44 mM and increased further with increased concentration of the drug; the depression was 14% at 0.44 mM, 22% at 0.88 mM and 35% at 2.2 mM. 30′ after exposure, the depression was 31% at 0.44 mM, 42% at 0.88 mM and 57% at 2.2 mM streptozotocin. Of interest is the observation that at 0.22 mM streptozotocin no depression of ATP content was observed despite the fact that inhibition of both insulin release and proinsulin synthesis was seen with 60 min of exposure to 0.22 mM streptozotocin (Figs. 3 and 4).

It would seem, therefore, that while decreased availability of ATP might be the cause of, or contribute to, the effects of streptozotocin on insulin release and synthesis at concentrations of 0.44 mM and higher, inhibition of insulin release and synthesis may also occur without changes in ATP content.

Specificity of the Effect of Streptozotocin

Whether or not the effect of streptozotocin on proinsulin synthesis is a specific one can be assessed by

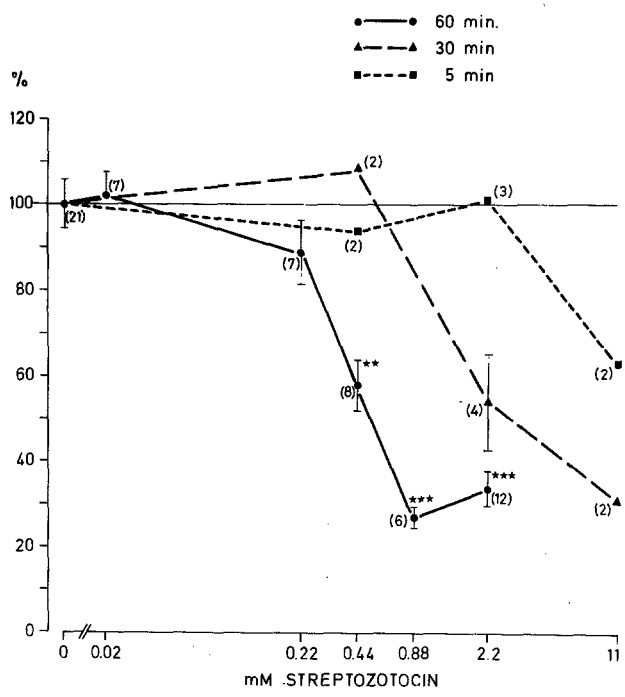

Fig. 7. Incorporation of ^{3}H-leucine into proinsulin, expressed as percent of the incorporation into total proteins. The 100% control value for ^{3}H-leucine incorporation into proinsulin was 33.3 ± 1.6% of the incorporation into total proteins

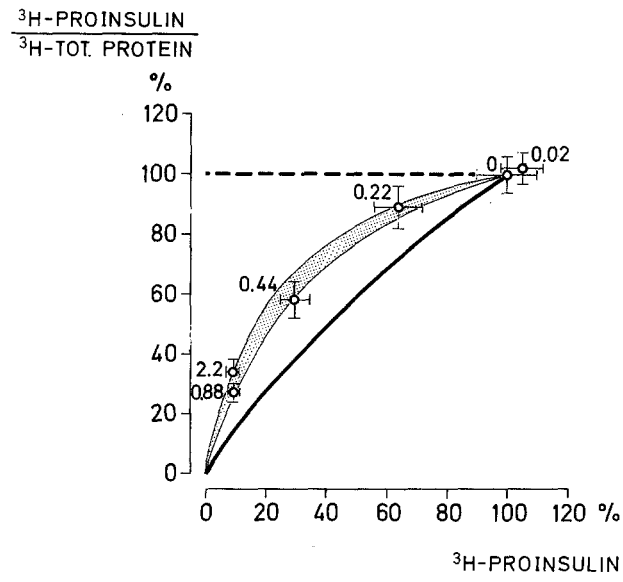

Fig. 8. Ratio of proinsulin incorporation over total protein incorporation (ordinate) as a function of proinsulin incorporation (abscissa), in presence of different concentrations (indicated by numbers) of streptozotocin (⊢⊣). Both indices are expressed as percent of control ± SEM, the 100% control value being 33.3 ± 1.6% for the ordinate and 2022 ± 179 cpm/islet for the abscissa. Also shown are the theoretical plots describing: (a) the complete lack of specificity versus overall islet protein synthesis (----); (b) the complete specificity of the effect for B-cells, without any preferential effect within them, assuming that B-cells account for 70–80% of islet cell mass (⁘); (c) the complete specificity for proinsulin versus other islet proteins (——)

examining its effects on the relative rates of proinsulin and total protein synthesis. Thus, in Figure 7, the ratio of proinsulin to total protein synthesis in the presence of streptozotocin has been expressed as a percentage of the control ratio. The results again show a complex pattern of streptozotocin effects. For instance, at 0.22 mM streptozotocin and 60 min exposure, no change in the $\frac{\text{proinsulin}}{\text{total protein}}$ ratio occurred despite a 36% inhibition of proinsulin synthesis under these conditions, thereby suggesting that proinsulin synthesis and total protein synthesis were affected to an equal extent. With increasing concentrations of streptozotocin, however, an apparently specific inhibition of proinsulin synthesis was noted since the $\frac{\text{proinsulin}}{\text{total protein}}$ ratio decreased from 90% (0.22 mM) to 60% (0.44 mM) to 24% (0.88 mM) i.e. proinsulin synthesis was more markedly affected by streptozotocin treatment than protein synthesis generally. Similarly, when exposure to streptozotocin was shorter (5 min or 30 min) there was evidence for an apparent specific effect on proinsulin synthesis (Fig. 7).

The situation is complicated however, by the fact that proinsulin is synthesized only in the B-cells. Thus the nature of the apparent specificity is not easily assessed by the ratio of proinsulin to total islet protein. Therefore, a technique was devised to test the specificity of the streptozotocin effect on proinsulin synthesis with respect to its effect on protein synthesis generally. Theoretically, this may be done by plotting the incorporation of ^{3}H-leucine into proinsulin in terms of, or relative to, the ^{3}H-leucine incorporation into total proteins. Thus if, as in Figure 8, one plots incorporation into proinsulin divided by incorporation into total protein against the incorporation into proinsulin, one sets up a nomogram which describes the specificity of the system [35].

Theoretical calculation permits the definition of the lines corresponding respectively to: (a) complete lack of specificity in the variation of the rate of total islet protein synthesis (broken, horizontal line); (b) non-specific effect on protein synthesis within the B-cells, without any effect on other islet cells (shaded area) assuming that the B-cells account for 70–80% of the islet cell mass [41, 42]; (c) extreme inhibitory specificity for proinsulin synthesis even as compared with other B-cell proteins, assuming that, in the control situation, proinsulin accounts for 30% of overall protein synthesis, an assumption that applies to our system (solid line). Plotting the results obtained in the

presence of different concentrations of streptozotocin into the nomogram of Figure 8, it becomes apparent that they coincide with the shaded area, thereby suggesting (or confirming) that the action *on B-cells* is indeed selective, but negating any further *intra-B-cell* specificity directed to the synthesis of proinsulin when compared with that of other B-cell proteins.

Discussion

The results reported confirm the well-known inhibitory effect of streptozotocin on insulin release. In addition, they establish that proinsulin synthesis, and therefore insulin synthesis, are also inhibited profoundly.

As to the effect of the drug upon IRI release, five minutes exposure of islets to 2.2 mM streptozotocin was sufficient to cause a 37% inhibition during the subsequent 30 min incubation period. Longer exposure and higher concentrations resulted in greater inhibition of release. These results agree with those of Golden et al. [29] who reported that during a one hour incubation, following preincubation for one hour with 0.44 mM streptozotocin, insulin release was inhibited by 40%, and by 85% when the exposure had been to 2.2 mM streptozotocin. Similarly, Renold et al. [16] reported that 1.1 mM streptozotocin inhibited IRI release into the medium of pancreatic monolayer cultures and that this effect was a function of duration of exposure to the drug (2 to 24 h).

In the present study, the inhibitory effect of streptozotocin on proinsulin synthesis resembled that on insulin release in that exposure for only 5 min was needed to produce inhibition and increasing the concentration and duration of exposure caused greater inhibitory effects. There was, however, a significant difference in the precise relationship between concentration and effect on the two processes. Comparison of Figures 3 and 4 shows that after 60 min exposure to 0.88 mM and 2.2 mM streptozotocin, proinsulin synthesis was inhibited to a greater extent than insulin release. Both insulin release and synthesis were protected from the effects of streptozotocin by the prior addition of nicotinamide or methylnicotinamide, a finding which suggests that at least the early effects of streptozotocin on NAD in B-cells are common to the inhibitory action on both release and synthesis. It can be seen that in this single experiment with nicotinamide and methyl-nicotinamide the streptozotocin-induced inhibition of proinsulin synthesis was not greater than the inhibition of insulin release, in apparent contradiction to Figures 3 and 4. This experiment was included, however, in the data for these two figures and in the statistical evaluation of the relative effectiveness of streptozotocin for synthesis and release.

An attempt to demonstrate a protective effect of glucose, perhaps through interference with specific uptake of streptozotocin by B-cells, failed to reveal any significant effect. In agreement with this observation is the report by Dulin et al. [19] who showed that in vivo 2-deoxyglucose, but not glucose, glucosamine or mannoheptulose protected against the diabetogenic action of streptozotocin. Yet Anderson et al. [25] have demonstrated that the glucose carrier of methyl-^{14}C-streptozotocin facilitates the uptake of its cytotoxic group, l-methyl-l-nitrosourea, into islets. These authors have also shown that a dose of streptozotocin, which was diabetogenic when administered intra-peritoneally, attained a peak plasma N-nitroso intact streptozotocin concentration of 0.22 mM and thus a concentration in the range of those used in vitro in the present studies.

Karunanayake et al [43] have established that labelled streptozotocin is rapidly cleared from the blood by kidney and liver, and that some pancreatic accumulation is demonstrable only for the 3-methylated form. This observation suggests that the specificity of streptozotocin for the B-cell is more likely to result from greater sensitivity of B-cells to the drug than from greater uptake. Because of this, and in order to establish whether inhibition of insulin release and synthesis may be dissociated from general deterioration of cell metabolism and survival, islet ATP content was measured under conditions similar to those used for the insulin experiments. The ATP content of the islets did decrease progressively as a function of both time and concentration of streptozotocin used. Of importance, however, was the fact that proinsulin synthesis and insulin release could be inhibited under conditions in which no decrease in ATP was observed. For example, after exposure to 0.22 mM streptozotocin, no decrease of ATP was observed, which contrasts with the effects of 60 min exposure to 0.22 mM streptozotocin on proinsulin synthesis and insulin release, inhibited by 36% and 48%, respectively. This dissociation suggests some degree of specificity of the streptozotocin effect in that it did not necessarily correlate with general B-cytotoxicity. As ATP content does decrease with higher concentrations of streptozotocin; however, inhibitions of insulin release and synthesis at these concentrations are likely to result from both "specific" and a more general effect revealed by decreased ATP.

The ratio $\frac{\text{proinsulin content}}{\text{total IRI content}}$ was chosen because it represented best the proinsulin content by overcoming sample-to-sample variation in the amount of islet material. Variations in this ratio should not be af-

fected by variation in the total IRI amount in any cell sample since proinsulin and insulin should be altered to the same extent. Indeed, it has been shown by Sando et al. [44] that the percentage of secreted proinsulin to insulin remains the same under stimulated and non-stimulated conditions and, even assuming an increase in IRI content due to decreased release (relative to controls), the ratio $\frac{\text{proinsulin}}{\text{total IRI}}$ would most likely remain the same, since proinsulin retention would also increase. It seems most reasonable to assume that the decrease in the $\frac{\text{proinsulin}}{\text{total IRI}}$ ratio reflects primarily a decrease in the proinsulin content, and that this is most likely due to streptozotocin-induced decrease in proinsulin biosynthesis. Proinsulin content thus appears to be as sensitive a marker of the rate of synthesis as incorporation of tritiated leucine, consistent with a small proinsulin pool turning over rapidly. Both the observation and our conclusions are in good agreement with those of Sando et al. [44].

Turning to the specificity of streptozotocin for proinsulin synthesis relative to overall protein synthesis, we have observed that at all inhibitory concentrations tested, the effect on proinsulin synthesis was greater than that on total protein synthesis. This could be anticipated from the knowledge that the effect of streptozotocin is specific for B-cells as opposed to A-cells and other islet cells. This is the likely explanation for the apparent specificity of streptozotocin for proinsulin synthesis relative to total protein synthesis expressed in Figure 8. Relating the ratio of proinsulin synthesis over total protein synthesis to proinsulin synthesis results in a curve sloping down to the left, i.e. showing apparent specificity. This curve fits almost exactly, however, a theoretical one in which streptozotocin is assumed to inhibit equally proinsulin synthesis and total protein synthesis in B-cells without affecting protein synthesis in non B-cells, assuming that B-cells account for about 70–80% of the islet cell mass [41, 42]. Thus we are led to conclude that there is no evidence for a specific effect of streptozotocin on proinsulin synthesis as such.

This conclusion was reached with caution, since it is not valid if more (or less) protein synthesis was taking place in non B-cells; the same results could also be obtained in a hypothetical "mixed" situation in which streptozotocin exerted both a preferential effect on proinsulin synthesis, and a preferential (but not exclusive) effect on B-cells.

Acknowledgements. The authors gratefully acknowledge the help of Dr. E. Cerasi, the expert technical assistance of Miss I. Korber and Miss. D. Kaelin, and the contribution of Mrs. M. Alberico.

References

1. Herr, R.R., Eble, T.E., Bergy, M.E., Jahnke, H.K.: Isolation and characterization of streptozotocin. In: Antibiotics annual, pp. 236–240. New York: Antibiotica Inc. 1959–1960
2. Vavra, J.J., DeBoer, C., Dietz, A., Hanka, L.J., Sokolski, W.T.: Streptozotocin: a new antibacterial antibiotic. In: Antibiotics annual, pp. 230–235. New York: Antibiotica Inc. 1959–1960
3. Evans, J.S., Gerritsen, G.D., Mann, K.M., Owen, S.P.: Antitumor and hyperglycemic activity of streptozotocin (NSC 37917) and its cofactor, U 15774. Cancer Chemother. Rep. **48**, 1–6 (1965)
4. Rakietene, N., Rakieten, M.L., Nadkarni, V.: Studies on the diabetogenic action of streptozotocin. Cancer Chemother. Rep. **29**, 91–98 (1963)
5. Herr, R.R., Jahnke, H.K., Argoudelis, A.D.: The structure of streptozotocin, J. Amer. chem. Soc. **89**, 4808–4809 (1967)
6. Junod, A., Lambert, A.E., Orci, L., Pictet, R., Gonet, A.E., Renold, A.E.: Studies on the diabetogenic action of streptozotocin. Proc. Soc. exp. Biol. (N.Y.) **126**, 201–205 (1967)
7. Junod, A., Lambert, A.E., Stauffacher, W., Renold, A.E.: Diabetogenic action of streptozotocin: Relationship of dose to metabolic response. J. clin. Invest. **48**, 2129–2139 (1969)
8. Brosky, G., Logothetopoulos, J.: Streptozotocin diabetes in the mouse and guinea pig. Diabetes **18**, 606–611 (1969)
9. Pitkin, R.M., Reynolds, W.A.: Diabetogenic effects of streptozotocin in rhesus monkeys. Diabetes **19**, 85–90 (1970)
10. Schein, P.S., Alberti, K.G.M.M., Williamson, D.H.: Effects of streptozotocin on carbohydrate and lipid metabolism in the rat. Endocrinology **89**, 827–934 (1971)
11. Sybulski, S., Maughan, G.B.: Use of streptozotocin as diabetic agent in pregnant rats. Endocrinology **89**, 1537–1540 (1971)
12. Lazarus, S.S., Shapiro, S.H.: Streptozotocin-induced diabetes and islet cell alterations in rabbits. Diabetes **21**, 129–137 (1972)
13. Dixit, P.K., Tam, B.B., Hernandez, R.E.: Comparison of the effect of diabetogenic agents on the microdissected pancreatic islet tissue of the rat. Proc. Soc. exp. Biol. (N.Y.) **140**, 1418–1423 (1972)
14. Zapf, J., Waldvogel, M., Froesch, E.R.: Protein kinase and cyclic-AMP binding activities in liver and adipose tissue of normal, streptozotocin-diabetic and adrenalectomized rats. FEBS Letters **36**, 253–256 (1973)
15. Reaven, E.P., Reaven, G.M.: Mechanisms for development of diabetic hypertriglyceridemia in streptozotocin-treated rats. J. clin. Invest. **54**, 1167–1178 (1974)
16. Renold, A.E., Rabinovitch, A., Wollheim, C., Kikuchi, M., Gutzeit, A., Amherdt, M., Malaisse-Lagae, F., Orci, L.: Spontaneous and experimental diabetic syndromes in animals. A re-evaluation of their usefulness for approaching the physiopathology of diabetes. Excerpta Medica International Congress Series **312**, pp. 22–38. Amsterdam 1974
17. Schein, P.S., Cooney, D.A., Vernon, M.L.: The use of nicotinamide to modify the toxicity of streptozotocin diabetes without loss of antitumoral activity. Cancer Res. **27**, 2324–2332 (1967)
18. Schein, P.S., Loftus, S.: Streptozotocin: Depression of mouse liver pyridine nucleotides. Cancer Res. **28**, 1501–1506 (1968)
19. Dulin, W.E., Wyse, B.M.: Studies on the ability of compounds to block the diabetogenic activity of streptozotocin. Diabetes **18**, 459–466 (1969)
20. Stauffacher, W., Burr, I., Gutzeit, A., Beaven, D., Veleminsky, J., Renold, A.E.: Streptozotocin diabetes: Time course of irreversible B-cell damage; further observations on prevention by nicotinamide. Proc. Soc. exp. Biol. (N.Y.) **133**, 194–200 (1970)

21. Stauffacher, W., Balant, L., Amherdt, D.P., Cameron, L., Orci, L.: Mode of action of streptozotocin: indication for accelerated NAD destruction. Diabetologia **8**, (Abstr.) 68 (1972)
22. Chang, A.Y.: On the mechanism for the depression of liver NAD by streptozotocin. Biochem. biophys. Acta (Amst.) **261**, 77–84 (1972)
23. Lazarus, S.S., Shapiro, S.H.: Influence of nicotinamide and pyridine nucleotides on streptozotocin and alloxan-induced pancreatic B-cell cytotoxicity. Diabetes **22**, 499–506 (1973)
24. Gunnarson, R., Berne, C., Hellerstrom, C.: Cytotoxic effects of streptozotocin and N-nitrosomethylurea on the pancreatic B-cells, with special regard to the role of nicotinamide adenine dinucleotide. Biochem. J. **140**, 487–494 (1974)
25. Anderson, T., Schein, P.S., McMenamin, M.G., Cooney, D.A.: Streptozotocin diabetes: Correlation with extent of depression of pancreatic islet nicotinamide adenine dinucleotide. J. clin. Invest. **54**, 672–677 (1974)
26. Rakieten, N., Gordon, B.S., Beaty, A., Cooney, D.A., Davis, R.D., Schein, P.S.: Pancreatic islet cell tumors produced by the combined action of streptozotocin and nicotinamide. Proc. Soc. exp. Biol. (N.Y.) **137**, 280–283 (1971)
27. Volk, B.W., Wellmann, K.F., Brancato, P.: Fine structure of rat islet cell tumors induced by streptozotocin and nicotinamide. Diabetologia **10**, 37–44 (1973)
28. Wyse, B.M., Dulin, W.E.: The effects of streptozotocin on glucose oxidation by isolated islets of Langerhans. Proc. Soc. exp. Biol. (N.Y.) **136**, 70–72 (1971)
29. Golden, P., Baird, L., Malaisse, W.J., Malaisse-Lagae, F.: Effect of streptozotocin on glucose-induced insulin secretion by isolated islets of Langerhans. Diabetes **20**, 513–518 (1971)
30. Taylor, K.W.: Regulation of insulin synthesis: International Congress of Endocrinology III, pp. 220–224. (1968)
31. Lacy, P.E., Kostianovsky, M.: Method for the isolation of intact islets of Langerhans from the rat pancreas. Diabetes **16**, 35–39 (1967)
32. Eagle, H.: Amino-acid metabolism in mammalian cell cultures. Science **130**, 432–437 (1959)
33. Davoren, P.R.: The isolation of insulin from a single cat pancreas. Biochim. biophys. acta (Amst.) **63**, 150–153 (1962)
34. Permutt, M.A., Kipnis, D.M.: Insulin biosynthesis. On the mechanism of glucose stimulation. J. biol. Chem. **247**, 1194–1199 (1972)
35. Pipeleers, D.G., Marichal, M., Malaisse, W.J.: The stimulus-secretion coupling of glucose-induced insulin release. XIV. Glucose regulation of insular biosynthetic activity. Endocrinology **93**, 1001–1011 (1973)
36. Herbert, V., Lau, K.S., Gottlieb, C.W., Bleicher, S.J.: Coated charcoal immunoassay of insulin. J. clin. Endocr. **25**, 1375–1384 (1965)
37. Greenwood, F.C., Hunter, W.M., Glover, J.S.: The preparation of I-131-labelled human growth hormone of high specific radioactivity. Biochem. J. **89**, 114–123 (1963)
38. Strehler, B.L., Totter, J.R.: Determination of ATP and related compounds: Firefly luminescence and other methods. In: Methods of biochemical analysis, Vol. 1, (ed. D. Glick), p. 341. New York: Interscience
39. Wettermark, G., Tegnér, L., Brolin, S.E., Borglund, E.: Photokinetic measurements of the ATP and ADP levels in isolated islets of Langerhans. In: The structure and metabolism of the pancreatic islets, Werner-Gren Symposium N. 16. (eds. G. Falkner, B. Hellman, I.B. Täljedal). Oxford: Pergamon Press 1970
40. Ashcroft, S.J.H., Weerasinghe, L.C.C. Randle, P.J.: Interrelationship of islet metabolism, adenosine triphosphate content and insulin release. Biochem. J. **132**, 223–231 (1973)
41. Hellman, B.: The relation between age and the B/A cell ratio in the islet tissue of the rat. Acta endocr. (Kbh.) **31**, 80–90 (1959)
42. Hellman, B.: The effect of ageing on the total volumes of the A and B cells in the islets of Langerhans of the rat. Acta endocr. (Kbh.) **32**, 92–112 (1959)
43. Karunanayake, E.H., Hearse, D.J., Mellows, G.: The synthesis of (^{14}C)-streptozotocin and its distribution and excretion in the rat. Biochem. J. **142**, 673–683 (1974)
44. Sando, H., Borg, J., Steiner, D.F.: Studies on the secretion of newly synthesized proinsulin and insulin from isolated rat islets of Langerhans. J. clin. Invest. **51**, 1476–1485 (1972)

Received: November 12, 1975, and in revised form: June 21, 1976

Dr. A Maldonato
Cattedra di Endocrinologia
c/o II Clinica Medica
Università di Roma
1-00100 Rome
Italy