Biochemical Pharmacology, Vol. 29, pp. 2625–2633.

0006-2952/80/1001-2625 $02.00/0

LONG-TERM EXPOSURE OF ISOLATED PANCREATIC ISLETS TO MANNOHEPTULOSE: EVIDENCE FOR INSULIN DEGRADATION IN THE β CELL

PHILIPPE A. HALBAN*, CLAES B. WOLLHEIM, BENIGNA BLONDEL and ALBERT E. RENOLD

Institut de Biochimie Clinique and Institut d'Histologie et d'Embryologie, University of Geneva, Geneva, Switzerland

(*Received* 6 *March* 1980; *accepted* 24 *April* 1980)

Abstract—The possibility that the β cell may degrade insulin to regulate pancreatic insulin stores was studied. Isolated rat islets were maintained in tissue culture for 6 days with 8.3 mM glucose and mannoheptulose (0.5, 5 and 20 mM), an inhibitor of glucose phosphorylation. Mannoheptulose inhibited glucose utilization, insulin accumulation in the culture medium, and insulin biosynthesis, and decreased islet insulin content. The total recoverable insulin from the culture system (islets plus culture medium, corrected for extracellular insulin degradation) was 251, 127, 56 and 24 per cent of the content of freshly isolated islets for 0, 0.5, 5 and 20 mM mannoheptulose, respectively. Thus both 5 and 20 mM mannoheptulose caused a net loss of insulin accounted for by intracellular degradation. After 6 days in culture with 20 mM mannoheptulose, apart from β cell degranulation, there were no ultrastructural changes indicative of the mechanism of intracellular insulin destruction. When the inhibition of insulin biosynthesis was taken into account, intracellular insulin degradation could even be demonstrated in the presence of 0.5 mM mannoheptulose. Since such degradation occurred under conditions allowing for a net increase in recoverable insulin, this may be a mechanism normally operative in islets to control insulin stores.

The hormone content of endocrine cells is thought to be a reflection of a finely regulated balance between hormone biosynthesis, release, storage and degradation. Of these four intracellular events, hormone degradation is the least well characterized, even though its importance in the control of hormone content is likely in, for example, parathyroid hormone producing cells [1]. Moreover, it has been suggested that lysosomal activity contributes to the regulation of cellular hormone levels [2]. For the pancreatic β cell, evidence for the participation of insulin degradation in the regulation of pancreatic insulin content is sparse, although ultrastructural studies have shown that inhibition of insulin release by certain agents is associated with an apparent increase in lysosomal activity together with loss of storage granules [3–5]. There is also evidence for the existence in pancreatic islets of proteolytic enzymes capable of insulin degration [6].

In an earlier study, intracellular degradation of insulin was inferred from the results of experiments in which pancreatic islets were exposed to the serotonin antagonist cyproheptadine [7]. It was considered likely that this degradation was related to the severe inhibition of insulin release due to cyproheptadine. This agent, however, causes a multitude of biochemical and ultrastructural changes to islets [5, 7–9], thereby complicating the interpretation of the results.

In the present investigation, an attempt was made to improve and extend the appraisal of insulin degradation in the β cell by a better definition of the interrelationship between insulin degradation and inhibition of insulin release and biosynthesis. To this end, isolated rat pancreatic islets were maintained under tissue culture conditions in the presence of various concentrations of mannoheptulose. This seven carbon sugar, which is known to inhibit insulin release and biosynthesis [10–13], acts as a competitive inhibitor of glucose phosphorylation [14].

The results show that stored insulin decreased following exposure of islets to mannoheptulose, a decrease in which the major part could be attributed to intracellular insulin destruction.

MATERIALS AND METHODS

Isolation and maintenance of islets in culture. Pancreatic islets of Langerhans were isolated from adult male Wistar rats by the collagenase method of Lacy and Kostianovsky [15]. Islets were separated from tissue debris and exocrine cells by the method of Offord and Halban [16] in which islets are washed in a large volume of buffer and harvested on a nylon mesh, thereby ensuring adequate removal of the collagenase and avoiding time-consuming hand picking of the islets. The islets were then maintained for 6 days in tissue culture in Dulbecco's modified Eagle's medium (glucose 8.3 mM, 10% heat inactivated calf serum) as described previously [7]. Islets (100–250) were maintained in 60 mm plastic Petri dishes (to which the islets did not attach) in 4 ml of

* To whom correspondence should be addressed. Institut de Biochimie Clinique, Sentier de la Roseraie, 1211 Geneva 4, Switzerland.

culture medium in a humidified atmosphere of 95% air–5% CO_2 at 37°. Mannoheptulose was prepared as a 100× concentrated stock solution in culture medium and was sterilized by filtration (0.2 μm pore size) prior to addition to the cultures.

Determination of immunoreactive insulin and glucagon, and of total protein. Following the maintenance period, the islets were washed three times in a modified Krebs–Ringer bicarbonate (KRB) buffer [17] containing 10 mM Hepes [2-(*N*-2 hydroxyethylpiperazin-*N'*-yl) ethanesulphonic acid], 5 mM $NaHCO_3$, 5 mg/ml bovine serum albumin (BSA) and 2.8 mM glucose, adjusted to pH 7.4. For determination of the islet insulin or glucagon content, batches of 10 islets were then transferred to 1 ml of acid ethanol (150:47:3, v/v; absolute ethanol: H_2O: concentrated HCl) and left overnight at 4°. Immunoreactive insulin (either of acid ethanol extracts or of culture medium samples) was measured by radioimmunoassay [18] with rat insulin as the standard and guinea pig anti-pork insulin serum. Radioimmunoassay of glucagon was carried out following the method of Unger *et al.* [19] using pork glucagon as the standard and rabbit 30 K antiglucagon serum. ^{125}I-insulin and ^{125}I-glucagon, used as tracers for the immunoassays, were prepared by the method of Hunter and Greenwood [20]. For the estimation of total protein content islets were washed three times in KRB–Hepes buffer without BSA and then digested in 1 M NaOH before estimating protein by the method of Lowry *et al.* [21].

Measurement of glucose utilization. Glucose utilization in either freshly isolated or maintained islets was determined by measuring the conversion of [5-^{3}H]glucose to ^3H_2O after Ashcroft *et al.* [22]. In brief, this method involves the incubation of batches of 10 islets in 40 μl KRB–Hepes–BSA buffer (with mannoheptulose where appropriate) containing 2 μCi [5-^{3}H]glucose (12 Ci/mmole) isotopically diluted to obtain a final glucose concentration of 8.3 mM. The [^{3}H]glucose was lyophilized overnight before use in order to remove any ^3H_2O. The islets were incubated in small plastic wells which were supported inside sealed glass scintillation vials containing 500 μl of water. After a 2-hr incubation period islet metabolism was arrested by the addition of 40 μl of 0.1 M HCl (which was injected through the rubber caps sealing the scintillation vials) to the inner well containing the islets. ^3H_2O formed by metabolism of the [^{3}H]glucose was then allowed to equilibrate with the 500 μl of water in scintillation vials by incubation overnight at 37°. The inner wells were then removed and the amount of ^3H_2O formed determined by measuring the radioactivity of the water in the scintillation vials in a liquid scintillation counter (Beckman model LS-8000) using Biofluor as the scintillant. The percentage recovery of ^3H_2O by this method was 85 per cent of the theoretically expected value. All incubations were corrected for non-specific radioactivity by carrying out control incubations with [^{3}H]glucose in the absence of islets.

Measurement of insulin degradation in the culture medium. In order to assess the amount of insulin degraded in the medium in the presence of islets during the culture period, 50 nCi of semisynthetic [^{3}H]insulin [23], a tracer known to be degraded at the same rate as native insulin [24], was added to the medium at the start of the culture period. The specific activity of the tracer was 5 Ci/mmole and the initial insulin concentration was thus 2.5 nM. At 2, 4 and 6 days, 200 μl of medium was taken aseptically from the culture dishes and subjected to immunoprecipitation (as described below) in order to determine the percentage intact [^{3}H]insulin remaining in the culture medium.

Insulin and total protein biosynthesis. For the study of insulin and protein biosynthesis, 100 islets were transferred, after washing three times with KRB–Hepes–5 mg/ml BSA, to 0.5 ml of Eagle's minimal essential medium (Earle's salts) containing 8.3 mM glucose and 5 mg/ml BSA but lacking leucine, to which was added 100 μCi [4,5-^{3}H]leucine (specific activity 53 Ci/mmole). When islets had been maintained in culture in the presence of mannoheptulose and biosynthesis was to be measured in the continued presence of the agent, the same concentration of mannoheptulose as during maintenance was present both in the washing buffer and in the biosynthesis incubation buffer. Alternatively, when biosynthesis was measured after removal of mannoheptulose, the agent was omitted from the washing and biosynthesis buffers. After 3 hr at 37° (humidified atmosphere of 95% air: 5% CO_2) the islets were washed three times in KRB–Hepes–5 mg/ml BSA and then transferred to 1 ml of 0.2 M glycine–2.5 mg/ml BSA, pH 8.8 (glycine–BSA buffer) and sonicated for 2 × 15 sec (Branson B-12 sonifier, setting 4). The sonicates were then centrifuged (30,000 *g*) for 30 min at 2–4° in a Beckman high speed centrifuge (Model L5-65). The supernatants were used for subsequent analyses. No loss of immunoreactive insulin was detectable following sonication and ultracentrifugation by this method [25].

Immunoprecipitable radioactive material (proinsulin and insulin) was measured using the following method. A 10 μl portion of the islet sonicate (maximum immunoreactive insulin content 50 ng) was dispensed into a 400 μl plastic microfuge tube. To this, 10 μl of guinea pig anti-pork insulin serum was added (total binding capacity 300–400 ng of insulin in this system) followed by 100 μl glycine–BSA buffer containing 0.5% NP40 (Nonidet P40). Binding of insulin to antibodies was allowed to proceed for 1 hr at room temperature. After this incubation period, 5 mg Protein A–Sepharose was added in 100 μl glycine–BSA–NP40 buffer. The tubes were mixed for 15 min at room temperature and then centrifuged for 30 sec in a microfuge (8000 *g*). The supernatant fluid (containing non-immunoprecipitable material) was withdrawn with a Pasteur pipette and placed in a liquid scintillation vial. The precipitate (protein A–Sepharose with the insulin–antibody complexes bound to it) was washed twice with 250 μl glycine–BSA–NP40. The supernatant fraction from each wash was added to the original supernatant fluid. The precipitate was finally resuspended in 250 μl 1 M acetic acid–2.5 mg/ml BSA and transferred to a separate counting vial. The microfuge tube was then rinsed out with a further 250 μl acetic acid which was added to the precipitate suspension. The radioactivity of all samples was then determined after addition of 10 ml of Biofluor. For determination of nonspe-

cific binding of radioactive material to either the antiserum or the Protein A–Sepharose, nonimmune guinea pig serum was used instead of anti-insulin serum. Alternatively a 100-fold excess of native insulin (relative to the total binding capacity of the antiserum) was added to the anti-insulin serum. Both methods gave a nonspecific binding component of less than 6 per cent of the total bound radioactivity and all results are expressed as specifically immunoprecipitable radioactivity (i.e. total bound radioactivity less the nonspecifically bound radioactivity as measured for each sample in duplicate). Immunoprecipitation carried out on several aliquots of the same sample gave less than 10 per cent variability. Every sample was assayed in triplicate, and for any given condition, three independent experiments were performed.

For the determination of total radioactive protein, 10 μl aliquots of the islet sonicate supernatant fractions were mixed with 500 μl glycine–BSA buffer to which 500 μl 10% trichloracetic acid (TCA) was then added. After mixing, the tubes were centrifuged for 15 min at 3000 *g* in the cold. The supernatant fraction was removed and the precipitate resuspended in 0.5 ml of glycine–BSA and 0.5 ml 10% TCA. Both the supernatant fraction and the resuspended precipitate were then transferred to liquid scintillation vials for measurement of radioactivity. Each individual sample was assayed in triplicate; there was less than 5 per cent variability in the measured TCA-precipitable radioactivity. In control experiments, it was found that washing the TCA precipitate did not significantly improve the precision of the method.

Electron microscopy. For electron microscopy, islets were fixed in a 2% solution of phosphate-buffered glutaraldehyde, pH 7.4. After postfixing in 2% phosphate-buffered osmium tetroxide, the islets were dehydrated in ethanol solutions and embedded in Epon. Thin sections were prepared with an LKB Ultratome and stained sequentially with uranyl acetate and lead before examination with a Philips 300 electron microscope.

Presentation of results and statistical methods. All results, unless otherwise stated, are presented as the mean ± S.E. All statistical comparisons were performed by Student's *t*-test for unpaired data.

Sources of material. The following were used in this study: collagenase (Serva Gmbh, Heidelberg, F.R.G.), culture medium and Hepes (Grand Island Biochemical Co., Grand Island, NY), Petri dishes (type 1007; Falcon, Oxnard, CA), mannoheptulose (Sigma, St. Louis, MO), BSA (fraction V; Behringwerke A.G., Marburg/Lahn, F.R.G.), rat insulin and pork glucagon (Novo Terapeutisk Laboratorium, Bagsvaerd, Denmark), anti-pork insulin serum (a generous gift of Dr. H. H. Schöne, Farbwerke Hoechst A.G., Frankfurt, F.R.G.), anti-pork glucagon serum (30K; Dr. Roger Unger, Department of Internal Medicine, University of Texas Southwestern Medical School at Dallas, TX), [5-^{3}H]glucose and [4,5-^{3}H]leucine (Radiochemical Centre, Amersham, Bucks., U.K.), Sephadex G-50 and Protein A-Sepharose (Pharmacia, Zürich, Switzerland), NP40 (Nonidet P40; Fluka A.G., Buchs, Switzerland), and Biofluor (New England Nuclear, Dreieich, F.R.G.).

RESULTS

Effects of mannoheptulose on glucose utilization by islets before and after 6 days in culture. In order to compare short and long-term effects of mannoheptulose on islet glucose utilization, utilization was measured in either freshly isolated islets or in islets which had been maintained for 6 days in the presence of different concentrations of mannoheptulose. Glucose utilization was assessed by measuring the conversion of [5-^{3}H]glucose to ^3H_2O during a 2 hr incubation period in the presence of 8.3 mM glucose.

In the absence of mannoheptulose (controls), glucose utilization by freshly isolated islets was 308 ± 26 pmoles/10 islets/hr (N = 5). Following 6 days of maintenance in the absence of mannoheptulose, glucose utilization was 136 ± 10 pmoles/10 islets/hr (N = 5). To facilitate comparison between the different groups, the results of the glucose utilization experiments are expressed as a percentage of these appropriate controls (Fig. 1). For freshly isolated islets, 0.5 mM mannoheptulose inhibited glucose utilization to 72 per cent of control. Mannoheptulose (5 mM) reduced utilization to 43 per cent of control, while 20 mM mannoheptulose had no further effect. When glucose utilization was measured in the presence of 0.5 mM mannoheptulose in islets which had been maintained for 6 days at the same mannoheptulose concentration, no inhibition of utilization was observed. For islets exposed continuously to 5 mM mannoheptulose, glucose utilization was inhibited to 72 per cent of the control. Finally, islets maintained for 6 days with 20 mM mannoheptulose showed inhibition of glucose utilization to 55 per cent of control. Thus, prolonged exposure to mannoheptulose appears to cause a shift in the dose–response curve without any major influence on the maximal percentage inhibition observed.

In order to test whether such prolonged exposure to mannoheptulose resulted in permanent alterations to the islets, we also studied the reversibility of the inhibition of glucose utilization. When glucose util-

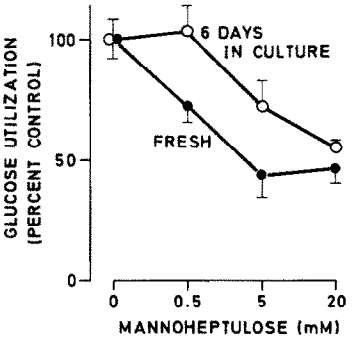

Fig. 1. Effects of different concentrations of mannoheptulose on glucose utilization by freshly isolated islets and islets maintained for 6 days in tissue culture. The maintained islets were exposed to the given mannoheptulose concentration during both the culture period and a 2 hr incubation with [5-^{3}H]glucose. The results (mean ± S.E.) are expressed as a percentage of the rate of glucose utilization of freshly isolated or maintained islets in the absence of mannoheptulose (see Results).

Table 1. Insulin, glucagon and total protein content of islets maintained for 6 days in the presence of mannoheptulose*

Mannoheptulose (mM)	Insulin (ng/10 islets) (N = 16)	P	Glucagon (ng/10 islets) (N = 16)	P	Total protein (μg/10 islets) (N = 8)	P
0	266 ± 12		18.6 ± 1.6		5.3 ± 0.5	
0.5	207 ± 13	<0.005	15.2 ± 0.8	>0.05	4.7 ± 0.4	>0.05
5	81 ± 5	<0.001	16.2 ± 1	>0.05	5.6 ± 0.4	>0.05
20	32 ± 4	<0.001	15.5 ± 1.2	>0.05	3.6 ± 0.2	<0.01

* After maintenance for 6 days in tissue culture, islets were extracted with acid ethanol for radioimmunoassay of insulin or glucagon content. Alternatively, the islets were digested with 1 M NaOH for estimation of the total protein content. Results are expressed as the mean ± S.E. The P values refer to the level of significance of the differences between islets exposed to mannoheptulose and controls (0 mannoheptulose).

ization was measured in the presence of 8.3 mM glucose but in the absence of mannoheptulose, islets which had been maintained for 6 days with 0.5, 5 and 20 mM mannoheptulose (and subsequently washed three times prior to the 2 hr glucose utilization incubation) showed glucose utilization values of 104 ± 8, 122 ± 14 and 104 ± 14 per cent of control, respectively (N = 6). Since these values are not significantly different from the control (maintained in the absence of mannoheptulose), the inhibitory effects of mannoheptulose exerted over 6 days are rapidly and completely reversible.

Effects of mannoheptulose on islet content of insulin, glucagon and total protein. Islets were maintained in culture for 6 days in the presence of different concentrations of mannoheptulose. Islet insulin, glucagon or total protein content was then determined (Table 1). The islet content of immunoreactive insulin decreased with increasing mannoheptulose concentration. At the highest dose used, 20 mM, islet insulin content was 12 per cent of control. By contrast, mannoheptulose was without significant effect on islet content of immunoreactive glucagon. Finally, total islet protein content was not affected by 0.5 or 5 mM mannoheptulose, whereas exposure for 6 days to 20 mM mannoheptulose resulted in a lowering to 68 per cent of control. The most pronounced changes induced by mannoheptulose were thus those found in insulin content.

Effects of mannoheptulose on insulin and total protein biosynthesis. Biosynthesis was followed by incubating islets for 3 hr in the presence of [^{3}H]leucine. Insulin biosynthesis was measured by immunoprecipitation of radioactive material. No attempt was made to distinguish between proinsulin and insulin biosynthesis, because under the conditions used for immunoprecipitation (> 100-fold excess of antibody binding capacity over antigen) both insulin and proinsulin are quantitatively precipitated. All results shown have been corrected for nonspecific precipitation (see Materials and Methods). Total protein biosynthesis was measured by trichloroacetic acid precipitation of aliquots of the radioactive material.

The results of experiments with freshly isolated islets are shown in the left hand panels of Fig. 2. Mannoheptulose (0.5 mM) had little effect on either insulin or protein biosynthesis. At 5 mM mannoheptulose, insulin biosynthesis was 24 per cent of control whereas protein biosynthesis was 84 per cent of control. At 20 mM mannoheptulose, these values were 8 and 77 per cent, respectively. Thus, at both these higher concentrations of the heptose, there was a preferential inhibition of insulin biosynthesis. In islets maintained for 6 days in the presence of mannoheptulose and then exposed to [^{3}H]leucine in the continued presence of the same concentration of the agent (Fig. 2, middle panels), 0.5 mM mannoheptulose only induced a small inhibition in insulin biosynthesis. The higher doses of mannoheptulose inhibited insulin biosynthesis to a comparable extent as that seen for fresh islets ($P > 0.2$), whereas total protein biosynthesis was more inhibited than in freshly isolated islets.

To test the reversibility of the inhibition of both insulin and protein biosynthesis, islets which had been maintained for 6 days in the presence of mannoheptulose were washed and then exposed to [^{3}H]leucine in the absence of the heptose (Fig. 2, right hand panels). The inhibition of insulin biosynthesis following exposure for 6 days to 0.5 and 5 mM mannoheptulose was fully reversible. Whilst removal of 20 mM mannoheptulose following 6 days of exposure significantly increased insulin biosynthesis, it was only restored to 54 per cent of control, and thus proved only partially reversible during the 3 hr biosynthesis incubation. Strikingly, total protein biosynthesis, following removal of 5 or 20 mM mannoheptulose, was 184 and 185 per cent of control, respectively.

Effects of mannoheptulose on total recoverable insulin from the culture system (islets plus culture medium). Mannoheptulose is known to inhibit insulin release [11]. This was reflected in an inhibition of the amount of insulin accumulated in the culture medium during 6 days of maintenance of islets. The results are shown in the left hand panel of Fig. 3. It can be seen that there was a progressive inhibition of insulin accumulation in the culture medium with increasing mannoheptulose concentration. At 20 mM mannoheptulose, insulin in the medium was only 9 per cent of control. In these experiments the islet insulin content was also measured (Fig. 3, right hand panel). In addition, the insulin content of representative islets prior to culture was assessed (Fig. 3, open bar). Islets maintained for 6 days in the absence of mannoheptulose showed a 19 per cent decrease in insulin content relative to the content of the appropriate fresh islets; this difference, however,

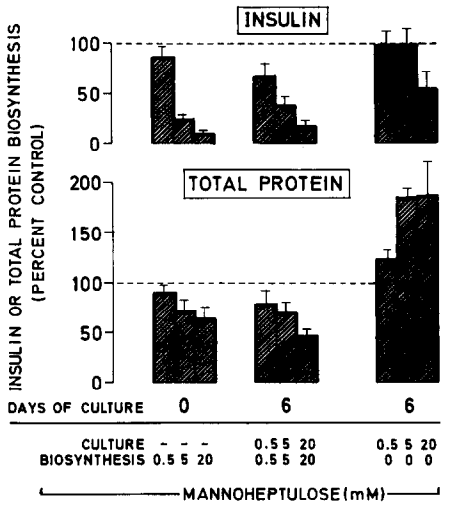

Fig. 2. Effects of different concentrations of mannoheptulose on insulin and total protein biosynthesis, and reversibility of such effects. The incorporation of [^{3}H]leucine into immunoprecipitable products (proinsulin plus insulin) and into TCA precipitable products (total protein) was assessed after a 3 hr incubation with the labelled amino acid of either freshly isolated islets (left hand panels) or of islets maintained for 6 days in the presence of mannoheptulose, the heptose being maintained at the same concentration during the biosynthesis incubation (middle panels). The right hand panels show results for islets maintained for 6 days with mannoheptulose followed by the 3 hr incubation for the study of biosynthesis in the absence of the heptose. The results are expressed as a percentage of the biosynthetic rates of control islets (dashed line). For freshly isolated islets these were 5.6 ± 1.3 ($\times 10^4$) and 31 ± 12 ($\times 10^4$) dpm/10 islets/3 hr (N = 3) for insulin and total protein biosynthesis, respectively. For 6-day-maintained islets, the biosynthetic rates were 2.5 ± 0.3 ($\times 10^4$) and 9 ± 1.4 ($\times 10^4$) dpm/10 islets/3 hr (N = 6) for insulin and total protein biosynthesis, respectively. The results are expressed as the mean ± S.E. (of three independent experiments, each experiment having been performed in triplicate). The mannoheptulose concentrations given at the bottom of the figure refer to the concentration present during either the 6 day maintenance period ('culture') or during the 3 hr incubation with [^{3}H]leucine ('biosynthesis')

was not statistically significant ($P > 0.05$). This series of experiments showed a similar decrease in islet insulin content with increasing mannoheptulose concentration as described above (Table 1).

Clearly, any insulin in the culture medium may be susceptible to degradation. In order to assess such extracellular degradation, [^{3}H]insulin was added to the culture system (in the presence of islets) at the start of the culture period, and aliquots of the medium taken for analysis of insulin degradation at various times. There was progressive, exponential degradation of insulin with time, and after 6 days 20 per cent of the original [^{3}H]insulin had been degraded. The presence of 5 or 20 mM mannoheptulose during the maintenance period had no effect on insulin degradation in the medium. Since both of these doses of the heptose markedly inhibited insulin accumulation in the culture medium, it would appear that insulin degradation in the presence of islets is independent of the insulin concentration in the medium. For the estimation of the total recoverable insulin from the culture system (islets plus culture medium) the extracellular degradation was taken into account by augmenting values for insulin accumulated in the culture medium by the 20 per cent correction factor. This factor is, in reality, an overestimated value, since it pertains to insulin present throughout the 6 day incubation. Clearly, insulin released towards the end of the culture period would be expected to be degraded to a lesser extent. The insulin content of cells which had detached from the islets during the culture period was never found to contribute significantly to the total recoverable insulin of the culture system and was thus not taken into account.

The total recoverable insulin for islets maintained in the absence of mannoheptulose was 1135 ng/10 islets (Fig. 3, right hand panel). This value shows a net increase of 683 ng/10 islets relative to the content of the islets before culture (i.e. the amount of insulin introduced into the culture system). Exposure of islets for 6 days to 0.5 mM mannoheptulose resulted in a reduction of the recoverable insulin to 569 ng/10 islets. This value is only 117 ng/10 islets higher than the insulin content of the freshly isolated islets. Thus, islets exposed to 0.5 mM mannoheptulose for 6 days only increased the insulin content of the culture system by 17 per cent compared to the increase seen in untreated islets. Insulin biosynthesis, however, was only inhibited by up to 33 per cent with this dose of mannoheptulose (Fig. 2). The apparently diminished generation of insulin cannot, therefore, be accounted for merely by inhibition of insulin biosynthesis.

When islets were treated for 6 days with 5 mM mannoheptulose, the total recoverable insulin was only 245 ng/10 islets. This value, it should be stressed, is 207 ng/10 islets lower than the initial fresh islet content. This effect is even more pronounced following exposure to 20 mM mannoheptulose where the total recoverable insulin fell to 107 ng/10 islets, a decrease of 345 ng/10 islets from the insulin content of the freshly isolated islets originally placed in culture. Although these two concentrations of mannoheptulose severely inhibit insulin biosynthesis (Fig. 2) this would not be expected to result in a net loss of insulin from the culture system (exceeding the 20 per cent insulin degradation in the culture medium already taken into account). It can therefore be concluded that treatment of islets for 6 days with mannoheptulose at the three doses studied (0.5, 5 and 20 mM) results in an intracellular loss of immunoreactive insulin. It is interesting to note that, as shown in Table 1, there was no significant decrease in islet glucagon content due to exposure of mannoheptulose. Furthermore, mannoheptulose had no effect on the accumulation of glucagon in the culture medium and, accordingly, no net loss of glucagon from the culture system due to mannoheptulose was observed (data not shown).

Effects of mannoheptulose on islet ultrastructure. Following 6 days of maintenance in culture in the absence of mannoheptulose, the ultrastructure of the islet cells was well preserved. As shown in Fig. 4a, the β cells were well granulated, the β granules retaining their typical appearance. The most striking

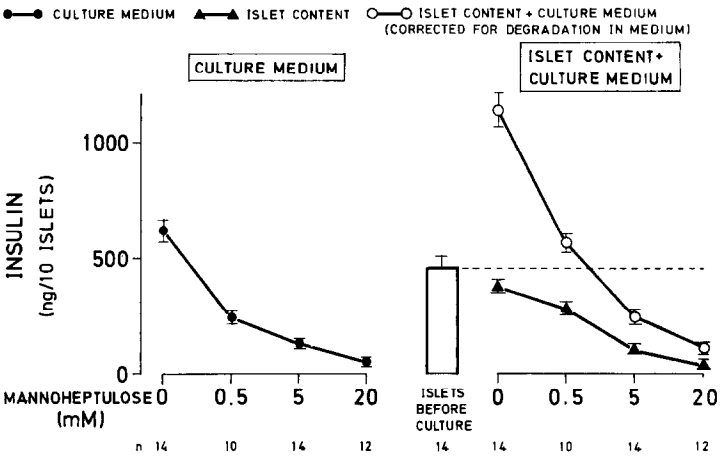

Fig. 3. Effects of 6 day exposure of islets to mannoheptulose on insulin accumulated in the culture medium and on the total recoverable insulin from the culture system. The insulin accumulated in the culture medium was assessed at the end of the 6 day maintenance period (shown in the left hand panel). The measured values were then corrected for the amount of insulin degraded in the culture medium during the culture period (see Results). These corrected values were then added to the values for insulin content of islets from the same culture vessel (shown in the lower line of the right hand panel). The sum of these values represents the total recovered insulin from the culture system corrected for extracellular insulin degradation (right hand panel, upper line). The bar and the dashed line refer to the insulin content of freshly isolated islets, i.e. the initial insulin content of the culture system. The results are expressed as the mean ± S.E. of the number of independent cultures indicated in the bottom line of the figure.

effect of 6 days of exposure to 20 mM mannoheptulose was a marked degranulation of the β cells (Fig. 4b), with a moderate dilatation of the cisternae of the rough endoplasmic reticulum which were filled with pale flocculent material.

DISCUSSION

Exposure of islets for 6 days to mannoheptulose resulted in a dose-related inhibition of glucose utilization, protein and insulin biosynthesis, and insulin release, as well as a decrease of islet insulin content. Glucose utilization by freshly isolated islets was inhibited even by 0.5 mM mannoheptulose in the presence of 8.3 mM glucose. For an agent thought to exert its effects as a competitive inhibitor of glucose phosphorylation [14] this is an unexpected result, although it does confirm earlier findings [11, 26]. Furthermore, even at 20 mM mannoheptulose, glucose utilization was inhibited only to approximately 50 per cent of control, suggesting the existence of a mannoheptulose-resistant component of glucose utilization, a conculsion reached, albeit tentatively, elsewhere [11, 22, 26]. Finally, long-term exposure of islets to mannoheptulose resulted in a greater inhibition of insulin release (as measured by insulin accumulation in the culture medium) than of glucose utilization. This has been previously observed following short-term exposure to the agent [27]. It can thus be concluded that mannoheptulose may affect β cell function by pathways distinct from glucose utilization.

Insulin biosynthesis was more severely inhibited by mannoheptulose than total protein biosynthesis under all the experimental conditions used. The inhibition of insulin biosynthesis was fully reversible following exposure for 6 days to 0.5 or 5 mM mannoheptulose and partially reversible after exposure to 20 mM mannoheptulose. For total protein biosynthesis, the inhibitory effects of mannoheptulose were fully reversible. In the case of 5 and 20 mM mannoheptulose, removal of the agent after 6 days resulted in an overshoot in protein biosynthesis to 185 and 184 per cent of control, respectively, possibly reflecting a compensatory mechanism for the prior inhibition of the synthesis of rapidly turning-over proteins. An alternative explanation could be that mannoheptulose affects intracellular leucine pools. This could result in a reduced incorporation of [^{3}H]leucine in the presence of mannoheptulose and an augmented incorporation following its removal. However, any such effects on leucine pools would not be expected to inhibit insulin biosynthesis preferentially and must thus be taken to play only a relatively minor role. Finally, there is a possibility that prolonged exposure to an agent known to interfere with glycolysis may change the energy status of the cell, in turn altering biosynthetic rates. Nonetheless, it should be stressed that insulin biosynthesis was inhibited to a similar extent whether islets had been exposed for 3 hr to mannoheptulose or for 6 days plus 3 hr. This suggests that prolonged interference with islet function by mannoheptulose does not exert an additional effect on insulin biosynthesis.

The main purpose of this study was to investigate degradation of insulin within islet cells. Since intracellular degradation in other tissues has been shown to be operative at all times [28, 29], resulting in a futile cycle, it was anticipated that careful manipulation of the balance between insulin release and biosynthesis could reveal or unmask any significant intracellular insulin degradation that might occur.

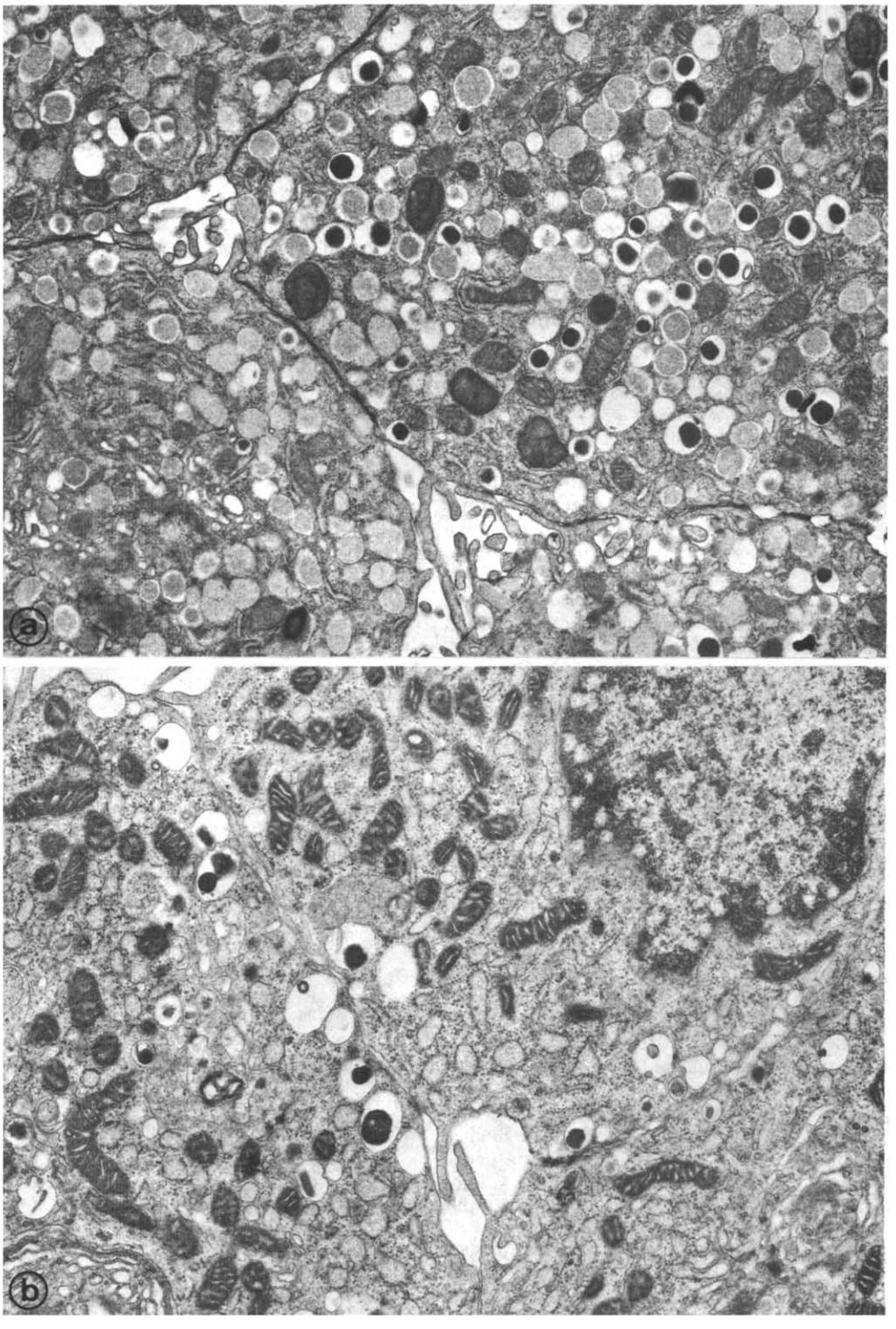

Fig. 4. Effects of mannoheptulose on islet ultrastructure. (a) β cells of islets maintained for 6 days in the absence of mannoheptulose. Magnification × 11,300. (b) β cells of islets maintained for 6 days in the presence of 20 mM mannoheptulose. Magnification × 13,800.

In addition, it has been suggested that inhibition of hormone release from various tissues may actually stimulate intracellular degradation [1, 4, 7, 30]. In this study, by changing the mannoheptulose concentration, it was possible to selectively affect insulin release with either only partial or more severe inhibition of insulin biosynthesis.

Knowing (a) the degradation of insulin in the culture medium, (b) the insulin content of the islets before culture (which is the starting content of the culture system), (c) the content of islets after culture, and (d) the amount of insulin accumulated in the culture medium, it is possible to calculate whether intracellular degradation of insulin occurs when islets are exposed to mannoheptulose. Considering the results of exposure for 6 days to 5 or 20 mM mannoheptulose, it is apparent that there is a net loss of insulin from the culture system (Fig. 3, right hand panel). Since degradation of insulin in the culture medium was measured and the results in Fig. 3 subsequently corrected for such extracellular degradation, the net loss of insulin must be due to insulin degradation within islet cells. However, merely comparing the net recoverable insulin with the insulin content of the system at the start of the culture period by necessity gives an underestimate of insulin degradation because none of the mannoheptulose concentrations used caused a complete inhibition of insulin biosynthesis. In order to assess intracellular insulin degradation with continuing, albeit reduced, insulin biosynthesis, the net production of insulin in the culture system rather than the net loss must be considered. This is achieved by comparing the total recoverable insulin from islets maintained in the absence of mannoheptulose with that measured following exposure to the agent. The expected insulin production relative to controls can be calculated knowing the percentage inhibition of insulin biosynthesis at any given dose. When this is done, it can be shown that even 0.5 mM mannoheptulose, which only marginally affected insulin biosynthesis and which allowed for a small increase in total recoverable insulin (Fig. 3), caused intracellular insulin degradation (see Results). Intracellular degradation estimated by this approach was 341 ± 36 ($N = 10$), 460 ± 20 ($N = 14$) and 454 ± 11 ($N = 12$) ng/10 islets/6 days for 0.5, 5 and 20 mM mannoheptulose, respectively. These values are similar in magnitude to the insulin content of the freshly isolated islets.

Interestingly, if the estimated values for intracellular insulin degradation are compared with the total recoverable insulin for the different mannoheptulose concentrations (Fig. 3, right hand panel) it becomes apparent that the estimated degradation varies much less. However, it should be noted that these estimations do not take into account the possibility of a major contribution of intracellular degradation normally occuring in the absence of mannoheptulose. Indeed, should such degradation occur in unperturbed islets and should its magnitude be much greater than the above estimates then the effects of mannoheptulose in absolute terms will be different. Because the techniques used here do not permit assessment of the rate constants for either insulin biosynthesis or intracellular degradation, further studies are required to answer this question.

The disposal of intracellular peptide hormone secretory granules is thought to proceed via a lysosomal pathway involving phagocytosis and subsequent destruction of granules by preexisting lysosomes (crinophagy) [3, 4, 31–33]. The ultrastructural findings presented here do not provide evidence for increased appearance of lysosomal figures following mannoheptulose treatment. Since the end products of lysosomal activity in islets have been shown to be released from the cell [34], the ultrastructure of islets maintained in the presence of mannoheptulose for only one day was also examined in order to determine whether lysosomal residual bodies could be detected prior to their release. No evidence for increased lysosomal activity due to mannoheptulose could be found even at this early time point (data not shown). Further studies are thus required in order to elucidate the mechanism of intracellular insulin degradation by the β cell, and to establish to what extent such degradation regulates insulin storage under both physiological and pathological conditions.

Acknowledgements—The authors are indebted to Miss Danielle Duvillard and Mrs. Anne-Sofie Annen for expert technical assistance, and to Drs. Orci and Meda for helpful discussions. This work was supported by the Swiss National Science Foundation (grant Nos. 3.774-0.76SR and 3.120.77).

REFERENCES

1. J. F. Habener, B. Kemper and J. T. Potts, Jr., *Endocrinology* **97**, 431 (1975).
2. R. E. Smith and M. G. Farquhar, *J. Cell Biol.* **31**, 319 (1966).
3. W. Creutzfeldt, C. Creutzfeldt and H. Frerichs, in *The Structure and Metabolism of Pancreatic Islets* (Eds. S. Falkmer, B. Hellman and I.-B. Taljedal), p. 181. Pergamon Press, Oxford (1970).
4. A. E. Renold, *Diabetes* (Suppl. 2) **21**, 619 (1971).
5. B. P. Richardson, M. L. McDaniel and P. E. Lacy, *Diabetes* **24**, 836 (1975).
6. H. Zühlke, K.-D. Kohnert, H. Jahr, S. Schmidt, H. Kirschke and D. F. Steiner, *Acta biol. med. germ.* **36**, 1695 (1977).
7. P. A. Halban, C. B. Wollheim, B. Blondel, E. Niesor and A. E. Renold, *Endocrinology* **104**, 1096 (1979).
8. K. L. Hintze, A. B. Grow and L. J. Fischer, *Biochem. Pharmac.* **26**, 2021 (1977).
9. H. G. Joost, J. Beckmann, S. Lenzen and A. Hasselblatt, *Acta endocrin. (Kbh.)* **82**, 121 (1976).
10. H. G. Coore, P. J. Randle, E. Simon, P. F. Kraicer and M. C. Shelesnyak, *Nature, Lond.* **197**, 1264 (1963).
11. W. S. Zawalich, A. S. Pagliara and F. M. Matschinsky, *Endocrinology* **100**, 1276 (1977).
12. D. G. Pipeleers, M. Marichal and W. J. Malaisse, *Endocrinology* **93**, 1001 (1973).
13. S. J. H. Ashcroft, J. Bunce, M. Lowry, S. E. Hansen and C. J. Hedeskov, *Biochem. J.* **174**, 517 (1978).
14. H. G. Coore and P. J. Randle, *Biochem. J.* **91**, 56 (1964).
15. P. E. Lacy and M. Kostianovsky, *Diabetes* **16**, 35 (1967).
16. R. E. Offord and P. A. Halban, *Biochem. biophys. Res. Commun.* **82**, 1091 (1978).
17. C. B. Wollheim, M. Kikuchi, A. E. Renold and G. W. G. Sharp, *J. clin. Invest.* **60**, 1165 (1977).
18. V. Herbert, K.-S. Lau, C. W. Gottlieb and S. J. Bleicher, *J. Endocrin. Metab.* **25**, 1375 (1965).

19. R. H. Unger, E. Aguilar-Parada, W. A. Muller and A. M. Eisentraut, *J. clin. Invest.* **49**, 837 (1970).
20. W. M. Hunter and F. C. Greenwood, *Nature, Lond.* **194**, 495 (1962).
21. O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. biol. Chem.* **193**, 265 (1951).
22. S. J. H. Ashcroft, L. C. C. Weerasinghe, J. M. Bassett and P. J. Randle, *Biochem. J.* **126**, 525 (1972).
23. P. A. Halban and R. E. Offord, *Biochem. J.* **151**, 219 (1975).
24. P. A. Halban, C. Karakash, J. G. Davies and R. E. Offord, *Biochem. J.* **160**, 409 (1976).
25. A. Maldonato, P. A. Trueheart, A. E. Renold and G. W. G. Sharp, *Diabetologia* **12**, 471 (1976).
26. S. J. H. Ashcroft, C. J. Hedeskov and P. J. Randle, *Biochem. J.* **118**, 143 (1970).
27. J. Sehlin, *Fedn Eur. biochem. Soc. Lett.* **30**, 45 (1973).
28. F. J. Ballard, in *Essays in Biochemistry* (Eds. P. N. Campbell and W. N. Aldrige), Vol. 13, p. 1. Academic Press, London (1977).
29. R. S. Bienkowski, B. J. Baum and R. G. Crystal, *Nature, Lond.* **276**, 413 (1978).
30. B. Ziegler, H.-K. Hahn, M. Ziegler and H. Fiedler, *Endokrinologie* **69**, 103 (1977).
31. M. G. Farquhar, *Mem. Soc. Endocrin.* **19**, 79 (1971).
32. L. Orci, A. Junod, R. Pictet, A. E. Renold and C. Rouiller, *J. Cell. Biol.* **38**, 462 (1968).
33. P. Meda, *Diabetologia* **14**, 305 (1978).
34. R. Zwahlen, B. P. Richardson and R. E. Hauser, *J. ultrastruct. Res.* **67**, 340 (1979).