Journal of Chromatography, 614 (1993) 185–204
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam

CHROMBIO. 6752

Review

Analysis of proinsulin and its conversion products by reversed-phase high-performance liquid chromatography

Susanne Linde*

Immunochemical Department, Novo Nordisk A/S, Novo Allé, DK-2880 Bagsværd (Denmark)

Benny S. Welinder

Department of Structural Chemistry, Novo Nordisk A/S, DK-2820 Gentofte (Denmark)

Jens H. Nielsen

Hagedorn Research Institute, DK-2820 Gentofte (Denmark)

(First received November 20th, 1992; revised manuscript received January 18th, 1993)

ABSTRACT

Proinsulin is synthesized in the β-cells of the endocrine pancreas, one of the four cell types found in the islets of Langerhans. Specific enzymatic cleavage of proinsulin results in the formation of equimolar amounts of insulin and C-peptide, via several intermediate split-proinsulin forms. Most mammals produce a single insulin, but in rodents two non-allelic insulin genes are expressed. There is an inverse ratio between the two insulins in rats and mice, the reason for this being unknown. It has been suggested that differences in transcription, translation (biosynthesis) and/or posttranslational processes (enzymatic conversion, intracellular degradation) could be possible explanations. Elevated amounts of proinsulin-immunoreactive material (PIM) have been described to occur in various conditions/diseases, suggesting alterations in β-cell function, but the composition of the secreted PIM (intact proinsulin or its intermediates) has been incompletely determined. Studies of the biosynthesis of proinsulins and their conversion with the purpose of revealing some of these points depend on accessible reversed-phase high-performance liquid chromatographic (RP-HPLC) analyses capable of separating all the relevant, closely related polypeptides involved. This review will deal with the optimization of the RP-HPLC separations as well as sample preparation and recovery. Applications of the selected methods in the study of proinsulin biosynthesis and its conversion will also be presented.

CONTENTS

* Corresponding author.

0378-4347/93/$24.00

LIST OF ABBREVIATIONS

β-TC	β-Tumor cell
C_1, C_2	C-Peptide I and II
cDNA	Copy deoxynucleic acid
ELISA	Enzyme-linked immunosorbent assay
HBSS	Hanks' buffered salt solution
hGH	Human growth hormone
HI	Human insulin
HPI	Human proinsulin
HSA	Human serum albumin
I_1, I_2	Insulin I and II
IDDM	Insulin-dependent diabetes mellitus
kD	Kilodalton
mRNA	Messenger ribonucleic acid
NEDH	New England Deaconess Hospital (inbred rat strain)
NIDDM	Non-insulin-dependent diabetes mellitus
NMRI	Naval Medical Research Institute (outbred mouse strain)
P_1, P_2	Proinsulin I and II
PAGE	Polyacrylamide gel electrophoresis
PIM	Proinsulin immunoreactive material
RER	Rough endoplasmatic reticulum
RIA	Radioimmunoassay
RIN	Rat insulinoma NEDH
RP-HPLC	Reversed-phase high-performance liquid chromatography
RPMI	Roswell Park Memorial Institute culture medium
R_s	Resolution
TEAP	Triethylammonium phosphate
TFA	Trifluoroacetic acid

1. INTRODUCTION

The islets of Langerhans in the pancreas are composed of four different endocrine cell types, each producing predominantly a single type of polypeptide hormone. The most abundant cell type is the β-cells, which synthesize insulin. The α-cells, the δ-cells and the PP cells synthesize the hormones glucagon, somatostatin and pancreatic polypeptide, respectively [1]. All the polypeptide hormones from the pancreatic islets are synthesized as larger precursor molecules that are post-translationally processed by specific enzymes to the mature hormones.

The initial product of translation of insulin mRNA is preproinsulin [2,3], the first 24 amino acids of which constitute the signal or prepeptide. The very hydrophobic prepeptide is thought to be removed while the preproinsulin traverses the membrane of the rough endoplasmatic reticulum (RER), presumably coincidentally with the translation [4]. The newly formed proinsulin chain then folds up and the disulphide bonds are established.

The conversion of proinsulin to insulin involves specific converting enzymes and occurs within clathrin-coated secretory vesicles coordinately with acidification [5]. Two classes of enzymes are involved: endopeptidases I and II cleave at the C-terminal side of the two pairs of basic amino acid residues between the C-peptide and the insulin B- and A-chains, respectively, resulting in the formation of two split-proinsulin intermediates [6]. Next carboxypeptidase H [6] removes the basic residues one at a time, resulting

in the formation of two des-proinsulin intermediates prior to the formation of the final conversion products, insulin and C-peptide (see Fig. 1).

Recently cDNAs encoding candidate prohormone-processing endopeptidases have been cloned and found to be related to the bacterial subtilisin family of serine proteases, as reviewed by Barr [7].

Rats and mice are unique among mammals in that they produce two different insulins (insulins I and II) [8], encoded by two non-allelic genes [9,10]. The conservation of both genes suggests that each provides some selective advantage, *e.g.* the capacity to produce twice as much insulin during foetal growth or prolongation of reproductive life [11], or that insulins I and II might play different roles as growth and regulatory factors during foetal development [12]. The two insulins are usually found in unequal amounts in whole pancreas as well as in isolated pancreatic islets of rats [8,13–16] and mice [14,16–18]. In rats, insulin I is always more abundant than insulin II, whereas the opposite is found in mice [16,17].

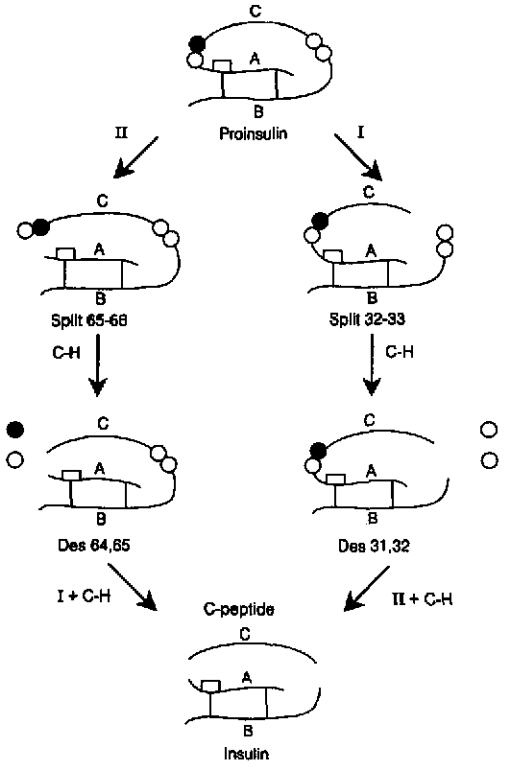

Fig. 1. Processing of proinsulin via the major conversion intermediates to insulin and C-peptide. The A- and B-chains and the C-peptide are designated A, B and C, respectively. The open and closed circles represent the basic amino acids arginine and lysine, respectively. Proinsulin is cleaved by endopeptidases I and II at the BC and AC junctions, respectively, followed by removal of the basic amino acids by carboxypeptidase H (C-H).

The reasons for the reported difference in the insulin I/II ratios in rat and mouse islets could be differences in transcription of the two genes, translation of the mRNAs, proinsulin conversion rates or intracellular degradation (crinophagy, *i.e.* fusion of granules with lysosomes) between the two species. Up to now the expression of the two genes in rats has been proposed to be independently regulated [13,14] or coordinately regulated in both rats [19,20] and mice [12].

Simultaneously with the isolation of proinsulin from the first crystals of insulin, intermediate derivatives of the proteolysis were observed by Steiner *et al.* [21]. Normally more than 99% of proinsulin in rats is processed via the regulatory pathway [22] to the final products, insulin and C-peptide, which are released from the β-cell on appropriate stimuli.

In healthy individuals the circulating levels of proinsulin-immunoreactive material (PIM) are mostly rather low, but in various forms of diabetes an elevated level of PIM has been found [23,24], suggesting that proinsulin or its intermediates could be predictive markers for the manifestation of diabetes. A heterogeneous composition of PIM in human plasma was demonstrated in 1978 using a combination of immunoaffinity, gel filtration and radioimmunoassay [25], but the physiological and clinical significance of proinsulin and its conversion intermediates in the circulation is not known. The lack of specific immunological analyses for the individual metabolites [26] demands further development or alternative methods based on an initial separation of the conversion intermediates prior to the immunological analysis.

During the past ten years reversed-phase high-performance liquid chromatography (RP-HPLC) has been the preferred technique for the

separation of closely related polypeptides, including insulin and insulin-related polypeptides [27–30]. The extremely high resolving power of RP-HPLC is exemplified by the successful separation of the four isomeric monoiodoinsulins having identical composition [31].

This review will deal with the use of carefully selected RP-HPLC methods in the evaluation of the proinsulin biosynthesis in rats, mice and transgenic mice as well as the composition of proinsulin-immunoreactive material in human serum.

2. RP-HPLC OF PROINSULINS, INSULINS AND C-PEPTIDES FROM RATS AND MICE

The two proinsulins in the rat differ in four of the 86 amino acids, two in the B-chain and two in the C-peptide [32]. The two mouse insulins (I and II) have amino acid sequences identical to those of rat insulins I and II [17]. Mouse C-peptide I differs in two of the 31 amino acids from rat C-peptide I and also has a two amino acid deletion, whereas mouse C-peptide II differs from rat C-peptide II in one of the 31 amino acids [16,33] (see Table 1).

The separation of the proinsulin biosynthesis products from rats and mice was previously performed by ion-exchange chromatography [8,17,34], polyacrylamide gel electrophoresis (PAGE) [8,13,14,17,34] or isoelectric focusing [35]. These methods all depend on differences in net charge, but none is selective enough to give the desired separation of all the components relevant to proinsulin biosynthesis.

TABLE 1

AMINO ACID DIFFERENCES IN RAT AND MOUSE PROINSULINS

Amino acid residue number[a]	Rat I	Rat II	Mouse I	Mouse II
B9	Pro	Ser	Pro	Ser
B29	Lys	Met	Lys	Met
C8	Pro	Ala	Glu	Ala
C15	Gly	Gly	Ser	Gly
C17	Glu	Gly	–[b]	Gly
C18	Ala	Ala	–[b]	Ala
C30	Arg	Arg	Arg	Gln

[a] B and C refer to the B-chain and C-peptide part of proinsulin, respectively.

[b] Deletion.

The successful use of RP-HPLC in polypeptide analyses is most of all the result of its superior selectivity, which can be optimized by variations in stationary and mobile phase composition. The quantitative aspects of the separation of the closely related polypeptides involved in proinsulin biosynthesis are also influenced by the sample preparation steps.

2.1. Sample preparation

Extraction of insulin and insulin-like components from pancreatic tissue and isolated islets has been performed by the classical industrial extraction in acid alcohol modified for laboratory scale [13,35], in acid sodium chloride solution [36] or in 3 *M* acetic acid [16]. When dealing with concentrations less than 10 μg/ml, 0.1% serum albumin is added to minimize adsorption to various surfaces [37,38].

Tissue disruption has been accomplished by sonication [22,34,39], by freezing–thawing cycles [40] or by a combination of both methods [41]. It is presumably important to avoid generation of heat [42], but a sonication step seems to be unnecessary in 3 *M* acetic acid, as the insulins left after passive extraction of isolated rat and mouse islets for 1 h at room temperature account for less than 5% [42].

A specific sample purification is the immunoprecipitation of insulins/proinsulins by the use of a large excess of anti-insulin serum incubated with sonicated islets [43]. Quantitative precipitation and recovery can be achieved with this technique [44], but the isolation of microgram amounts of insulins requires very large amounts of anti-insulin serum. Even if the immunoprecipitation is not quantitative, it has been found that the ratios of insulins to proinsulins in biosynthetically labelled rat islets do not change significantly when compared with an acetic acid-extracted

sample of an identical pool of islets [42]. On the other hand, the detergent used in this technique (Nonidet P-40) changed the surface characteristics of the HPLC stationary phase, leading to a deterioration in separations [42]. The C-peptides will not be precipitated using anti-insulin serum, and the evaluation of their significance in the biosynthetic sequence is therefore rendered impossible.

Reduction of the sample volume has often been performed on a disposable C_{18} cartridge [45,46], but this procedure may lead to oxidation of methionine residues (Met B29 in insulin II and proinsulin II) to methionine sulphoxide derivatives [47]. Since the oxidized polypeptides have reduced retention times in RP-HPLC [48], this oxidation should be minimized in order to prevent misleading chromatograms and problematic quantitation, *e.g.* the Met-O derivative of mouse proinsulin II partly co-elutes with proinsulin I [18]. The oxidation of methionine residues can be minimized by the addition of reducing agents [47,49].

Adsorption to tube walls can be a problem if the samples are lyophilized to dryness, as illustrated in Fig. 2. Extracts of biosynthetically labelled rat islets were analysed by RP-HPLC directly or after lyophilization and redissolving in various solvents, and the recoveries after lyophilization/redissolving were in most cases differentially decreased [42]. It seems that 3 *M* acetic acid containing 0.1% human serum albumin (HSA) is the best solvent to obtain an equal dissolution of all groups of the polypeptides.

Finally, the removal of particulate matter is important to secure the performance and lifetime of the RP-HPLC column. Centrifugation (2 min at 10 000 *g*) is preferred over filtration because of the risk of differential adsorption of individual sample compounds to the filter.

2.2. *Effects of the stationary phase*

Evaluation of the performance of RP-HPLC systems for the separation of all the components involved in insulin biosynthesis is based on the

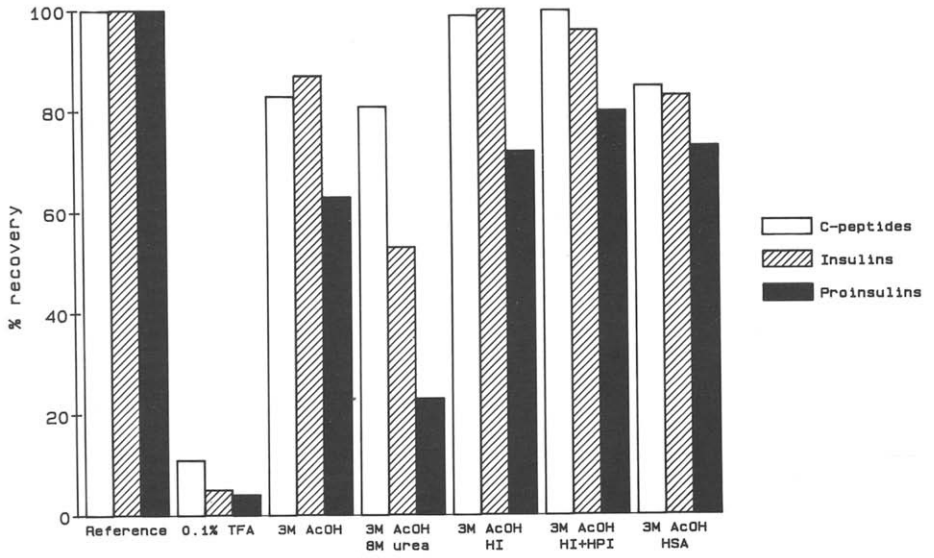

Fig. 2. Identical samples of 50 biosynthetically labelled rat islets (60 min with [^{3}H]leucine and [^{35}S]methionine [16]) were analysed by RP-HPLC directly or after lyophilization and redissolving in 0.1% TFA (trifluoroacetic acid) or 3 *M* AcOH (acetic acid) containing 8 *M* urea, HI (human insulin, 0.1 mg/ml), HI plus HPI (human proinsulin, 0.1 mg/ml) or HSA (human serum albumin, 1 mg/ml). The RP-HPLC conditions and the measurements of the radioactivity are described in the legend to the upper panel of Fig. 3. The content of C-peptides, insulins and proinsulins (radioactivity) in the samples is calculated relative to that of the reference sample directly analysed (~100%) as a measurement of binding to the tube walls.

resolution (R_s) of closely related pairs of biosynthetically labelled C-peptides (I and II), insulins (I and II) and proinsulins (I and II) calculated as $2 \times (t_2 - t_1)/(w_1 + w_2)$, where t_1 and t_2 are the retention times of the two adjacent peaks and w_1 and w_2 their base widths. R_s values between 0.5 (12.5% overlap) and 1.0 (baseline separation) are considered satisfactory [50].

Table 2 shows a simplified evaluation of the RP-HPLC separations performed with these goals in mind. Details of the individual separations can be found in the cited references, but in general shallow linear acetonitrile gradients were used. From the table it can be seen that the separation of the two rat C-peptides and insulins is easy to achieve, whereas only a few systems are

TABLE 2

RP-HPLC SEPARATION OF RAT PANCREATIC ISLET POLYPEPTIDES

Bonded phase	Stationary phase	Mobile phase buffer[a]	C-peptides	Insulins	Proinsulins	Ref.
C_{18}	Ultrasphere ODS	TEAP–perchlorate pH 3.0	Not detected	Separated	Co-eluted	39
C_{18}	Ultrasphere ODS	TEAP–perchlorate pH 3.0	Separated	Separated	Co-eluted	15
C_{18}	LiChrosorb RP-18[b]	TFA	Separated (R_s = 0.8)	Separated (R_s = 1.7)	Separated (R_s = 0.7)	16
C_{18}	LiChrosorb RP-18	TEAP pH 4.0	Separated (R_s = 0.8)	Separated (R_s = 1.0)	Co-eluted	16
C_{18}	Ultrasphere ODS	TEAP pH 4.0	Separated (R_s = 1.3)	Separated (R_s = 1.6)	Separated (R_s = 0.5)	16
C_{18}	LiChrosorb RP-18	Ammonium sulphate pH 4.0	Separated (R_s = 0.6)	Separated (R_s = 1.1)	Co-eluted	51
C_{18}	LiChrosorb RP-18	Ammonium sulphate pH 3.0	Separated (R_s = 1.3)	Separated (R_s = 3.0)	Separated (R_s = 0.8)	51
C_{18}	Nucleosil 120-5C_{18}	TEAP pH 4.0	Separated (R_s = 1.6)	Separated (R_s = 1.2)	Separated (R_s = 0.4)	50
C_{18}	Ultrasphere ODS	TEAP–perchlorate pH 3.0	Not detected	Separated	Separated	52
C_8	PEP-RP1	TFA morpholine	Not detected	Separated	Co-eluted	53
C_8	LiChrosorb RP-8	TEAP pH 4.0	Separated (R_s = 1.3)	Separated (R_s = 1.3)	Co-eluted	50
C_4	Nucleosil 300-5C_4	TEAP pH 4.0	Separated (R_s = 1.2)	Separated (R_s = 0.5)	Separated[c] (R_s = 0.4)	50
C_4	Nucleosil 300-5C_4	TFA	Separated (R_s = 1.2)	Separated (R_s = 1.1)	Separated (R_s = 0.4) or co-eluted	50, 51
C_4	Bakerbond WP Butyl	TEAP pH 4.0	Separated (R_s = 1.1)	Separated (R_s = 1.1)	Co-eluted	50
C_4	Bakerbond WP Butyl	TFA	Separated (R_s = 1.2)	Separated (R_s = 1.1)	Co-eluted	50
C_3	Zorbax Protein Plus	TEAP pH 4.0	Separated (R_s = 1.3)	Separated (R_s = 1.0)	Co-eluted	50
C_3	Zorbax Protein Plus	TFA	Separated (R_s = 1.3)	Separated (R_s = 1.0)	Co-eluted	50

[a] Organic modifier: acetonitrile.

[b] Recent batches bind insulins and proinsulins irreversibly.

[c] Eluted in reverse order.

able to separate the two rat proinsulins [16,51]. Problems with irreversible binding of insulins and proinsulins to certain batches of LiChrosorb columns, specifically when using the preferred mobile phase trifluoroacetic acid (TFA)–acetonitrile, were attributed to the poor ion pairing of diluted TFA in combination with the hydrophobic C_{18} (and C_8) ligands [51]. This was never seen when, for example, triethylammonium phosphate (TEAP) was used as buffer.

Low yields and poor reproducibility were reported when a LiChrosorb RP-18 column was used for the separation of large and hydrophobic proteins [54], in accordance with the assumption that this column is probably one of the most hydrophobic of all commercially available C_{18} columns [55]. Irreversible protein adsorption has also been attributed to the denaturing mobile phase in combination with too long alkyl-bonded phases [56].

Insulins and proinsulins are eluted with ideal peak shapes from the less hydrophobic C_4 and C_3 stationary phases, however, the selectivity of these stationary phases eluted with TFA–acetonitrile gradients is too low to separate the two rat proinsulins [51].

The separation of C-peptides, insulins and proinsulins from mouse is in fact easier to achieve (see Table 3). The two mouse proinsulins can even be resolved in one of the RP-HPLC systems unable to separate the two rat proinsulins, *i.e.* LiChrosorb RP-18/TEAP pH 4.0–acetonitrile, presumably reflecting the two amino acid deletion in the C-peptide part of mouse proinsulin I (see Fig. 1). An example of a successful RP-HPLC separation on a LiChrosorb RP-18 column eluted with TFA–acetonitrile is shown in Fig. 3, upper panel.

By using carefully selected silica-based RP columns it is possible to achieve the separation of all rat and mouse polypeptides related to the biosynthesis of insulin. However, since several of these stationary phases suffer from far from ideal behaviour (pronounced non-specific binding and lack of column-to-column reproducibility), ascribed to the presence of unmasked silanol groups on the silica surface [51], the use of polymer-based RP columns for this critical separation has been evaluated [50]. Polymer-based RP columns offer superior chemical stability (pH 1–13) compared with silica-based RP columns [59], and no potential interfering silanol groups exist [55]. The excellent properties of the polyvinyl alcohol-based Asahipak columns have been demonstrated for standard proteins, as well as for complicated mixtures of pancreatic proteins and polypeptides, including insulin [60].

Non-ideal peak shapes and irreversible binding

TABLE 3

RP-HPLC SEPARATION OF MOUSE PANCREATIC ISLET POLYPEPTIDES

Bonded phase	Stationary phase	Mobile phase buffer[a]	C-peptides	Insulins	Proinsulins	Ref.
C_{18}	Vydac	TFA or TEAP pH 2.25	Not detected	Separated	Separated	18
C_{18}	LiChrosorb RP-18	TEAP pH 4.0	Separated[b] (R_s = 1.1)	Separated (R_s = 1.2)	Separated (R_s = 0.8)	57
C_{18}	LiChrosorb RP-18	TFA	Separated (R_s = 1.1)	Separated (R_s = 2.3)	Separated (R_s = 1.1)	58
C_4	Nucleosil 300-5C_4	TFA	Not detected	Separated (R_s = 1.1)	Separated (R_s = 0.8)	42

[a] Organic modifier: acetonitrile.

[b] Reversed elution order.

of insulins and proinsulin have not been observed when these columns are eluted with shallow acetonitrile gradients in TFA, as is the case for several C_{18} and C_8 silica-based columns, however, only the Asahipak C_4 column is able to separate all insulin biosynthesis polypeptides, including the rat proinsulins, satisfactorily in TFA–acetonitrile, whereas both the C_4 and C_8 column resolve all components in TEAP–acetonitrile [51], suggesting an advantageous use of polymeric RP columns over silica RP columns.

2.3. *Effects of the mobile phase*

The selection of mobile phase organic modifier is based on solubility and viscosity, and in spite of its toxicity acetonitrile is most widely used because of its excellent performance as well as lower backpressure and lower UV cut-off compared with other modifiers (*i.e.* ethanol, 2-propanol).

The majority of reversed-phase separations of insulin and insulin-related compounds have been performed using mobile phases based upon two groups of buffer substances: acid or neutral salts; or other ion-pairing substances, such as TEAP or TFA [30], which increase polypeptide hydrophobicity, consequently increasing the retention.

The use of acids, *e.g.* phosphoric acid [61], and salts, *e.g.* ammonium sulphate [30,62], results in high resolution and excellent peak shapes (narrow, symmetrical). TFA [63] is the most popular acid because of its excellent solubilizing properties, low UV cut-off and volatility. However, the peak shapes of polypeptides are often inferior to those resulting from the use of TEAP (also allowing low UV detection) as mobile phase buffer [64]. The successful use of TEAP as mobile phase buffer at pH values between 2.25 and 4.0, [16,18,50,57], occasionally with the addition of perchlorate [15,39,52], can be seen in Tables 2 and 3.

The use of these buffer components minimizes the general problem encountered with silica-based stationary phases, the free silanol groups. The primary function of the buffer is ion pairing to the charged groups in the sample molecules and to the free silanol groups [49,65], thereby hopefully eliminating non-specific binding of peptide/proteins to the stationary phase in general and to the free silanol groups in particular. Despite secondary derivatization (end-capping) procedures (performed with the purpose of obtaining maximal carbon coverage of the silica support), it is unavoidable that silanol groups to a greater or lesser extent remain unmasked [66], and additional silanols are constantly created during reversed-phase chromatography owing to chemical degradation of the silica-based stationary phases in the commonly used acidic mobile phases [49,59,65,67]. Since unreacted silanol groups have been blamed for most of the undesirable effects on reversed-phase silicas (such as reduced recovery and non-ideal peak shapes [49,67–73]), shielding of these groups is extremely important in analytical RP-HPLC.

Ideal peak shapes have been obtained using the alkylammonium salts, *i.e.* TEAP and TEAP–perchlorate, as well as neutral salts such as ammonium acetate [74], sodium phosphate [36] and ammonium sulphate [51] as mobile phase additives (see also references in Tables 2 and 3), but the separation of the two rat proinsulins proved difficult to achieve (Table 2). The pH value is an important parameter, *e.g.* a mobile phase containing ammonium sulphate pH 4.0 cannot separate the two rat proinsulins, but this is possible at pH 3.0 [51].

Another parameter of importance for the resolution of polypeptides is the separation temperature. This has a marked influence on the resolution and retention times of several of the polypeptides involved in the insulin biosynthesis, resulting in the desired separation of the two rat and mouse proinsulins at elevated temperature. Typical increases in R_s for the two rat proinsulins are from 0.4 to 0.8 [50] and for the two mouse proinsulins from 0.8 to 1.7 [42]. An example of the separation at 45°C on the Nucleosil 300-5C_4 column is shown in Fig. 3, lower panel.

Interestingly, the retention times of individual islet polypeptides are differently affected on silica- and polymer-based columns [50], reflecting the complexity of the influence of the temperature. It has been reported that the use of elevat-

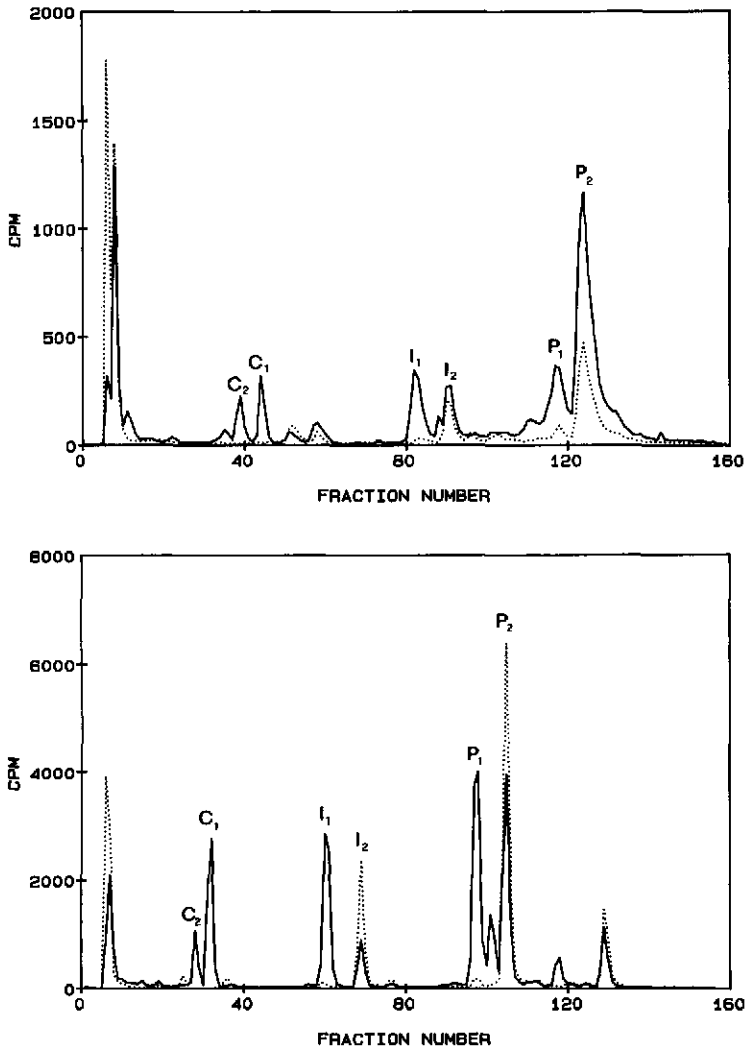

Fig. 3. RP-HPLC separation of a 3 *M* acetic acid extract of 50 mouse islets (upper panel), biosynthetically labelled for 20 min (pulse) with [^{3}H]leucine and [^{35}S]methionine (25 μCi of each), followed by 60 min incubation without radioactive amino acids (chase). The column was a LiChrosorb RP-18, 5 μm particle size, 250 mm × 4.0 mm I.D., eluted at 1.0 ml/min with a linear acetonitrile gradient (30–36%) in 0.1% TFA during 60 min. The lower panel shows an RP-HPLC separation of a 3 *M* acetic acid extract of 50 rat islets, labelled for 60 min with [^{3}H]leucine and [^{35}S]methionine using a Nucleosil 300-5C_4 column, 5 μm particle size, 250 mm × 4.0 mm I.D., eluted at 1.0 ml/min with a linear acetonitrile gradient (26–32%) in 0.1% TFA during 60 min at 45°C. The radioactivity was measured in the collected 0.5-min fractions after addition of 4 ml of Optiphase "HiSafe" (LKB). The solid lines represent [^{3}H]leucine and the dotted lines [^{35}S]methionine counts per minute, respectively. Peaks: C_1 = C-peptide I; C_2 = C-peptide II; I_1 = insulin I; I_2 = insulin II; P_1 = proinsulin I; P_2 = proinsulin II.

ed temperatures is not feasible with silica-based stationary phases [67], but the performance of the Nucleosil C_4 column at 45°C in TFA–acetonitrile does not seem to deteriorate during six months of use [42].

The elution order of polypeptides is related to the overall hydrophobicity, and their retention times may be predicted from the amino acid sequence [75,76]. Peptides larger than fifteen to twenty residues tend to elute more rapidly than predicted from hydrophobic considerations alone unless a peptide chain-length correction is

used [77]. Comparing the two pairs of C-peptides, insulins and proinsulins, their actual elution order is in accordance with the predicted elution order [42], but in some cases a reverse elution order has been observed for the C-peptides from mice [57], the insulins from rats and mice [41,74] and the proinsulins from rats [50] (see Tables 2 and 3). This behaviour, taken together with altered retention times and elution orders at elevated separation temperature [50], suggests that the adsorption/desorption kinetics depends upon experimental parameters other than hydrophobicity and chain length.

Transgenic mice with the human insulin gene inserted express, besides the two mouse insulins, human insulin. The RP-HPLC system used for evaluation of the biosynthesis and conversion should be able to separate human insulin and proinsulin from mouse insulins and proinsulins. An overview of the possible systems (using only TFA–acetonitrile as mobile phase) for the separation of the polypeptides involved in insulin biosynthesis in transgenic mice is given in Table 4. Only systems previously shown to separate the two mouse proinsulins [42] are evaluated.

2.4. Recovery

The recovery of the polypeptides during extraction, sample preparation and RP-HPLC analysis is of major importance when a quantitative calculation of the biosynthesis and conversion of the two proinsulins to the corresponding C-peptides and insulins is the goal. The tendency of insulins and proinsulins to bind to plastic and glass surfaces [78] may result in changed ratios of individual polypeptides, as mentioned in Section 2.1 (see Fig. 2), unless albumin is added to the solution.

The overall recovery of rat insulins/proinsulins after RP-HPLC has been determined immunologically to be 90–110% [39] or 78–93% [41], and that of mouse insulins/proinsulins to be 90–100% [79], but since the proinsulins only amount to a few per cent of the insulins, the total recovery of proinsulins may very well be lower, as reported previously [80]. By injecting identical samples onto the column as well as after bypassing the column with a capillary, the absolute recoveries of individual biosynthetically labelled rat C-peptides, insulins and proinsulins in the mass range

TABLE 4

RP-HPLC SEPARATION OF PANCREATIC POLYPEPTIDES FROM TRANSGENIC MOUSE ISLETS

Bonded phase	Stationary phase	Mobile phase buffer[a]	Temperature (°C)	HI/I_1[b]	HPI/P_1[b]	Ref.
C_{18}	LiChrosorb RP-18	TFA	25	Separated (R_s = 1.1)	Separated (R_s = 0.8)	58
C_4	Nucleosil 300-5C_4	TFA	25	Separated (R_s = 1.3)	Separated (R_s = 1.4)	50
C_4	Nucleosil 300-5C_4	TFA	45	Poorly separated (R_s = 0.4)	Separated (R_s = 0.9)	50
C_{18}	Asahipak ODP-50	TFA	45	Co-eluted	Separated[c] (R_s = 0.8)	42
C_4	Asahipak C_4P-50	TFA	25	Separated[d] (R_s = 1.5)	Separated (R_s = 0.7)	50

[a] Organic modifier: acetonitrile.

[b] HI = human insulin; I_1 = insulin I; HPI = human proinsulin; P_1 = proinsulin I.

[c] HPI co-eluted with insulin II.

[d] Eluted in reverse order.

from micrograms to picograms have been measured to be in the range 89–110% using both silica- and polymer-based C_4 columns eluted with a shallow acetonitrile gradient in 0.1% TFA [50].

Several reports support these quantitative recoveries for peptides [64,81,82], whereas reduced recoveries have also been reported [72,83,84]. The molecular mass/hydrophobicity of the polypeptide is presumably also of importance for the recovery [30,85], as discussed previously in connection with free silanol groups on the stationary phase surface. The influence of temperature on recovery seems to be controversial [27,51,67].

It is evident that any chromatographic system that results in less than quantitative recovery of all the components of a mixture cannot be used as a quantitative analytical tool.

3. RP-HPLC OF HUMAN PROINSULIN AND ITS CONVERSION INTERMEDIATES

It is well known that the limited digestion of proinsulin by trypsin leads to the generation of conversion intermediates cleaved either between the B-chain and the C-peptide (BC junction) or between the C-peptide and the A-chain (CA junction), resulting in two split-proinsulin intermediates still carrying basic amino acids at their C-termini [86]. Within the β-cell such basic residues are rapidly removed by carboxypeptidase H [87], resulting in two des-proinsulin intermediates (see Fig. 1). Intermediates lacking the basic residues are much more abundant in pancreatic extracts, presumably because carboxypeptidase-like proteases are present in relative excess over trypsin-like endoproteolytic enzymes in the maturing secretory vesicles [86].

Early attempts to separate bovine proinsulin intermediates by ion exchange and electrophoresis were inadequate [88], leaving RP-HPLC as the only alternative for successful separation.

3.1. Sample preparation

Owing to the very low content of human proinsulin (HPI) and intermediates in serum (often less than 1 pmol/l), specific sample preparations are required. At least 10 ml of serum were applied to an anti-insulin IgG–Sepharose 4B column [89,90] or to an empty siliconized Econo column followed by addition of an anti-insulin immunobead slurry using polyclonal antibodies with intermediate affinity and high capacity [91]. In both cases it is important that the immunobinding capacity exceeds the content of proinsulin, intermediates as well as insulin in the sample. After a washing step the serum insulin/proinsulin-related material was eluted and lyophilized.

The recoveries exceeded 90% [90] or 76–105% [92], but the procedure had to be performed twice with the immunobead slurry to exceed 90% [93]. These results were mainly based on experiments with ^{125}I-labelled proinsulin and insulin. The recovery of unlabelled HPI and insulin (radioimmunoassay, RIA) was 88 ± 6% [92] and 90–95% [28], respectively. It cannot be excluded that the recoveries of the intermediates could deviate from the insulin recovery. However, preliminary results show similar recoveries of proinsulin and the four intermediates [93].

3.2. Optimization of the stationary and mobile phases

The separation of insulin, proinsulin and intermediates has been evaluated using a range of silica-based RP-HPLC stationary phases, covering bondings with different hydrophobicity and elution with some of the mobile phase combinations used for the separation of rat and mouse proinsulins, insulins and C-peptides (section 2); the results are gathered in Table 5.

Insulin is easily separated from proinsulin and intermediates because of its lower hydrophobicity, but only a very few of the RP-HPLC systems can satisfactorily separate proinsulin and all four intermediates. Since the split forms have exactly the same amino acid compositions as proinsulin, it is likely that cleavage of the BC or CA junctions results in conformational changes that permit a separation.

Given *et al.* [90] were the first to obtain a reasonable separation using an Ultrasphere ODS column eluted with TEAP–perchlorate–acetoni-

TABLE 5

RP-HPLC SEPARATION OF HUMAN PROINSULIN AND PROINSULIN INTERMEDIATES

Abbreviations: human proinsulin (HPI), split(32–33)HPI (S-BC), des(31,32)HPI (D-BC), split(65–66)HPI (S-CA) and des(64,65)HPI (D-CA).

Bonded phase	Stationary phase	Mobile phase	Results	Ref.
C_{18}	Altex Ultrasphere	TEAP–perchlorate pH 3.0–acetonitrile	Separation of proinsulin and des intermediates (R_s = 0.7) Elution order: D-BC, D-CA, HPI	89
C_{18}	Altex Ultrasphere	Phosphoric acid, perchlorate hexane sulphonic acid pH 3.0–acetonitrile	Baseline separation of proinsulin and des intermediates (R_s = 1.3) Elution order: D-BC, D-CA, HPI	89
C_{18}	Ultrasphere ODS	TEAP–perchlorate pH 3.0–acetonitrile	Separation of proinsulin and all four intermediates ((R_s = 0.4, 0.7 and 1.5) Elution order: S-BC, D-BC, S-CA, D-CA, HPI	90
C_{18}	LiChrosorb RP-18	Phosphoric acid, perchlorate heptane sulphonic acid pH 3.0–acetonitrile	Baseline separation of proinsulin and all four intermediates ($R_s \geq$ 1.0) Elution order: D-BC, D-CA, S-BC, S-CA, HPI	94
C_{18}	LiChrosorb RP-18	TEAP pH 4.0–acetonitrile	One intermediate eluted with proinsulin	91
C_{18}	Lichrosorb RP-18	Ammonium sulphate pH 4.0–acetonitrile	Baseline separation of proinsulin and all four intermediates (R_s = 0.9–1.4) Elution order: S-CA, S-BC, D-CA, D-BC, HPI	91
C_{18}	Nucleosil 100-5C_{18}	TEAP pH 4.0–acetonitrile	Two intermediates eluted with proinsulin	91
C_{18}	Ultrasphere Ion-pair	TEAP–perchlorate pH 3.0–acetonitrile	Separation of proinsulin and all four intermediates (R_s = 1.1, 0.4 and 1.7) Elution order: S-BC, D-BC, S-CA, D-CA, HPI	95
C_4	Nucleosil 300-5C_4	TFA–acetonitrile	Baseline separation of proinsulin and all four intermediates (R_s = 1.0–2.3) Elution order: S-BC, D-BC, HPI, S-CA, D-CA	91
C_3	Zorbax Protein Plus	TFA–acetonitrile	One des-proinsulin eluted with proinsulin	91

trile pH 3.0. The beneficial addition of hexane- or heptanesulphonic acid was demonstrated by several groups [89,94], leading to baseline separation of proinsulin and all four intermediates [94]. Also, the use of ammonium sulphate–acetonitrile pH 4.0 could resolve the components using the same LiChrosorb RP-18 column [91]. The often-preferred mobile phase, TFA–acetonitrile was successful only when using a Nucleosil 300-5C_4 column [91], as shown in Fig. 4.

The separations in Table 5 were performed at ambient temperature (20–25°C), except the separation by Yano *et al.* [95] (45°C). The success of the separation using Nucleosil C_4/TFA–acetonitrile (Fig. 4) deteriorated if the separation temperature was increased to 45°C, as the R_s values for des(31,32)HPI and HPI were reduced from 1.9 to 0.8 [42].

Interestingly, the elution order of proinsulin and intermediates is highly variable (Table 5). In most cases proinsulin elutes last, suggesting that it is more hydrophobic, although the use of ion-pairing agents in HPLC buffers precludes an easy assessment of relative peptide hydrophobicity [90]. The chromatogram shown in Fig. 4 reveals an unusual elution order, *i.e.* proinsulin elutes between the two pairs of split/des intermediates.

The use of polymer-based stationary phases for this separation has not been described but could be an interesting alternative because of their excellent properties when used for the separation of rat and mouse proinsulins (see Section 2.2). Also, the extended pH range of use with these columns could be an additional advantage with the possibility of cleaning the columns with a strong base, a very efficient treatment for removal of precipitated polypeptides/proteins.

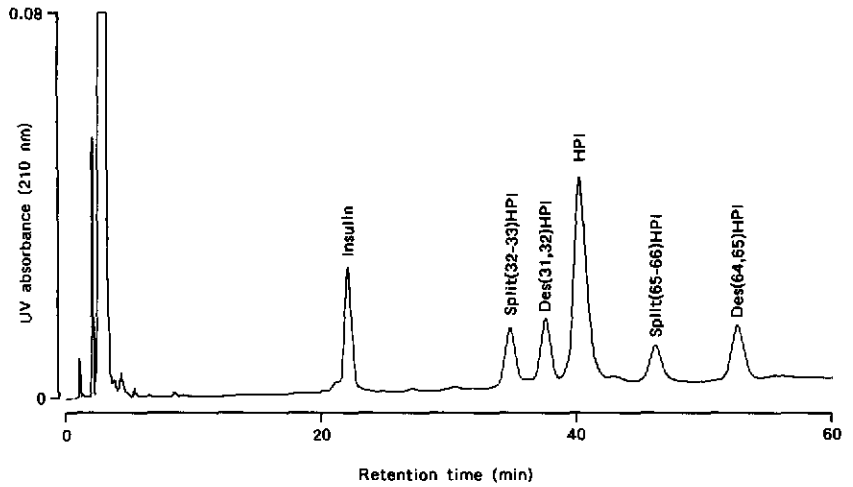

Fig. 4. RP-HPLC separation of human insulin (*ca.* 1 μg), proinsulin (*ca.* 3 μg) and the four proinsulin intermediates (*ca.* 1 μg of each) using a Nucleosil 300-5C_4 column (5 μm particle size, 250 mm × 4.0 mm I.D.) eluted at 1.0 ml/min with a linear acetonitrile gradient (29.4–30.6%) in 0.1% TFA during 60 min (reprinted from ref. 91 with permission).

The quantitative recovery of insulin and proinsulin after RP-HPLC of picogram to nanogram amounts has been evaluated using radioactively labelled insulin and proinsulin as described in Section 2.4. The yields of immunoreactive insulin recovered range from 70 to 90% of the material applied [90]. In order to minimize the adsorption of material to the fraction tubes, bovine serum albumin was added and the pH adjusted to neutral [90,91]. No direct measurement of the recoveries of the four proinsulin intermediates has been described. As their hydrophobicities and molecular masses are very similar to those of proinsulin their recoveries would be expected to be similar.

4. APPLICATIONS OF THE RP-HPLC ANALYSES

4.1. Regulation of insulin biosynthesis in rodents

As mentioned in the Introduction, rats and mice are unique among mammals in expressing two non-allelic insulin genes. Ever since the existence of the two genes was discovered it has been a puzzle why the ratio of the two insulins deviates from unity. As there is a discrepancy in the ratio as reported by various authors, it has been speculated that there may be differential regulation of the expression of the two genes or a difference in translation, processing of the precursor or stability/degradation of the products.

The ratio of the two insulins has been evaluated on insulin extracted from the whole pancreas as well as from isolated pancreatic islets and transformed β-cells. In most cases the ratio of insulins I and II in the rat pancreas has been reported to be between 1.0 and 1.6, with the exception of pregnant (4.7) and growth hormone tumor-bearing rats (5.5–7.7), reported by Kakita and co-workers [13,14], who used electrophoretic separation of the two insulins followed by transfer to nitrocellulose and immunological staining. Studies on isolated rat islets using more accurate RP-HPLC methods combined with RIAs and UV area measurements confirm the ratio to be between 1.0 and 1.6, as shown in Table 6. However, an increased ratio in islets cultured in high glucose and in the presence of human growth hormone (hGH) has also been found, which may support the findings of Kakita and co-workers [13,14]. An increased ratio in foetal pancreas (2.7) [36] may be due to the culture conditions, as is the case for islets (Table 6).

There is also controversy in the literature regarding insulins in mouse pancreas/islets. Ratios between 0.4 and 3.3 have been reported in the

TABLE 6

INSULIN I/II RATIOS IN RAT AND MOUSE ISLETS AND IN TRANSFORMED β-CELLS

Source	Strain	Age	Culture	Condition	Ratio insulin I/II	Ref.
Rat islets	Sprague–Dawley	–	–	–	Approx. 1.5	8
Rat islets	Long–Evans	Adult	No	Freshly isolated	1.40	41
Rat islets	Sprague–Dawley	–	No	Freshly isolated	1.26	15
Rat islets	Sprague–Dawley	–	2 days	11 m*M* Glucose	1.44	15
Rat islets	Sprague–Dawley	–	3 days	11 m*M* Glucose	1.58	15
Rat islets[a]	Wistar	1 day	No	Freshly isolated	1.07 ± 0.03	42
Rat islets[a]	Wistar	3–5 days	21 days	11 m*M* Glucose	2.06 ± 0.06	96
Rat islets[a]	Wistar	3–5 days	21 days	11 m*M* Glucose + 1 μg/ml hGH	2.53 ± 0.04	96
Rat islets[a]	Wistar	3–5 days	1–14 days	11 m*M* Glucose	2.07 ± 0.17	42
Rat insulinoma[b]	NEDH	–	No	Freshly isolated	10	97
Rat insulinoma[c]	NEDH	–	No	Freshly isolated	4.6	42
Rat insulinoma cell line NHI 6F[d]	NEDH	(Passage 17)	Yes	11 m*M* Glucose	9.4	98
Rat insulinoma cell line NHI 6F[d]	NEDH	(Passage 21)	Yes	11 m*M* Glucose	15.3	98
Rat insulinoma cell line NHI 6F[d]	NEDH	(Passage 25)	Yes	11 m*M* Glucose	20.0	98
Rat insulinoma cell line NHI 6F[d]	NEDH	(Passage 50)	Yes	11 m*M* Glucose	Only I_1	98
Mouse islets[a]	B6D2 F1/J	Neonatal	Yes	11 m*M* Glucose	0.62	57
Mouse islets[a]	B6D2 F1/J	4–5 weeks	No	Freshly isolated	0.52	99
Mouse islets[a]	B6D2 F1/J	4–5 weeks	Yes	11 m*M* Glucose	0.44	99
Mouse islets[a]	B6D2 F1/J	13 weeks	Yes	11 m*M* Glucose	0.46	99
Mouse islets[a]	NMRI	3 weeks	No	Freshly isolated	0.51 ± 0.01	42
Mouse islets[a]	NMRI	3 weeks	1–3 days	11 m*M* Glucose	0.55 ± 0.01	100
Mouse islets[a]	DBA/2	Adult	2 days	2.8 m*M* Glucose	0.32	79,101
Mouse inlets[a]	DBA/2	Adult	2 days	16.7 m*M* Glucose	0.43	79,101
Mouse insulinoma cell line β-TC1[c]	B6D2 F1/J	(Passage 39–44)	Yes	11 m*M* Glucose	0.5	57
Mouse insulinoma cell line β-TC3[c]	B6D2 F1/J	(Passage 45)	Yes	11 m*M* Glucose	0.3	42
Mouse insulinoma cell line β-TC3[c]	B6D2 F1/J	(Passage 50)	Yes	11 m*M* Glucose	0.1	42

[a] The insulin I/II ratio was determined by RIA or UV area after RP-HPLC separation of extracted islets and the results are expressed as mean ± S.E.M., $n \geq 4$ when possible.

[b] The ratio was determined by PAGE of the immunoprecipitated preproinsulins.

[c] The insulin I/II ratio was determined by RIA or UV area after RP-HPLC separation of granules/cells.

[d] The ratio was calculated from the ratio of cells stained for C-peptide I and II.

pancreas [12,14,35,42,79,102] and, although strain differences may exist, nutritional state may play a role. A ratio lower than 1 has been reported in crystalline mouse insulin [17], but was ascribed to selection by the separation procedure. However, studies on isolated mouse islets, uncultured or cultured under various conditions, agree that the ratio is between 0.3 and 0.6 (Table 6), suggesting that methodological differences in the extraction or separation of insulins from the whole pancreas may influence the results.

Underestimation of the amount of insulin II,

and consequently an artificially high insulin I/insulin II ratio, may result if unwanted oxidation of the methionine residue in insulin II occurs during extraction [53] or sample preparation [47,49].

Another explanation of the disproportionate amounts of the two insulins in rats and mice could be differences in stability caused by intracellular degradation [15,103,104]. Insulins and C-peptides are normally secreted from the pancreatic β-cell in equimolar amounts both in humans *in vivo* [105] and in rat islets in culture [8]. However, the amount of biosynthetically labelled C-peptide remaining within the islets has been observed to decrease slowly relative to that of labelled insulin, which indicates some selective degradation [8,15]. This increased insulin/C-peptide ratio has been attributed to slower degradation of insulin owing to its storage as crystals [104], in agreement with an increased degradation reported for some insulin species unable to crystallize, *i.e.* Asp B10 insulin [18,40].

RP-HPLC analyses of the stability of the C-peptides in rat and mouse islets [100] have shown an increased degradation of mouse C-peptides compared with rat C-peptides, which is also influenced by the culture conditions. Degradation products of the rat C-peptides have been detected in rat islets and identified by sequence analyses as C-peptide 1–24 from both C-peptides I and II [42], in accordance with the described cleavage at the chymotryptic Leu-24–Ala-25 site in the C-peptides [106]. Although no selective insulin degradation was observed during culture, it might suggest that the degradation of insulin in the mouse pancreas is more aggressive than in the rat.

When insulin biosynthesis was studied in isolated islets biosynthetically labelled with radioactive amino acids and analysed by RP-HPLC, it was found that the conversion of proinsulin II to insulin II is slower in both rat and mouse islets [16,100] (Fig. 5), in transgenic mouse islets also expressing human insulin [18] as well as in β-tumor cell (β-TC) lines from insulinomas derived from transgenic mice carrying a hybrid insulin-promoted simian virus tumor antigen gene [57]. Indirectly, the relatively faster appearance of labelled insulin I than insulin II in both rats [41] and mice [100] (Fig. 3, upper panel) suggests the same.

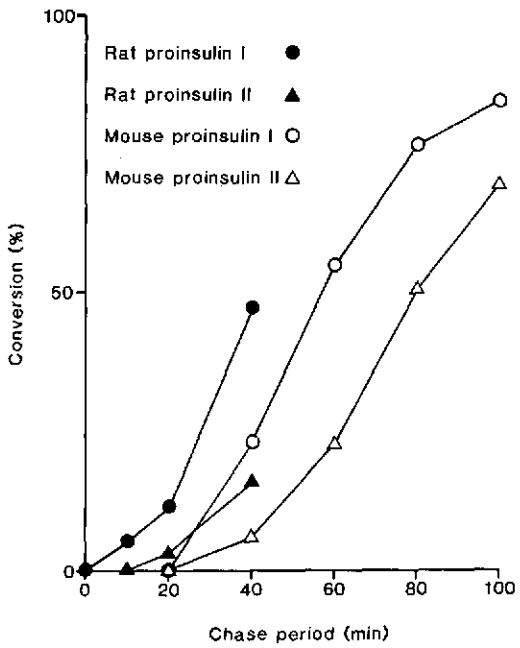

Fig. 5. Conversion of proinsulin to insulin in rat (filled symbols) and mouse (open symbols) islets determined from pulse-chase experiments using [^{3}H]leucine and [^{35}S]methionine. The pulse period was 20 min, the chase period increasing from 0 to 100 min. The conversion (%) was calculated from [^{3}H]leucine counts in insulin I and II and proinsulin I and II, as determined after RP-HPLC separation using the same conditions as described for the upper panel in Fig. 3.

As previously suggested for the rat proinsulins [8], the presence of the bulky methionine residue in B29 in proinsulin II probably decreases the enzymatic cleavage rate at the neighbouring peptide bond between the B-chain and the C-peptide. Alternatively, it was recently suggested that certain endoproteases preferentially cleave at a site with a basic residue in position 4 from the dibasic site, as in proinsulin I (Lys B29) [107].

The ratios between the newly synthesized proinsulins (before conversion begins, *i.e.* after a 20-min pulse [16,100]; see Fig. 6) are identical to that of the stored insulins, *i.e.* around 1.7 and 0.5 in rat and mouse islets, respectively, suggesting

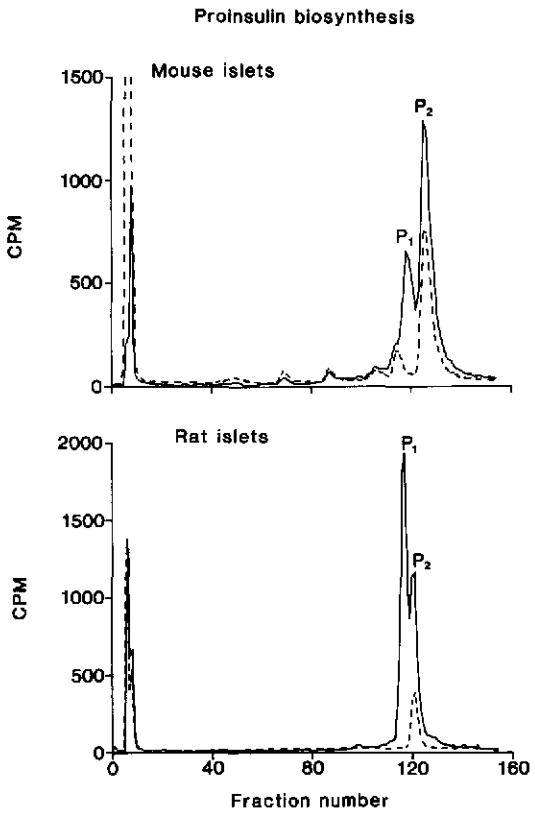

Fig. 6. RP-HPLC separation of a 3 *M* acetic acid extract of 50 rat and mouse islets labelled for 20 min with [^{3}H]leucine and [^{35}S]methionine (solid and dashed lines, respectively). RP-HPLC-conditions, measurement of radioactivity and identity of peaks as in Fig. 3 (upper panel). The ratio P_1/P_2 for rats and mice was 1.7 and 0.5, respectively.

that the differences occur either at the level of preproinsulin synthesis or processing in the RER or at the transcriptional level. The former possibility has not yet been pursued because of the extreme short half-life of the preproinsulins [108], but it has been proposed that the amino acid composition of mouse prepeptide I might favour a less efficient transport of mouse preproinsulin I into the RER, resulting in less efficient production of insulin I in mouse islets [101], but the proof for this explanation has, however, not been reported. It is worthwhile noting that in transformed cell lines the difference in the expression of the two insulin genes is exaggerated (see Table 6). Thus the X-ray-induced rat insulinoma cell lines almost exclusively produce insulin I, whereas the mouse β-TC lines produce much more insulin II than insulin I, especially in the later passages. Although the ratio between the two insulins is fairly constant in normal islets, recent studies on islet tumor cells indicate that the two genes are regulated differentially. Thus, in rat insulinoma cell lines only a small fraction of the cells express both insulin I and II, whereas a majority only express insulin I [98] in correlation with the mRNA levels of the two genes, suggesting that the regulation occurs at the transcriptional level. This was confirmed both in normal rat and mouse islets and in the cell lines RIN 5AH and β-TC [109], in contrast to some reports showing higher levels of insulin I mRNA in the mouse [79,101] and equal amounts of mRNA for insulins I and II in rat insulinoma cells [110], suggesting a defective maturation or translation of the mouse insulin I and rat insulin II mRNAs, respectively. It was recently reported that the two insulins are differentially expressed during embryonic development [111].

In conclusion, these studies suggest that in rats and mice the two non-allelic genes are differentially expressed, but in the stable mature β-cell the ratio of insulin I to II is 1.5–2.0 in the rat and 0.3–0.5 in the mouse. The use of RP-HPLC has greatly facilitated the elucidation of the biosynthesis and conversion of the two gene products, which supports the idea that the ratios are determined at the level of gene transcription, although the mechanisms involved are not yet known.

4.2. Proinsulin conversion intermediates as markers for β-cell function in man

It has been reported that under prolonged glucose infusion the amount of circulating proinsulin is increased [23], suggesting that the conversion of proinsulin to insulin cannot be completed when the secretory rate is high. Elevated levels of proinsulin and its conversion intermediates have been reported in insulin-dependent diabetes mellitus (IDDM), non-insulin-dependent diabetes mellitus (NIDDM), in patients with cystic fibrosis and also in healthy siblings and monozygotic

twins of IDDM patients, as reviewed in ref. 112. This may be caused by the increased demand for insulin when the β-cell mass is reduced or a passive loss of proinsulin and intermediates from the damaged β-cells, or it may be the result of a genetic trait.

In vitro studies on isolated rat islets do not suggest that the conversion process is rate-limiting for the release of insulin, since prolonged exposure to high glucose concentrations in culture does not result in release of significant amounts of proinsulin. However, when islets are damaged by exposure to interleukin 1, the conversion of proinsulin is reduced [113]. Although these studies were performed on rat islets, the results suggest that certain agents may influence the conversion process, resulting in the release of incompletely processed proinsulin intermediates in man.

As mentioned above, at least two endopeptidases and a carboxypeptidase are involved in the proinsulin conversion, indicating that the composition of the circulating intermediates may give a clue to the etiology of the phenomenon.

The preferential appearance of intermediates cleaved at the BC junction besides intact proinsulin has been demonstrated directly in sera from NIDDM patients using two-site immunoradiometric assays [114,115], but the concentrations quoted for "32–33 split proinsulin" are in fact the sum of split(32–33)- and des(31,32)proinsulin, since these assays do not distinguish between the split and des forms from the same cleavage site.

As the existing immunological methods do not permit sufficiently specific and sensitive assays for the intermediates as well as intact proinsulin (for a review, see ref. 26), RP-HPLC is at present the method of choice, although the capacity is limited.

The composition of serum PIM was analysed in eight fasting healthy controls and twelve NIDDM patients using RP-HPLC in combination with an enzyme-linked immunosorbent assay (ELISA) for PIM [116,117]. In the control sera intact proinsulin as well as des(31,32)proinsulin was demonstrated, and intact proinsulin constituted approximately two thirds of total PIM. In one subject des(31,32)proinsulin was the predominant compound, and in four of the controls minor amounts of des(64,65)proinsulin were found. Also, split(32–33)proinsulin was demonstrated in one subject. The RP-HPLC pattern of PIM in the sera from the NIDDM patients comprised in some cases 100% intact proinsulin. Two normal-weight and four obese patients had intact and des(31,32)proinsulin in their serum; in others three to five peaks were demonstrated, intact proinsulin being the major peak. These results demonstrate substantial heterogeneity of serum PIM in normal and diabetic subjects.

It can thus be concluded that recent studies have revealed that a major part of circulating PIM consists of metabolites. It has been suggested that the heterogeneity could be genetically determined [116], *e.g.* as a result of variation in the amount or activity of the individual enzymes involved in the conversion. As the genes for the enzymes have now been cloned, it has become possible to test this hypothesis.

5. CONCLUSIONS

Several RP-HPLC systems have been evaluated in order to study the biosynthesis and conversion of the two non-allelic proinsulins expressed in rats and mice, as well as the composition of serum proinsulin and conversion intermediates in humans. No universal set of chromatographic conditions has been found to result in satisfactory resolution of all the polypeptides involved, but in each case several systems have been found to be satisfactory, with temperature being an important parameter.

A key to good resolution and recovery is correct sample preparation. To avoid selective adsorption as well as oxidation of methionine groups (proinsulin II and insulin II from rats and mice only) it is recommended that the samples are not lyophilized to dryness prior to RP-HPLC. Addition of serum albumin as a carrier when handling very low amounts of proinsulin/insulin is of great importance for the recovery.

The optimized RP-HPLC methods have been used to examine the regulation of insulin biosyn-

thesis in rodents and the significance of proinsulin conversion intermediates as markers for β-cell function in humans and should be useful in the future development of appropriate methods for the study of biosynthesis and conversion of other polypeptides of interest.

6. ACKNOWLEDGEMENTS

The authors thank Ragna Jørgensen, Linda Larsø and Ingelise Fabrin for excellent technical assistance. The helpful discussions and suggestions from Dr. Michael E. Røder are gratefully acknowledged. Lisbeth Brandt-Møller is thanked for typing this review.

REFERENCES

1 C. Patzelt, H. S. Tager, R. J. Carroll and D. F. Steiner, *Nature*, 282 (1979) 260.
2 S. J. Chan, P. Keim and D. F. Steiner, *Proc. Natl. Acad. Sci. U.S.A.*, 73 (1976) 1964.
3 S. J. Chan and D. F. Steiner, *Trends Biochem. Sci.*, 2 (1977) 254.
4 H. S. Tager, D. F. Steiner and C. Patzelt, *Methods Cell Biol.*, 23 (1981) 73.
5 L. Orci, M. Ravazzola, M. Amherdt, O. D. Madsen, A. Perrelet, J.-D. Vassalli and R. G. W. Anderson, *J. Cell Biol.*, 103 (1986) 2273.
6 J. C. Hutton, *Curr. Opin. Cell Biol.*, 2 (1990) 1131.
7 P. J. Barr, *Cell*, 66 (1991) 1.
8 J. L. Clark and D. F. Steiner, *Proc. Natl. Acad. Sci. U.S.A.*, 62 (1969) 278.
9 B. Cordell, G. Bell, E. Tischer, F. M. DeNoto, A. Ullrich, R. Pictet, W. J. Rutter and H. M. Goodman, *Cell*, 8 (1979) 533.
10 P. Lomedico, N. Rosenthal, A. Efstratiadis, W. Gilbert, R. Kolodner and R. Tizard, *Cell*, 18 (1979) 545.
11 S. J. Giddings and L. Carnaghi, *J. Biol. Chem.*, 264 (1989) 9462.
12 L. Koranyi, M. A. Permutt, J. M. Chirgwin and S. J. Giddings, *Mol. Endocrinol.*, 3 (1989) 1895.
13 K. Kakita, S. Giddings and M. A. Permutt, *Proc. Natl. Acad. Sci. U.S.A.*, 79 (1982) 2803.
14 K. Kakita, K. O'Connell and M. A. Permutt, *Diabetes*, 31 (1982) 841.
15 C. J. Rhodes and P. A. Halban, *Biochem. J.*, 251 (1988) 23.
16 S. Linde, J. H. Nielsen, B. Hansen and B. S. Welinder, *J. Chromatogr.*, 462 (1989) 243.
17 J. Markussen, *Int. J. Pept. Protein Res.*, 3 (1971) 149.
18 R. J. Carroll, R. E. Hammer, S. J. Chan, H. H. Swift, A. H. Rubenstein and D. F. Steiner, *Proc. Natl. Acad. Sci. U.S.A.*, 85 (1988) 8943.
19 R. L. Cate, W. Chick and W. Gilbert, *J. Biol. Chem.*, 258 (1983) 6645.
20 S. J. Giddings and L. R. Carnaghi, *J. Biol. Chem.*, 263 (1988) 3845.
21 D. F. Steiner, O. Hallund, A. Rubenstein, S. Cho and C. Bayliss, *Diabetes*, 17 (1968) 725.
22 C. J. Rhodes, C. A. Lucas and P. A. Halban, *FEBS Lett.*, 215 (1987) 179.
23 J. Ludvigsson and L. G. Heding, *Acta Diabet. Lat.*, 19 (1982) 351.
24 M. E. Mako, J. I. Starr and A. H. Rubenstein, *Am. J. Med.*, 63 (1977) 865.
25 C. de Haën, S. A. Little, J. M. May and R. H. Williams, *J. Clin. Invest.*, 62 (1978) 727.
26 R. Temple, P. M. S. Clark and C. N. Hales, *Diabetic Med.*, 9 (1992) 503.
27 J. Rivier and R. McClintock, *J. Chromatogr.*, 268 (1983) 112.
28 S. Shoelson, M. Haneda, P. Blix, A. Nanjo, T. Sanke, K. Inouye, D. F. Steiner, A. Rubenstein and H. Tager, *Nature*, 302 (1983) 540.
29 R. L. Patience and L. H. Rees, *J. Chromatogr.*, 352 (1986) 241.
30 B. S. Welinder, H. H. Sørensen and B. Hansen, *J. Chromatogr.*, 361 (1986) 357.
31 B. S. Welinder, S. Linde and B. Hansen, *J. Chromatogr.*, 265 (1983) 301.
32 L. F. Smith, *Am. J. Med.*, 40 (1966) 662.
33 B. M. Wentworth, I. M. Schaefer, L. Villa-Komaroff and J. M. Chirgwin, *J. Mol. Evol.*, 23 (1986) 305.
34 L. B. Rall, R. L. Pictet and W. J. Rutter, *Endocrinology*, 105 (1979) 835.
35 G. Trump, W. H. Hildemann and G. B. Tebow, *Anal. Biochem.*, 138 (1984) 298.
36 M. De Gasparo and M. Faupel, *J. Chromatogr.*, 357 (1986) 139.
37 C. H. Suelter and M. DeLuca, *Anal. Biochem.*, 35 (1983) 112.
38 H. Kotaniguchi and M. Kawakatsu, *Int. Lab.*, Dec. (1988) 36.
39 P. A. Halban, C. J. Rhodes and S. E. Shoelson, *Diabetologia*, 29 (1986) 893.
40 D. J. Gross, P. A. Halban, C. R. Kahn, G. C. Weir and L. Villa-Komaroff, *Proc. Natl. Acad. Sci. U.S.A.*, 86 (1989) 4107.
41 M. L. Gishizky and G. M. Grodsky, *FEBS Lett.*, 223 (1987) 227.
42 S. Linde, unpublished results.
43 P. A. Halban and C. B. Wollheim, *J. Biol. Chem.*, 255 (1980) 6003.
44 P. A. Halban, C. B. Wollheim, B. Blondel and A. E. Renold, *Biochem. Pharmacol.*, 29 (1980) 2625.
45 R. M. Cohen, B. D. Given, J. Licinio-Paixao, S. A. Provow, P. A. Rue, B. H. Frank, M. A. Root, K. S. Polonsky, H. S. Tager and A. H. Rubenstein, *Metabolism*, 35 (1986) 1137.
46 D. Gross, A. Skvorak, G. Hendrick, G. Weir, L. Villa-Komaroff and P. Halban, *FEBS Lett.*, 241 (1988) 205.

47 S. Linde, J. H. Nielsen, B. Hansen and B. S. Welinder, *J. Chromatogr.*, 530 (1990) 29.
48 U. Gether, H. V. Nielsen and T. W. Schwartz, *J. Chromatogr.*, 447 (1988) 341.
49 J. Rivier and F. McClintock, in A. R. Kerlavage (Editor), *The Use of HPLC in Receptor Biochemistry*, Alan R. Liss, New York, 1989, p. 77.
50 S. Linde and B. S. Welinder, *J. Chromatogr.*, 548 (1991) 195.
51 S. Linde and B. S. Welinder, *J. Chromatogr.*, 536 (1991) 43.
52 V. Sizonenko and P. A. Halban, *Biochem. J.*, 278 (1991) 621.
53 G. Gold, M. D. Walker, D. L. Edwards and G. M. Grodsky, *Diabetes*, 7 (1988) 1509.
54 B. Presnell, A. Conti, G. Erhardt, I. Krause and J. Godovac-Zimmermann, *J. Biochem. Biophys. Methods*, 20 (1990) 325.
55 C. T. Wehr, in W. S. Hancock (Editor), *CRC Handbook for the HPLC Separation of Amino Acids, Peptides and Proteins*, CRC Press, Boca Raton, FL, 1984, p. 31.
56 R. M. Chicz and F. E. Regnier, *Methods Enzymol.*, 182 (1990) 392.
57 S. Efrat, S. Linde, H. Kofod, D. Spector, M. Dalannoy, S. Grant, D. Hanahan and S. Bækkeskov, *Proc. Natl. Acad. Sci. U.S.A.*, 85 (1988) 9037.
58 M. Fromont-Racine, D. Bucchini, O. Madsen, P. Desbois, S. Linde, J. H. Nielsen, C. Saulnier, M.-A. Ripoche, J. Jami and R. Pictet, *Mol. Endocrinol.*, 4 (1990) 669.
59 J. F. Kennedy, Z. S. Rivera and C. A. White, *J. Biotechnol.*, 9 (1989) 83.
60 B. S. Welinder, *J. Chromatogr.*, 542 (1991) 83.
61 W. S. Hancock, C. A. Bishop, R. L. Prestidge and D. R. K. Harding, *J. Chromatogr.*, 153 (1978) 391.
62 A. Dinner and L. Lorenz, *Anal. Chem.*, 51 (1979) 1872.
63 H. P. J. Bennett, A. M. Hudson, C. McMartin and G. E. Purdon, *Biochem. J.*, 168 (1977) 9.
64 J. E. Rivier, *J. Liq. Chromatogr.*, 1 (1978) 343.
65 F. Lottspeich and A. Henschen, in A, Henschen, K.-P. Hupe, F. Lottspeich and W. Voelter (Editors), *High Performance Liquid Chromatography in Biochemistry*, VCH, Weinheim, 1985, p. 139.
66 T. Welsch, H. Frank and G. Vigh, *J. Chromatogr.*, 506 (1990) 97.
67 C. T. Wehr, R. P. Lundgard and K. D. Nugent, *LC · GC Int.*, 2 (1989) 24.
68 W. S. Hancock and D. R. K. Harding, in W. S. Hancock (Editor), *Handbook of HPLC for the Separation of Amino Acids, Peptides and Proteins*, Vol. II, CRC Press, Boca Raton, FL, 1983, p. 3.
69 H. Engelhardt, H. Müller and U. Schön, in A. Henschen, K.-P. Hupe, F. Lottspeich and W. Voelter (Editors), *High Performance Liquid Chromatography in Biochemistry*, VCH, Weinheim, 1985, p. 15.
70 J. Nawrocki and B. Buszewski, *J. Chromatogr.*, 449 (1988) 1.
71 R. E. Majors, *LC · GC Int.*, 3 (1989) 12.
72 P. C. Sadek, P. W. Carr, L. D. Bowers and L. C. Haddad, *Anal. Biochem.*, 153 (1986) 359.
73 M. Andre, *J. Chromatogr.*, 351 (1986) 341.
74 U. Damgaard and J. Markussen, *Horm. Metab. Res.*, 11 (1979) 580.
75 T. Sasagawa, T. Okuyama and D. C. Teller, *J. Chromatogr.*, 240 (1982) 329.
76 C. A. Browne, H. P. J. Bennett and S. Solomon, *Anal. Biochem.*, 124 (1982) 201.
77 C. T. Mant, T. W. L. Burke, J. A. Black and R. S. Hodges, *J. Chromatogr.*, 458 (1988) 193.
78 S. Sato, C. D. Ebert and S. W. Kim, *J. Pharm. Sci.*, 72 (1983) 228.
79 D. Gross, B. Wentworth, C. Rhodes, B. Schnetzler, P. Halban and L. Villa-Komaroff, *Diabetologia*, 33 (1990) A94, P329.
80 B. S. Welinder, H. H. Sørenson and B. Hansen, *J. Chromatogr.*, 408 (1987) 191.
81 P. Böhlen, F. Castillo, N. Ling and R. Guillemin, *Int. J. Pept. Protein Res.*, 16 (1980) 306.
82 H. P. J. Bennett, C. A. Browne and S. Solomon, *Biochemistry*, 20 (1981) 4530.
83 D. M. Desiderio, S. Yamada, F. S. Tanzer, J. Horton and J. Trimble, *J. Chromatogr.*, 217 (1981) 437.
84 C. T. Wehr, L. Correia and S. R. Abott, *J. Chromatogr. Sci.*, 20 (1982) 114.
85 M. W. T. Hearn, in Cs. Horváth (Editor), *High Performance Liquid Chromatography*, Vol. 3, Academic Press, New York, 1983, p. 87.
86 W. Kemmler, J. D. Peterson and D. F. Steiner, *J. Biol. Chem.*, 246 (1971) 6786.
87 J. C. Hutton, *Diabetologia*, 32 (1989) 271.
88 J. Markussen and L. G. Heding, *J. Peptide Protein Res.*, 8 (1976) 597.
89 D. C. Robbins, S. E. Shoelson, A. H. Rubenstein and H. S. Tager, *J. Clin. Invest.*, 73 (1984) 714.
90 B. D. Given, R. M. Cohen, S. E. Shoelson, B. H. Frank, A. H. Rubenstein and H. S. Tager, *J. Clin. Invest.*, 76 (1985) 1398.
91 S. Linde, M. E. Røder, S. G. Hartling, C. Binder and B. S. Welinder, *J. Chromatogr.*, 548 (1991) 371.
92 R. M. Cohen, T. Nakabayashi, P. M. Blix, P. A. Rue, S. E. Shoelson, M. A. Root, B. H. Frank, R. R. Revers and A. H. Rubenstein, *Diabetes*, 34 (1985) 84.
93 M. R. Røder, H. Cheng, S. G. Hartling, B. Dinesen, S. Lanng and C. Binder, in preparation.
94 H. W. Davidson and J. C. Hutton, *Biochem. J.*, 245 (1987) 575.
95 H. Yano, N. Kitano, M. Morimoto, K. S. Polonski, H. Imura and Y. Seino, *J. Clin. Invest.*, 89 (1992) 1902.
96 J. H. Nielsen, S. Linde, B. S. Welinder, N. Billestrup and O. D. Madsen, *Mol. Endocrinol.*, 13 (1989) 165.
97 B. Cordell, D. Diamond, S. Smith, J. Pünter, H. H. Schöne and H. M. Goodman, *Cell*, 31 (1982) 531.
98 N. Blume, J. S. Petersen, L. C. Andersen, H. Kofod, T. Dyrberg, B. K. Michelsen, P. Serup and O. D. Madsen, *Mol. Endocrinol.*, 6 (1992) 299.
99 S. Linde and S. Bækkeskov, unpublished results.

100 S. Linde, J. H. Nielsen, B. Hansen and B. S. Welinder, *Diabetologia*, 32 (1989) 510A, No. 291.
101 B. M. Wentworth, C. Rhodes, B. Schnetzler, D. J. Gross, P. A. Halban and L. Villa-Komaroff, *Mol. Cell. Endocrinol.*, 86 (1992) 177.
102 B. Schnetzler, R. F. Selden and P. A. Halban, *Diabetologia*, 33 (Suppl.) A36 (1990) 99.
103 L. Orci, M. Ravazzola, M. Amherdt, C. Yanaihara, N. Yanaihara, P. Halban, A. E. Renold and A. Perrelet, *J. Cell Biol.*, 98 (1984) 222.
104 P. A. Halban, R. Mutkoski, G. Dodson and L. Orci, *Diabetologia*, 30 (1987) 348.
105 D. L. Horwitz, J. I. Starr, M. E. Mako, W. G. Blackard and A. H. Rubenstein, *J. Clin. Invest.*, 55 (1975) 1278.
106 J. Markussen and F. Sundby, *Eur. J. Biochem.*, 25 (1972) 153.
107 F. Vollenweider, J.-C. Irminger, D. J. Gross, L. Villa-Komaroff and P. A. Halban, *J. Biol. Chem.*, 267 (1992) 14629.
108 S. L. Howell and G. St. J. Bird, *Br. Med. Bull.*, 45 (1989) 19.
109 K. Lund, personal communication.
110 N. Itoh, K. Nose and H. Okamoto, *Eur. J. Biochem.*, 97 (1979) 1.
111 L. Deltour, P. Leduque, N. Blume, O. D. Madsen, P. Dubois, J. Jami and D. Bucchini, *Proc. Natl. Acad. Sci. U.S.A.*, 90 (1993) 527.
112 S. Madsbad, S. G. Hartling and O. K. Faber, in K. G. M. M. Alberti, R. A. DeFronzo, H. Keen and P. Zimmet (Editors), *International Textbook of Diabetes Mellitus*, Wiley, Chichester, 1992, p. 303.
113 B. S. Hansen, J. H. Nielsen, S. Linde, G. A. Spinas, B. S. Welinder, T. Mandrup-Poulsen and J. Nerup, *Biomed. Biochim. Acta*, 47 (1988) 305.
114 R. C. Temple, C. A. Carrington, S. D. Luzio, D. R. Owens, A. E. Schneider, W. J. Sobey and C. N. Hales, *Lancet*, i (1989) 293.
115 W. J. Sobey, S. F. Beer, C. A. Carrington, P. M. S. Clark, B. H. Frank, I. P. Gray, S. D. Luzio, D. R. Owens, A. E. Schneider, K. Siddle, R. C. Temple and C. N. Hales, *Biochem. J.*, 260 (1989) 535.
116 S. G. Hartling, M. E. Røder, S. Linde, B. S. Welinder, B. H. Frank and C. Binder, *Pancreatic Beta-cell*, Joslin Diabetes Center at Harvard University, Boston, MA, July 1991.
117 M. E. Røder, S. G. Hartling, S. Linde, B. S. Welinder and C. Binder, *Diabetologia*, 34 (1991) A15.