REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC ANALYSES OF INSULIN BIOSYNTHESIS IN ISOLATED RAT AND MOUSE ISLETS*

S. LINDE*, J. H. NIELSEN, B. HANSEN and B. S. WELINDER
Hagedorn Research Laboratory, Niels Steensensvej 6, DK-2820 Gentofte (Denmark)
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SUMMARY

Two RP-HPLC systems were developed for the separation of the products of the conversion of proinsulin into insulin in rat and mouse islets, including proinsulin I and II. Peaks were identified by microsequencing and radiosequencing. It was confirmed that mouse C-peptide I has a two amino acid deletion compared to rat C-peptide I. A marked species difference in the ratio between insulin I and II was observed, *i.e.*, 2:1 in the rat and 1:2 in the mouse. Pulse-chase experiments in rat islets have demonstrated that the ratio between insulin I and II in newly synthesized insulin is higher than that of the stored insulin, indicating a slower conversion rate of proinsulin II compared to proinsulin I.

INTRODUCTION

Several reports have described the use of reversed-phase high-performance liquid chromatography (RP-HPLC) for the separation of insulin, insulin-related and non-insulin-related polypeptides from species producing a single insulin (see ref. 1 for a review). The rat and mouse endocrine pancreas secrete two insulins (I,II)[2,3] coded for by two non-allelic genes[4,5]. Their biosynthesis involves rapid removal of a signal peptide from the precursor preproinsulin, and under normal conditions only the two proinsulins can be detected as precursors in the islets.

The two rat proinsulins differ in 4 of the 86 amino acid residues, 2 in the B-chain and 2 in the C-peptide[2]. Mouse insulin I and II have amino acid sequences identical to those of the rat insulins[6]. Recently, Wentworth *et al.*[7] cloned and sequenced the two mouse preproinsulin genes confirming the sequence for the two mouse insulins, but the nucleotide sequence indicated that the mouse C-peptide I has a deletion of two amino acids compared to the mouse C-peptide II.

Investigation of the rat and mouse insulin biosynthesis requires a method sep-

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arating both the two proinsulins and the conversion products, insulin I and II and C-peptide I and II. The separation of proinsulins and insulins was previously attempted using polyacrylamide gel electrophoresis (PAGE) at pH 4.4[3], 8.9[8] and HPLC[9–11]. Only acid PAGE can separate the two proinsulins, but none of the systems is capable of resolving all conversion products.

The aim of the present study was to develop an RP-HPLC system which allows separation of all six main products of the insulin biosynthesis in rat and mouse islets in order to study the regulation of the biosynthesis of the two non-allelic insulin gene products which occurs in these species. The identification of the individual peaks after RP-HPLC fractionation of extracts from 4700–9500 islets was based upon amino acid sequencing using microsequencing and radiosequencing. The conversion of the two rat proinsulins into insulin I and II and C-peptide I and II was elucidated in pulse-chase experiments.

MATERIALS AND METHODS

RP-HPLC

The HPLC system consisted of Waters M6000A pumps, a WISP 710A, a 660 solvent programmer, a 730 data module and a Pye Unicam LC-UV detector. The columns were LiChrosorb RP-18, 5 μm, 250 mm $\times$ 4.0 mm I.D. (Merck) and Ultrasphere ODS, 5 μm, 250 mm $\times$ 4.6 mm I.D. (Beckman). Acetonitrile was used as the organic modifier in 0.125 M triethylammonium phosphate (TEAP), pH 4.0 or 0.1% trifluoroacetic acid (TFA). The columns were eluted at 1 ml/min using linear acetonitrile gradients from 25 to 30% and 30 to 36%, respectively. The column eluate was monitored at 210 nm and collected in 0.5- or 0.3-min fractions (FRAC 300 fraction collector, Pharmacia). All separations were performed at room temperature.

Reagents

Phosphoric acid (p.a.) was from Merck, trifluoroacetic acid (Peptide Synthesis Grade) from Applied Biosystems, triethylamine (99%) from Janssen Chimica and acetonitrile (HPLC grade S) from Rathburn Chemicals. All other chemicals were of analytical reagent grade. Distilled water was drawn from a Millipore Milli Q plant and all buffers were filtered (0.45 μm, Millipore) and vacuum/ultrasound degassed before use.

Standards

Medium from cultured newborn rat islet cells containing 44 μg/ml insulin I + II as well as equimolar amounts of C-peptide I and II, rat pancreatic polypeptide (Penninsula) and porcine glucagon (NOVO) were used.

Islet isolation and culture

Islets from newborn (3–5 days) Wistar rats and 3-weeks-old NMRI mice fed with 5% glucose overnight were isolated by the collagenase method [12] and cultured at 37°C in RPMI 1640 supplemented with 10% newborn calf serum.

Pulse-chase labelling

One hundred newborn rat islets were precultured for 2 weeks and pulse-labelled

with 50 μCi [^{3}H]leucine (130 Ci/mmol) and 50 μCi [^{35}S]methionine (> 1000 Ci/mmol) from Amersham for 20 min followed by chase periods with non-radioactive amino acids from 0 to 40 min. For preparative purposes, 5000–9500 rat islets were precultured for 2–7 days and labelled for 60 min with 250 μCi [^{3}H]leucine and 250 μCi [^{35}S]methionine. All pulse-chase experiments were performed at 37°C.

Sample preparation

Harvested islets were homogenized by sonication or extracted in 3 *M* acetic acid containing 0.1% human serum albumin, and centrifuged at 10 000 *g* to remove any particulate content.

Radioactivity measurement

A 4-ml volume of Optiphase "HiSafe" (LKB) was added to each fraction followed by counting in a Packard Tri-Carb liquid scintillation counter (Model 460 C).

Radioimmunoassay

Collected fractions containing TFA–acetonitrile were dried in a Speed-Vac Concentrator (Savant). Radioimmunological determination of insulin was performed using rat insulin (NOVO) as a standard and anti-mouse insulin antibodies (developed in this laboratory) as described[13]. Radioimmunological determination of glucagon was performed using a kit from NOVO[14].

Amino acid analysis

Amino acid analysis was performed using the PICO-TAG method (Waters) as described by the manufacturer.

Amino acid sequencing

Amino acid sequencing was performed using a gas-phase protein sequencer (Applied Biosystems, model 475A) equipped with on-line HPLC analysis (120A Analyser). In radiosequencing an aliquot (60%) from each step was collected and the radioactivity counted as described above.

RESULTS

Separation of polypeptides from rat islets

The separation of rat insulins (I and II) and C-peptides (I and II) secreted to the culture medium from rat islets using two different RP-HPLC columns and buffer systems is shown in Fig. 1. The peaks shown with dashed lines are due to standards of rat pancreatic polypeptides (peak 5) and porcine glucagon (peak 6) (same amino acid sequence as rat glucagon[15]). The identities of the C-peptide I (peak 2), C-peptide II (peak 1), insulin I (peak 3) and insulin II (peak 4) were verified by amino acid analysis and amino acid sequencing. The three chromatograms all show baseline separation of the analyzed polypeptides.

Preparative RP-HPLC fractionation of 1400 rat islets is shown in Fig. 2. The positions of the insulin and glucagon peaks were confirmed using radioimmunological analyses of the individual fractions. The later peaks F, G, H and I were insulin immunoreactive, and preliminary amino acid analysis and sequencing indicates a
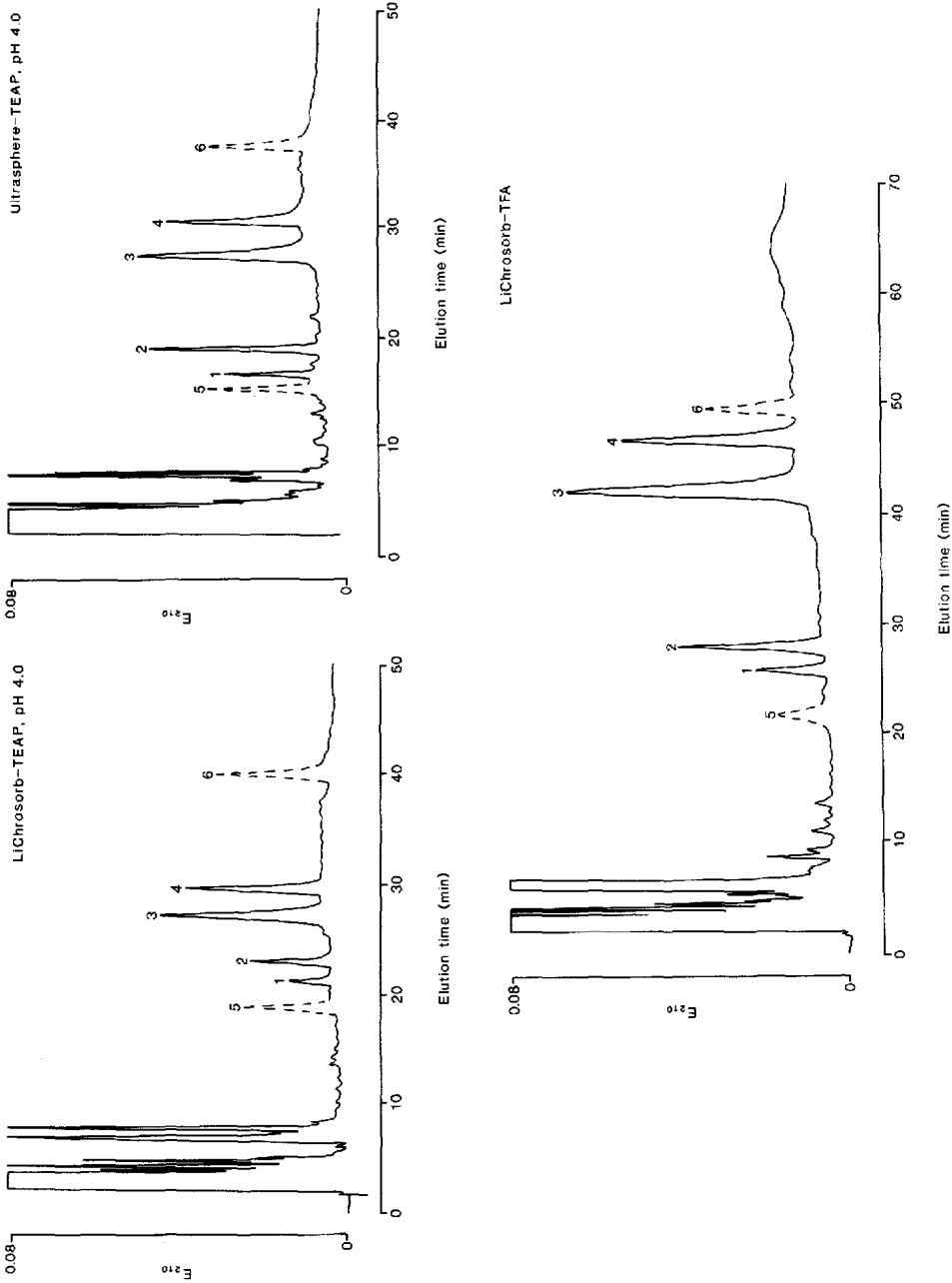
Fig. 1. RP-HPLC separation of rat islet cell culture medium containing insulin I (peak 3), insulin II (peak 4), C-peptide I (peak 2) and C-peptide II (peak 1) plus added rat pancreatic polypeptide (peak 5) and porcine glucagon (peak 6). Upper left and lower panel: LiChrosorb RP-18, 5 µm, 250 mm × 4.0 mm I.D. Upper panel right: Ultrasphere ODS, C_{18}, 5 µm, 250 mm × 4.6 mm I.D. The columns were eluted with a linear acetonitrile gradient (25 to 30%) in 0.125 M TEAP, pH 4.0 during 30 min (upper panels) or 30 to 36% in 0.1% TFA during 60 min (lower panel). Flow-rate 1.0 ml/min.
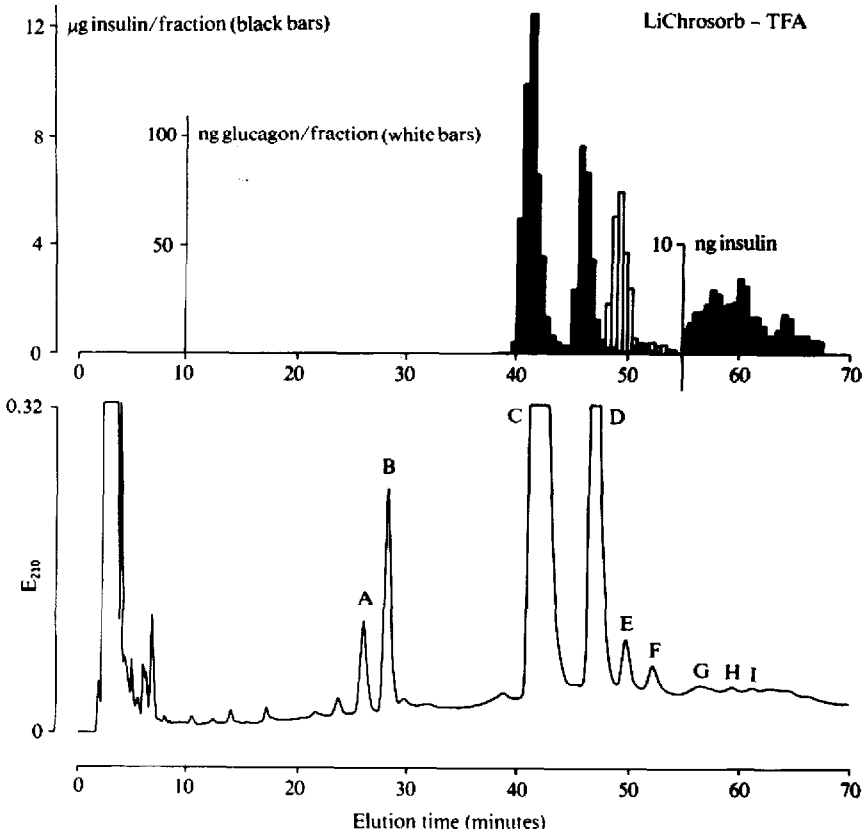
Fig. 2. RP-HPLC separation of 1400 rat islets extracted in 3 M acetic acid using a LiChrosorb RP-18, 5 μm, 250 mm × 4.0 mm I.D. column eluted at 1.0 ml/min with a linear acetonitrile gradient (30 to 36%) in 0.1% TFA during 60 min. Black bars represent insulin immunoreactivity, white bars represent glucagon immunoreactivity measured in the 0.5-min fractions collected. Peaks: A = C-peptide II; B = C-peptide I; C = insulin I; D = insulin II; E = glucagon; F and G = proinsulin-like; H = proinsulin I; I = proinsulin II.

proinsulin-like amino acid composition. Peaks H and I show B-chain sequences corresponding to 18 and 20 steps of proinsulin I and II, respectively. The amino acid differences between rat proinsulin I and II are shown in Fig. 3.
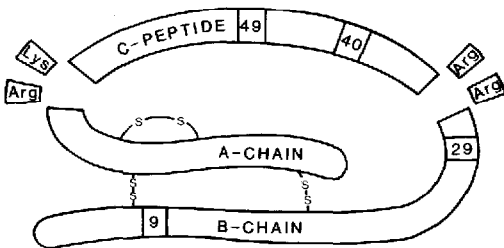

Fig. 3. Products of the conversion of proinsulin into insulin in rat islets. Amino acid differences in rat proinsulin I and II are in position 9 = Pro/Ser, position 29 = Lys/Met, position 40 = Pro/Ala and position 49 = Glu/Gly, respectively.
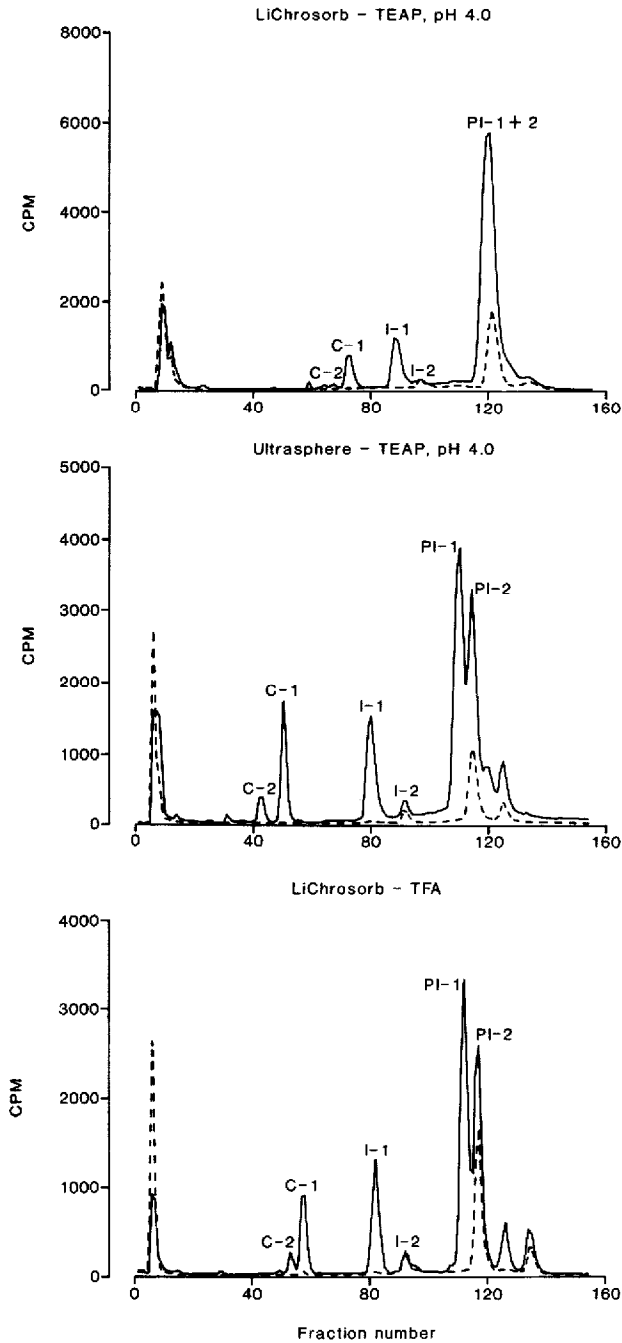
Fig. 4. RP-HPLC separation of rat islets pulse-labelled for 60 min with [³H]leucine and [³⁵S]methionine as described in the legend to Fig. 1. The solid line represents ³H-radioactivity, the dashed line ³⁵S-radioactivity. The peaks are C-1 and C-2 (C-peptides I and II), I-1 and I-2 (insulins I and II), PI-I and PI-2 (proinsulins I and II).

Radioactive labelling of rat islets

Rat islets, 5000 and 9500 were pulse-labelled for 60 min with [^{3}H]leucine (present in both proinsulin and insulin I and II) and [^{35}S]methionine (present only in proinsulin and insulin II). Aliquots of acetic acid extracts of the labelled islets were fractionated using the same HPLC columns and buffers as in Fig. 1. The resulting radioactivity patterns are shown in Fig. 4. The radioactivity in the first fractions is due to remaining free amino acids. The next four peaks correspond to the C-peptides and insulin peaks identified in Fig. 1. The expected proinsulin peaks eluted around fractions 110–120 were separated in two of the systems.

The labelled islets were fractionated preparatively in the LiChrosorb–TFA system (lower panel in Fig. 4) and pooled fractions from the two "proinsulin" peaks were subjected to microsequencing. The correct amino acids were identified in 39 and
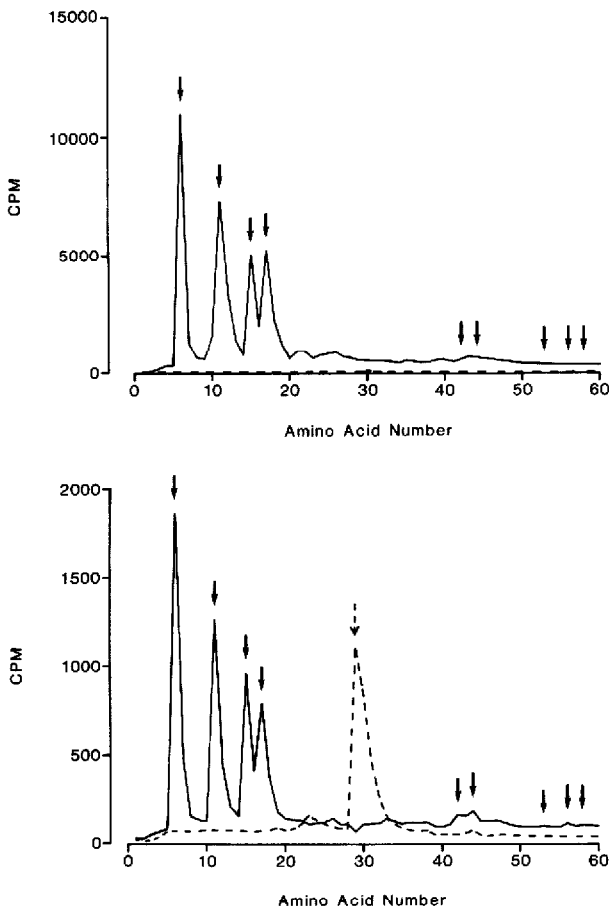

Fig. 5. Radiosequencing of pooled proinsulin fractions from preparative RP-HPLC fractionation of 5000 or 9500 rat islets pulse-labelled for 60 min with [^{3}H]leucine and [^{35}S]methionine. The solid line represents ^{3}H radioactivity, the dashed line ^{35}S-radioactivity. The solid arrows indicate the known positions of leucines, the dashed arrow the position of methionine. Upper panel shows the sequencing of proinsulin I, lower panel that of proinsulin II.

36 steps for proinsulin I and II, respectively, corresponding to 9 and 6 steps in the C-peptide regions of proinsulin I and II, respectively.

Taking advantage of the [^{3}H]leucine and [^{35}S]methionine labelling, it was possible to demonstrate the correct position of Leu-6, -11, -15, -17, and even Leu-42 and -44 can be detected, see Fig. 5. The presence of [^{35}S]methionine in position 29 in proinsulin II was also clearly demonstrated.

Separation of polypeptides present in mouse islets

Fig. 6 shows the RP-HPLC fractionation of acetic acid extracts of isolated mouse islets compared to extracted rat islets. It is seen that the retention times for the
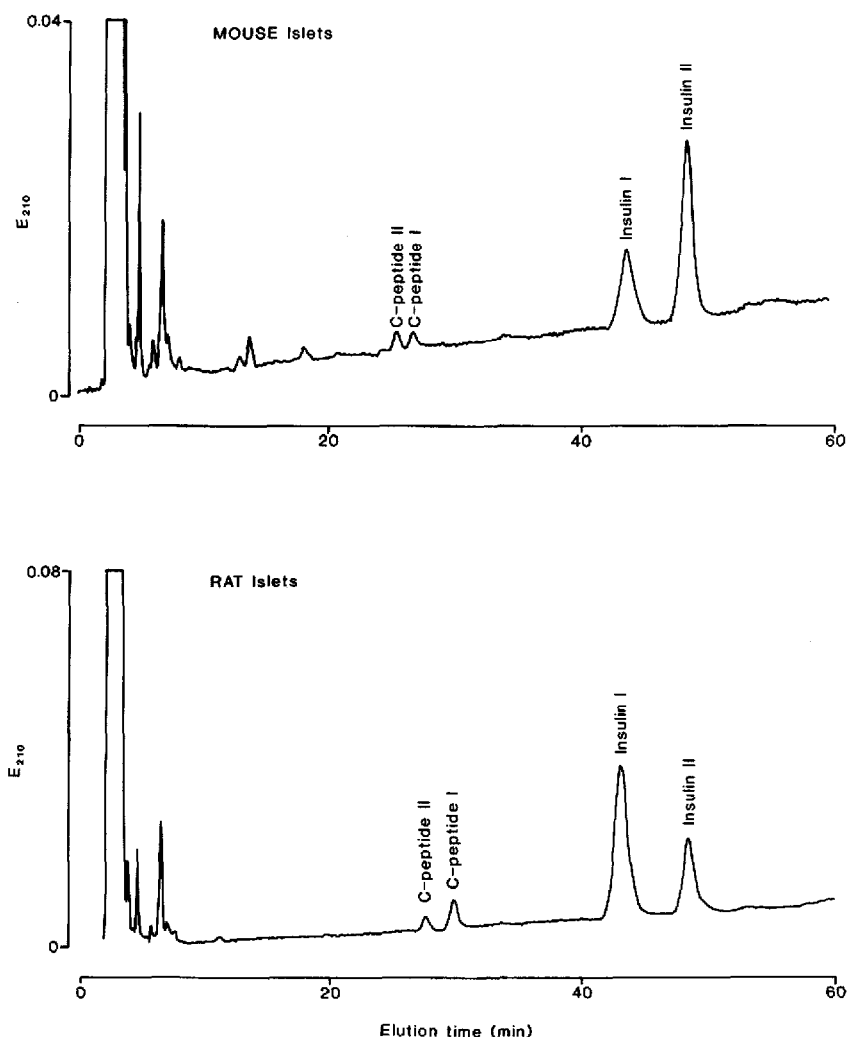

Fig. 6. RP-HPLC separation of acetic acid extracts of isolated mouse and rat islets using a LiChrosorb RP-18, 5-μm column, 250 × 4.0 mm I.D. eluted at 1.0 ml/min with a linear acetonitrile gradient (30 to 36%) in 0.1% TFA during 60 min.

presumed mouse C-peptides are somewhat lower than those for the rat C-peptides. Mouse islets (4700) were extracted with 3 M actic acid and the extract separated using the LiChrosorb–TFA system, analogously with the separation in Fig. 6. The expected C-peptide fractions were pooled and subjected to microsequencing. The resultant sequences are shown in Table I in comparison with the rat C-peptide sequences.

Biosynthesis of insulin in rat islets

Pulse-chase labelling was performed at 37°C with [³H]leucine and [³⁵S]methionine in samples containing 100 rat islets. The pulse period was 20 min, ensuring that only proinsulins were labelled. The chase periods were 0, 10, 20 and 40 min. RP-HPLC fractionation of the media showed that no radioactive insulin and C-peptide were secreted during the pulse-chase experiment. RP-HPLC fractionation of the islet extracts separated proinsulin I and II, insulin I and II as well as C-peptide I and II

TABLE I

AMINO ACID SEQUENCES OF RAT AND MOUSE C-PEPTIDES

Amino acid number	Rat C-I	Mouse C-I	Rat C-II	Mouse C-II
1	Glu	Glu	Glu	Glu
2	Val	Val	Val	Val
3	Glu	Glu	Glu	Glu
4	Asp	Asp	Asp	Asp
5	Pro	Pro	Pro	Pro
6	Gln	Gln	Gln	Gln
7	Val	Val	Val	Val
8	Pro	Glu*	Ala	Ala
9	Gln	Gln	Gln	Gln
10	Leu	Leu	Leu	Leu
11	Glu	Glu	Glu	Glu
12	Leu	Leu	Leu	Leu
13	Gly	Gly	Gly	Gly
14	Gly	Gly	Gly	Gly
15	Gly	Ser*	Gly	Gly
16	Pro	Pro	Pro	Pro
17	Glu	*	Gly	Gly
18	Ala	*	Ala	Ala
19	Gly	Gly	Gly	Gly
20	Asp	Asp	Asp	Asp
21	Leu	Leu	Leu	Leu
22	Gln	Gln	Gln	Gln
23	Thr	Thr	Thr	Thr
24	Leu	Leu	Leu	Leu
25	Ala	Ala	Ala	Ala
26	Leu	Leu	Leu	Leu
27	Glu	Glu	Glu	Glu
28	Val	Val	Val	Val
29	Ala	Ala	Ala	Ala
30	Arg	Arg	Arg	Gln*
31	Gln	Gln	Gln	Gln

* Difference in amino acid sequence of the mouse C-peptides compared to the rat C-peptides.

TABLE II

INSULIN BIOSYNTHESIS IN RAT ISLETS

Conversion of proinsulin I and II into insulin I and II in pulse-chase experiments with [^{3}H]leucine and [^{35}S]methionine.

Pulse (min)	Chase (min)	Proinsulin I to insulin I (%)	Proinsulin II to insulin II (%)	Insulin I/insulin II	
				UV	cpm
20	0	0	0	2.1	–
20	10	4.8	0	2.1	–
20	20	11.6	3.4	2.2	5.4
20	40	47.4	16.1	1.9	6.0

(same separation pattern as in Fig. 4, lower panel). The ^{3}H- and ^{35}S-radioactivities in the fractions corresponding to proinsulin I and II and insulin I and II were added and the molar amounts of proinsulin and insulin were calculated taking account of the difference in leucine content (11 leucine and 6 leucine, respectively). The calculated conversions of proinsulins into insulins are shown in Table II.

DISCUSSION

The presence of two non-allelic insulin genes in mouse and rat has raised the question how the expression of the two genes is regulated. A prerequisite for a detailed analysis of the latter is a separation system which allows identification of the individual biosynthesis and processing prodcuts, *i.e.*, the two proinsulins, insulins and C-peptides.

Two HPLC methods were recently described for the study of insulin biosynthesis in rat islets[10,11]. Neither of these systems separated the two proinsulins and the C-peptides were not identified.

By comparing different RP-HPLC systems which have been applied for the separation of insulin and related peptides, we have developed two systems which permit separation of these six major peptides (Figs. 1 and 2). Compared with the Ultrasphere–TEAP system we found that the LiChrosorb–TFA system gave a better separation of the two rat proinsulins and as the TFA buffer can be lyophilized the fractions can be subjected to amino acid analysis and sequencing without buffer exchange.

By a combination of microsequencing and radiosequencing we have identified the individual peaks by comparison with published sequence data: rat C-peptide I and II, rat insulin I and II and rat proinsulin I and II (partial sequences).

The elution order of insulin I and II was reversed in the HPLC system used by Gishizky and Grodsky[11] compared to out findings in spite of the similarity in buffers, but was perhaps caused by the use of another HPLC column (Dupont PEP-R C$_8$ instead of LiChrosorb). We also noticed a reversal of the elution order of mouse C-peptide I and II on the LiChrosorb column when changing from TFA to TEAP, pH 4.0[16], demonstrating the versatility of he HPLC systems.

Both after preparative fractionation and after labelling with radioactive amino

acids, two peaks with higher retention times than those of the proinsulins were observed. Since they showed insulin-like immunoreactivity and proinsulin-like amino acid composition, appeared after the initial proinsulin synthesis and could be converted into the two insulins by trypsin and carboxypeptidase B (ref. 17), they probably represent intermediary split products of the proinsulins, although human split proinsulins appear earlier than human proinsulin in this system (data not shown). The final identity of these peaks must await isolation of more material.

During this study we noted that the molar ratio between insulin and C-peptide was 1 in the culture medium and the newly synthesized products of the islets, but always much higher in the stored insulin (compare Fig. 1, lower panel and Fig. 4, lower panel with Fig. 6, lower panel). Whether this is due to a more rapid intracellular degradation of the C-peptides or to insufficient extraction is not known at present.

The sequences of the two mouse insulins have long been known to be identical to those of the rat insulins[6], but it was only recently that the sequences of the C-peptides were found to differ as predicted by the nucleotide sequences of the c-DNA[7]. These differences give a change in retention time of the mouse C-peptides from those of the rat (Fig. 6). By amino acid sequencing of the isolated peaks we confirmed the two amino acid deletion in the mouse C-peptide I, and two and one amino acid differences from rat C-peptide I and II, respectively (Table I).

The RP-HPLC separation was used to study the conversion of the two rat proinsulins into the corresponding insulins (Table II). It was observed that the ratio between insulin I and II was about 2 in the stored insulin (UV absorption at 210 nm) and as high as 5–6 in the newly synthesized insulins (^{3}H-radioactivity) as also reported by Gishizky and Grodsky[11]. The calculated conversion rates showed an higher conversion rate of proinsulin I than of proinsulin II (Table II) as already suggested by Clark and Steiner[3]. This is also supported by our previous finding that inhibition of the insulin biosynthesis with interleukin-1 resulted in an increased ratio between newly synthesized insulin I and II which can be explained by a more pronounced attenuation of the conversion of proinsulin II[18].

We also observed that the usual ratio between insulin I and II in the rat, about 2:1, was reversed in the mouse, about 1:2 (Fig. 6). A similar ratio was reported by Markussen[6] in crystalline mouse insulin, but was assumed to be due to a selection by the separation procedure. By immunoelectrophoresis, Kakita et al.[19] always found more insulin I than II in the pancreas of both rats and mice. Variations in the ratio with age and metabolic state have been reported[19–21] but the effect of glucose was not confirmed by HPLC[22]. In rat insulinomas a ratio of 10:1 has been found although the two genes were equally transcribed[23]. Recently, neither age, glucose nor growth hormone were found to change the ratio between transcription of the two genes in rats in vitro[24], indicating that if a change in the ratio between the two insulins occurs, it is probably due to a variation in translation, conversion or degradation. Further studies on the insulin biosynthesis in mouse islets may reveal by which mechanism the odd ratio occurs.

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