

Development of a Radioimmunoassay for Plasma C-Peptide in Sheep: Kinetics of C-Peptide and Effects of Exogenous Growth Hormone and Glucose on Insulin and C-Peptide

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Abstract

An antiserum to purified bovine C-peptide was used to develop a sensitive radioimmunoassay for C-peptide in sheep. The assay was used to measure kinetics of C-peptide and insulin in non-pregnant and non-lactating sheep. Injected, purified C-peptide was distributed in pools comprising c. 11.4% of liveweight, the half time of C-peptide was estimated as 13.7 min and its clearance rate was c. 5 ml kg⁻¹ min⁻¹. In lactating ewes exogenous recombinant bovine growth hormone (rebGH) increased both plasma insulin and C-peptide as did glucose challenge given before and during administration of rebGH. Estimates of insulin secretion rate in lactating ewes were c. 7 × 10⁻³ and 8.5 × 10⁻³ nmol kg⁻¹ min⁻¹ before and after glucose challenge prior to injections of rebGH. After 4 days of injection of rebGH, corresponding values were c. 8 × 10⁻³ and 10 × 10⁻³ nmol min⁻¹ kg⁻¹.

Introduction

Insulin secretion rate may be estimated in surgically modified animals by measuring portal venous/arterial differences for insulin together with portal blood flow rate (Brockman and Bergman 1975). Such procedures are invasive and alternative procedures have been sought. Since newly secreted insulin is extracted, to varying degrees, by the liver before being mixed with systemic blood (Field 1973; Brockman and Bergman 1975) it is not valid to assess insulin secretion rate from plasma concentrations of the hormone in peripheral blood. An alternative approach is to measure peripheral blood concentrations of C-peptide and its clearance rate. This approach provides valid estimates of insulin secretion since insulin and C-peptide are secreted in equi-molar proportions and there is negligible hepatic extraction of C-peptide (Polonsky *et al.* 1984; Polonsky and Rubenstein 1984; Radziuk and Morishima 1984).

The above procedure has been used to measure insulin secretion rate in humans, but no similar measurements have been made in ruminants. Accordingly, the objectives of the present study were to develop an assay for C-peptide in the sheep and to utilise the assay to measure kinetics of C-peptide, and thereby insulin secretion rate. Exogenous glucose and rebGH were administered to alter insulin secretion rate.

Materials and Methods

Assay for Bovine C-Peptide

Preparation of C-peptide

Bovine C-peptide was isolated from 'mother liquor', a waste product from the extraction of insulin from the bovine pancreas, obtained from Commonwealth Serum Laboratories (CSL), Melbourne. The C-peptide was isolated and purified by ion exchange chromatography using minor modifications

of the procedure described by Sundby and Markussen (1970). Briefly, a crude precipitate of C-peptide (20 g) was obtained from 30 l of the mother liquor by precipitation with trichloroacetic acid (TCA) followed by acetone. A portion of this crude preparation (2.5 g) was partially purified on QAE-Sephadex A25 (Pharmacia, Uppsala, Sweden) equilibrated with 0.05 M ethylene diamine/acetic acid buffer (pH 5.0), yielding c. 100 mg of partially purified material. The C-peptide was eluted by establishing a linear pH gradient (pH 5.0–4.0) and fractions containing C-peptide identified by precipitation of the C-peptide in aliquots of 100 μ l of each fraction with 1 ml of 11% (w/v) TCA and subsequent measurement with Folin-Ciocalteu's reagent (Lowry *et al.* 1951). Fractions reacting with this reagent, but not absorbing u.v. light (280 nm), were considered to contain C-peptide.

The selected fractions were then dialysed against 0.1 M acetic acid at 4°C before lyophilization and subsequently further purification. A total of 100 mg of partially purified C-peptide was fractionated on a column (c. 5 \times 100 cm) of Sephadex G50 fine (Pharmacia) equilibrated with 1 M acetic acid. The C-peptide peak was identified as outlined by Sundby and Markussen (1970) and appropriate fractions were pooled and lyophilized after extensive dialysis against 0.1 M acetic acid. A total of 85 mg of purified C-peptide were obtained.

The amino acids making up the purified C-peptide were determined and quantified using an amino acid analyser (Dionex, Model D-300; Dionex, California, U.S.A.) after acid hydrolysis of the C-peptide for 24 h at 110°C.

Labelled C-peptide

As there is no tyrosine residue in bovine C-peptide (Clark *et al.* 1969) tyrosylated-C-peptide was prepared as described by Markussen *et al.* (1970). The method of Hunter and Greenwood (1962) then was used to iodinate tyrosylated-C-peptide. Briefly, 0.25 M (pH 7.4) phosphate buffer was used to prepare solutions of tyrosylated-C-peptide (1 mg ml⁻¹), chloramine-T (1 mg ml⁻¹) and ¹²⁵I (3.7 \times 10⁹ Bq ml⁻¹). These solutions were rapidly mixed in the proportions 2 μ l : 5 μ l : 10 μ l, then after 30 s the reaction was quenched by adding 20 μ l of a solution of L-tyrosine (0.1 mg ml⁻¹ in 0.05 M phosphate buffer, pH 7.4). Free and coupled ¹²⁵I (tracer) were separated as described by Starr *et al.* (1979).

Antisera to C-peptide

Purified C-peptide was coupled to bovine serum albumin (Calbiochem, San Diego, California, U.S.A.) using 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride (coupling reagent; Sigma, St Louis, U.S.A.) and the method of Beischer *et al.* (1976). Amounts of albumin and purified C-peptide used were 50 mg and these were added to 500 mg of coupling reagent.

Four rabbits were immunized in multiple intramuscular sites, up to 8 times and at intervals of 4 weeks. At each immunization, rabbits were injected with aliquots of 1 ml (0.5 mg) of coupled C-peptide emulsified with 2 ml of Complete Freund's Adjuvant (Difco Laboratories, Michigan, U.S.A.). The rabbits were bled immediately before each immunization and sera were stored at -20°C pending titration of antibody activity. All antisera were assessed for binding capacity using a single batch of tracer and sera with titres of 4000 or greater were considered useful for assay purposes. The titre was taken as the reciprocal of the highest dilution of antiserum which bound 50% of the added tracer.

Standard bovine pro-insulin

The starting material used for purification of bovine pro-insulin was a paste of material prepared from the fractions immediately before the peak of insulin during purification of crude insulin, by gel filtration on an industrial scale. This paste was obtained from CSL, Melbourne, and was subjected to isoelectric precipitation (J. Considine, CSL, personal communication) and lyophilization prior to filtration, on Sephadex G50 fine gel (Pharmacia) equilibrated with 1 M acetic acid. A crude preparation of pro-insulin was obtained in the fractions preceding the main insulin peak (Steiner *et al.* 1968). This crude preparation of pro-insulin was purified by separation on QAE-Sephadex A25 (Pharmacia) equilibrated with a solution at pH 8.4 and containing 0.1 M NH₄Cl, 0.2 M NH₃ and 60% (v/v) ethanol, see Heding *et al.* (1974).

Details of radioimmunoassay

A modification of the assay procedure described by Heding (1975) was used. Phosphate buffer (0.04 M, pH 7.4) containing bovine serum albumin (1 g l⁻¹; Commonwealth Serum Laboratories,

Melbourne) was used for dilution of standard C-peptide solutions, dilution of antiserum (DRU3, 18/12; final titre 4000) and for dilution of tracer.

The standard curve for the assay was prepared by mixing aliquots of 100 μl each of standard solution of C-peptide (0–5 nM), plasma from an alloxan diabetic sheep extracted with talc, antiserum (final dilution 1/4000) and C-peptide tracer. For measurements of C-peptide in plasma, aliquots of 100 μl of buffer, unknown plasma, antiserum and tracer were mixed. After incubation for 24 h at 4°C 'antibody-bound' and 'free' tracer were separated by alcohol precipitation (Heding 1975).

The cross reactivity of the antiserum was assessed by measuring displacement of tracer from antibody in the presence of pro-insulin and insulin. The sensitivity (detection limit) of the assay was assessed as outlined by Burger *et al.* (1972). The inter-assay coefficient of variation of the assay was assessed using a single pooled plasma sample analysed in triplicate in 11 assays. The intra-assay coefficient of variation was assessed from the variation between replicate measurements of standard preparations of C-peptide in single assays.

Analytical Procedures

All blood samples collected for analyses were kept in ice-chilled tubes containing heparin (c. 100 iu ml^{-1} blood) and plasma was prepared within 15 min of collection of blood. Plasma samples were stored at -20°C pending analyses.

Concentrations of plasma insulin were measured with the radioimmunoassay previously described by Leenanuruxsa *et al.* (1988). Concentrations of insulin were expressed as nmol l^{-1} , rather than mU l^{-1} , to allow comparison of molar ratios of insulin : C-peptide. Plasma concentrations of GH were measured by the assay described by Gow *et al.* (1981). This assay has been found to measure ovine and bovine GH with equal avidity (Leenanuruxsa and McDowell, unpublished data).

The half-time of C-peptide administered to sheep was calculated as $0.693/K$, where K is the slope of the straight line decay curve of C-peptide, over the first 40 min following intravenous administration, plotted on a semilogarithmic scale. Clearance rate of plasma C-peptide was measured as either infusion rate divided by plasma concentration (when C-peptide was administered by continuous infusion) or the dose of injected C-peptide divided by area under the decay curve of plasma C-peptide (when the C-peptide was administered as a single injection), see Riggs (1963).

Polynomial equations were fitted to data pairs of plasma insulin *v.* time and plasma C-peptide *v.* time, using the method of least squares, to smooth the respective curves as suggested by Radziuk *et al.* (1978). The significance of differences between mean values for parameters measured were assessed using the paired t-test (Steel and Torrie 1960).

Experimental Procedures

Kinetics of C-peptide were measured in four multiparous, non-pregnant and non-lactating crossbred ewes (Border Leicester \times Merino) with liveweights varying from 39.1 to 44.7 kg. Each ewe was fitted with indwelling, polyvinyl chloride catheters (1.0 mm i.d. \times 1.5 mm o.d.; Dural Plastics, Sydney) in both external jugular veins 4–5 days before the experiment. Ewes were deprived of food from 48 h before and for the duration of the experiment.

A continuous intravenous infusion of purified C-peptide (4.2 $\mu\text{mol l}^{-1}$ in saline–9g l^{-1} NaCl), at rates varying from 78.6 to 81.0 nmol h^{-1} for individual sheep, was administered for 150 min via one jugular catheter. Blood samples (5 ml) were collected from the contra-lateral jugular catheter at –10, 0, 120, 130, 140 and 150 min from the start of infusion.

Commencing 4 h after cessation of continuous infusion of C-peptide ewes were given a rapid single injection of C-peptide at doses varying from 99.5–105.6 nmol ewe^{-1} , via one jugular catheter. Blood samples (5 ml) were collected from the contra-lateral jugular catheter at –10, 5, 10, 15, 20, 30, 40, 60, 80, 100 and 120 min from injection of C-peptide.

Changes in plasma C-peptide and insulin in response to exogenous glucose were measured in three multiparous, lactating, crossbred ewes (Border Leicester \times Merino) with liveweights varying from 59.2–66.7 kg. Lactating ewes were used for this study because of the marked increase in glucose requirements during lactation (Annison and Linzell 1964) and presumed concomitant changes in insulin secretion. Ewes had been lactating for c. 60 d and they had been milked by hand twice daily at 0830 and 1630 h from the day of parturition. They were fed continuously with belt feeders a good quality ration containing rolled barley : chopped lucerne (50 : 50 w/w air dry; 9.6 MJ metabolizable energy and 218 g crude protein per kg dry matter) in sufficient amounts to satisfy requirements for metabolizable energy for maintenance plus milk production (Anon. 1975). Water was available *ad*

libitum. Each ewe had been fitted with catheters (see above) in both external jugular veins 1–2 d before experiments commenced.

Measurements were made on day 4 of successive periods of 4 d during which ewes received daily subcutaneous injections of pH 9.0 bicarbonate buffer (control) or 0.1 mg kg⁻¹ liveweight rebGH, (donated by American Cyanamid Company, New Jersey, U.S.A.) dissolved in bicarbonate buffer. On each of these days ewes were given intravenous infusions of glucose (500 g l⁻¹; Abbott Australia Pty Ltd), via one jugular catheter, commencing c. 2 h after the daily control or rebGH injection. Blood samples (5 ml) were obtained, from the contra-lateral jugular catheter and at intervals of 10 min, over the period from -40 to 120 min after commencement of glucose infusion.

Results

Yield and Purity of C-Peptide

The molar ratios of constituent amino acids were 6.4:7.7:3.0:2.1:3.1 for glutamic acid:glycine:alanine:valine:leucine. The analytical procedure did not allow measurement of proline, the other amino acid including C-peptide.

The specific radioactivity of the tyrosylated-C-peptide labelled with ¹²⁵I was 6.48–9.25 × 10⁶ Bq μg⁻¹.

Antisera to C-Peptide and Radioimmunoassay Characteristics

Serum collected from one of the immunized rabbits did not satisfy the criterion of having a titre of 4000. The other three rabbits responded to the immunizations and suitable sera were collected from these animals on one, six and seven occasions respectively. The antiserum chosen for use in the assay (viz. DRU3: 18/12) showed no cross reaction with insulin which failed to displace C-peptide tracer from antibody when added at concentrations of up to 10 nmol ml⁻¹ insulin. There was cross reaction between the antiserum and pro-insulin, varying from 40–47%, such that the displacement of tracer at 1, 2 or 3 nmol l⁻¹ of C-peptide was similar to displacement when 2.5, 4.5 or 6.4 nmol l⁻¹ of pro-insulin were added respectively.

The sensitivity of the assay was 0.13 nmol l⁻¹ and intra- and inter-assay coefficients of variation were <10 and <13% respectively.

Kinetics of C-Peptide

From the response to single injection of C-peptide to non-pregnant and non-lactating ewes the volume of distribution was measured as 11.36 ± 0.711% of liveweight (mean ± s.e.m., *n* = 4), the half-time of injected C-peptide was 13.71 ± 0.547 min and the clearance rate of C-peptide was 5.01 ± 0.305 ml kg⁻¹ min⁻¹. This latter value for clearance rate was not significantly different (*P* > 0.05) from the clearance rate of 4.88 ± 0.319 ml kg⁻¹ min⁻¹ measured by continuous infusion of C-peptide.

Responses of Lactating Ewes to rebGH and Exogenous Glucose

Plasma growth hormone

Plasma concentrations of growth hormone increased significantly (*P* < 0.05) from c. 1.4 μg l⁻¹ before to c. 7.1 μg l⁻¹ during injection of rebGH. Glucose infusions did not affect plasma growth hormone.

Plasma C-peptide

Changes in plasma concentrations of C-peptide in response to exogenous glucose are depicted in Fig. 1. During treatment with rebGH, basal concentration of C-peptide increased non-significantly (*P* > 0.05) from c. 1.4 to c. 1.6 nmol l⁻¹. Plasma C-peptide tended to increase (*P* > 0.05) following glucose infusion during the control period and increased significantly (*P* < 0.05) following exogenous glucose during treatment with rebGH.

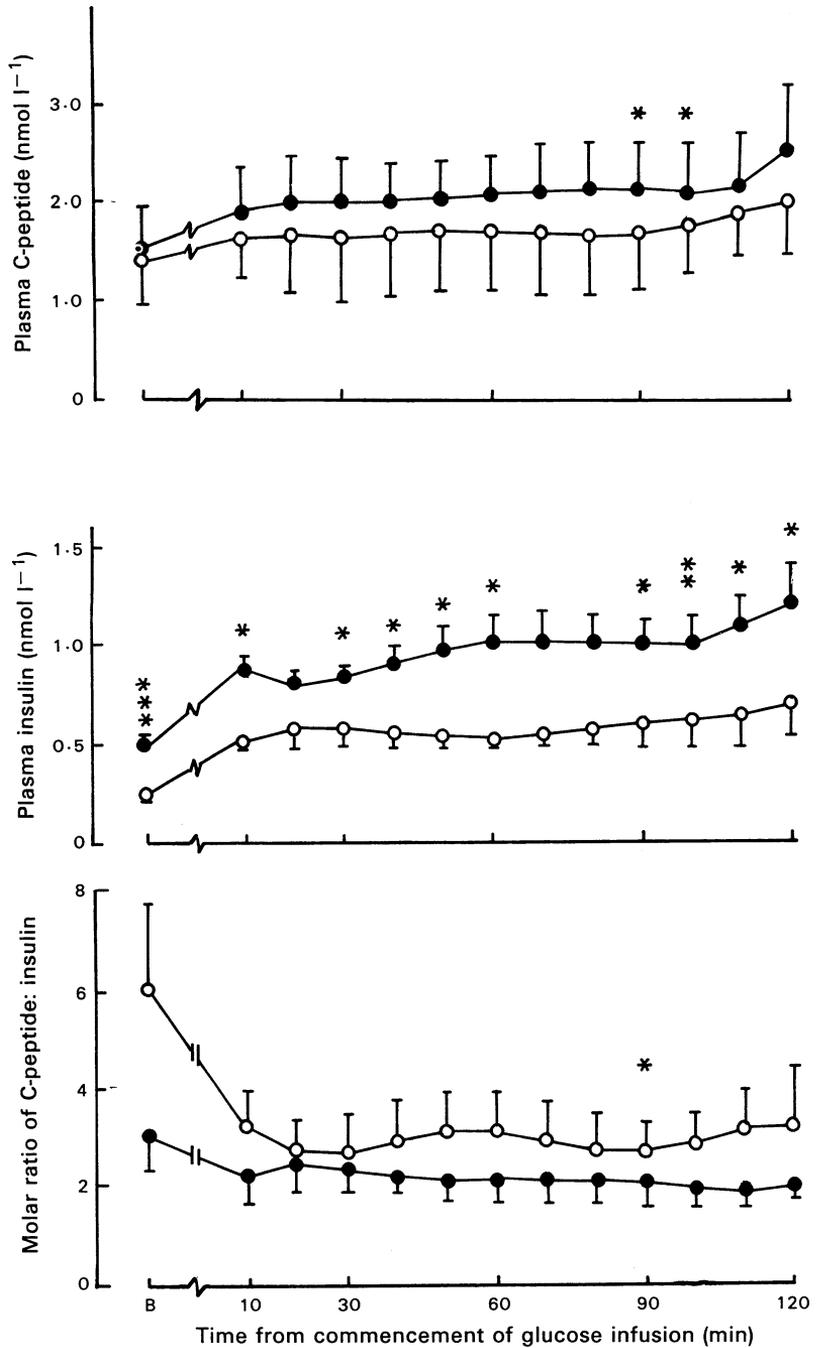


Fig. 1. Plasma concentrations of C-peptide, insulin and the ratio of C-peptide:insulin before and after glucose challenge in lactating ewes given control injections (○) or injections of rebGH (●). Plotted points represent mean values for three ewes and standard errors are shown as vertical bars. Values shown before glucose challenge (B) were derived from the means of three measurements for each ewe. Values for the period when rebGH was injected which differ significantly from corresponding values when the control injections were given are indicated thus: **P* < 0.05, ***P* < 0.01, ****P* < 0.001.

Although concentrations of C-peptide were consistently higher during injection of rebGH than during control injections, significant differences ($P < 0.05$) between periods were measured at 90 and 100 min after commencing glucose infusions.

Plasma insulin

Basal concentrations of plasma insulin were significantly elevated ($P < 0.01$) during injection of rebGH. In response to exogenous glucose, concentrations of insulin increased significantly ($P < 0.05$) during both periods and concentrations were significantly lower ($P < 0.05$) during control than rebGH injections (see Fig. 1).

Molar ratio of C-peptide : insulin

The basal value for the molar ratio of C-peptide : insulin was lower, but not significantly ($P > 0.05$), during injection of rebGH than during control injections. The ratio was consistently lower throughout the period of observation when ewes were treated with rebGH, being significantly lower ($P < 0.05$) at 90 min after commencing exogenous glucose. During both control injections and injections of rebGH, the ratio was lower, but not significantly so ($P > 0.05$) when glucose was infused (see Fig. 1).

Discussion

In previous studies bovine C-peptide has been measured by radioimmunoassays in which antisera to pro-insulin were used (Rubenstein *et al.* 1970; Heding *et al.* 1974). The present report is the first in which an assay specific for C-peptide has been developed and used to quantify C-peptide in the sheep. The observation that bovine and ovine C-peptide have identical sequences of amino acids (Peterson *et al.* 1972) enables development of an assay for measurement of C-peptide in both species.

The C-peptide prepared for use as standard and for preparation of antisera in rabbits was highly purified as shown by the molar ratios of constituent amino acids measured. The theoretical ratios of constituent amino acids, glutamic acid : glycine : alanine : valine : leucine are 6 : 8 : 3 : 2 : 3 (Clark *et al.* 1969) and it is clear that the measured ratios of 6.2 : 7.7 : 3.0 : 2.1 : 3.1 were remarkably similar to theoretical values. The yield of C-peptide after ion exchange chromatography was similar to that previously reported by Sundby and Markussen (1970) and the final purification on Sephadex G-50 gel removed the traces of lysine, threonine, serine and aspartic acid measured by these workers.

It has been reported that isolated C-peptide is poorly immunogenic because of its size and its configuration as a random coil (Markussen *et al.* 1971; Markussen and Schiff 1973). Other workers have raised antisera, of high titre, against human C-peptide using material coupled to albumin, but responses to immunization with this complex antigen have been reported to be very variable (Beischer *et al.* 1976; Faber *et al.* 1976). The observation that three of four rabbits yielded suitable antisera at only some bleeding times is consistent with this reported variability in responsiveness to immunization with C-peptide.

It is clear that the assay developed and reported here allowed quantification of C-peptide in ovine plasma. There was significant cross reactivity with pro-insulin but no cross reaction with insulin. The latter confirms the purity of the isolated C-peptide.

Cross reactivity with pro-insulin would be expected as the pro-insulin molecule contains the C-peptide moiety (Faber *et al.* 1978). This cross reaction with pro-insulin could be corrected for by quantifying pro-insulin using the procedure of Starr *et al.* (1979), or alternatively by removal of pro-insulin from plasma by addition of antibody to insulin and subsequent removal of the complexed pro-insulin prior to assay for C-peptide (Krause *et al.* 1981). In practice, concentrations of pro-insulin measured as outlined by Heding *et al.* (1974) were sufficiently low (< 10% of C-peptide as pro-insulin; Leenanuruksa, unpublished data) to obviate the need to correct for this entity.

The rates of clearance of C-peptide measured in the present study by constant infusion

and single injection ($c. 5 \text{ ml kg}^{-1} \text{ min}^{-1}$) were similar to estimates of clearance of C-peptide in normal and diabetic humans of 4.4 and $4.7 \text{ ml kg}^{-1} \text{ min}^{-1}$ respectively (Faber *et al.* 1979). Estimates of kinetics of C-peptide in dogs made by Polonsky *et al.* (1984) showed the volume of distribution to be 5.3% of liveweight, a half time of 6.6 min and clearance rate of $13 \text{ ml kg}^{-1} \text{ min}^{-1}$. The present estimates of 11.4% of liveweight, 13.7 min and $5 \text{ ml kg}^{-1} \text{ min}^{-1}$ respectively for these parameters indicate a higher volume of distribution, a longer half time and a correspondingly slower clearance rate. The latter differences may be correlated with metabolic size. The similar estimates of clearance rate of C-peptide in sheep and humans (see above) might be expected if clearance rate were to be related to metabolic size.

The changes in plasma C-peptide and insulin in response to administration of glucose in normal ewes and in ewes following treatment with rebGH are of interest in view of previous observations (Hart 1983; Hart *et al.* 1984). In our laboratory it has been found that exogenous GH exerts apparently paradoxical effects on insulin. On one hand exogenous growth hormone appears to increase insulin secretion whereas on the other hand the sensitivities of body tissues to insulin are reduced (Leenanuruksa and McDowell 1988; Leenanuruksa *et al.* 1988). Furthermore, the hyperinsulinaemia observed in sheep treated with growth hormone may be associated with reductions in the proportions of secreted insulin extracted by and degraded in the liver (Leenanuruksa, personal observations).

In the present study, concentrations of insulin were higher before and after glucose challenge during injection of rebGH than during control injections. Similar, but less pronounced changes were measured for plasma C-peptide. This observation, together with increased plasma C-peptide following glucose challenge when control injections were administered, is consistent with increased secretion of insulin from the pancreatic β -cells.

Injections of rebGH tended to decrease the molar ratio of C-peptide:insulin and the ratio decreased in response to glucose challenge whether ewes were given control or rebGH injections. These changes would be consistent with reduced extraction of insulin by the liver as has been reported for many investigations in humans (see Polonsky and Rubenstein 1984). However, as discussed by Polonsky and Rubenstein (1984) possible differences in the kinetics of C-peptide and insulin require that caution be exercised when interpreting changes in the ratio of C-peptide:insulin, particularly in relation to hepatic extraction of insulin.

The kinetics of C-peptide have been used to estimate insulin secretion rate (see Polonsky and Rubenstein 1984; Radziuk and Morishima 1984). Although insulin and C-peptide make up the pro-insulin molecule and are released in equimolar proportions from pancreatic β -cells (Rubenstein *et al.* 1969), considerable and variable amounts of insulin are extracted by the liver (Brockman and Bergman 1975). Accordingly, peripheral concentration of insulin will not reflect insulin secretion rate (Radziuk 1986). On the other hand, it has been shown that there is negligible hepatic extraction of C-peptide in rats (Stoll *et al.* 1970), normal dogs (Polonsky *et al.* 1983) and dogs given oral glucose (Polonsky *et al.* 1984). Furthermore, the clearance of C-peptide has been found to be constant under a wide range of physiological conditions and at varying concentrations of plasma C-peptide in rats (Katz *et al.* 1975), dogs (Polonsky *et al.* 1983; Polonsky *et al.* 1984) and humans (Faber *et al.* 1979).

Given that the above are true in the sheep, the secretion rate of C-peptide (equivalent to the product of clearance rate and peripheral concentration) will be equal to insulin secretion rate (Polonsky and Rubenstein 1984; Polonsky *et al.* 1984; Radziuk and Morishima 1984). Thus in the lactating ewes basal rates of insulin secretion of $c. 7 \times 10^{-3} \text{ nmol min}^{-1} \text{ kg}^{-1}$ during the control period and of $c. 8.5 \times 10^{-3} \text{ nmol min}^{-1} \text{ kg}^{-1}$ during injection of rebGH increased to $c. 8 \times 10^{-3}$ and $c. 10.5 \times 10^{-3} \text{ nmol min}^{-1} \text{ kg}^{-1}$ respectively when glucose was administered.

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