Metabolism and Placental Transfer of ^{125}I-Proinsulin and ^{125}I-Tyrosylated C-Peptide in the Pregnant Rhesus Monkey

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Abstract

^{125}I-Proinsulin or ^{125}I-tyrosylated-C-peptide (^{125}I-tyr-CP) was administered to pregnant Rhesus monkeys by bolus followed by constant infusion to examine placental transfer of these peptides. At the end of each infusion, fetuses were exsanguinated in situ via the umbilical vein. The bolus-constant infusion technique produced a steady state in maternal plasma of immunoprecipitable label, measured using excess insulin or C-peptide antiserum. In animals infused with ^{125}I-proinsulin, analysis of umbilical venous plasma revealed no apparent transfer to the fetus of immunoprecipitable label. In animals infused with ^{125}I-tyr-CP, 3–13% of the umbilical venous plasma radioactivity was immunoprecipitable, representing 1.4–5.8% of the immunoprecipitable radioactivity in maternal plasma at delivery. Gel filtration chromatography of umbilical venous plasma revealed that the immunoprecipitated moiety was a fragment of ^{125}I-tyr-CP. Analysis of maternal plasma showed that the predominant peak of radioactivity represented intact C-peptide. A peak corresponding to the fetal immunoprecipitable peak was also present. Analysis of simultaneous maternal arterial and uterine vein plasma samples showed that degradation of ^{125}I-tyr-CP occurred across the uterus. Studies in one nonpregnant and three postpartum animals indicated that pregnancy increased the rate of metabolism of ^{125}I-tyr-CP. When ^{125}I-tyr-CP was incubated with trophoblastic cells in culture, degradation to a species corresponding on gel filtration to the immunoprecipitable fetal metabolite was found. We conclude that proinsulin, like insulin, does not traverse the placenta. Immunoreactive fragments of C-peptide do cross, however, and pregnancy alters the metabolism of ^{125}I-tyr-CP, probably owing to placental degradation.

Introduction

The biosynthesis of insulin involves the posttranslational proteolytic processing of its precursor, proinsulin (1, 2). This results in the secretion of equimolar amounts of C-peptide, a product of this processing, and insulin (1). Proinsulin itself has recently emerged as a pharmacologic agent (3, 4). C-peptide, by contrast, seems to be physiologically inert, a characteristic that has resulted in the use of plasma C-peptide concentration as an indirect means of assessing insulin secretion by adult humans (for review see 2, 5), human fetuses and newborns (6–8), and pregnant women (8, 9). The need for such an indirect measure of insulin secretion is dictated by the ability of the liver to extract ~ 50% of secreted insulin on the first pass (10–12). Furthermore, hepatic extraction of insulin is variable and under physiologic control (13–15). Unlike insulin, it appears that C-peptide is extracted by the liver to a negligible degree (16–18), although contradictory data do exist (19, 20). The measurement of C-peptide also permits an assessment of insulin secretion in situations where the presence of insulin antibodies precludes direct interpretation of insulin concentration (2, 5–9). Most recently, the peripheral kinetics of biosynthetic human C-peptide have been studied in normal and diabetic adults (21) allowing a more accurate estimation of insulin secretion.

The use of proinsulin as a therapeutic agent in diabetes during pregnancy and the use of C-peptide measurements as an indication of fetal beta cell function require that these peptides not cross the placenta. Data are not available for proinsulin while evidence for a placental barrier to C-peptide is indirect (6, 8), being largely based on an apparent lack of correlation between maternal and fetal plasma C-peptide concentrations. The use of C-peptide measurements in the study of maternal beta cell function is still limited by the absence of kinetic data in pregnant subjects. Extrapolation from the nonpregnant state assumes the absence of any role for the conceptus in C-peptide metabolism.

The present study was undertaken to directly demonstrate the presence or absence of placental transfer of proinsulin and C-peptide from the maternal to fetal circulation. A bolus-constant infusion to the mother of ^{125}I-human biosynthetic proinsulin (^{125}I-PI)[1] or ^{125}I-tyrosylated-biosynthetic human C-peptide (^{125}I-tyr-CP) was utilized, as has previously been done to demonstrate the placental barrier to insulin (22), growth hormone (23), and glucagon (24).

Methods

Animal studies. Pregnant rhesus monkeys (*Macaca mulatta*) at 140–154 d of gestation (term being 165 d) were supplied and maintained by the New England Regional Primate Research Center, as were nonpregnant monkeys. After an overnight fast, catheters were placed under ketamine anesthesia in an antecubital vein for infusion and (except where noted) a saphenous vein for blood sampling. Pregnant animals were maintained under halothane inhalation anesthesia for

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1. *Abbreviations used in this paper:* MEM, Eagle's minimal essential medium; MOPS, 3[*N*-morpholino]propanesulfonic acid; ^{125}I-PI, ^{125}I-biosynthetic human proinsulin; ^{125}I-tyr-CP, ^{125}I-tyrosylated-biosynthetic human C-peptide; V_E, elution volume.

the duration of each study. Nonpregnant animals were maintained with ketamine anesthesia.

Animals received 20–430 μCi of ^{125}I-PI or ^{125}I-tyr-CP during a given study. The specific dose used was based on the purpose of a particular study; for example, studies aimed at characterizing metabolites present in the fetal circulation required administration of a higher dose. In each study, the initial bolus consisted of 10–40% of the total dose. The subsequent constant infusion (6–10 ml/h) was delivered by syringe pump (model 355, Sage Instruments, Div. Orion Research, Inc., Cambridge, MA). Infusates consisted of the labeled peptide, 1.0 ml of autologous heparinized plasma (to minimize nonspecific adsorption to surfaces of the syringe and tubing) and normal saline. Final volume was 25 ml. Gel filtration chromatography of ^{125}I-PI and ^{125}I-tyr-CP infusates showed elution of a single peak of radioactivity for each that was identical in elution volume to the positions of the respective labeled peptides (data not shown).

Maternal plasma samples were obtained at 5, 10, 15, 20, and 30 min after completion of the bolus, and every 15 min thereafter until completion of the study. Studies in the pregnant animals were terminated by cesarean section. Before removal of the fetus, amniotic fluid was obtained. The fetus was then exsanguinated in situ via the umbilical vein. Analyses were done on the initial sample of umbilical venous blood so as to minimize the effects of placental anoxia. Fetuses weighed 514±80 g (mean±1 SD).

^{125}I-PI and ^{125}I-tyr-CP were prepared as previously described (25). Following purification by high pressure liquid chromatography, the preparations were lyophilized to remove acetonitrile. The specific activities of the preparations used were 194 μCi/μg for ^{125}I-PI and 535–562 μCi/μg for ^{125}I-tyr-CP.

Analytical methods. All plasma samples were heparinized and immediately centrifuged and separated upon collection. Analyses included a determination of ^{125}I total counts per minute, TCA-precipitable counts and immunoprecipitable counts.

To determine TCA-precipitable counts, 0.1 ml sample was mixed with 0.1 ml 20% TCA, vortexed, and allowed to stand on ice for 10 min. After centrifugation (3,000 *g* for 15 min at 4°C) the supernatants were removed and the pellets counted.

For studies in which ^{125}I-tyr-CP was infused, immunoprecipitable counts were determined with goat antiserum to C-peptide (lot E08-7B2-159-4-G, courtesy of M. Root, Eli Lilly & Co.). This antibody is predominantly aimed at the central to NH_2-terminal portion of C-peptide with the immunoreactive area largely confined to residues within the 33-56 chymotryptic fragment of C-peptide. (The residue number is based on the position in proinsulin [2].) Immunoprecipitation was carried out by the addition of 0.1 ml antiserum at a 1:1,000 dilution to 0.1-ml sample. After incubation at 4°C overnight, separation of bound from unbound label was accomplished by the addition of 0.05 ml bovine gamma globulin (2.5 g/dl) and 1.0 ml 20% polyethylene glycol (PEG). The incubation mixture was allowed to stand for 30 min on ice and was then centrifuged at 3,000 *g* for 15 min at 4°C. After removal of the supernatant, the pellet was washed with 1.0 ml 20% PEG. Following recentrifugation and removal of the supernatant, the pellet was counted. Measurement of nonspecific immunoprecipitation utilized guinea pig antiporcine insulin antiserum (Miles Yeda Ltd., Elkhart, IN) in the place of anti-C-peptide antiserum and was ~ 1% (0–2%) of the total counts. Analysis of ^{125}I-tyr-CP infusates by this method showed > 90% of the counts were specifically immunoprecipitable.

Immunoprecipitation for studies in which ^{125}I-PI was infused utilized the guinea pig antiporcine insulin antiserum in a similar procedure. The nonspecific immunoprecipitation measurement, performed on plasma with added ^{125}I-tyr-CP and without labeled proinsulin, resulted in precipitation of 3% of the total counts per minute.

Gel filtration chromatography was carried out using Fractogel TSK HW-50S (EM Science, Gibbstown, NJ). Samples (0.5 or 1.0 ml) were applied to a 1.6 × 50 cm column equilibrated in 20 mM 3[*N*-morpholino]propanesulfonic acid (MOPS), 150 mM NaCl, 10% glycerol, pH 7.4. The column was eluted at 0.4 ml/min with a pump (model 110A, Beckman Instruments, Inc., Fullerton, CA) and 1.0-ml fractions were collected. Calibration of this column was accomplished using a partial chymotryptic digest of ^{125}I-tyr-CP. Chymotrypsin (Worthington Biochemicals, Freehold, NJ, 48 U/mg) was incubated with 100 μg/ml purified C-peptide with 50,000 cpm ^{125}I-tyr-CP in 20 mM MOPS, 10% glycerol, pH 7.5. Incubation was for 30 min at 27°C with a ratio of chymotrypsin to C-peptide of 1:1,000 (wt/wt). Analysis of the mixture by gel filtration performed as described above (data not shown) revealed six peaks, corresponding to the six chymotryptic, NH_2-terminal peptides predicted by the amino acid sequence of C-peptide. These respective peptides were of 26, 24, 21, 12, 7, and 5 residues plus ^{125}I-tyrosine. A semilogarithmic plot of K_{AV} versus predicted chain length was used to estimate the size of peptides derived from infusion of ^{125}I-tyr-CP in the subsequent animal studies. The peptides that were 12 residues in length or longer were immunoprecipitable in the procedure described above.

Results

Maternal to fetal transfer of ^{125}I-PI. Two pregnant animals were given boluses (4 and 6 μCi) followed by constant infusions of ^{125}I-PI (8 and 6.5 μCi/h, respectively). The infusions were of 4 h duration, resulting in a steady state of at least 2 h duration. At the completion of the infusions the total and immunoprecipitable counts per minute in the maternal plasma for the two animals were: total cpm/ml = 29,650 and 21,740; immunoprecipitable cpm/ml = 13,520 and 12,490, respectively. Analysis of the umibilical venous plasma revealed the absence of any immunoprecipitable label in excess of the nonspecific immunoprecipitation. These results were interpreted as indicating that, like insulin, intact proinsulin does not traverse the placenta.

Maternal to fetal transfer of ^{125}I-tyr-CP and its metabolites. Four pregnant animals were given boluses (4 to 15 μCi) followed by 2 h constant infusions (8 to 45 μCi/h). Two additional animals were given boluses (28 and 15 μCi) followed by 4-h constant infusions (100 and 35 μCi/h, respectively). The bolus plus constant infusion technique achieved a steady state for immunoprecipitable counts in the maternal plasma within 30–45 min of the start of the infusion. Total counts and TCA-precipitable counts rose slightly throughout the infusion period, indicating ongoing metabolism of the ^{125}I-tyr-CP. Umbilical venous blood gas analysis demonstrated bicarbonate concentrations of 19.8 to 22.5 meq/liter, indicating the absence of significant fetal metabolic acidosis.

Umbilical venous plasma samples in all cases contained immunoprecipitable counts above the level of nonspecific immunoprecipitation (Table I). The level of immunoprecipitable counts in the fetal circulation ranged from 1.4 to 5.8% of the immunoprecipitable counts in the maternal circulation (Table I). Comparison of the data from the 2-h vs. the 4-h infusions revealed no apparent differences, indicating that a steady state for immunoprecipitable counts may have been reached by 2 h of infusion.

Gel filtration chromatography of the umibilical venous plasma samples from animals 3–6 revealed that little, if any, of the labeled material was present at the elution volume (V_E) for native C-peptide (38–40 ml). A representative gel filtration chromatogram of umbilical venous plasma is shown in Fig. 1. An indistinct peak beginning at the void volume, designated *a*, eluted earlier than C-peptide and was precipitated by PEG alone with 42–48% efficiency, as well as by the usual immu-

Table I. ^{125}I-Tyrosylated-C-Peptide Infusions: Maternal and Umbilical Venous Plasma Immunoprecipitation Data

Animal no.	Infusion duration	Maternal plasma cpm*	Umbilical venous plasma cpm	Fetal:maternal ratio‡
	h	*% total*	*% total*	*%*
1	2	5,340 (60)	307 (13)	5.8
2	2	7,430 (65)	229 (7)	3.1
3	2	46,210 (62)	880 (5)	1.9
4	4	33,330 (52)	1,180 (5)	3.5
5	4	19,900 (66)	1,130 (8)	5.7
6	2	54,580 (52)	780 (3)	1.4

Maternal data are from samples obtained at the termination of each study.

* cpm, counts per minute per 1 ml. All data are corrected for nonspecific immunoprecipitation done on individual samples.

‡ Fetal:maternal ratio. The ratio of umbilical venous plasma to maternal plasma immunoprecipitable cpm.

noprecipitation procedure (44–56%). This peak may represent the binding of fragments to larger proteins or the aggregation of labeled peptides. Peak *b* eluted at 49–52 ml indicating a size of ~ 25 amino acids plus ^{125}I-tyrosine. Peak *b* material was immunoprecipitated in excess of the control (anti-insulin antiserum) immunoprecipitation. Approximately 50% of the counts in this peak (41–55%) were immunoprecipitable. This can probably be attributed to a decrease in affinity for the C-peptide antiserum owing to partial proteolysis, as would be expected based on the specificity of the C-peptide antiserum. Peak *c*, with an apparent size of ~ 12 residues plus ^{125}I-tyrosine, was not immunoprecipitable. Peak *d* eluted at the total column volume. The percentage of total counts recovered from each column present in each peak is as follows (n = 4): *a:* 5–12%; *b:* 8–27%; *c:* 57–63%, and *d:* 8–13%. These results indicate that the immunoreactive species present in the umbilical venous plasma was not intact ^{125}I-tyr-CP, but was a partially proteolyzed, NH_2-terminal (and, hence, still labeled) fragment that retained an immunoreactive site.

Analysis of amniotic fluid revealed the presence of barely detectable levels of both TCA-precipitable and immunoprecipitable ^{125}I. The low level of radioactivity prevented further analysis by gel filtration.

Metabolites of ^{125}I-tyr-CP in the maternal circulation and comparison to the nonpregnant state. To investigate the metabolism of ^{125}I-tyr-CP by the mother, the final maternal sample from one 2-h (Fig. 2 *A*) and one 4-h infusion were analyzed by gel filtration chromatography. The results were similar, with the predominant immunoreactive species (> 85% immunoprecipitable) eluting at the expected position for native C-peptide. A second peak that was ~ 50% immunoprecipitable eluted in the position (V_E = 47–50 ml) of peak *b* of the umbilical venous plasma chromatograms. Gel filtration chromatography of a sample from a nonpregnant animal (No. 4, 10 wk postpartum; Fig. 2 *C*) was similar to the results obtained for the maternal mixed venous or arterial samples. Immunoprecipitation of ^{125}I-labeled material eluting near 50 ml resulted in recovery of 40–50% of the total cpm, as was the case for material from the umbilical venous plasma chromatogram with similar V_E.

The maternal sample for the analysis depicted in Fig. 2 *A* was actually obtained through a femoral artery catheter. A second sample obtained simultaneously at cesarean section from a uterine vein was subjected to gel filtration chromatography (Fig. 2 *B*). Comparison between the chromatograms of the arterial and uterine vein samples showed a relative increase in the peak at V_E = 50 ml compared with the peak that eluted at the volume expected for C-peptide. Further analysis of these samples revealed both a relative and absolute decrease in immunoprecipitable counts across the placenta. The data are as follows: arterial plasma, total cpm/0.1 ml = 10,590, immunoprecipitable cpm/0.1 ml = 5,760 (54% of total); uterine venous plasma – total cpm/0.1 ml = 9,506, immunoprecipitable cpm/0.1 ml = 3,466 (36% of total). These data indicate that ^{125}I-tyr-CP was metabolized to nonimmunoprecipitable forms and/or removed from the circulation in traversing the uterus.

The rate of metabolism of ^{125}I-tyr-CP was examined indirectly by comparing the percentage of total cpm that was precipitated by C-peptide antiserum in the 2-h samples from six pregnant versus four nonpregnant animals (Fig. 3), three of which were postpartum animals (2–4 mo) that had been previously studied. These results indicated that after a 2-h infusion, a higher percentage of the ^{125}I-tyr-CP was degraded to nonimmunoreactive forms in pregnant animals than in the nonpregnant animals (unpaired t test with all subjects, $P < 0.005$).

In vitro metabolism of ^{125}I-tyr-CP by trophoblastic and hepatic cells in culture. The above data led us to hypothesize that limited proteolysis of ^{125}I-tyr-CP occurred in the placenta and resulted in accumulation of immunoreactive fragments in the fetal circulation. To confirm that placental tissue might have the capacity for degrading ^{125}I-tyr-CP to the observed immunoreactive, labeled fragment recovered from plasma on gel filtration, incubation with trophoblastic cells (human choriocarcinoma, JEG, American Type Culture Collection) was carried out. Since in vivo metabolism of C-peptide by the liver may occur (19, 20), parallel incubation with human hepatoma cells (HepG2, American Type Culture Collection) was also performed.

Cells were cultured in Eagle's minimal essential medium (MEM) with 10% fetal bovine serum and grown to near confluence in 1.5-cm wells. After the cells were washed with unlabeled incubation medium (MEM, 10 mM Hepes, 0.1 mg/ml purified beef heart cytochrome *c*, pH 7.3), 0.5 ml incubation medium containing 15,000 cpm ^{125}I-tyr-CP and unlabeled C-peptide (0–2.5 ng) was added to each well. At 2 h, the medium from each well was recovered for analysis by gel filtration chro-

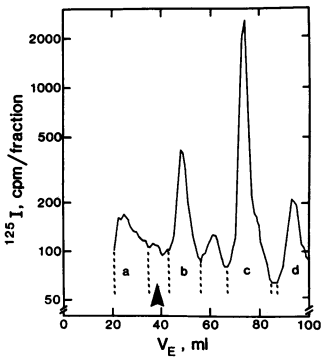

Figure 1. Gel filtration chromatography of 1.0 ml umbilical venous plasma from animal 3. Immunoprecipitation was carried out on four to six peak tubes from peaks *a–d*. The limits used to determine the percentage of total radioactivity per peak are shown by the broken lines. The position at which ^{125}I-tyr-CP elutes is marked by the arrow.

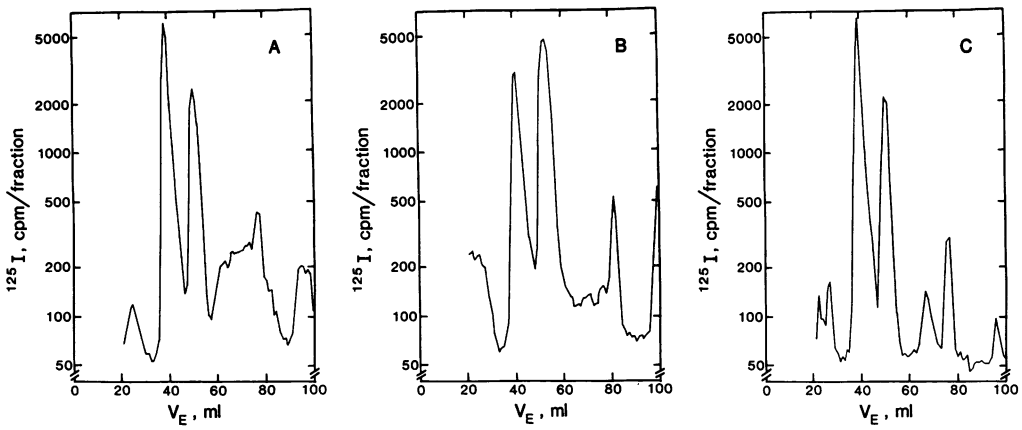

Figure 2. Gel filtration chromatography of 0.5 ml of maternal arterial plasma (*A*), maternal uterine vein plasma (*B*), and venous plasma from a nonpregnant animal (*C*). The predominant peak of radioactivity at V_E = 38–40 ml corresponds to the position of ^{125}I-tyr-CP. The peak at V_E = 48–52 ml corresponds to the position of peak *b* in Fig. 2.

matography. Medium containing ^{125}I-tyr-CP was incubated in wells without cells as a control.

Gel filtration chromatography of medium incubated with trophoblastic cells (Fig. 4 *A*) revealed the presence of multiple peaks of labeled material. The predominant peaks eluted at (V_E = 39 ml) or just after (V_E = 42 ml) the position of intact C-peptide. A peak coinciding with the location of peak *b* from the umbilical venous plasma chromatogram (V_E = 50–52 ml) was also present. The addition of 2.5 ng unlabeled C-peptide did not alter the profile. Gel filtration chromatography of medium incubated with hepatoma cells (Fig. 4 *B*) was similar to that obtained with the trophoblastic cells. The medium incubated without cells (data not shown) exhibited a large peak at V_E = 38–40 ml accounting for > 95% of the radioactivity eluted from the column.

Discussion

The data from the present study are relevant to the therapeutic administration of proinsulin to pregnant diabetics and the application of C-peptide concentration as an indicator of fetal or maternal insulin secretion and metabolism. First, we have demonstrated that the placenta presents a barrier to proinsulin as it does for insulin. With respect to C-peptide, the barrier is not absolute. It appears that immunoreactive fragments of ^{125}I-tyr-CP do cross the placenta, although the concentration in fetal plasma relative to the maternal plasma was low. It seems unlikely that maternal-to-fetal transfer of immunoreactive C-peptide fragments would be sufficient to alter interpretation of fetal C-peptide concentrations. The possibility of fetal-to-maternal transfer, however, is not examined in the present study and must be considered potentially important.

Our data demonstrate that ^{125}I-tyr-CP is metabolized in pregnant and nonpregnant animals, resulting in heterogeneity of circulating immunoreactive, labeled peptides. This finding is consistent with the earlier finding that C-peptide immunoreactivity in the circulation of adult humans is, indeed, heterogeneous (26). Furthermore, it appears that the rate of metabolism of ^{125}I-tyr-CP was increased by pregnancy. We also conclude that the placenta is capable of the extraction and metabolism of C-peptide. A gradient across the uterus for immunoreactive material was directly demonstrated. Gel filtration chromatography demonstrated that this was associated with a decrease in size of the labeled peptides with an increase in lower-molecular weight species relative to the intact label. The in vitro study confirmed that placental cells have the potential for metabolism of ^{125}I-tyr-CP. Of note, hepatoma cells in culture metabolized ^{125}I-tyr-CP in a manner similar to placental cells. This indicates that in vivo metabolism of C-peptide by the liver (19, 20) may occur, thereby accounting, at least in part, for the heterogeneity of immunoreactive C-peptide seen in the nonpregnant monkey.

A limitation in interpreting the data from the C-peptide studies is inherent in the use of ^{125}I-tyr-CP. This labeled moiety

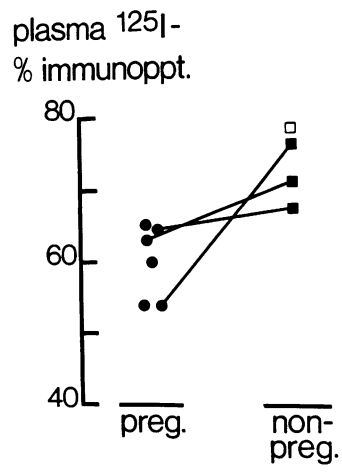

Figure 3. Comparison of the metabolism of ^{125}I-tyr-CP in pregnant vs. nonpregnant animals. The percentage of total maternal plasma cpm at 2 h in each study that was precipitated by C-peptide antiserum is shown. Three animals were studied 2–4 mo postpartum (*closed squares*). One nonpregnant animal was also studied (*open square*).

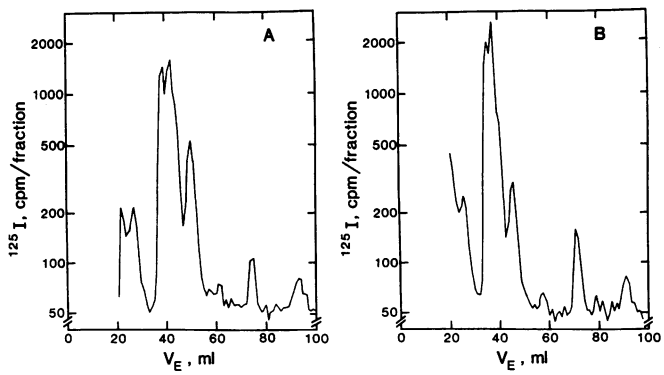

Figure 4. Gel filtration chromatography of incubation medium containing ^{125}I-tyr-CP that was incubated with trophoblastic cells (*A*) or hepatoma cells (*B*) in culture.

permits detection of fragments that retain the N-terminus only. For each fragment detected in the present study, there is at least one and probably multiple fragments that go undetected. Immunoreactive fragments not possessing the iodinated site are not detected by this approach although they may be important to the effect of C-peptide metabolism on the interpretation of C-peptide concentration measurements. The alteration in the primary structure that occurs with tyrosylation is probably less important. Since the metabolism of C-peptide has not been shown to involve an interaction with high-affinity binding sites, and since its metabolism is independent of its concentration (27, 28), it seems unlikely that tyrosylation and iodination would alter its metabolism by nonspecific endoproteases.

The mechanism by which proinsulin and C-peptide might be metabolized by the placenta may be considered in light of the available studies on placental protein metabolism and transport. This has been best studied in the yolk sac placentas of rabbits, rats, and guinea pigs. A hypothesis first put forth by Brambell (29) proposed a nonselective uptake into yolk sac vesicles. After uptake, specific receptors, such as those for the Fc portion of IgG, protected certain proteins from digestion in lysosomes, while proteins without specific receptors were degraded. Hemmings and Williams (30), who studied placental transport of ^{125}I-tyr-IgG, also proposed that proteins enter rabbit yolk sac endodermal cells by nonselective pinocytosis, and that the presence of a specific receptor for a given protein would result in transportation rather than degradation. Although carrier mediated transport has not been directly demonstrated in human (chorioallantoic) placentas, IgG and, more specifically, Fc receptors have been demonstrated in human placental membranes (31, 32). Regarding plasma proteins for which there appear to be no high-affinity placental receptors (e.g., albumin, fibrinogen), transfer to the fetal circulation may be by diffusion and influenced by size (33). This is likely to be the category that includes C-peptide for which high-affinity receptors have not been described. This is in contrast to proinsulin, which binds to insulin receptors and, like insulin, can be expected to be degraded through a receptor-mediated mechanism.

The metabolism of C-peptide by the placenta has implications for interpretation of C-peptide concentrations. Since there is a difference between pregnant and nonpregnant animals in the percentage of administered ^{125}I-tyr-CP metabolized to nonimmunoreactive forms, extrapolation from the nonpregnant to the pregnant state probably is not valid. Furthermore, the effect of the placenta would presumably be changing throughout gestation. Use of C-peptide measurements to estimate the hepatic extraction of insulin and insulin secretion rate in pregnant subjects must take this into account. Application to estimates of fetal insulin secretion or fetal hepatic extraction must similarly be considered in light of the possible metabolism of fetal C-peptide by the placenta. The apparent ability of the placenta to extract and metabolize C-peptide must therefore be added to the list of variables (20) that must be considered in the interpretation of peripheral C-peptide concentrations.

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