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Gonadotropin releasing hormone in first trimester human placenta: Isolation, partial characterisation and *in vitro* biosynthesis

N. MATHIALAGAN and A. JAGANNADHA RAO
Department of Biochemistry, Indian Institute of Science, Bangalore 560 012. India

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Abstract. Using a specific radioimmunoassay for gonadotropin releasing hormone, the presence of gonadotropin releasing hormone like material in the first trimester human placenta has been demonstrated. The material has been partially characterized using carboxy methyl cellulose chromatography, high pressure gel permeation chromatography and reverse phase C18 high pressure liquid chromatographic analysis. Analysis for bioactivity revealed that placental gonadotropin releasing hormone is much more active than synthetic gonadotropin releasing hormone in *in vitro* rat pituitary lutinising hormone release assay. *In vitro* biosynthetic studies using labelled precursors and immunoaffinity chromatography indicated that first trimester human placenta synthesizes gonadotropin releasing hormone like material.

Keywords. Placenta; gonadotropin releasing hormone; receptor assay; biosynthesis; immunoaffinity.

Introduction

The presence of extra hypothalamic gonadotropin releasing hormone (GnRH) like material has been reported in placenta (Gibbons *et al.,* 1975; Khodr *et al.,* 1980; Nowak *et al.,* 1984), testes (Hedger *et al.,* 1985), ovary (Aten *et al.,* 1986) and adrenal cortex (Gautron *et al.,* 1981). Though the precise role of the extra hypothalamic GnRH is not well established, it has been suggested that this exerts its action locally, as an autocrine system (Hsuesh *et al.,* 1982). Thus several studies carriedout using human term placenta have suggested that placental GnRH has an important rolein the regulation of chorionic gonadotropin (CG) secretion by placenta (Khodr *et al.,*1978; Siler–Khodr *et al.,* 1986; Rao *et al.,* 1984; Das *et al.* , 1983; Malik *et al.,* 1986). However, it is known that human term placenta is minimally active as far as CG production is concerned and placental CG production is maximal during the first 6–8 weeks of pregnancy. Thus studies using placenta from early stages can be more meaningful and recently we have demonstrated that GnRH exerts a differential effect (Rao *et al.,* 1985) on CG production during short term incubation of first trimester human placental minces. This observation as well

Abbreviations used: GnRH, Gonadotropin releasing hormone; CG, chorionic gonadotropin; IgG, immunoglobulin G; CM cellulose, carboxy methyl cellulose; BSA, bovine serum albumin; EMEM, Eagle's minimum essential medium; EBSS. Earle's balanced salt solution; HP-GPC. high pressure gel permeation chromotography; HPLC, higher pressure liquid chromatography; LH, lutinising hormone; HCG, human chorionic gonadotropin; CNBr, cyanogen bromide; RP C 18, reverse phase C 18; RIA, radioimmunoassay; GAP, GnRH associated peptide.

as the fact that so far only term human placental GnRH has been well characterized (Tan *et al.,* 1982; Seeberg *et al.,* 1984) prompted us to look for the presence and biosynthesis of GnRH like material in the first trimester human placenta.

Materials and methods

Synthetic GnRH was obtained as a gift from Ayerst and Wyeth laboratories, New York, USA. The source of various analogues used in the study, the development and validation of radioimmunoassay (RIA) of GnRH used in following the purification of placental GnRH have been described in the preceeding paper (Mathialagan *et al.,* 1986). Antiserum to GnRH raised in goat, used in the immunoaffinity column, was generously provided by Dr. H. M. Fraser, MRC Reproductive Biology Unit, Edinburgh, UK. Coupling of anti GnRH immunoglobulin G was done according to the procedure given by the suppliers (Pharmacia, Sweden). Carboxy methyl cellulose (CM cellulose), cyanogen bromide activated sepharose 4B, cycloheximide and bovine serum albumin (BSA) were obtained from Sigma Chemicals Co., St. Louis, Missouri, USA. [^{3}H]-Leucine (specific activity 100 Ci/mmol) and [^{125}I]-sodium iodide were obtained from Amersham International, England. [^{14}C]-Chlorella hydrolysate (specific activity 20 mCi/m atom C) was supplied by Bhabha Atomic Research Centre, Bombay. Eagle's minimum essential medium (EMEM) was purchased from Gibco Laboratories, New York, USA. Earle's balanced salt solution (EBSS) was obtained from Hi Media, Bombay. Highly purified hCG (CR-123, 12,000 IU/mg) used for iodination was generously provided by NIAMDD, USA.All other chemicals used in the study were of analytical grade procured from local sources.

Collection of human placenta

Placenta was collected from cases of medical termination of pregnancy (6-10 weeks) from local hospital. The placenta along with blood was collected in EBSS and quickly transported on ice to laboratory. After decanting the blood, placenta was extensively washed with EBSS.

Extraction and fractionation of GnRH

Placental villi were minced and processed essentially according to the procedure of Guillemin *et al.* (1963). The minces were homogenized with 2 N acetic acid and the homogenate was spun at 10,000 *g* for 20 min at 4°C. The clear supernatant was lyophilized (fraction A) and stored at – 20°C until further processing. Fraction A was reconstituted (5 mg protein/ml) in distilled water and loaded on CM cellulose column (10 × 1·5 cm) equilibrated with 0·01 M ammonium acetate, pH 4·5. Flow rate was maintained at 8 ml/h to ensure complete retention of GnRH like material on to the column. The column was washed extensively with equilibration buffer until there was no detectable absorbance at 280 nm, in flow through fractions Elution was carried out with 0·1 M ammonium acetate, pH 7·5 and 2·0 ml fractions were collected. The optical density was monitored at 280 nm and immunoreactivity in each fraction was assayed by GnRH specific RIA. The fractions which showed

immunoreactivity and eluted at a position corresponding to standard GnRH were pooled and lyophilized (fraction B).

High pressure liquid chromatographic analysis

High pressure gel permeation chromatography (HP-GPC) of fraction B was carried out on a TSK G2000 SW ultrapac column (LKB, 7·5 × 600 mm, Fr. Range 0·5-60 K). The chromotography system was equipped with two pumps, a solvent programmer attached to UV absorbance detector set at 226 and 280 nm. The equilibration buffer was 0·05 M phosphate buffer pH 6·8 containing 0·1 M NaCl. The column was calibrated with standard molecular weight markers (GnRH 1·1 K, ACTH 4·5 K, ß-LPH 9·8 K, Cyt. C 12·4 K, RNase 13·7 K, trypsin inhibitor 20 K, ovalbumin 44 K and BSA 65 K). Synthetic GnRH eluted at an elution volume of 35 ml and fractions collected were monitored at 226 and 280 nm and also for immunoreactivity by GnRH specific RIA.

Fraction B was also subjected to reverse phase high pressure liquid chromatography (HPLC) analysis using C18 column according to the procedure described earlier (Nikolics *et al.,* 1983). The isocratic system used in the analysis consisted of 58% (v/v) ammonium acetate. 0·05 M and 42% (v/v) methanol (pH 4·2). Synthetic GnRH had a retention time of 13 min in this system. Gradient elution was carried out using the following solvents: (A) 90% (v/v) methanol and 10% (v/v) phosphate buffer; 0·1 M, pH 2·0, (B) 10% (v/v) methanol and 90% (v/v) phosphate buffer, 0·1 M, pH 2·0. In the gradient elution synthetic GnRH eluted with a retention time of 22·5 min.

Bioactivity of placental GnRH

The biological activity of placental GnRH was determined using the *in vitro* rat pituitary lutinising hormone (LH) release bioassay as described by Sairam *et al.* (1981). Pituitaries from 20 day old Wistar male rats were incubated at 37°C with varying concentrations of GnRH (1-10 ng/ml) in EMEM for 4 h under 95% O_2 and 5% CO_2. Medium was separated by centrifugation at 3000 *g* for 20 min at 4°C and analysed for LH by human chorionic gonadotropin (hCG) radioreceptor assay as described by Sairam *et al.* (1981). Iodination of highly purified hCG was done according to the method of Greenwood *et al.* (1963) and the specific activity ranged from 40–50 μCi/μg. The assay range was from 0·5-50 ng/tube and minimum detectable quantity of rat LH equivalent was 1 ng/tube. The inter and intra assay variation of the assay were 12·4% and 9·5%, respectively.

In vitro biosynthesis of GnRH

Placental minces were incubated with 100 μCi of [^{14}C]-chlorella protein hydrolysate or [^{3}H]-leucine in the presence of 0·01% bacitracin in 1 ml of EBSS for 2 h at 37°C under 95% O_2 and 5% CO_2 in a Dubnoff shaker. Where appropriate, cycloheximide was used at a concentration of 500 μM. After the incubation, 5 volumes of 2 N acetic acid were added to the incubation flask and the minces were homogenized using ground glass homogenizer. The homogenate was centrifuged in cold at 10,000 *g* and the clear supernatant was lyophilized and fractionated on CM cellulose column as described earlier. The radioactive fractions which eluted at a

position corresponding to that of synthetic GnRH were loaded on a column of anti GnRH IgG coupled to cyanogen bromide (CNBr) activated sepharose. Equilibration and washing of the immunoaffinity column was performed using 0·01 M phosphate buffer pH 7·4 containing 0·9% (v/v) NaCl. GnRH was eluted from the column using 1 M acetic acid (Akanxuma *et al.,* 1974). Fractions were monitored for radioactivity and absorption at 280 nm. In the experiment where [^{3}H] -leucine was used as a precursor approximately 25,000 cpm and in case of [^{14}C]-chlorella hydrolysate, approximately 4,00,000 cpm were loaded on the affinity column. In both cases recovery of the radioactivity was 90–95% with about 3% of the radioactivity being specifically eluted with 1 M acetic acid.

Results

Detection of GnRH like material in placenta by RIA

In order to ascertain the presence of GnRH like material, 2 N acetic acid extract (fraction A) of the first trimester human placenta was assayed in the GnRH specific RIA. It can be seen from the data presented in figure 1 that fraction A gave a dose dependent displacement. In acetic acid extraction and CM cellulose chromatography of homogenate caused significant enrichment of placental GnRH like activity (table 1).

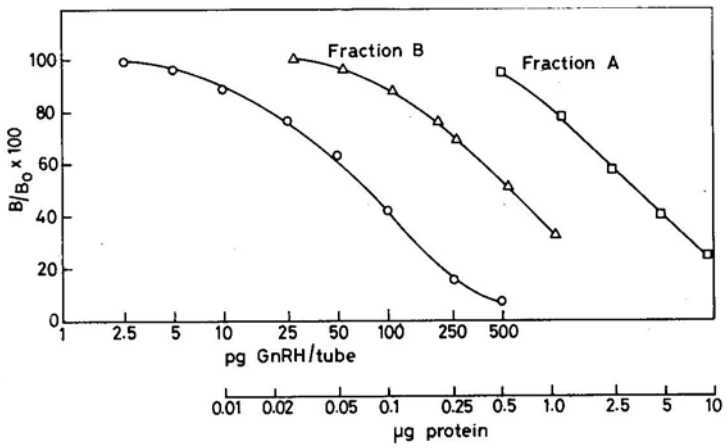

Figure 1. Standard inhibition plot for GnRH; parallel displacement by fraction A and B.

CM cellulose chromatography

The specific elution profile of synthetic GnRH on CM cellulose column is shown in figure 2A. This elution profile is characteristic of synthetic GnRH (Guillemin *et al.,* 1963) and is highly reproducible under standardised conditions. It can be seen that following fractionation on CM cellulose column, fraction A gave a peak whose elution pattern was exactly similar to that of standard GnRH (fraction B). Analysis of the fractions from this peak for immunoreactivity by GnRH RIA (figures 1 and

Table 1. Purification of placental GnRH.

Fraction	Qty. of immunoreactive GnRH/mg protein	Fold purification
Homogenate	116·6 pg	1
2 N Acetic acid extraction (fraction A)	21·0 ng	180
CM cellulose chromatography (fraction B)	123·0 ng	1054

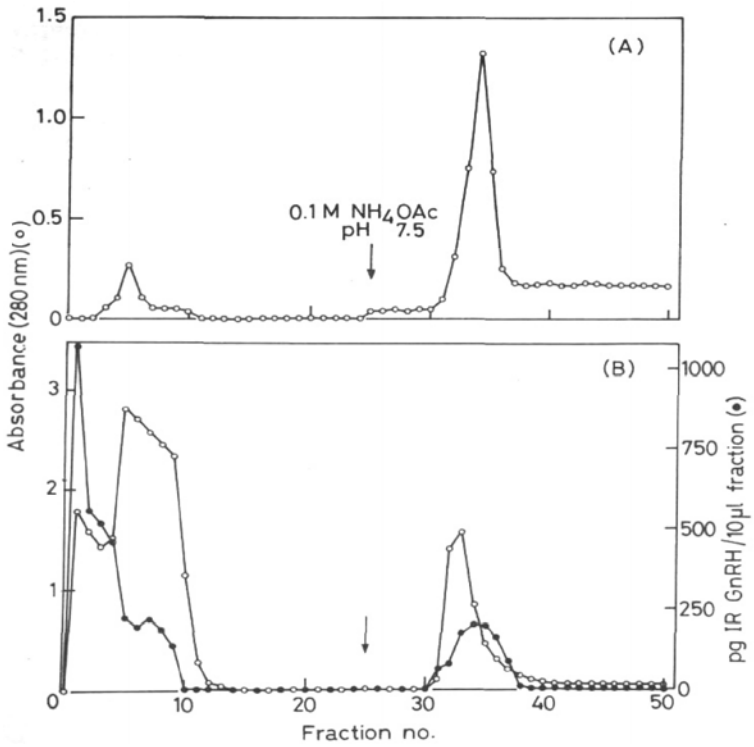

Figure 2. Elution profile on CM cellulose column. The column (1·5×7 cm) was equilibrated with 0·01 M ammonium acetate, pH 4·5. Elution was done with 0·1 M ammonium acetate, pH 7·5. (A). Synthetic GnRH; (B). fraction A.

2) indicated that there is a significant immunoreactivity associated with this peak. It should be pointed out that fraction A gave in addition to the peak corresponding to GnRH, another peak which did not bind to the column with which considerable immunoreactivity is also associated. However, in view of the fact that elution

profile of synthetic GnRH is a very characteristic feature, only fraction B was subjected to HPLC analysis.

HPLC analysis

The profile obtained following analysis of fraction B on HP-GPC is shown in figure 3. It can be seen that the fraction B showed an absorption peak (figure 3B) at an elution volume of 35 ml which corresponded well with the immunoreactivity (figure 3C) as well as standard elution profile of GnRH (figure 3A). As in the case of CM cellulose analysis, a peak of immunoreactivity in the fractions which eluted very early (elution volume less than 8 ml) was noticeable. The results of analysis of fraction B on reverse phase C18 (RP C18) column using isocratic system and gradient elution are shown in figures 4 and 5, respectively. It can be seen that in the isocratic system, a clear peak corresponding exactly to the retention time 13 min of standard GnRH was observed. In the case of gradient elution (figure 5) a peak corresponding to the retention time 22·5 min of standard GnRH was noticed and it can also be seen that the peak heights were concentration dependent. All these results together strongly

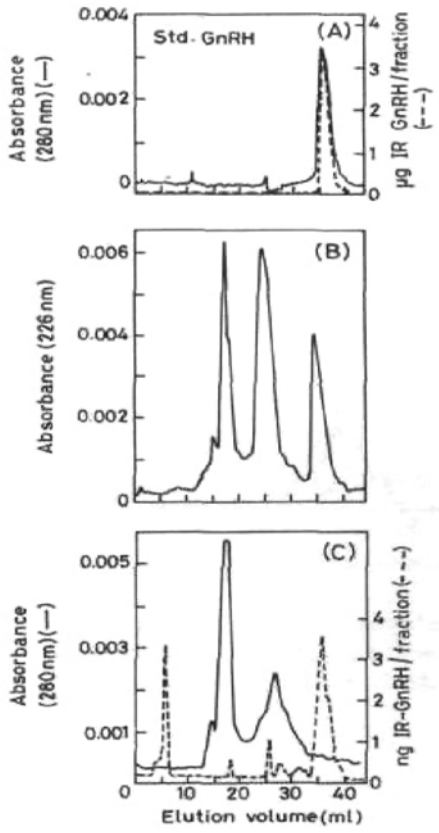

Figure 3. HPGPC analysis of fraction B. See methods for details of column and buffer used. Elution profile of (A) standard GnRH, (B) and (C) fraction B.

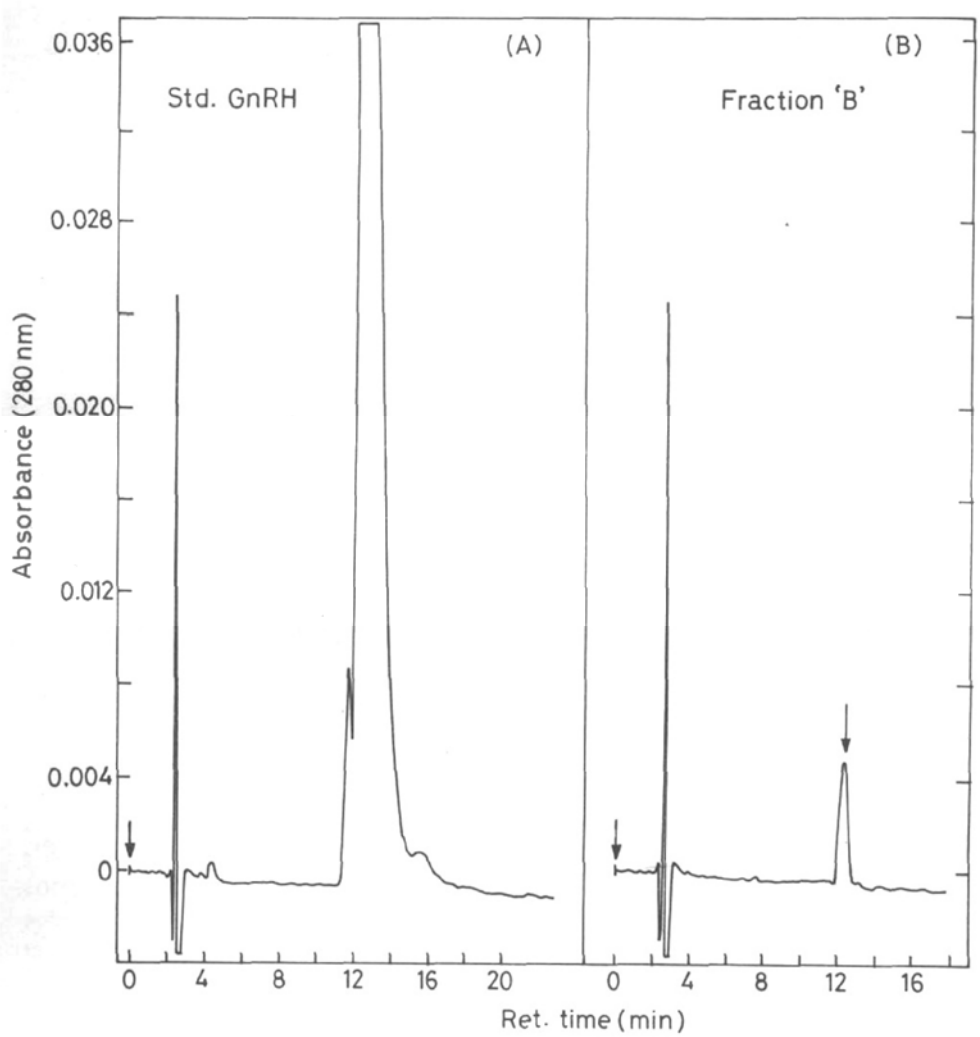

Figure 4. RP C18 HPLC analysis: Isocratic system.

support the conclusion that there is a material which is very similar to hypothalamic GnRH in the first trimester human placenta.

Biological activity of placental GnRH

The results presented in figure 6A show that following addition of 1-10 ng of synthetic GnRH there was a significant, dose dependent increase in the hCG equivalent of rat LH released into the medium, thus validating the radioreceptor assay. The results of the effect of addition of fraction A and fraction B on the release of LH into the medium is shown in figure 6B. It can be seen that both fractions showed significant, dose dependent stimulation. Interestingly both fractions exhibited much higher activity than standard GnRH. The highest quantity of

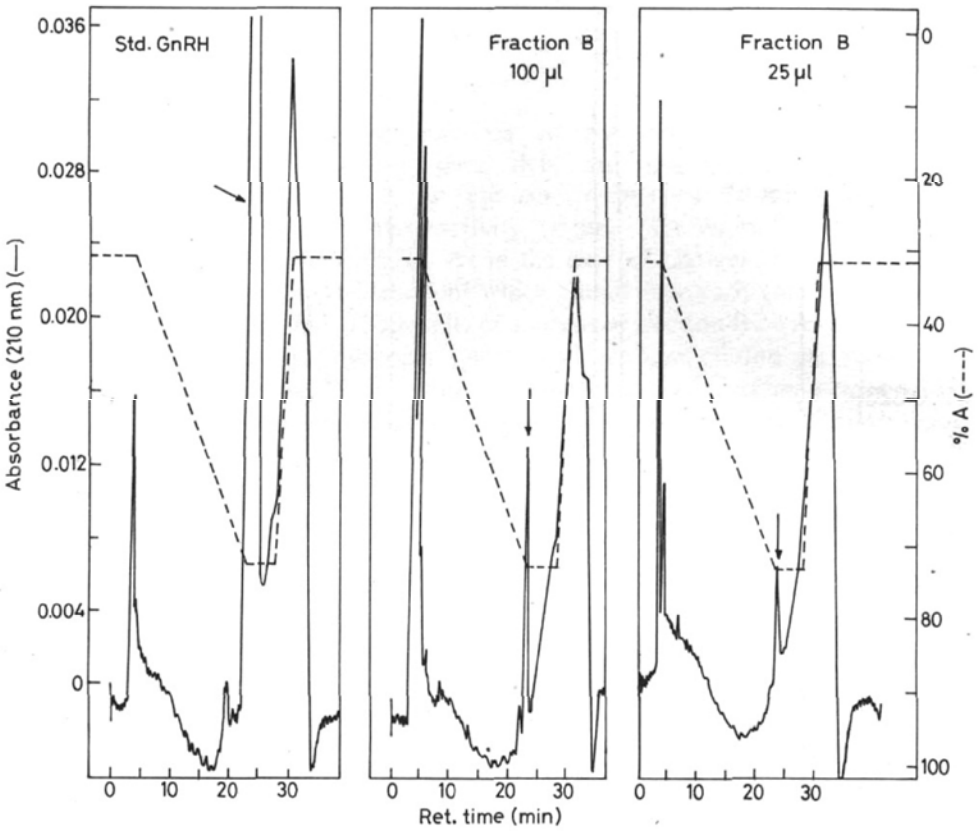

Figure 5. RP C18 HPLC analysis: Gradient elution.

fraction A and B tested corresponded to 0·5 ng and 0·25 ng of immunoreactive GnRH and at these concentrations, they were 3 and 4 fold more active respectively than synthetic GnRH used in the study.

In vitro biosynthesis of placental GnRH

Biosynthesis of placental GnRH was monitored by following the incorporation of [^{3}H]-leucine or [^{14}C]-chlorella hydrolysate into 2 N acetic acid extractable peptides which were fractionated on CM cellulose column. The results of this study are presented in figure 7. It is clear that the incorporation of both [^{14}C] and [^{3}H] radioactivity corresponding to the elution of standard GnRH on CM cellulose column could be observed. Analysis by specific RIA indicate that immunoreactivity is also associated with the radioactivity peak suggesting that the peak represents the newlysynthesized GnRH. Experiments carried out in presence of cycloheximide (500 μm) indicate that the synthesis of GnRH is dependent upon protein synthesis as there is a considerable decrease in the incorporation of labelled precursor into this fraction (figure 8).

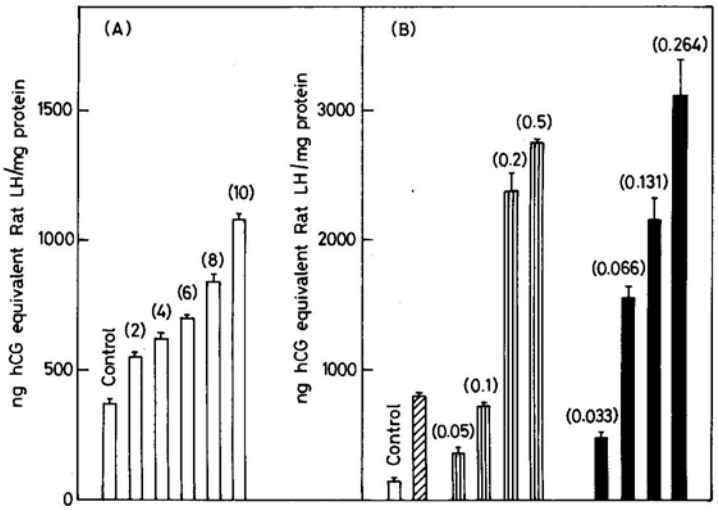

Figure 6. Bioactivity of placental GnRH. (A). Dose response with synthetic GnRH Quantity (ng) of GnRH added per tube indicated in parenthesis. (B) Bioactivity of (1) synthetic GnRH (l0 ng/tube) ▨; (2), fraction A ▥; (3), fraction B ■, Figures in the parenthesis indicate the quantities (ng/tube) of immunoassayable GnRH added. Each point presents Mean ± SEM of triplicate determinations.

To further establish the identity of the radioactive peak corresponding to GnRH. this material was applied to GnRH immunoaffinity column and the results are presented in figure 9. It can be seen that in both experiments where [^{3}H]-leucine or [^{14}C]-chlorella hydrolysate was used as a precursor, a peak of radioactivity which corresponded to the elution profile of standard GnRH was noticeable, though the specific radioactivity recovered in the fraction was only about 3% of the total radioactivity applied to the column.

Discussion

The presence of GnRH like material in extrahypothalamic sources has been reported using immunochemical methods or the conventional isolation procedures or a combination of both (Khodr *et al.,* 1980; Gautron *et al.,* 1981; Tan *et al.,* 1982; Hedger *et al.,* 1985). We have been able to conclusively demonstrate the presence of GnRH like material in human placenta using a combination of immunological, biochemical and biosynthetic approaches. The RIA employed in the study has been validated for its specificity (Mathialagan *et al.,* 1986). As mentioned earlier the elution profile of hypothalamic GnRH on CM cellulose column was characteristic and we have successfully employed this in following the purification of placental GnRH. The purification achieved at various steps is also a clear indication that we have purified a GnRH like material. HPLC analysis provided an additional evidence for the identity of a placental GnRH like peptide. Most importantly the material isolated from placenta had biological activity similar to that of GnRH in the *in vitro* rat pituitary assay. We have also consistently observed that placental

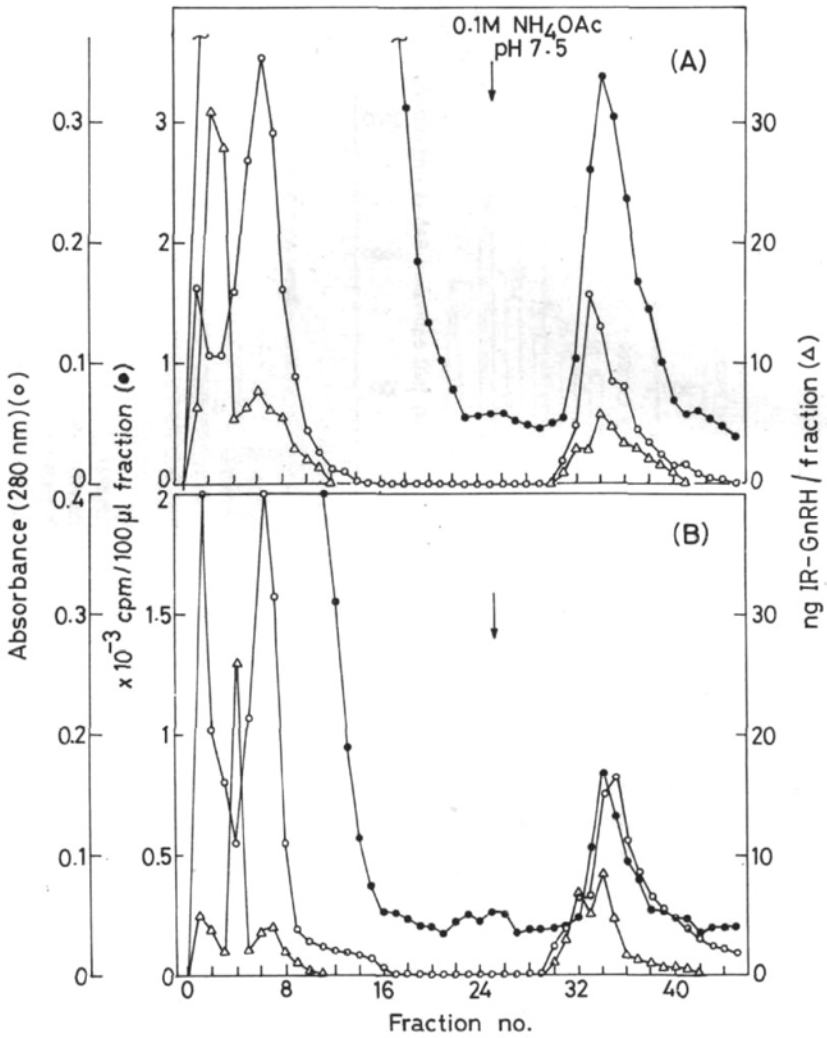

Figure 7. *In vitro* biosynthesis of placental GnRH: Elution profile on CM cellulose column of 2 N acetic acid extractable peptides. (A), [^{14}C]-chlorella hydrolysate; (B), [^{3}H]-leucine.

GnRH like material was found to be more active than synthetic GnRH in the bioassay and interestingly a similar observation has been made by Siler-Khodr *et al.* (1986). However, as the material has not been purified to homogenity, it is possible that the placental material may have in addition to native GnRH other materials which possess GnRH like activities. This conclusion is supported by the fact that HP-GPC analysis reveals multiple immunoreactive peaks in addition to the peak corresponding to standard GnRH (figure 3C). Recently Nowak *et al.* (1984) have suggested that the presence of significant part of GnRH like activity is due to factors other than GnRH in rabbit placenta.

It is known that the proteolysis of GnRH in tissues occurs between Gly^{6}-Leu^{7} bond (Koch *et al.,* 1974) and the half life of GnRH is extremely low (Elkind-Hirsch

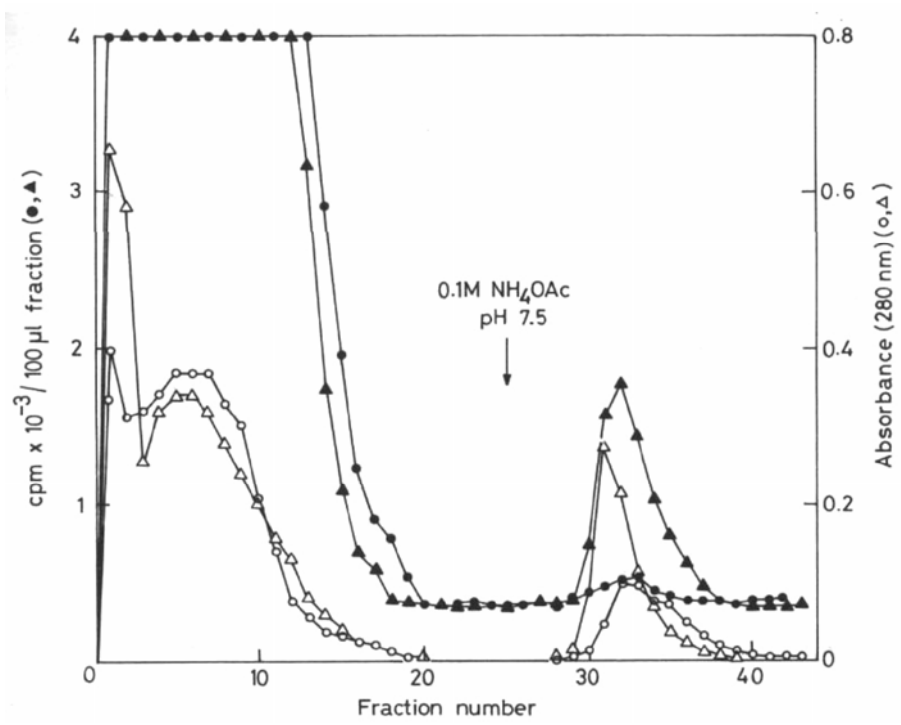

Figure 8. *In vitro* biosynthesis of placental GnRH: Effect of cycloheximide, 500 μM (O, ●).

et al., 1982). Thus use of [^{3}H]-leucine alone as a precursor in *in vitro* biosynthetic studies of GnRH will result in a decreased incorporation of label. In order to eliminate this uncertainty we have employed [^{14}C]-chlorella protein hydrolysate also as a precursor. Studies using immunoaffinity chromatography also support the conclusion that GnRH is biosynthesized in the first trimester human placenta. The immunological identity of the synthesized GnRH is further established by the fact that we have employed a rabbit antiserum to GnRH in RIA and a goat antiserum to GnRH for immunoaffinity chromatography. The foregoing results establish that placental GnRH also has similar immunological and biological characteristics of the hypothalamic counterpart.

During the course of CM cellulose analysis of fraction A we have consistently observed a peak of immunoreactive material in the flow through fractions. At the moment we are not clear as to the identity of this fraction. It may be pertinent to point out that post translational processing of the precursor of GnRH results into two peptide fragments (Nikolics *et al.,* 1985), one of which is a 56 amino acid C-terminal peptide (GnRH-associated peptide, GAP) having a prolactin release inhibiting activity as well as gonadotropin releasing activity in pituitary cell cultures. It may be possible that the immunoreactive CM cellulose flow through fraction represents the GAP peptide. Additional information as to the immunological reactivity of GAP with GnRH antiserum and its chromatographic fractionation will be of help in establishing the identity of the CM cellulose column flow through peptide observed in our studies.

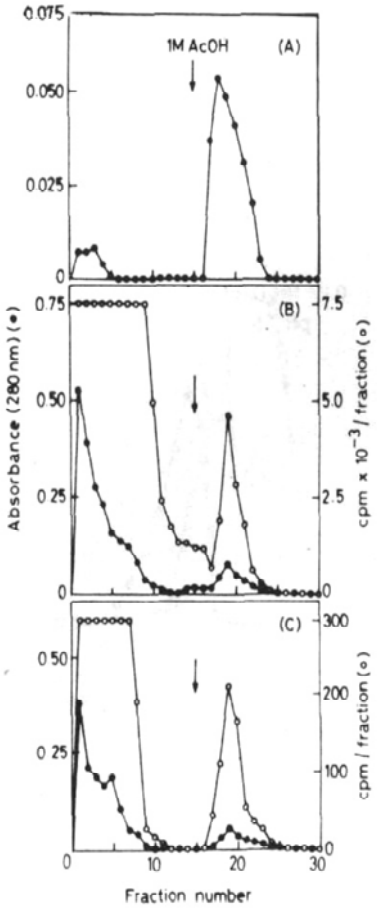

Figure 9. *In vitro* biosynthesis of placental GnRH: Immunoaffinity chromatographic analysis of fraction B. (A), Standard GnRH; (B), [^{14}C]-labelled fraction B; (C), [^{3}H]-labelled fraction B.

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