Journal of Chromatography, 397 (1987) 379–387
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMSYMP. 1180

HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF AMINO ACIDS, PEPTIDES AND PROTEINS

LXXV*. ISOLATION OF ISOHORMONES OF BOVINE THYROTROPIN AND LUTROPIN

PETER G. STANTON and MILTON T. W. HEARN*
Department of Biochemistry, Monash University, Clayton, Victoria 3168 (Australia)

SUMMARY

Purification of bovine thyrotropin and lutropin by conventional soft-gel chromatographic methods is shown to yield microheterogeneous products. High-performance ion-exchange chromatography (HPIEC) can be used to purify these isohormones further for investigations into the structure/function role of glycoprotein microheterogeneity. Hormonal microheterogeneity is shown to be reflected in differences in surface-accessible charged groups, as demonstrated by HPIEC, and differences in net charge, as indicated by isoelectric focusing. Optimal separation of the glycoprotein hormones, based on protein charge, can therefore be achieved by a combination of these two methods.

INTRODUCTION

The pituitary-derived glycoprotein hormones, thyrotropin (TSH), lutropin (LH), and follitropin (FSH), are a family of proteins composed of non-covalently associated α- and β-subunits. Within any one species, the α-subunit is common to all the glycoprotein hormones, whilst hormonal specificity is conferred by the β-subunit[1]. Purification of the glycoprotein hormones to homogeneity has traditionally presented a variety of technical difficulties, due to their structural microheterogeneity, arising from differences in carbohydrate moieties[1,2], charged groups[3], extent of deamidation[4], and extent of sulphation and phosphorylation[1]. Examination of *in vivo* bioactivities or immunological and receptor-binding activities of partially fractionated isoforms of these glycoprotein hormones shows that they are intrinsically different in their biological activities and metabolic clearance rates[5,6], and this variation is reflected in the endocrinological status of the individual[5–7]. Almost all information available on the chemical, biological, and immunological properties of bovine TSH (bTSH) and bovine LH (bLH) originates from preparations that are still not characterised by the physico-chemical methods now available for high-resolution

* For Part LXXIV, see ref. 25.

0021-9673/87/$03.50

protein purification or structural analysis. Early techniques, based on open-column ion-exchange chromatography, have proved time-consuming, or of low resolving power. For example, most commercial or research-grade preparations of LH or TSH, as traditionally isolated by open-column ion-exchange chromatography, are contaminated with other pituitary proteins, due to inadequate resolution.

The extent of microheterogeneity observed for a particular glycoprotein hormone preparation is dependent on the level of resolution of the purification techniques employed. For example, Robertson *et al.*[8] demonstrated that seven different fractions of human LH (hLH), obtained after preparative isoelectric focusing, contained at least twenty discernible species of hLH by bioassay[8]. Similarly, Roos *et al.*[9], and Wide[10] have demonstrated that traditionally isolated human FSH (hFSH) preparations or samples could be resolved by preparative isoelectric focusing or moving-boundary electrophoresis into multiple components with different biological/immunological activity (B/I) ratios.

The purpose of this study was to explore further the purification of the isoforms of conventionally purified bTSH and bLH by application of high-performance anion-exchange chromatographic (HPIEC) techniques. Analysis of the derived products by a combination of sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and analytical isoelectric focusing confirms that HPIEC on Mono Q supports provides a rapid and efficient technique for the fractionation of these proteins.

MATERIALS AND METHODS

Purification of bovine TSH

Fresh bovine pituitaries were collected from the local abattoir and frozen at −20°C until processed. Glycoprotein hormones were extracted with ammonium sulphate and concentrated by batch adsorption on Bio-Rex 70, as described by Reichert[11]. Protease inhibitors (benzamidine, phenylmethylsulphonyl fluoride, ethylenediaminetetraacetic acid) were included during the extraction. The crude extract obtained was chromatographed on Sephadex G-100 (100 cm × 5 cm I.D.) in 100 mM ammonium bicarbonate, and the 20–40 kilodalton molecular mass range, containing the hormonal activity, was concentrated by lyophilisation. A concanavalin A-Sepharose 4B column (34 cm × 2.6 cm I.D.) was pre-equilibrated in 10 mM Tris–HCl, 300 mM sodium chloride, 1 mM magnesium chloride 1 mM manganese chloride, 1 mM calcium chloride (pH 7.40) prior to loading the active fraction. Non-bound proteins were eluted with the equilibration buffer, and the glycoproteins were eluted with the equilibration buffer containing 200 mM α-methyl-D-mannoside. The column was washed with equilibration buffer containing 1 M sodium chloride and regenerated. Protein fractions were dialysed against 50 mM ammonium bicarbonate and lyophilised. The glycoproteins (containing hormonal activity) were then loaded onto a DEAE-Sepharose (Fast-Flow) column (35 cm × 1.6 cm I.D.), pre-equilibrated in buffer A (5 mM sodium glycinate, pH 9.60) and eluted stepwise with the following buffers, B (50 mM sodium glycinate, pH 9.60), C (200 mM sodium glycinate, pH 9.60), D (200 mM sodium glycinate, 100 mM sodium chloride, pH 9.60), E (200 mM sodium glycinate, 400 mM sodium chloride, pH 9.60), F (200 mM sodium glycinate, 1 M sodium chloride, pH 9.60). Recovered fractions were dialysed against 100 mM

ammonium bicarbonate and lyophilised, prior to final gel chromatography on Sephadex G-100 (Superfine) (100 cm × 1.5 cm) in 100 m*M* ammonium bicarbonate. Fractions corresponding to a molecular mass of 28 kilodalton were pooled and lyophilised. All chromatographic steps were carried out at 4°C. Protein recoveries were calculated on the basis of the Bradford dye-binding assay[12] for all steps prior to affinity chromatography, and by amino acid analysis for all later steps. The percent carbohydrate content of purified bTSH and bLH was estimated on the basis of the results of Liao and Pierce[13].

Electrophoretic analysis

SDS-PAGE under non-reducing conditions, was carried out in 12.5% gels with 5% stacking gels by the method of Laemmli[14] and were silver-stained by the Wray procedure[15]. Isoelectric focusing was carried out in 6% gels using Ampholines (LKB, Bromma, Sweden) to generate a broad-range pH gradient. Gels were silver-stained by the method of Kruse *et al.*[16].

High-performance ion-exchange chromatography

HPIEC was carried out with the Pharmacia FPLC system (Uppsala, Sweden), including a Mono Q column (Hr 5/5), equilibrated in 20 m*M* piperazine (pH 9.60) at 20°C. Samples were loaded in the same buffer and eluted 5 min after injection with a linear gradient to 20 m*M* piperazine 300 m*M* sodium chloride (pH 9.60) at a flow-rate of 1 ml/min; gradient time, 34.3 min. Individual fractions were monitored by absorbance at 278 nm, and assayed for hormone activity.

Biological assays

LH activity was monitored with a LH radioreceptor assay, with iodinated human chorionic gonadotropin (hCG) as tracer and the 2nd International Reference Preparation of Pituitary FSH and LH (78/549) (provided by the WHO International Laboratory for Biological Standards and Control, London, U.K.), as described elsewhere[17]. TSH activity was assayed with a radioimmunoassay using rabbit anti-bovine-TSH antiserum (NIADDK-bTSH-I-1) and bTSH (1 I.U./mg) as a standard preparation. Parallelism between logit log–dose transformed response lines was observed between the standard and the samples in each of the above assays.

RESULTS

Fractionation of bovine thyrotropin and lutotropin

The mass yield of bLH and bTSH, obtained as a lyophilised powder from the bovine pituitaries by the ammonium sulphate and Bio-Rex 70 extraction procedures, was consistently *ca.* 2.7 g/kg wet weight starting material ($n = 23$). This was reduced to 1.0 g/kg, following the initial gel chromatography. A typical result for the chromatography of the crude material on concanavalin A Sepharose 4B is shown in Fig. 1. More than 80% of the loaded material was retained on the column and eluted with the α-methyl-D-mannoside. This is in contrast to the yield of glycosylated protein from bovine pituitaties of less than 30% found by Bloomfield *et al.*[18]. This dichotomy probably reflects the additional Bio-Rex 70 and Sephadex G-100 purification steps used in our procedure. The lectin affinity purified material gave a TSH specific activity

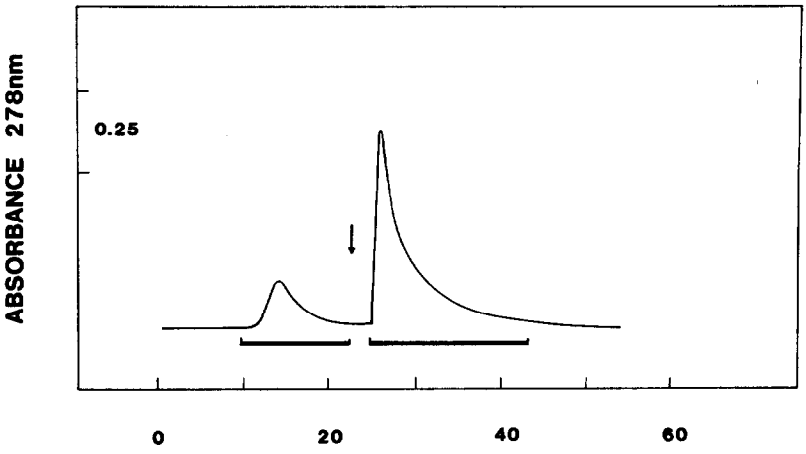

Fig. 1. Affinity chromatography on concanavalin A Sepharose 4B. Column dimensions, 34 cm × 2.6 cm I.D. Approximately 720 mg pituitary extract was loaded and eluted at a flow-rate of 0.7 ml/min, as described in Materials and methods. Buffer changes were made, as indicated by the arrows. Fractions were pooled, as indicated by the bars, and dialysed against ammonium bicarbonate, prior to lyophilisation.

TABLE I

RECOVERIES OF THYROTROPIN AND LUTROPIN

Fraction/purification step	*Specific activity (I.U./mg)*		*Recovery of total activity (%)*	
	LH	*TSH*	*LH*	*TSH*
Con A Sepharose glycoprotein fraction	5370	5.57	100	100
DEAE Sepharose				
Pool I	7585	0	2.1	0
Pool II	10863	0.20	57.2	1.0
Pool III	7702	0.31	16.2	0.6
Pool IV	4230	5.11	15.5	18.1
Pool V	645	14.3	0.9	20.0
Pool VI	214	16.3	0.7	53.1
Sephadex G-100S				
Pool I	ND*	–	–	–
Pool II	7896	0.19	71.3	96
Pool III	ND	–	–	–
Pool IV	9948	6.7	100	82
Pool V	826	19.4	81	88
Pool VIa**	217	16.6	33	30
Pool VIb**	159	27.5	21	32

* ND = not done.

** Individual fractions corresponding to the leading and trailing sections of the active peak were pooled to give VIa and VIb.

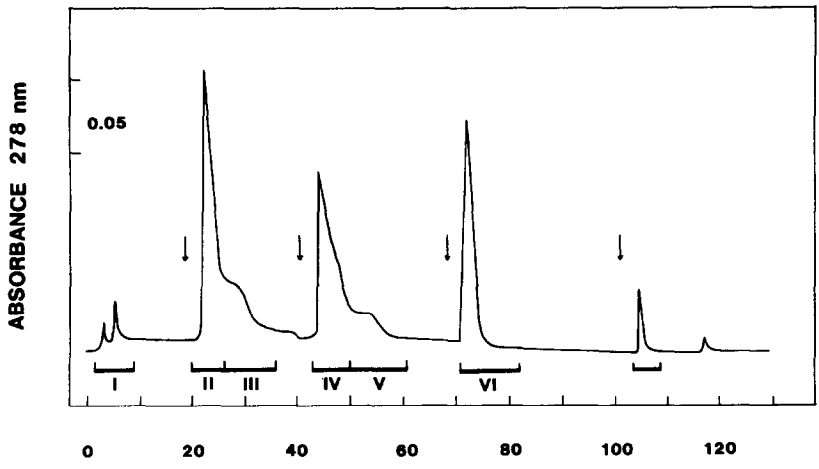

Fig. 2. Anion-exchange chromatography on DEAE Sepharose (Fast-Flow). Column dimensions, 35 cm × 1.6 cm I.D. Approximately 350 mg of the glycoprotein fraction was loaded and eluted stepwise at a flow-rate of 1.6 ml/min, as described in Materials and methods. Buffer changes were made as indicated by the arrows. Fractions were pooled as shown.

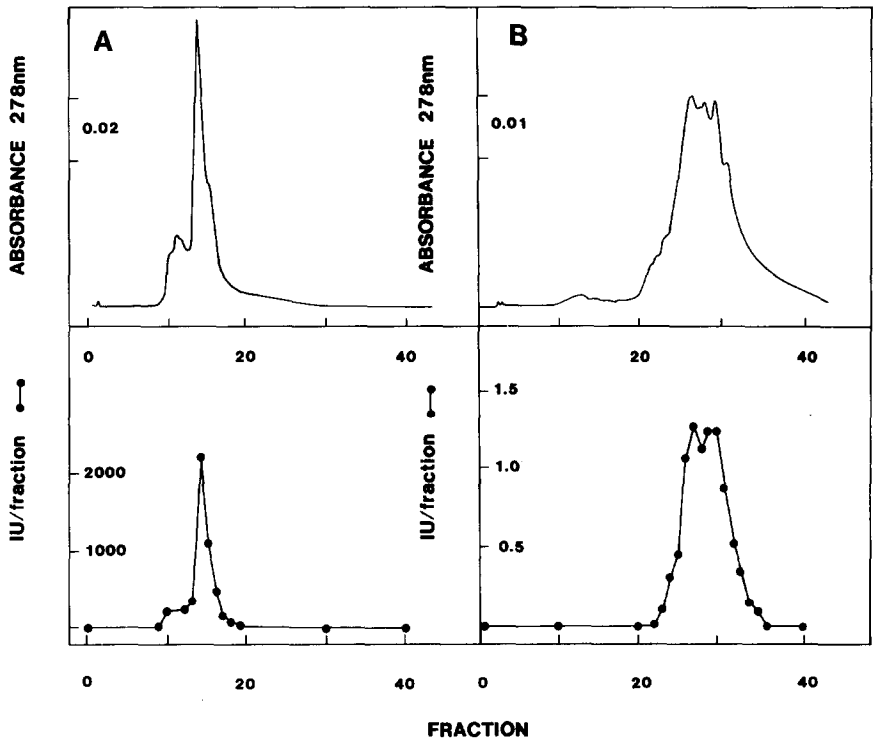

Fig. 3. HPIEC of bLH and bTSH. Approximately 750 μg each protein was loaded and eluted from a Mono Q column as described in Materials and methods. Fractions of 1.0 ml each were collected and stored at −20°C prior to assay. (A) Protein sample, = bLH, pool II from Sephadex G-100S column (Table I); (B) protein sample, = bTSH, pool VIb from Sephadex G-100S column (Table I).

of 5.6 I.U./mg and a LH specific activity of 5370 I.U./mg (Table I), with a yield of protein (by mass) of 95%.

Chromatography of the bovine LH/TSH-active fraction on DEAE-Sepharose (Fast Flow) yielded a profile with multiple protein peaks (Fig. 2), LH of highest specific activity being eluted in the initial steps containing minor TSH contamination (< 1.0% total TSH activity, see Table I). Each successive buffer change eluted LH and TSH of varying specific activity (Table I, designated as pools I–VI, see also Fig. 2) indicating significant charge heterogeneity for these hormones (see also electrophoretic results below). TSH of greatest specific activity (16.3 I.U./mg) was eluted by the third buffer change and contained less than 1% of the total LH activity. The recoveries of lutropic and thyrotropic activities were 92% and 93%, respectively (Table I). Each hormonally active fraction (pools I–VI) was chromatographed on Sephadex G-100 (Superfine) to remove aggregated material and any dissociated subunits of the glycoprotein samples. The final specific activities of LH and TSH were increased up to two-fold with this step and were comparable with those of highly purified preparations from other groups.

Characterisation of bovine thyrotropin and lutropin by HPIEC

Fractionated bTSH preparations (pool VIb) containing less than 1% contamination of bLH, and a bLH preparation (pool II) containing less then 1% bTSH, could be further resolved by high-performance ion-exchange chromatography on a Mono Q strong-anion-exchange column. Initial experiments showed that optimal resolution was obtained with a linear gradient and a rate of change of $[Cl^-]$ of 8.75 m*M*/min at a flow-rate of 1 ml/min. At pH 9.60 under these conditions, bLH (pool II from Sephadex G-100S) was shown to consist of one major UV-absorbing peak with several shoulders (Fig. 3A). LH receptor-binding activity yielded a similar profile (Fig. 3A). TSH activity (pool VIb from Sephadex G-100S) was eluted as a much broader peak, consisting of at least three major protein species with several shoulders, (Fig. 3B), this heterogeneity being also reflected in the receptor-binding profile (Fig. 3B).

Polyacrylamide gel electrophoretic analysis

The pooled fractions containing LH activity from the purification procedure (pools I, II, and III) gave characteristic α- and β-subunit band patterns following SDS-PAGE (Fig. 4A) with a third, contaminating protein band of lower molecular weight. This band, with an apparent molecular mass of 16 800 under non-reducing conditions, may correspond to the fast-migrating component observed by Reichert and Lawson[19] and Stockell-Hartree *et al.*[20] in highly purified bLH preparations. As LH activity decreased across the DEAE-Sepharose profile (pools IV, V and VI) the relative concentration of this component also decreased, and was not present in pools VIa or VIb (Fig. 4A). Highly purified bTSH (pools V and VIa, VIb) yielded three bands on SDS-PAGE, corresponding to the α-subunit, and two bands in the β-subunit region (Fig. 4A). We have observed this pattern previously, where the band of higher apparent molecular mass could be removed by affinity chromatography on TSH receptors[21]. Further, Giudice and Pierce[22] have noted the presence of two β-subunit bands in bTSHβ preparations, only one of which will recombine with free α-subunit[22], supporting this observation.

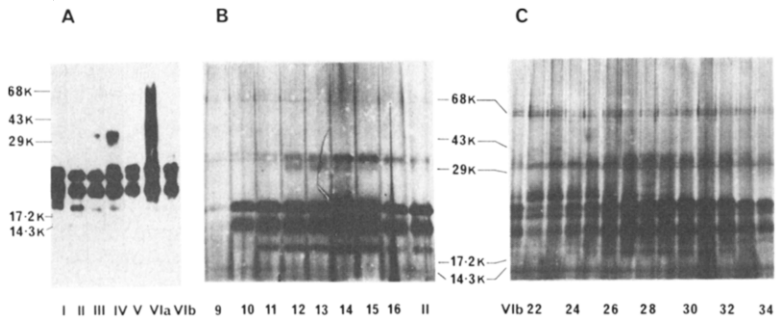

Fig. 4. SDS-PAGE of TSH/LH fractions. Samples (approximately 400 ng) of each protein were boiled in SDS for 2 min, and electrophoresed at a constant current of 25 mA at room temperature. (A) TSH/LH pools from Sephadex G-100S chromatography, (B) LH samples from HPIEC of LH pool II (Fig. 3), (C) TSH samples from HPIEC of TSH pool VIb (Fig. 3).

SDS-PAGE of the LH fractions, collected across the HPIEC profiles, provided similar results. All fractions contained the α- and β-subunits of LH, the band of lower molecular weight being present in fractions 11–16 (Fig. 4B). SDS-PAGE of the TSH fractions collected from HPIEC (Fig. 4C) demonstrated that the α-subunit and two β-subunit bands were present in every case, with a fourth band of apparent molecular mass of 24 500 present in the first six fractions.

Analytical isoelectric focusing of the LH fractions collected from the Mono Q column demonstrated the presence of at least six diffuse bands of differing pI values in the pH range 8.45–9.00 (Fig. 5A). Furthermore, the six bands were present in every collected fraction, no particular band being concentrated under the main chromatographic peak (Fig. 5A). Similarly, focusing of the TSH fractions from the Mono Q column yielded multiple bands (Fig. 5B), at least 40 individual bands being present

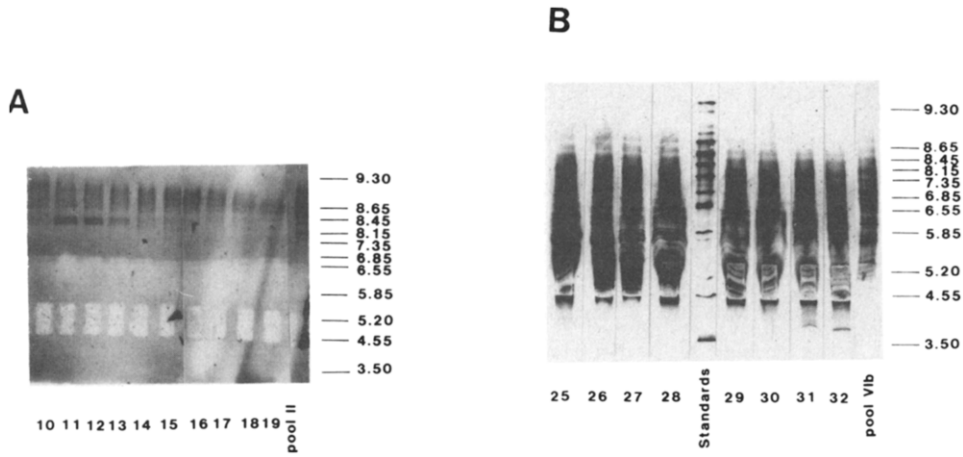

Fig. 5. IEF of TSH/LH fractions. LH samples (A) from HPIEC of LH pool II, and TSH samples (B) from HPIEC of TSH pool VIb (*cf.* Fig. 3) were electrophoresed for *ca.* 100 min at 25°C, 15 W maximum power, in a broad-range pH gradient (pH 3.0–9.5), generated with Ampholines. The pH gradient was measured by using standard proteins of known p*I*.

in the broad pH range of 5.0–8.4. As seen for bLH above, the band patterns observed for bTSH in each chromatographic fraction were all similar, no particular charge species being present in any fraction (Fig. 5B).

DISCUSSION

The above results demonstrate that bovine thyrotropin and lutropin, as prepared by lectin affinity chromatography and conventional ion-exchange chromatography on soft gels, consist of multiply charged species. This heterogeneity was also reflected in the TSH and LH activity profiles. A number of studies have previously been carried out on the heterogeneity of bovine thyrotropin preparations, including separation by charge electrophoresis[3], isoelectric focusing[2,3] and lectin affinity chromatography[2]. Despite these earlier investigations, the structural changes resulting in hormonal heterogeneity remain unclear. The charge heterogeneity of the human glycoprotein hormones is in part due to the presence of variable concentrations of sialic acid[1]. However, this mono-saccharide derivative is absent from the bovine hormones[1]. Davy *et al.*[4] isolated five isohormones of bTSH by isoelectric focusing, and attributed the observed charge differences entirely to changes in amido contents of the isohormones. However, other workers have noted changes in sulphation of carbohydrate residues[1,24] and C-terminal processing of the β-subunit[1]. In addition, Joshi and Weintraub[2] have probed the surface accessibility of various hexose groups using lectins, and found that isohormones exist which have differing surface-accessible carbohydrate structures.

From the results presented here it is clear that bLH and bTSH can be further purified by HPIEC. Although these isoforms migrate as unique surface-charge species on, *e.g.*, the Mono Q column, it can be anticipated that these species will still exhibit heterogeneity with respect to net charge. Optimal separation, based on charge, would therefore be achieved by a combination of at least two methods: HPIC and isoelectric focusing. In addition, purification of isohormones based on this approach permits distinguishing proteins that differ in surface structure as a result of alterations in neutral structural entities, such as neutral amino acids or carbohydrate groups. Such alterations in protein structure are still expected to influence the way the protein interacts with the ion-exchanger. Under these circumstances with non-charged residue replacements, *e.g.*, with heterozygotic proteins, HPIEC thus provides a powerful method for probing the overall charge asymmetry of the protein surface. The above results clearly indicate that by using a combination of HPIEC on the Mono Q column and preparative isoelectric focusing, the charge heterogeneity of bTSH and bLH may be analysed in terms of surface-accessible charge (charged groups on the surface of the protein which interact with the Mono Q anion exchanger) and net charge (protein charge, as determined by isoelectric focusing).

ACKNOWLEDGEMENTS

This investigation was funded by a project grant from the National Health and Medical Research Council of Australia to M. T. W. Hearn. The excellent technical assistance of Ms. R. Johnston is also acknowledged.

REFERENCES

1 J. G. Pierce and T. F. Parsons, *Ann. Rev. Biochem.*, 50 (1981) 465.
2 L. R. Joshi and B. D. Weintraub, *Endocrinology*, 113 (1983) 2145.
3 N. A. Takai, S. Filetti and B. Rapoport, *Endocrinology*, 109 (1981) 1144.
4 K. M. M. Davy, J. S. Fawcett and C. J. O. R. Morris, *Biochem. J.*, 167 (1977) 279.
5 L. Wide and B. M. Hobson, *J. Clin. Endocrinol. Metab.*, 56 (1983) 371.
6 L. Wide and M. Wide, *J. Clin. Endocrinol. Metab.*, 58 (1984) 426.
7 D. M. Robertson, L. M. Foulds and S. Ellis, *Endocrinology*, 111 (1982) 385.
8 D. M. Robertson, M.-P. Van Damme and E. Diczfalusy, *Mol. Cell. Endocrinol.*, 9 (1977) 45.
9 P. Roos, L. Nyberg, L. Wide and C. Gemzell, *Biochem. Biophys. Acta*, 405 (1975) 363.
10 L. Wide, *J. Clin. Endocrinol. Metab.*, 55 (1981) 682.
11 L. E. Reichert, Jr., *Methods Enzymol.*, 38 (1975) 360.
12 M. M. Bradford, *Anal. Biochem.*, 72 (1976) 248.
13 T. H. Liao and J. G. Pierce, *J. Biol. Chem.*, 245 (1970) 3275.
14 U. K. Laemmli, *Nature*, 227 (1970) 680.
15 W. Wray, T. Boulikas, V. P. Wray and R. Hancock, *Anal. Biochem.*, 118 (1981) 197.
16 H. Kruse, H. Reiber and H. I. Schipper, *LKB Technical and Diagnostic Bulletin*, LKB Bromma, Sweden.
17 F. Leidenberger and L. E. Reichert, Jr., *Endocrinology*, 91 (1972) 901.
18 G. A. Bloomfield, M. R. Faith and J. G. Pierce, *Biochem. Biophys. Acta*, 533 (1978) 371.
19 L. E. Reichert Jr. and G. M. Lawson Jr., *Endocrinology*, 92 (1973) 1034.
20 A. Stockell-Hartree, J. B. Lester and R. C. Shownkeen, *J. Endocrinol.*, 105 (1985) 405.
21 P. G. Stanton and M. T. W. Hearn, *Endrocrinology*, 119 (1986) 887.
22 L. C. Giudice and J. G. Pierce, *Endocrinology*, 101 (1977) 776.
23 J. S. Fawcett, M. L. Dedman and C. J. O. R. Morris, *FEBS Lett.*, 3 (1969) 250.
24 T. F. Parsons and J. G. Pierce, *Proc. Natl. Acad. Sci. U.S.A.*, 77 (1980) 7089.
25 M. T. W. Hearn, M. Guthridge and J. Bertolini, *J. Chromatogr.*, 397 (1987) 371.