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Purification and characterisation of prolactin from sheep and buffalo pituitaries†

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Abstract. A study of the problem of structural variants of proteins and their relative contribution to the expressed immunological and biological activity has been initiated using sheep and buffalo prolactins as models. The feasibility of obtaining immunologically and biologically active prolactin in high yields from the discarded 'acid pellet' of sheep and buffalo pituitaries has been demonstrated. This permits use of the same batch of glands for purifying lutropin, follitropin and prolactin as side fractions. The major component in preparations of buffalo prolactin has a molecular size of 24 kDa. The preparations were active in a radioligand binding inhibition assay and in a rat liver based radioreceptor assay. Charge and size isomers of sheep prolactin and buffalo prolactin have been observed. The reference sheep prolactin did not, in preliminary work, give any indication of being glycosylated. However radioactive sulphate was found to be incorporated into prolactin-rich fractions of sheep and buffalo pituitaries *in vitro*. By physico-chemical and immunochemical criteria the [^{35}S]-labelled material was similar to standard reference prolactin. The structural implications of sulphation have been probed.

Introduction

The pituitary polypeptide hormone prolactin (PRL) plays an important but enigmatic physiological role in all vertebrates from fish to mammals (Ensor, 1978). Over 80 biological effects have been ascribed to PRL (Nicoll *et al.,* 1986). Inspite of an astonishingly large body of information on its structure, biosynthesis, secretion and physiological action, nothing is understood regarding either the mechanism of its action or the relation of its structural domains to biological effects (Nicoll *et al.,* 1986).

A number of reports have claimed detection of multiple forms of PRL. These reports have identified size differences among these structural variants. Recently, Lewis *et al.* (1984) reported the detection and purification of a glycosylated form of ovine PRL. However, a detailed study of the origin and relative contribution of the different polymorphic forms of PRL to its expressed biological and immunological activity has not been made till now.

We have recently initiated a programme of study of PRL from buffalo. Our aim in this study was to relate the structural domains of buffalo PRL to physiologically and immunologically important epitopes especially from an evolutionary perspective. The bewildering variety of its actions—osmoregulation in fish, somatotropic action in amphibians, parental and migratory behaviour in birds, growth and tissue regene ration promotion in reptiles and luteotropic and luteolytic effects in mammals—

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Abbreviations used: PRL, Prolactin; RIA, radioimmunoassay; Con A, concanavalin A; LH, lutropin; FSH, follitropin; SDS, sodium dodecyl sulphate; PAGE, Polyacrylamide gel electrophoresis; RRA, radio receptor assay; EIA, enzyme immuno assay; kDa, kilo Daltons.

justified such a study. We report here the purification and physico-chemical characterisation of PRL from frozen pituitaries of sheep and buffalo.

Materials and methods

Hormones, chemicals, antisera and animals

Sheep PRL for radioiodination and rabbit anti-ovine PRL serum for radioimmunoassay (RIA) were obtained through the courtesy of Dr. S. Raiti, National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK, NIH) Bethesda, Maryland, USA. Enzyme (penicillinase)—human PRL conjugate, rabbit anti-human PRL and goat anti rabbit γ-globulin were provided by Dr. G. L. Kumari of the National Institute of Health and Family Welfare, New Delhi. Ovine PRL (average of 35 IU/mg), reference marker proteins for sodium dodecyl sulphate (SDS)-polyacrylamide gel electrophoresis (PAGE), bovine serum albumin (BSA), Sephadex-G50, DEAE Sephadex-A50, acrylamide, bis acrylamide, TEMED, Freund's complete adjuvant, ammonium persulphate, Coomassie blue R-250, concanavalin A (ConA)-Sepharose and α-methyl-D-mannoside were purchased from Sigma Chemical Company, St. Louis, Missouri, USA. Carrier-free Na ^{125} I and carrier-free ^{35} SO_4^{2-} were obtained from Bhabha Atomic Research Centre, Bombay. PPO and dimethyl POPOP were purchased from SISCO research laboratories, Bombay. The WHO reference standard ovine PRL (22 IU/mg) was obtained through the National Institute of Biological Standards, England. Adult rats employed in this study were of Holtzman strain, and were maintained in our colony under standard 14 h L: 10 h D schedule. They were given food (Hindustan Lever Ltd., Bombay) and water *ad libitum.* Male albino rabbits weighing 2·5 kg were purchased from Maulana Azad Medical College, New Delhi. All chemicals used in this study, unless otherwise mentioned were of Excelar/GR grade.

Pituitary incubations in vitro

Incubations for examining $^{35}SO_4^{2-}$ incorporation were carried out using minces of sheep and buffalo pituitaries collected fresh from a local abattoir. All incubations were done at 37°C in a metabolic shaker and in modified KRBG buffer (Arunasmithasri *et al.,* 1983). In sulphate incorporation studies $MgSO_4$ was replaced by $MgCl_2$. $_{All}$ media included amino acid and vitamin mixture as in Medium 199 (Morgan *et al.,* 1950).

Purification of PRL

Glands were collected from sheep and buffaloes which were being slaughtered at a local abattoir. They were frozen in liquid N_2 within 1 h of slaughter and transported to the laboratory in liquid N_2 for processing. The procedures were adaptations of those of Papkoff *et al.* (1965), Li (1974), NaiSiang-Jiang and Wilhelmi (1965) and Bell *et al.* (1985) with a few modifications. Briefly, the glands were minced and homogenised in 0·15 M $(NH_4)_2SO_4$ in a Waring blender and the homogenate centrifuged at 3000 *g* for 20 min at 4°C. The pH of the supernatant was successively

adjusted to pH 4 and pH 3 followed each time by centrifugation at 3000 *g* for 15 min at 4°C. The pellets, called acid pellet, were tested as sources of PRL. The supernatants were used for the purification of lutropin (LH) and follitropin (FSH). In the adaptation of the NaiSiang-Jiang and Wilhelmi (1965) procedure, chilled (–20°C) alkaline ethanol (pH 9·5) was added to the pH 4 acid pellet and the two were mixed well. The suspension was centrifuged. To the supernatant at pH 5·5 were added 2 volumes of chilled ethanol to precipitate enriched PRL (P-l fraction). This precipitate, collected by centrifugation, was dissolved and dialysed against weak ammonium bicarbonate and lyophilised. The material was then subjected to chromatography on DEAE Sephadex equilibrated with 0·01 M phosphate-0·0065 M borate buffer, pH 7. The bound proteins were eluted with *a* step wise gradient of NaCl. In the adaptation of the procedure of Li (1974), the pH 4 acid pellet was extracted with acidic acetone followed by the precipitation of PRL with a further 5 volumes of chilled acetone. The precipitate was dissolved and subjected to differential isoelectric precipitation by gradual adjustment of pH (figure 1). The

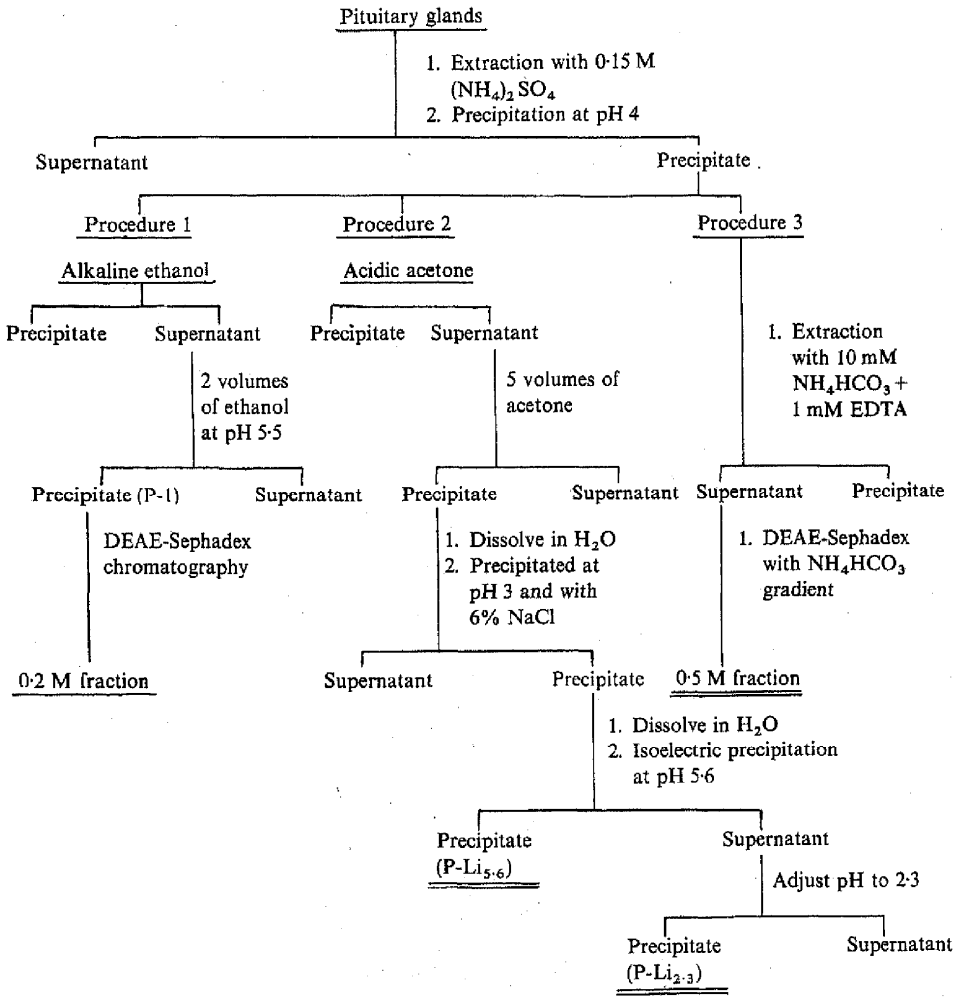

Figure 1.

procedure of Bell *et al.* (1985) involved extraction of the pH 4 acid pellet with 10 mM NH_4HCO_3-1 mM EDTA followed by chromatography of the extract directly on DEAE Sephadex.

Disc electrophoresis

Electrophoresis under denaturing conditions was carried out essentially according to the procedure of Laemmli (1970) using 11% resolving gels. Disc electrophoresis in native gels was carried out according to the method of Davis (1964). Electrophoretic transfer of proteins from gels onto nitrocellulose paper was carried out according to the procedure of Towbin *et al.* (1979) except that 80 mA current was used for 12 h and Whatman No. 1 filter sheets were employed for padding. Immuno-staining was carried out by the use of biotinylated second antibody and biotinylated peroxidase-avidin system as described by Vector Laboratories, USA (Hsu *et al.,* 1981). A 1:100 dilution of the rabbit antibody (local) was used.

Con A-Sepharose chromatography

Affinity chromatography on Con A-Sepharose was performed according to the Standard procedure of Bloomfield *et al.* (1978) at pH 6.

Antiserum

Antiserum to commercial ovine PRL (Sigma Chemicals, USA) was raised in rabbits essentially according to the procedure of Vaitukaitis *et al.* (1971).

Immunoprecipitation

Incubation of minces from sheep and buffalo pituitary glands (4–5/flask) was carried out in 10–20 ml of modified KRBG in the presence of radioactive sulphate (200 μCi/ml). At the end of the incubation the tissue was homogenised and processed as in the case of bulk preparation of PRL. The dialysed P-l fraction (figure 1) was taken for immunoprecipitations. ^{35}S-Labelled proteins from this fraction (165–200 μg protein containing 2000–2500 cpm) were incubated with excess of rabbit antiserum to PRL or normal rabbit serum for 1 h at 37°C and 3 days at 4°C. The buffer used was 0·01 M phosphate buffered saline, pH 7·5. The immunoprecipitate was collected by centrifugation at 3000 g for 15 min at 4°C, washed with chilled physiological saline by centrifugation, and dissolved in a small volume of 0·05 N NaOH. Aliquots were then taken for radioactivity measurement. In the case of the incubation medium, it was dialysed extensively and then lyophilised. The lyophilised powder was then subjected to immunoprecipitation as above.

Radioiodination and radioligand binding inhibition test

Radioiodination of ovine PRL (NIH Standard) was performed essentially according to the method of Greenwood *et al.* (1963). A specific activity of 25-30 μCi/μg was

maintained for both RIA and radioreceptor assay (RRA). Radioligand binding inhibition test was carried out using ^{125}I-labelled ovine PRL (NIH standard) and rabbit antiserum to ovine PRL (NIH product) at 1:25,000 initial dilution. Reference (ovine) and buffalo PRL were used for competitive binding inhibition.

RRA

This was carried out using rat liver homogenate as a source of PRL receptors essentially according to the method of Taga (1982) with slight modifications. Briefly, 300 μl of rat liver homogenate, 100 μl of ^{125}I-labelled ovine PRL (80,000 cpm) and 100 μl of a serially diluted ovine PRL standard or the unknown sample were incubated in 0·025 M buffer, pH 7·6/10 mM $CaCl_2$/0·1 % BSA at 37°C for 2h followed by the addition of 2 ml of the RRA buffer. The tubes (10 × 75 mm) were centrifuged in a Sorvall RC-2B centrifuge at 3000 *g* for 15 min at 4°C. The supernatants were discarded and after wiping the sides free of adhering liquid, the pellets were counted for radioactivity in an ECIL type manual counter at 70% efficiency. Suitable controls were included.

Enzyme immunoassay

The enzyme immunoassay (EIA) was performed according to a method standardized in the laboratory. Briefly 100 μl of anti human PRL serum (1:50,000 dilution) and 100 μl of reference human PRL (serially diluted, 100-4000 pg/tube) or the unknown were incubated for 16 h in 0·01 M phosphate-buffered saline, pH 7 containing 0·05% BSA, 0·1% sodium azide and 0·05% (v/v) Tween 20. At the end of the incubation, 100 μl of 1:2000 diluted enzyme (penicillinase)—PRL conjugate (1 mg/ml stock) was added, and incubation continued for 6 h at 37°C. Then goat anti-rabbit γ-globulin serum (1: 3 0 dilution) was added to all tubes followed by incubation for 36 h. The tubes were centrifuged and supernatants discarded. The substrate solution (1 mM) followed 1 h later by the starch-I_2 reagent were added. After 10 min, the reaction was terminated by the addition of 5 N HCl (1 ml/tube) and the colour developed was read at 620 nm in a spectrophotometer.

Results and discussion

We had initially examined the feasibility of purifying PRL from freshly frozen buffalo pituitary glands. As there was no previous work on PRL from this animal species, we tested procedures published for sheep, cattle and pigs (NaiSiang Jiang and Wilhelmi, 1965; Li, 1974; Bell *et al.,* 1985). We had earlier shown that the procedure of Papkoff *et al.* (1965) for the purification of ovine LH was applicable with a few modifications to buffalo pituitary glands (Arunasmithasri *et al.,* 1983; Muralidhar and Rajendrakumar, 1986). In the course of the work on LH, the chance observation was made that the acid pellet—the fraction usually discarded in the procedure employed for LH purification—was reacting positively with a specific rabbit antiserum to ovine PRL (Neeraja Chadha *et al.,* 1987). Surprisingly, the pH 4 acid pellet gave a precipitin line against anti-PRL serum in Ouchterlony immunodiffusion test while the pH 3 acid pellet did not (data not shown). The antiserum was raised against a

commercially available ovine PRL and was decomplemented and then absorbed with normal sheep serum (Muralidhar *et al.,* 1974) before use; the absorbed antiserum did not give a line against bovine growth hormone (data not shown). This result indicated that the pH 4 acid pellet could be a convenient source of PRL. This was indeed true for buffalo glands also. We obtained highly enriched PRL from this fraction by simply applying the published procedures for pituitaries of sheep, cattle and pigs with a few modifications to the pH 4 acid pellet (figure 1). Procedures 1, 2, 3 in the figure 1 refer to combination of the procedure of Papkoff *et al.* (1965) with those of NaiSiang Jaing and Wilhelmi (1965), Li (1974) and Bell *et al.* (1985), respectively. These preparations are referred to respectively as bu-P-W-P-1, bu-P-Li and bu-P-B in table 1. The procedure of Bell *et al.* (1985) did not yield any immunoreactive material from the acid pellet of sheep pituitary glands but was useful for the buffalo acid pellet. The procedures of NaiSiang Jiang and Wilhelmi (1965) and of Li (1974) were equally applicable to the acid pellets of sheep and buffalo glands. The material designated as P-l was highly enriched in PRL as indicated by RIA estimatable PRL which was obtained in high yields (table 1). The combination of the procedures of Papkoff *et al.* (1965) and NaiSiang Jiang and Wilhelmi (1965) worked well for obtaining PRL from both sheep and buffalo acid pellet. It was, for buffalo and sheep pituitary glands, a single fractionation scheme which yielded LH, FSH and PRL as different fractions. NaiSiang Jiang and Wilhelmi (1965) who combined the classical scheme of Ellis (1961) with a few additional steps reported good yields of PRL from ovine, bovine and porcine pituitary glands. However they did not report purification of LH and FSH also from the same batch of glands. We have established recently that their scheme of purification starting from Ellis (1961) fractionation procedure also yields highly enriched PRL from buffalo pituitary glands (table 1, bu-E-W-P-1).

Table 1. Yield of PRL (immunoreactive) in the procedures using acid pellet as starting material.

Fraction	Oüchterlony test against rabbit a/s to ovine PRL	Yield (g/kg glands)
o-P-W-P-1	+	1·0–1·5
o-P-$Li_{5·6}$	+	0·4–0·5
bu-P-W-P-1	+	2·0–2·5
bu-P-$Li_{5·6}$	+	0·04–0·05
bu-P-$Li_{2·3}$	+	0·06–0·07
bu-P-B	+	0·16–0·18
E-W-bu-P-1	+	0·5–0·6

o, ovine; bu, buffalo; P, Papkoff *et al.* (1965) procedure; Li, procedure of Li (1974); B, procedure of Bell *et al.* (1985); W, Wilhelmi; E, Ellis (1965) procedure.

All the preparations were active in a radioligand binding inhibition test using ^{125}I-labelled ovine PRL, rabbit anti-ovine-PRL serum and ovine-PRL as reference standard (table 2). Though they were tested at the same dose, the degrees of inhibition were different. As there was obviously no relation to purity (for example the NIDDK sample is iodination grade but has only 66% activity), the difference in

Table 2. Effectiveness of different buffalo PRL preparations in a radioligand binding inhibition test. ^{125}I-ovine PRL (NIDDK sample) and different PRLs (5 ng/tube level) were incubated with anti-ovine PRL serum and bound radioactivity measured.

Sample	$B/B_0 \times 100$
—	100
Sigma PRL	0
bu-E-W-P-1*	27·7
bu-P-$Li_{5·6}$	33·4
NIDDK ovine PRL	33·7
bu-P-W-P_1-0·2 M fraction	63·0

*E, Ellis procedure. For other abbreviations see table I.

degree of inhibition reflects the different avidities of the antiserum for these preparations. This in turn reflects the differences in antigenic epitope. If they indeed represent the *in vivo* situation, it would be extremely interesting. We are presently trying to develop a homologous RIA system for buffalo PRL. The buffalo pituitary PRL's (all the preparations) did not cross react with human PRL when tested in an EIA. The results are given in table 3. The absorbance values obtained for the

Table 3. Absence of cross reaction between buffalo and human PRL in an EIA.

Sample	Absorbance (620 nm)
Control (NRS)	2·016
Antiserum	0·285
Antiserum + 4 ng human PRL.	1·715
Antiserum + 100 ng buffalo PRL (bu-P-W-P-1 0·2 M)	0·229

different preparations of PRLs were around the same as that indicated in table 3 for bu-P-W-P-$l_{0·2}$M. Aston and Ivanyi (1985) in a study of human PRL using monoclonal antibodies have shown that two of them could bind both human and bovine/ovine PRL in a labelled antibody competition test. This would mean that even without selection by using ^{125}I-labelled ovine PRL for screening hybridoma supernatants containing secreted antibodies against human PRL, one can obtain cross-reaction between human and non-primate PRL. In our test we used a polyclonal antiserum, and it is possible that the cross-reacting antibodies were in too low a concentration to be detected in a competitive test.

The buffalo PRL preparations were also active in a rat liver based RRA (Taga, 1982). Figure 2 illustrates the results with reference PRL and with buffalo PRL prepared by the combination of the procedures of Papkoff *et al.* (1965) and Li (1974). The results also indicate that bovine growth hormone (GH) not exhibit significant activity in the range tested. Approximately 5000 ng of GH were needed to cause 50% inhibition of the binding of ^{125}I-labelled ovine PRL while bu-P-$Li_{5·6}$ and ovine PRL could do so at approximately 84 and 25 ng, respectively. This indicates that GH has

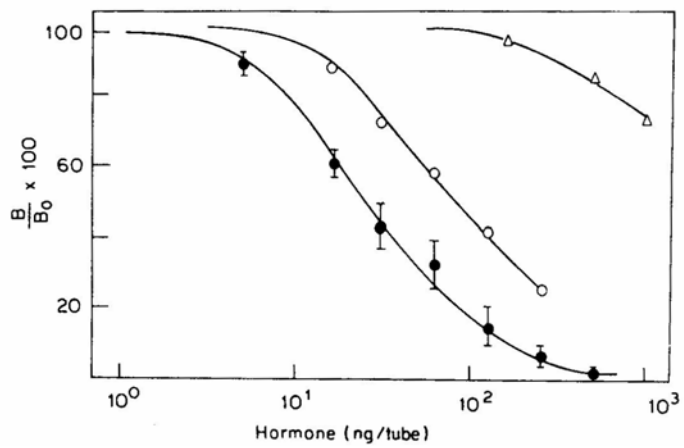

Figure 2. Radioreceptor assay for PRL using rat liver homogenate as a source of receptors. (●), ovine PRL (Sigma); (O), buffalo PRL (bu-P-Li$_{5.6}$); (Δ), bovine GH.

less than 0·5% activity in this assay system. It also proves that although the purification of buffalo PRL was monitored by immunological activity, the preparation was biologically active in a receptor system specific to PRL and hence is not a cross-reacting substance.

It was possible to further purify the P-l preparation by subjecting it to DEAE-Sephadex chromatography. A representative chromatographic profile for the bu-P-W-P-l preparation is given in figure 3. It can be seen that PRL gets bound to the column and is eluted with 0·2 M NaCl.

Although we do not have evidence it is possible that there was a biologically active but immunologically inactive PRL in other fractions. Immunologically poor

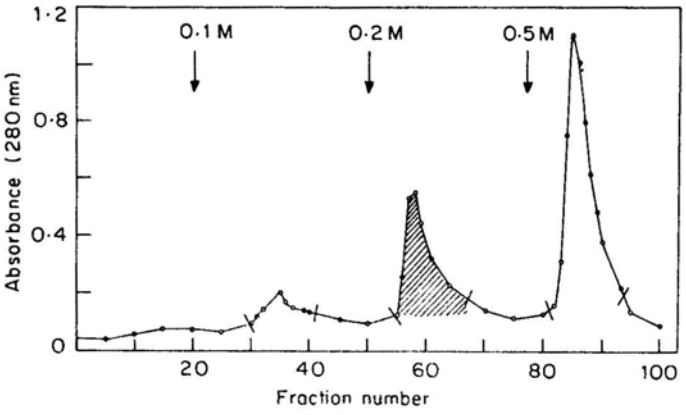

Figure 3. DEAE-Sephadex chromatographic profile of buffalo PRL (bu-P-W-P-1), 40 mg of bu-P-W-P-1 was loaded on DEAE-Sephadex column (1 × 40cm) at 4°C and the flow rate was 30 ml/h. Arrows indicate the points of start of salt gradient (stepwise). The shaded area represents the purified PRL. Sample was loaded in 0·01 MPO_4-0·0065 M borate pH 7.

but biologically very active forms of mouse PRL, for example, have been reported (Sinha and Baxter, 1979).

It must also be mentioned that only the Papkoff–Wilhelmi procedure gave a PRL which was eluted from DEAE Sephadex at 0·2 M NaCl. The procedure of Bell *et al.* (1985) gave a PRL which was eluted from DEAE Sephadex only with 0·5 M NH_4 HCO_3. It is possible that prolonged exposure to NH_4 HCO_3 in this procedure caused some deamidation giving rise to more acidic forms of PRL. However, isoelectric focusing has not been done yet to substantiate this idea. In the procedure of Li (1974) also, while the sheep acid pellet gave us a PRL which precipitated at pH 5·6, the buffalo acid pellet gave one at 5·6 (bu-P-$Li_{5.6}$) and another immunoreactive PRL at pH 2·3 (bu-P-$Li_{2.3}$). These results indicate the possible presence of charged isomers of PRL. Many reports in the literature do point to such a situation based on electrophoretic analysis of PRL in native gels. We have also found that the reference standard PRL (Sigma) and our 0·2 M fraction of bu–P–W–P-l show multiple bands on electrophoresis in native gels (figure 4). However, by

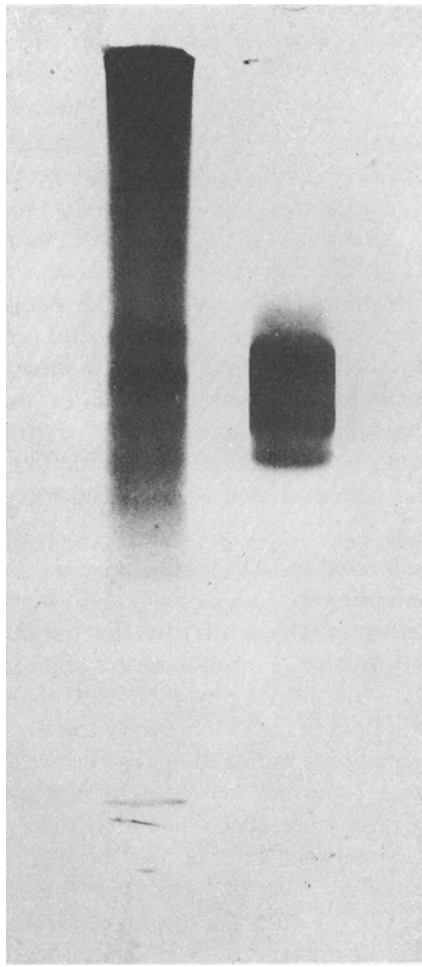

Figure 4. Electrophoretic profile (Coomassie blue bands) of P-l (left lane) and reference PRL (right lane) in native gels.

Western blotting, these forms were found to be immunologically same (data not shown). Direct evidence that these are deamidated forms has been provided by the study of Graf *et al.* (1970) who isolated two forms of PRL, a normal and a faster moving band. Peptide maps showed that the two forms differed only in one peptide spot. The difference was found to be attributable to Asn/Asp content. Whether deamidation occurs within cells or in the blood (enzymatic or otherwise) and if so, its physiological significance, are not known. Reports of altered immunological and receptor binding properties of the deamidated form of mouse PRL, however, do exist in the literature (Haro and Talamantes, 1985).

In view of the report of Lewis *et al.* (1984) of the existence of glycosylated forms of ovine PRL, we examined the commercial preparation of ovine PRL. We found no evidence for the presence of glycosylated forms. Neither the hormone nor even the ^{125}I-labelled ovine PRL was found to be retained on a Con A-Sepharose affinity column. Less than 1 % of the loaded material was found retained. There was no directly estimatable sugar in the dialysed preparation (Neeraja Chadha and K. Muralidhar, unpublished results). It gave two closely moving Coomassie blue stainable bands in SDS-PAGE indicating a difference in molecular size of about 1500-2000. The 0·2 M fraction from DEAE-Sephadex chromatography also gave a similar pattern (data not shown). However on immunoblotting after electrophoretic transfer, both the bands reacted with the antibody (figure 5, left and right lanes).

Based on our previous experience with the glycoprotein hormone LH, metabolic studies were conducted to throw additional light on the problem of size variation due to glycosylation. We had earlier demonstrated by physico-chemical, immunological and biological criteria that pituitary LH has sugar-bound sulphate (Arunasmithasri *et al*., 1983; Rajyalakshmi *et al*., 1983; Muralidhar and Rajendrakumar, 1986). A chance observation that significant radioactivity was present in the PRL rich fraction made us investigate this further. To our surprise we found that pituitary minces from sheep and buffaloes incorporate $^{35}SO_4^{2-}$ from the incubation medium into immunoprecipitable PRL-like material. Both tissue and incubation medium yielded labelled immuno-precipitate (Rita Kohli *et al.,* 1987). The material could be subjected to a fractionation scheme like that for purification of PRL and radioactivity was still associated with purified PRL (Rita Kohli *et al.,* 1987).

The ^{35}S-labelled proteins from the sheep pituitary tissue were extracted, purified to the P-l stage and then subjected to SDS-PAGE analysis. Parallel strips were taken for staining, slicing and counting for radioactivity and also for slicing and extraction for immunodiffusion tests. From table 4 it is obvious that there are a number of ^{35}S-labelled proteins in the P-l fraction. Two of these are of interest. They had molecular size in the region of 25 kDa but differing by 1500–2000 (figure 5). Interestingly both the proteins gave precipitin lines in the Ouchterlony test (figure 6). Protein from slice two (table 4) which had significant radioactivity did not react with anti-PRL serum. The Ouchterlony data was confirmed by Western blot analysis (figure 5, middle lane). These observations raise more questions rather than provide answers.

The nature of the linkage between sulphur and the PRL peptide backbone is not clear. A number of possibilities exist: (i) both these bands (figure 5, middle lane) contain PRL with sugar-SO_4, (ii) both the forms are tyrosine-SO_4-containing PRL with the larger protein having a chemical modification leading to the size increase, and (iii) the larger protein is a sugar-SO_4 4containing PRL while the smaller protein is a tyrosine-SO_4 containing PRL. In the experiment demonstrating the absence of

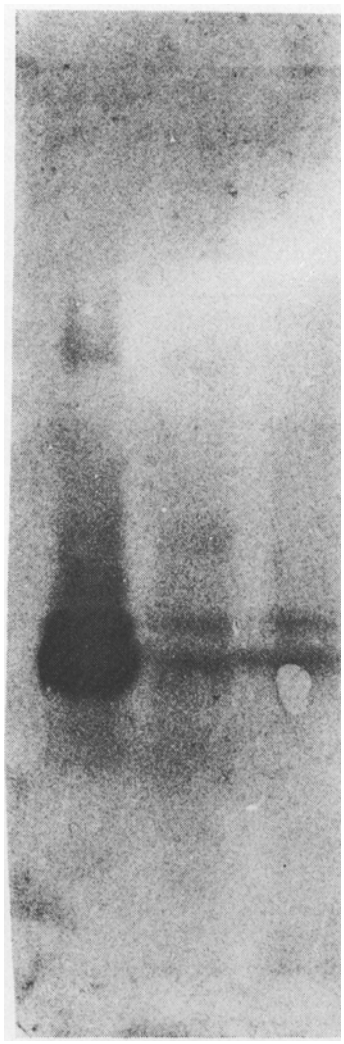

Figure 5. Western blot picture of standard PRL (left lane), ^{35}S-labelled P-l (middle lane) and 0·2 M fraction from DEAE-Sephadex chromatography (right lane). These were subjected to SDS-PAGE and then transferred to nitrocellulose sheet.

adsorption on Con A Sepharose, a commercial preparation of sheep PRL was used. The reason for invoking the presence of sugar-SO_4 is that the ^{35}S-labelled immuno reactive proteins may differ from the commercial preparation. However, none of these alternatives point to the existence of the non-glycosylated and non-sulphated PRL and 24 kDa known for over 40 years (Li, 1980). A 20 kDa PRL has been reported to be present in human pituitary extracts (Aston *et al.,* 1984). Aston and Ivanyi (1985) have shown that even affinity purified human PRL, *i.e.*, PRL purified on monoclonal antibody affinity columns, gives multiple bands of Western blots. Two bands, at 26 and 24 kDa positions, were predominant. Minor bands at 18, 16 and 8 kDa positions were also noticed. The generation of these polypeptides was ascribed to proteolysis. Ovine PRL also gave the 26 and 24 kDa bands on similar

Table 4. Molecular size variation among ^{35}S-labelled sheep pituitary proteins (P-l fraction).
Sheep pituitary minces were incubated *in vitro* with ^{35}SO and at the end of the incubation the tissue was fractionated to yield P-l fraction. This was subjected to SDS-PAGE as per the procedure of Laemmli (I970) using II % preparative gels,

Slice No.	Radioactivity (cpm)*
1	512
2	1148
3	518
4	394
5	258
11	80
13	5584
14	2626
15	118

*After subtracting background radioactivity.

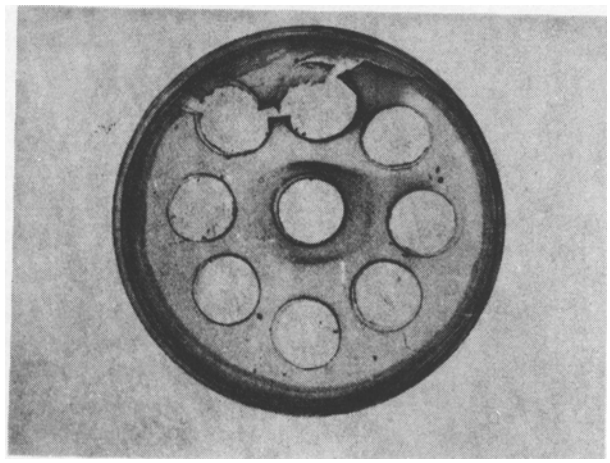

Figure 6. Ouchterlony immunodiffusion test results, The central well had rabbit anti-ovine PRL serum and the surrounding wells had materials as follows: I2-O′ clock well, Standard ovine PRL; 3-O′ clock well, slice no. I3 extract; 6-O′ clock well, slice no. I4 extract. All the other wells had extracts from control slices taken arbitrarily from other portions of the SDS-PAGE slab gel.

analysis. Bovine PRL gave a less intense 26 kDa band. There have been other reports of the existence of size variants of PRL. These include aggregates (Squire *et al.,* 1963; Nyberg *et al.,* 1980; Niall, 1981), monomers with chemical modifications such as glycosylation, phosphorylation etc. (Lewis *et al.,* 1984; Butnev and Pankov, 1985;

Oetting *et al.,* 1986) and biosynthetic precursors (Lingappa *et al.,* 1977). The relative contributions of these forms to the biological activity of the hormone have not been fully investigated. On the other hand, these forms may be experimental artifacts (*e.g.* due to lyophilisation). However, size variants of proteins are known to arise as a result of alternative processing of mRNA, post-translational modifications, or proteolytic in circulation.

In conclusion, it has been possible to obtain highly enriched preparations of PRL from the discarded acid pellets of sheep and buffalo pituitary glands. It was possible to obtain the PRL in a homogeneous form. Further purification of the PRL preparations from sheep and buffalo glands is in progress. During these studies a number of polymorphic forms of PRL were noticed. Some were shown to be charged isomers while others were shown to differ in degree of sulphation and hence to be size isomers. The nature of the linkage between polypeptide and sulphur is being investigated. It could be sugar -SO_4 or tyrosine- SO_4. Further work is in progress.

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