Incorporation of radioactive $^{35}SO_4^{2-}$ into immunoreactive pituitary lutropin

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The terminal hexosamines of bovine pituitary lutropin are thought to contain a sulfate moiety. In order to test this, a biosynthetic approach was adopted. When rat and buffalo (bovine) pituitaries were incubated with radioactive $^{35}SO_4^{2-}$ for 2 h in vitro it was observed that radioactivity gets incorporated into trichloroacetic acid-precipitable proteins. When the radioactive proteins were treated with an anti-sheep lutropin serum, radoactivity was found in the immunoprecipitate. The incorporation into rat lutropin like material was very marginal while it was very significant in the case of buffalo lutropin.

Lutropin *Buffalo pituitary* *Sulfate incorporation* *LH antiserum* *Rat LH* *Immunoprecipitation*

1. INTRODUCTION

Pituitary glycoprotein hormones like lutropin (LH), follitropin (FSH) and thyrotropin (TSH) have been subject to extensive investigations regarding their structure, function and biosynthesis [1]. Structural studies on their carbohydrate portion have led to the proposal that the terminal hexosamine residues in bovine LH could be blocked [2], possibly with sulfate moiety [3] thus making them resistant to chemical and enzymatic degradation. We have observed that radioactive $^{35}SO_4^{2-}$ could get incorporated into immunoreactive LH in both rat and buffalo (bovine) pituitary glands under in vitro conditions.

2. MATERIALS AND METHODS

2.1. *Hormones and chemicals*

Luteinizing hormone (NIH-LH-S20) was a gift from the National Institute of Arthritis, Metabolic and Digestive Diseases (NIH, Bethesda MD). Radioactive $^{35}SO_4^{2-}$ (carrier free) was purchased from Bhabha Atomic Research Centre (Trombay, Bombay). Bovine serum albumin (BSA), aprotinin and thimerosal (grade II) were obtained from Sigma (St Louis MO). Freund's complete adjuvant was purchased from DIFCO Lab. (Detroit MI), 2,5-diphenyloxazole (PPO) and 1,4-bis-2-(4-methyl-5-phenyl DM-oxazolyl-benzene (DM-POPOP) was bought from the CSIR Centre for Biochemicals (V.P. Chest Institute, Delhi). All other chemicals were of AnalaR grade.

2.2. *Antisera and animals*

Healthy adult male rabbits were immunized against NIH-LH-S20 essentially as in [4]. Equal volumes of the hormone solution and adjuvant were mixed, emulsified and administered to the animals through 50–60 intradermal injections. The animals were boosted (500 μg LH) 1 month later. A week after the booster injection, animals were bled through the ear vein, serum separated and stored frozen with thimerosal (1:10000) as a preservative. Sera were decomplemented before use.

2.3. *Immunoabsorption*

The antiserum was found to contain antibodies against normal sheep serum proteins. Hence it was absorbed to remove all such antibodies. To 1 ml antiserum, 2 drops of a 1:10 diluted normal sheep

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serum (NSS) were added. After mixing, the tube was incubated for 1 h at room temperature and overnight at 4°C. Next day the contents were centrifuged (2000 × g at 4°C) and the supernatant decanted. The process of addition of NSS, incubation, centrifugation and supernatant collection was repeated until no visible precipitate with NSS could be observed [5].

2.4. *Titre and crossreaction of antibody to sheep LH*

The titre of the absorbed antiserum was obtained by performing a quantitative precipitin test [6] against sheep LH. For checking crossreaction both Ouchterlony double-diffusion test and quantitative precipitin test were employed. The pituitary extract was prepared by homogenising fresh or frozen rat pituitaries in 0.01 M phosphate-buffered saline (PHS) with 1 or 2 drops of the protease inhibitor (aprotinin), the homogenate centrifuged (10 000 × g for 15 min at 4°C) and the supernatant used as a source of LH for Ouchterlony double diffusion and quantitative precipitin tests. Protein was estimated in the immunoprecipitates as in [7].

2.5. *Pituitary incubations*

Pituitaries were removed from either freshly killed rats which were castrated a week before or from freshly slaughtered Indian buffaloes. After cleaning with incubation medium to get freed of surrounding tissue and blood, the rat pituitaries were cut into halves while the buffalo pituitaries were sliced. The tissue was incubated in screw capped vials or conical flasks in a modified Krebs–Ringer bicarbonate (KRB) buffer (pH 7.35) in which $MgSO_4$ was replaced by $MgCl_2$ and which contained 0.2% glucose and 0.1% BSA. Radioactive $^{35}SO_4^{2-}$ was neutralized with dilute NaOH and added to the incubation medium at 25 μCi/ml for the rat system and 100 μCi/ml for the bovine system. At the end of the incubation in a metabolic shaker at 37°C, tissue halves or slices were rinsed with fresh modified KRB buffer and homogenised either in a Potter–Elevehjem tissue grinder or a Waring blendor using fresh KRB buffer.

2.6. *Trichloroacetic acid and immunoprecipitation*

To an aliquot of the tissue homogenate obtained as above, 15% trichloroacetic acid was added (2 vol.), the precipitate formed was washed with cold 10% trichloroacetic acid (1 ml × 3) and collected by centrifugation. The pellet was dissolved in 0.05 N NaOH and suitable aliquots taken for radioactive counting using 10 ml Bray's solution in a Beckman scintillation spectrometer.

For immunoprecipitation of the $^{35}SO_4^{2-}$ labelled LH, the incubated pituitary slices or halves were homogenised as above. The homogenate was centrifuged (10 000 × g for 15 min at 4°C) and the supernatant was incubated with anti-sheep lutropin serum. For control similar experiment was done but using normal rabbit serum (NRS). Incubation was conducted for 1 h at 37°C by 3 days and nights at 4°C. The formed immunoprecipitate was washed with chilled saline (1 ml, 3 ×) by centrifugation and resuspension and the final pellet

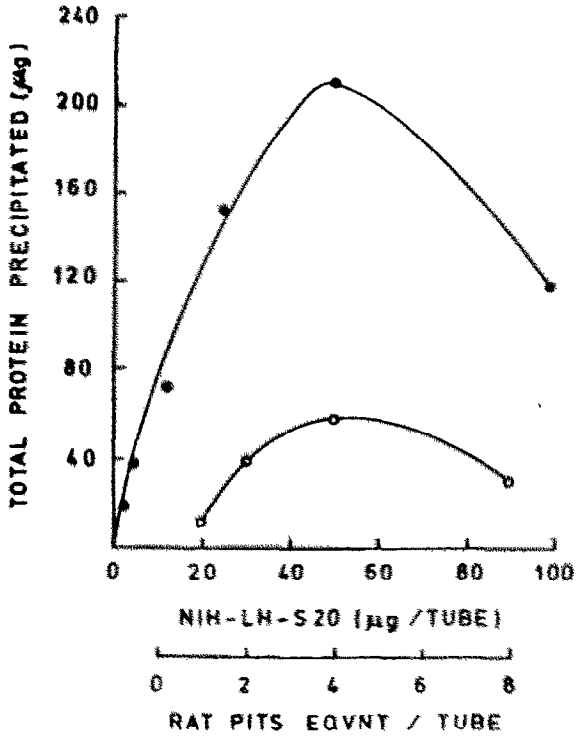

Fig. 1. Quantitative precipitin curve for anti-sheep lutropin serum against sheep lutropin (NIH-LH-S20) and rat pituitary extract. Serial dilutions of an LH solution (100 ug/ml) or rat pituitary extract (8 pituitary equiv./ml) was taken and incubated with 100 μl of anti-sheep lutropin serum for 1 h at 37°C followed by 3 days and nights at 4°C. At the end of incubation contents were centrifuged and protein estimated in the washed pellets: (○—○) rat pituitary extract; (●—●) NIH-LH-S20.

was dissolved in 0.05 N NaOH and taken for radioactive counting.

3. RESULTS AND DISCUSSION

The rabbit immunized against sheep LH produced antibodies which were found to crossreact with rat pituitary LH to the extent of 20–25% as seen from the results of quantitative precipitin test (fig. 1). The antiserum had to be absorbed with NSS in order to remove all non-specific antibodies. The absorbed antiserum gave precipitin lines against sheep LH, buffalo and rat pituitary extracts in an Ouchterlony double diffusion test (not shown). In a direct radioligand binding test also, the antiserum could bind ^{125}I- sheep and ^{25}I-labelled rat LH, exhibiting a crossreaction of 20% (not shown). Thus, by these tests the specificity of antibodies to LH and its ability to crossreact with rat and buffalo pituitary LH was ensured before its use in immunoprecipitations.

The question of presence of sulphate in the oligosaccharide chains of LH was investigated next by using a biosynthetic approach employing the above incubation conditions. Initially, the incorporation of $^{35}SO_4^{2-}$ into trichloroacetic acid-precipitable proteins was shown to be appreciable by 2 h incubation (table 1). The biosynthetic nature of the incorporated radioactivity was established by the following observation. The oligosaccharide-bound sulfate is known to be acid-labile [3]. Hence

Table 1

Incorporation of $^{35}SO_4^{2-}$ into rat pituitary proteins

Duration of incubation time (min)	Hemipituitaries/ flask	Trichloroacetic acid-precipitable radioactivity (cpm/mg protein)
0	7	814 ± 84
120	7	32 007 ± 6470

Rat pituitary halves were incubated with radioactive $^{35}SO_4^{2-}$ in Krebs–Ringer bicarbonate buffer and later processed for trichloroacetic acid precipitable radioactivity; see section 2 for details

in another experiment, the incubated pituitary halves were homogenised and the proteins precipitated with cold trichloroacetic acid. When the precipitate was exposed to hot trichloroacetic acid (by immersion in boiling water bath for 15 min) no radioactivity could be found retained in the precipitate, thus proving the organic nature of the bound sulfate (not shown). The results of immunoprecipitation indicate that radioactive sulfate could get incorporated into immunoreactive LH-like material in both rat and buffalo systems (table 2). The use of a heterologous antiserum was necessitated because of non-availability of homologous anti-rat LH and anti-bovine (buffalo) LH sera in sufficient quantities. The incorporation was marginal in the case of rat pituitary LH but considerable in the case of bovine (buffalo) LH.

Table 2

Synthesis of immunoreactive LH in in vitro incubations of rat and buffalo pituitaries with $^{35}SO_4^{2-}$

Expt. no.	No. of flasks	Pituitaries/ flask	$^{35}SO_4^{2-}$ in medium	Trichloroacetic acid-precipitable radioactivity (cpm/mg. protein)	Immunoprecipitable radioactivity[a] (cpm/flask)
1	6	3	25 μCi/ml	32 007 ± 6470	55
2	4	5	25 μCi/ml	-	87
3	6	8	25 μCi/ml	-	Nil[b]
4	1	25	800 μCi/ml	46 875	820[c]
5	5	1	100 μCi/ml	506 ± 52	6320 ± 742

[a] Radioactivity precipitated with LH antiserum minus radioactivity precipitated with normal rabbit serum
[b] NIH-LH-S20 (25 μg) was added during precipitation
[c] An anti-LH serum with 50–60% crossreaction, with rat LH

Rat hemipituitaries (exp. 1–4) or buffalo pituitary slices (exp. 5) were incubated with radioactive sulphate for 2 h as indicated below. At the end of the incubation, the pituitary halves or slices were processed for both trichloroacetic acid-precipitable and immuno-precipitable radioactivity, see section 2 for details

When the number of glands incubated per flask was increased and also the concentration of radioactive sulfate in the medium, the total incorporation into proteins was still in the same range. However, when an anti-sheep lutropin, which crossreacted with rat LH to 50–60%, was employed, the incorporation into LH could be increased to more appreciable extent (table 2, exp. 4). The specificity of the precipitation was indicated by the fact that when excess of competing sheep LH was added during immunoprecipitation, no radioactivity was detected in the precipitate (table 2, exp. 3). The identity of the precipitated radioacitivity with LH has been further established by detailed chromatographic and electrophoretic analysis of the labelled buffalo pituitary LH-like material preparation.

These results would therefore provide biosynthetic evidence that pituitary LH of rats and buffaloes could have sulfate moieties in them. After the completion of this manuscript, we discovered reports [8,9] with results similar to ours. We have observed that even in the case of rabbit pituitary, sulfate can be incorporated into LH. Therefore, the present results confirm the observations in [9,10]. Direct colorimetric estimation of sulfate released by acid hydrolysis from sheep LH also supports such a conclusion (in preparation). An SDS electrophoretic and autoradiographic study [10] of ^{35}S-labelled rat pituitary proteins did not reveal any sulfated protein in the M_r range of LH subunits; i.e., 15–17 kD a. More detailed structural analyses could clarify the situation.

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