J. Biosci., Vol. 5, Number 4, December 1983, pp. 355-363.

On the presence of sulphate in pituitary lutropin

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MS received 16 July 1983; revised 24 October 1983

Abstract. The presence of sulphate in the carbohydrate of pituitary lutropin from different species has been investigated using a biosynthetic approach. Pituitaries from rats, rabbits, goats, and buffaloes were incubated in the presence of $^{35}SO_4^-$ and the $^{35}SO_4^-$ -labelled proteins in the tissue immunoprecipitated with a well characterized anti-sheep lutropin serum. The incorporation into immunoreactive lutropin was low in the case of rat, rabbit and goat pituitaries while, it was considerable in the case of buffalo pituitaries. Hence further characterization studies were carried out on $^{35}SO_4^-$-labelled proteins of buffaloes. The physico-chemical, immunological and biological properties of radio-labelled buffalo pituitary material were shown to be similar to those of standard lutropin. In *in vitro* conditions of incubations, most of the incorporation of $^{35}SO_4^-$ was observed into tissue lutropin while under similar conditions of incubation, [^{14}C]-amino acids were found to get incorporated mostly into medium lutropin. The physiologically specific releasing hormone, lutropin-releasing hormone was found to stimulate the release of $^{35}SO_4^-$-labelled lutropin from the rabbit pituitaries into the medium. These results give indirect evidence that sulphate could be present in pituitary lutropin.

Keywords. Sulphate incorporation; pituitary hormone; lutropin; gonadotropin; immunoprecipitation.

Introduction

Structural work on the carbohydrate portion of bovine pituitary lutropin (LH) has revealed the presence of sulphate on the terminal hexosamine residues (Parsons and Pierce, 1980). This was based on direct spectrophotometric estimation of sulphate released from the hormone by acid treatment. Supporting evidence for the presence of sulphate moiety in the oligosaccharide chains of LH has come from metabolic studies in which radioactive $^{35}SO_4^-$ has been shown to be incorporated into pituitary LH (Hoshina and Boime, 1982; Kalyan Rao and Bahl, 1983; Rajyalakshmi *et al.*, 1983). The significance of this sulphate moiety is not known. The placental glycoprotein hormone human chorionic gonadotropin (hCG) on the other hand does not have any acid labile sulphate (Parsons and Pierce, 1980).

We are interested in the study of regulation of secretion of LH (Muralidhar *et al.*, 1977). We have characterised the biosynthetic precursor of bovine pituitary α-subunit (Muralidhar and Bahl, 1981) with an idea of locating regulatory sites in the

Abbreviations used: LH, Luteinizing hormone or lutropin; LH-RH, lutropin-releasing hormone; hCG, human chorionic gonadotropin; ECF, ethyl chloroformate; a/s, antiserum; NRS, normal rabbit serum; Da, Dalton (molecular weight); KRBG, Krebs-Ringer Bicarbonate Buffer with 0.2% glucose pH 7.4.

enzymatic pathway leading to the secretion of intact LH. In an extension of this study we recently observed that radioactive $^{35}SO_4^-$ could get incorporated into immunoreactive LH from rat and buffalo (bovine) pituitaries (Rajyalakshmi *et al.*, 1983). We undertook the studies reported here in order to obtain more convincing evidence for this and to determine whether sulphate is present in LH of other species as well as to probe whether sulphation/desulphation have any role to play as storage/secretion signals.

Materials and methods

Hormones, chemicals, animals and antiserum

Lutropin (NIH-LH-S18) and LH-RH were gifts from the National Institute of Arthritis, Metabolic and Digestive Diseases (NIAMDD), NIH, Bethesda, USA. [^{14}C]-Algal protein hydrolysate (Sp. activity 20–30 mCi/m.atom) and $^{35}SO_4^-$ (Carrier free) were purchased from Bhabha Atomic Research Centre, Trombay, Bombay, India. Bovine serum albumin (BSA), Aprotinin and Thimerosal (Grade II) were obtained from Sigma. Chemical Company, St. Louis, Missouri, USA. Dulbecco's modified Eagle's medium (DMEM) was bought from GIBCO laboratories, Grand Island, USA. Freund's complete adjuvant was purchased from DIFCO laboratories, Detroit, USA. All other chemicals were of AnalaR grade.

Antiserum to NIH-LH-S 18 was obtained by immunizing healthy male adult rabbits against LH by the procedure of Vaitukaitis *et al.* (1971). Blood was collected from an ear vein, serum separated, decomplemented and stored frozen with 0.01% azide as a preservative. The rabbit was periodically boosted with a saline solution of LH. The antiserum was purified by absorption with 1:10 diluted normal sheep serum (NSS) to remove nonspecific antibodies against tissue and serum antigens. The cross-reaction of this antiserum with pituitary LH of different species was checked by Ouchterlony double diffusion test (Moudgal and Li, 1961). An immunoadsorbent was prepared from this antiserum by cross linking the antiserum proteins by the use of the bifunctional reagent, ethyl chloroformate (ECF). The procedure was essentially same as that of Avrameas and Ternynck (1967).

Pituitary incubations

Pituitaries were quickly removed from rats (castrated a week earlier), rabbits, goats, or buffaloes at the time of slaughter, cleansed and were cut into quarters or sliced. Pituitaries in all cases were ready for incubation within 1–2 h after killing of animals. These were pooled and randomly distributed into incubation flasks. Incubations were conducted for 2–3 h in a metabolic shaker in either DMEM medium or a modified Krebs-Ringer Bicarbonate buffer (KRB) containing $MgCl_2$ instead of $MgSO_4$, 0.2% glucose and 0.2% BSA, where necessary neutralized [^{14}C]-algal protein hydrolysate (5 µCi/ml) or $^{35}SO_4^-$ (25–100 µCi/gland) were included.

Processing of tissue and medium

The tissue, at the end of the incubation was rinsed three times with the KRBG buffer to get free of incubation medium and then homogenised in KRBG buffer. The

total radioactive proteins were precipitated with 10% trichloroacetic acid. The precipitate was washed 3 times with cold trichloroacetic acid and dissolved in 0.05 N NaOH and the aliquots counted for radioactivity.

For immunoprecipitation, two procedures were followed. In one, aliquots of homogenate after centrifugation (9000 *g*/15 min/4° C) were incubated with antiserum (a/s) or normal rabbit serum (NRS) for 1 h at 37° C followed by three days and nights at 4° C. Protease inhibitor (Approtinin) was included during immunoprecipitation. The immunoprecipitate was washed with chilled saline, dissolved in 0.05 N NaOH and aliquots counted for radioactivity. The media were similarly processed except that they were dialysed extensively and lyophilised before processing for trichloroacetic acid precipitation or immunoprecipitation. In the second procedure a water insoluble polymer of a/s or NRS proteins was used instead of antiserum or normal rabbit serum (Avrameas and Ternynck, 1967).

Chromatography and electrophoresis

The chromatography of $^{35}SO_4^-$ -labelled proteins on SP-Sephadex was done essentially according to the procedure of Papkoff *et al.* (1965). Electrophoresis was performed in 10% gels essentially according to the procedure of Reisfeld *et al.* (1962). At the end of the run, gels were removed, proteins fixed and cut into 2 mm slices. The slices were dissolved in H_2O_2 and counted for radioactivity using 10 ml of Bray's solution (Bray, 1960). Standard sheep LH was also electrophoresed and stained with Coomassie blue.

Bioassay and immunoassay

The biological activity of standard lutropin and the [^{35}S]-labelled material from bovine pituitaries was tested by the Parlow's ovarian ascorbic acid depletion assay (Parlow, 1961). The ascorbic acid content of the ovaries was estimated according to the procedure given in Oser (1976). The immunological identity of LH and [^{35}S]-labelled material was tested by Ouchterlony double diffusion test (Kabat and Mayer, 1972).

Results and discussion

Sulphated proteins in pituitary glands of different species

The specific activity of the sulphated pituitary proteins in different species is given under table 1. Thus the rat pituitary proteins were labelled to the highest specific activity among the species investigated in this study while the buffalo pituitary proteins had the lowest specific activity (table 1). This could be due to different number of sulphated glycoproteins in different animals or due to existence of variation in the degree of sulphation within a limited number of sulphated glycoproteins. From results published by Rosa and Zanini (1981) and Kalyan Rao and Bahl (1983) it could be seen that at least 6–8 sulphated proteins are made by rat and sheep pituitaries respectively (counted from their figures).

We had earlier shown that increasing the concentration of $^{35}SO_4^-$ in the medium had no effect on the specific activity of the tissue protein (Rajyalakshmi *et al.*,1983). Supporting evidence for the organic nature of the bound sulphate was the observation that when the sulphated material is incubated in hot acid (4 M HCl

Table 1. Pituitary halves or quarters or slices were incubated with $^{35}SO_4^-$ in modified KRBG buffer for 3 h. The tissues were processed for total TCA precipitable radioactivity. See Methods section for details. Results are average of two experiments only.

Species	Pituitaries per flask	Approximate weight of the pituitaries (mg)	$^{35}SO_4^-$ in the medium (μCi/flask)	Radioactivity TCA (cpm/mg protein)	Precipitated Antiserum* (cpm/flask)
Rat	5	1	25	32,000	71
Goat	2	150	200	24,000	143
Rabbit	2	3	200	15,390	184
Buffalo	2	1000	200	3,020	4,040

*Radioactivity precipitated with LH a/s minus the radioactivity precipitated with normal rabbit serum.

in boiling water bath), no radioactivity was retained in the material (data not shown). Others have shown that exhaustive pronase digestion of the $^{35}SO_4^-$-labelled material results in quantitative recovery of label in the oligosaccharide chains (Kalyan Rao and Bahl, 1983).

Immunoprecipitation of $^{35}SO_4^-$-labelled proteins of rat, rabbit, goat, and buffalo pituitaries

When the $^{35}SO_4^-$-labelled proteins, synthesized under *in vitro* incubations of pituitaries with $^{35}SO_4^-$ were subjected to immunoprecipitation with the well characterised anti-sheep lutropin serum (Rajyalakshmi *et al*., 1983), all the species studied gave positive results of incorporation of $^{35}SO_4^-$ into immunoreactive LH (table 1). In the case of goat pituitaries, an immunoadsorbent was used instead of a/s. The degree of incorporation was however different in these species. In the case of the rat, rabbit and goat, the incorporation was marginal or low, while significant amounts of radioactivity was incorporated into buffalo pituitary LH (table 1). When computed as per cent of total trichloroacetic acid precipitable radioactivity the value ranged from 0.1% in the case of goat to 3% in the case of rabbit calculated on a mg protein basis. The specificity of the immunoprecipitation was established as described earlier (Rajyalakshmi *et al*., 1983). The studies of Rosa and Zanini (1981) and Kalyan Rao and Bahl (1983) have reported failure to detect incorporation of $^{35}SO_4^-$ into rat pituitary LH using autoradiographic techniques. Our results, where immunoprecipitate was directly taken for radioactive counting show marginal incorporation. Hence it might be too low for autoradiographic detection. However, Hoshina and Boime (1982) have been successful in demonstrating $^{35}SO_4^-$ incorporation into rat pituitary LH. The discrepancy between the results of Hoshina and Boime (1982), Rosa and Zanini (1981) and Kalyan Rao and Bahl (1983) is difficult to explain.

Similarities between [^{35}S]-labelled buffalo pituitary protein and LH

In order to obtain more convincing data on the presence of sulphate in LH, buffalo pituitary glands were incubated with $^{35}SO_4^-$, and [^{35}S]-labelled proteins were subjected to certain purification procedures. We have earlier established that these

steps of purification which are essentially those of Papkoff *et al.* (1965) yield a homogeneous preparation of buffalo LH (T. Rajendrakumar, A. S. Arunasmithasri and K. Muralidhar–unpublished work). Thus, the [^{35}S]-labelled buffalo pituitary proteins were extracted with 0.15 M $(NH_4)_2$ SO_4 solution, nonspecific proteins precipitated with metaphosphoric acid and the supernatant made 50% saturated with respect to $(NH_4)_2$ SO_4. The 50% $(NH_4)_2$ SO_4 precipitate was dialysed and subjected to immunoprecipitation. About 10% of this radioactivity could be precipitated as [^{35}S]-labelled immunoreactive LH. When the same 50% $(NH_4)_2$ SO_4 precipitate after dialysis, was chromatographed on a SP-Sephadex column in 0.01 M Na_2 -HPO_4, 20% of the [^{35}S]-labelled proteins was held up on the column and could be eluted with 0.05 M Na_2-HPO_4. These are exactly similar to the conditions under which LH is purified reinforcing the idea that pituitary LH possibly has sulphate moiety.

The [^{35}S]-labelled material from the 50% $(NH_4)_2$ SO_4 precipitate was checked for immunological and biological activity of LH. As shown in table 2, the material was immunologically active against anti-sheep LH serum and biologically active

Table 2. Immunological and biologica properties of $^{35}SO_4^-$-labelled buffalo pituitary protein fraction.

	Ouchterlony test*	Bioassay† (ascorbate/100 mg ovary)
Saline	−	93 ± 25
NIH-LH-S20	+	52 ± 5
$^{35}SO_4^-$ material	+	34 ± 9.9

* Against rabbit anti-sheep lutropin.

† Ovarian ascorbic acid depletion assay.

in ovarian ascorbic acid depletion assay, specific for LH (Parlow, 1961). When subjected to electrophoresis in 10% acrylamide gels, the major band of radioactivity from among the $^{35}SO_4^-$-labelled proteins of the 50% $(NH_4)_2$ SO_4 precipitate was found to comigrate with the Coomassie blue stainable LH band (figure 1). Hence these results indicate that the $^{35}SO_4^-$-labelled buffalo pituitary protein(s) has physico-chemical, immunochemical and biological properties similar to those of Standard sheep LH. These results do also indicate that there are other sulphated proteins in the pituitary which behave like LH during purification at least upto the SP-Sephadex chromatography step. Similar results are obtained in the case of sheep pituitary glands incubated with $^{35}SO_4^-$ (T. Rajendrakumar, A. S. Arunasmithasri and K. Muralidhar–unpublished work).

Differential labelling of tissue and medium LH

In our studies using [^{14}C]-amino acids as precursors for the synthesis of immunoreactive rat LH, we found that most of the incorporated radioactivity was found in the medium with negligible amounts in the tissue (data not shown). This observation that newly synthesized hormone was secreted preferentially agrees with the

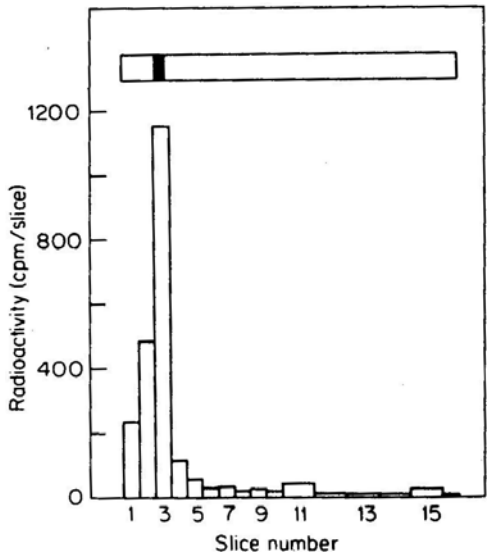

Figure 1. Buffalo pituitary slices were incubated with $^{35}SO_4^-$ The slices were then homogenised and the homogenate subjected to purification steps as described by Papkoff *et al.* (1965). The 50% $(NH_4)_2$ SO_4 precipitate was subjected to electrophoresis in a 10% polyacrylamide gel according to Reisfeld *et al.* (1962). Standard LH (100 µg) was run in another gel and stained with Coomassie Blue. The radioactive gel was sliced and the slices counted for radioactivity.

observations of other workers (Labrie *et al.,* 1971). Under similar conditions of incubation with $^{35}SO_4^-$ as precursor, we found most of the incorporation into tissue LH with negligible amounts into medium LH (table 3). This is in contrast to what we observed in the case of [^{14}C] -amino acids. Hoshina and Boime (1982) reported

Table 3. Pituitary quarters were incubated with $^{35}SO_4^-$ modified KRBG buffer for 2 h. The tissue and media were separately processed for immunoreactive LH. See Methods for details. Results are average of two experiments only.

Species	Pituitaries per experiment	$^{35}SO_4^-$ in the medium (µCi/flask)	Radioactivity precipitated* (cpm)	
			Tissue	Medium
Rat	25	800	1950	Nil.
Rabbit	2	200	400	85

★Radioactivity precipitated with LH a/s minus radio activity precipitated with NRS.

recently that they could measure $^{35}SO_4^-$-labelled free α-subunit in the medium of bovine pituitary incubations while detection of $^{35}SO_4^-$-labelled β-subunit or whole LH was reported to be difficult. In the studies of Kalyan Rao and Bahl (1983), the immunoreactive LH and $^{35}SO_4^-$-labelled protein from the medium did not co-chromatograph on Sephadex G-100, while from the tissue both behaved identically on Sephadex .G-100. Most of the medium radioactivity was eluting at a posi-

tion characteristic of α-subunit of LH. Our antiserum against whole LH has no significant cross reaction with α-subunit (data not shown) and hence we cannot measure this constituent specifically in our experiments. While in our studies we measured radioactivity directly in the immunoprecipitates, Hoshina and Boime (1982) tried to detect $^{35}SO_4^-$-labelled LH, α-subunit or β-subunit by electrophoresis and autoradiography subsequent to immunoprecipitation. In view of the low degree of incorporation into medium constituents, it is not surprising that they could not measure with ease [^{35}S]-labelled medium LH or β-subunit.

Effects of LH-RH

We have consistently observed that the incorporation of $^{35}SO_4^-$ into medium LH was negligible. That the specific activity of medium LH is much lower than tissue LH has been observed by others also (Kalyan Rao and Bahl, 1983). We thought it would be interesting to enhance the release of the hormone by LH-RH. In these experiments, rabbit pituitary quarters were first incubated with $^{35}SO_4^-$ for 2 h, rinsed free of initial medium and incubated in a fresh medium free of radioactivity in the absence or presence of fixed dose of LH-RH for an additional 30 min. The content of $^{35}SO_4^-$-LH in the tissue and media was measured. The content of free sulphate in tissue and media was also measured. We found that while there was no significant increase in $^{35}SO_4^-$ incorporation into tissue LH, LH-RH caused enhanced release of $^{35}SO_4^-$-LH into the medium (figure 2). When free $^{35}SO_4^-$ was measured in tissue and media and the results expressed as ratio of medium

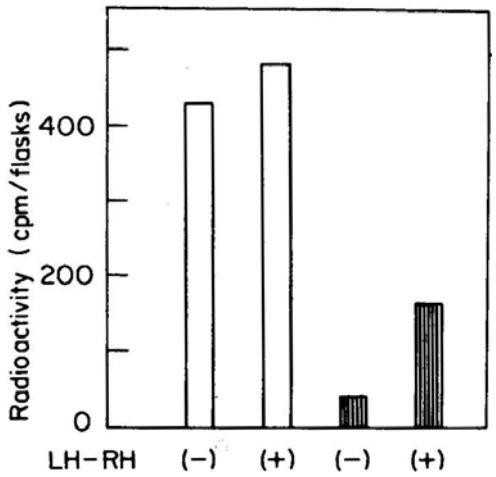

Figure 2. Rabbit pituitary quarters were incubated first in the presence of $^{35}SO_4^-$ At the end of 2 h, they were rinsed free of radioactivity and incubated for an additional 30 min in radioactivity free KRBG buffer pH 7.4. At the end of the incubation, $^{35}SO_4^-$-labelled LH was measured in tissue and medium. Results of a representative experiment is given, (□), $^{35}SO_4^-$-labelled LH in the tissue; (▨), $^{35}SO_4^-$-labelled LH in the medium.

radioactivity over tissue radioactivity, LH-RH was found to cause a consistent drop in the ratio at all the doses tested (table 4). These results would indicate that LH-RH could have enhanced the rate of utilization of $^{35}SO_4^-$ for sulphation. However, this has to be confirmed by actual assay of the enzymes involved in the activation and incorporation of sulphate into oligosaccharides of LH.

Table 4. Rabbit pituitary quarters were incubated with $^{35}SO_4^-$. They were then incubated in a fresh radioactivity free medium in the presence of LH-RH. Total radioactivity was measured in tissue and media.

LH-RH (ng/ml)	Radioactivity (cpm)		M/T
	Tissue (T)	Medium (M)	
0	99660	215090	2.1
100	92100	99000	1.07
400	125480	152370	1.2
1600	64680	105160	1.6
3200	76280	111200	1.4

Evidence for the presence of sulphate in pituitary LH has come from two approaches. In one, direct estimation of acid released sulphate from the hormone was undertaken (Parsons and Pierce, 1980). In the second approach metabolic studies indicated that $^{35}SO_4^-$ could get incorporated into pituitary immunoreactive LH (Hoshina and Boime, 1982; Kalyan Rao and Bahl, 1983; Rajyalakshmi *et al.*, 1983). The latter evidence would still be circumstantial. Recently, Kalyan Rao and Bahl (1983) reported the purification of $^{35}SO_4^-$-labelled protein from sheep pituitaries incubated with $^{35}SO_4^-$ to apparent homogeneity and the establishment of the exact location of bound sulphate. In both our present report and the studies of Kalyan Rao and Bahl (1983), the absolute biological identity (100 % recovery) of $^{35}SO_4^-$-labelled pituitary protein with LH has not been proved while physico-chemical and immuno-chemical similarities between the two have been demonstrated. One way to do this would be to prove that $^{35}SO_4^-$-labelled pituitary protein purified to homogeneity is active fully in receptor binding assays. Our present results, showing that an agent like LH-RH which is known to enhance the release of immunoreactive LH from pituitaries could, also cause enhanced release of $^{35}SO_4^-$-labelled material (which was precipitated by the same antiserum), is a physiological proof that pituitary LH could have sulphate.

The role of this sulphate moiety is not known at the present. It is possible that it might serve as a signal for storage of the hormone. Thus there might be two pools of LH, one for immediate secretion, and another for storage and secretion in response to acute demand. The latter pool may represent the sulphated portion. We do not as yet know of any role for sulphate either in contributing to plasma half life or in hormone action. It could also contribute to the structural heterogeneity of LH like sialic acid does in the case of hCG.

Acknowledgement

Work reported here was supported by funds from the University Grants Commission, New Delhi, India.

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