IMMUNOCHEMICAL COMPARISONS BETWEEN
A LOW MOLECULAR WEIGHT ADENOHYPOPHYSEAL
CONSTITUENT AND THE GONADOTROPHINS

By

H. Hall and M. P. Dubois

ABSTRACT

A biologically active peptide (called sperm-releasing substance, abbreviated SRS) with a molecular weight of about 5000, prepared from bovine adenohypophyses, has been compared with the known adenohypophyseal glycoprotein hormones using immunological methods. By means of immunofluorescence it can be seen that antisera against SRS have an affinity for the gonadotrophic cells in the anterior pituitary. Using complement fixation assay and immunodiffusion, no similarity between SRS and FSH can be found, while there are great resemblances between the reactions of LH and those of SRS. From the radioimmunoassay studies, it can be seen that the α-subunit of LH reacts in the same way as SRS in the different systems. However, using immunofluorescence, the antiserum against SRS has an affinity for the gonadotrophic cells only, like anti-LHβ, and not for the thyrotrophic cells, as has anti-LHα. The reasons for these different results are discussed.

The release of spermatozoa from the testes of frogs has often been used in the study of the physiology of the gonadotrophins. It has thus been used for comparative studies of the vertebrate gonadotrophins (Dodd 1960) and for bioassay of the gonadotrophins of fish (Burszawa-Gérard 1971). It has also been used for the diagnosis of pregnancy (Galli-Mainini 1947). Licht (1973) studied the spermiation response using highly purified mammalian pituitary gonadotrophins and their subunits. Kihlström & Danninge (1970) discovered
a factor present in the pituitary with rapid effects on the spermiation. This factor was considered not to be a gonadotrophin, and the molecular weight was later estimated to be approximately 5000 (Lakomaa, subm. for publ.). Further studies of this factor have shown that it is present in species from many different phyla, and in mammalian species including man (Kihlström et al. 1971) and that it probably also has some effects on the mammalian reproductive system (Lakomaa & Kihlström 1972). Some studies on the distribution of the factor in the organism show a similarity between this factor and the two gonadotrophic hormones, i.e. an accumulation in both the ovary and the epididymis (Kihlström et al. 1975; Hall et al. 1975). The present study deals with an immunological comparison between this factor, called sperm-releasing substance (abbreviated SRS) by Lakomaa (1974) and the two gonadotrophins and the subunits of the luteinizing hormone.

MATERIAL

1. Hormones and peptides

Sperm-releasing substance. – Two different preparations of the sperm-releasing substance, called SRSITF and SRSIELF, have been used. The preparations were performed by Drs. E. Lakomaa and H. Hall, according to Lakomaa (subm. for publ.). Acetone powder from bovine adenohypophyses (Biofac A/S, Copenhagen, Denmark), was first treated in dilute acetic acid (0.25%) at 100°C, and the lyophilized supernatant was then treated in water at 100°C. The supernatant was fractionated on Sephadex G50 Fine, and the fractions containing SRS were further purified using isotachophoresis on polyacrylamide gel. SRS was now found as a single peak, eluted last in the system. After desalting this fraction was used in this investigation (SRSITF). After purification using isoelectric focusing in a sucrose density gradient and subsequent desalting, the preparation called SRSIELF was obtained. The yields were approximately 0.1 mg SRSITF or 0.01–0.02 mg SRSIELF per bovine hypophysis.

LH (bovine). – Prepared by Dr. C. Courte (Paris). Activity approximately 2 NIH LH-B5-units per mg.


FSH (ovine). – Prepared by Prof. M. Jutisz. Activity approximately 1 NIH FSH-S3-unit per mg.

2. Antisera

Rabbit-anti-SRSITF. – Prepared by Dr. H. Hall. Antisera from two different rabbits, two different bleedings of each, have been used.

Rabbit-anti-SRSIELF. – Prepared by the authors. Only the antiserum from one rabbit has been used, although from different bleedings.

Rabbit-anti-bovine LH. – Prepared by Dr. M. Dubois, using bovine LH, prepared by Dr. C. Courte (activity approximately 2 NIH LH-B5-units per mg).

Rabbit-anti-porcine LHa. – Prepared by Dr. M. Dubois, using porcine LHa, prepared by Dr. C. Courte.

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Rabbit-anti-porcine LHβ. – Prepared by Dr. M. Dubois using porcine LHβ, prepared by Dr. C. Courte.

Rabbit-anti-ovine FSH. – Prepared by Dr. M. Dubois, using FSH-M2, prepared by Prof. M. Jutisz (activity approximately 8 NIH FSH-S3-units per mg).

Rabbit-anti-bovine TSH. – Prepared by Dr. M. Dubois, using NIH TSH-B4. The antiserum has been saturated with 400 µg NIH LH-B5 per ml concentrated antiserum.

Rabbit-anti-PMSG. – Prepared by Dr. M. Dubois, using PMSG, prepared by Organon (Holland) (activity approximately 6000 IU per mg).

Rabbit-anti-HCG. – Prepared by Dr. M. Dubois, using HCG, prepared by Clin-Byla (France) (activity approximately 3000 IU per mg).

Rabbit-anti-sheeperythrocytes (“hemolysin”). – Prepared by Institut Pasteur, Paris (France).


Sheep-anti-rabbitgammaglobulin. – (coupled with fluoresceinisothiocyanate). Prepared by Institut Pasteur, Paris (France).

The antisera were diluted with bidistilled glycerin (1:1) and stored at −20°C (except for the sera to be used in immunodiffusion studies, which were stored at −20°C, unglycerinated) or were stored unglycerinated at +4°C (sodium azide to the final concentration of 0.1 % added).

3. Other material

Complement. – From guinea-pigs. Produced by Institut Pasteur, Paris or Institut Merieux, Lyon (France).

Na125I. – Prepared by The Radiochemical Centre, Amersham (England).

METHODS

1. Immunizations

Preparation of anti-SRSITF. – The immunizations were carried out without coupling of SRSITF to a carrier molecule. One hundred µg or 250 µg of SRSITF was emulsified in Freund’s complete adjuvant and was injected intradermally into several sites in the back of the rabbit. One half ml of the vaccine Bordetella pertussis (Statens Bakteriologiska Laboratorium, Solna, Sweden) was injected simultaneously at one site (Vaitukaitis et al. 1971). Re-immunizations were carried out after 11 and 16 weeks respectively. The antisera used in this study were collected from the ear vein 11 and 18 days after the third immunization.

Preparation of anti-SRSIELF. – The antigen was coupled to a carrier, thyroglobulin, according to Vance et al. (1969). Approximately 200 µg SRSIELF coupled to 2.5 mg thyroglobulin in phosphate buffered saline was used each time, emulsified in Freund’s complete adjuvant. Half of the dose was injected into the spleen and the other half intradermally into several sites in the back of the rabbit. Re-immunizations (the whole dose intradermally) were carried out after three and seven weeks. The other rabbit-antisera were produced in a manner similar to that of the preparation of anti-SRSIELF.

2. The immunofluorescence study was carried out on sections of bovine or porcine pituitaries according to Dubois (1971a) using the double antibody technique.

3. The radioiodination was carried out according to Greenwood et al. (1963) using 0.5 mCi 125I and 5 µg SRSIELF each time.
Fig. 1.
Immunofluorescence on subsequent, porcine pituitary sections incubated with anti-SRNS1ELF (upper left), anti-LHa (upper right), anti-LHβ (lower right) and anti-TSH (lower left), respectively. The arrows show areas with thyrotrophic cells (anti-TSH) and with gonadotrophic cells (anti-LHβ).
4. The radioimmunoassay was performed with the double antibody technique. The specific antibody was incubated with the tracer (approximately 10 000 cpm corresponding to roughly 0.4 ng SRSIELF) and inhibiting antigen for three days at +4°C, after which the second antibody was added for an additional incubation over night at +4°C. After centrifugation the supernatant was discarded and the activity of the precipitate was counted in a gammacounter.

5. The complement fixation assay was performed according to Dubois (1971b).

6. The immunodiffusion study was performed according to Ouchterlony (1948). Five to 10 µl of concentrated antiserum was added to one basin in the agar gel, and 5 µl of the antigen solution was added to basins, 7 mm from the basin with the antiserum.

RESULTS

1. Immunofluorescence (Fig. 1 and Table 1)

On the basis of qualitative studies (Fig. 1), it is apparent that anti-SRSIELF has only an affinity for the gonadotrophic cells. This can be seen when comparing the affinities of anti-SRSIELF, anti-LHα, anti-LHβ and anti-TSH. No thyrotrophic cells are fluorescent using anti-SRSIELF or anti-LHβ, while the use of anti-LHα or anti-TSH gives more or less fluorescent thyrotrophic cells. The gonadotrophic cells are also fluorescent when using anti-LH and anti-SRSITF. Since anti-SRSITF also has an affinity for the thyrotrophic cells, there is a distinct difference between the two SRS-antisera. Anti-LH also has an affinity for the thyrotrophic cells although much weaker than the affinity of anti-SRSITF. In Table 1, the lowest amount of inhibiting antigen giving total inhibition of the fluorescence is shown for a number of different antibodies

<table>
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<th>anti-SRSIELF 1:1000</th>
<th>anti-SRSITF 1:400</th>
<th>anti-LH 1:1000</th>
<th>anti-LHα 1:200</th>
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The numbers show the lowest amount of antigen needed for total inhibition (µg per 500 µl diluted antibody). The dilutions of the antisera were chosen to give 4–8 times more concentrated antisera, than that dilution, which does not give fluorescence without the addition of inhibiting antigen (final dilutions of the antisera shown).
The ability of different antisera to bind iodinated SRSIELF, shown in per cent of total radioactivity added as a function of the final antibody dilution.

and antigens. Small amounts of LHα as well as of LHβ inhibit the reaction of anti-SRSITF and anti-SRSIELF, and each of the two preparations of SRS can inhibit the reactions of all these antibodies. Assuming that the serum with anti-SRSIELF contained two immunologically active systems, one anti-SRS of low titer and one anti-LH of higher titer, and that only the activity of anti-LH was seen, higher concentrations of the serum were incubated with different amounts of LH in order to saturate the anti-LH-part of the serum. The result of this was that no specific antibodies against SRS, which did not react with LH, could be found at the highest concentration used, 1:12.5.

2. Radioimmunoassay (Figs. 2–4)

The ability of different antisera to react with the iodinated SRSIELF is shown in Fig. 2. The antisera against LH, LHα and FSH have great affinities for SRSIELF, while the antisera against LHβ, PMSG and HCG have much lower affinities. Using homologous systems, however, the antiserum against LHβ has a much higher titer than that of LHα (50 % binding of added tracer with anti-LHα, dilution 1:20 000, and with anti-LHβ, dilution 1:250 000, re-
respectively). The two antisera against the subunits are raised against the porcine peptides, but they both react well with LH in bovine pituitaries (immunofluorescence). The other antisera used in this experiment (anti-LH, anti-FSH, anti-HCG, anti-PMSG) are all antisera with high specificities and high titers. Fig. 3 shows the standard curves of anti-SRSITF and of anti-SRSIELF using iodinated SRSIELF as tracer and SRSIELF, LH, FSH, LHα and LHβ as inhibiting antigens. There are similarities between the curves of LH, LHα and SRSIELF, while the curves of FSH and LHβ differ. There is also a difference between the two antisera against SRS when FSH is the inhibiting antigen. Using anti-SRSITF (Fig. 3 a) the slope is close to the slope of the others until the level of 1 pmol of added antigen, while using anti-SRSIELF (Fig. 3 b) the slope differs from the others, excluding that of LHβ, with a factor 50 times the amount of the antigen added. The lowest amount of antigen added showing a difference from 100 % is approximately 1 pmol, which gives a decrease of about 10 % in the amount of tracer bound. In the [125I]LH-anti-

**Fig. 3 a.**

Heterologous standard standard curves using iodinated SRSIELF and anti-SRSITF (1:8000 final dilution) with various antigens as inhibiting antigens, shown in per cent of B₀ (100 % = amount of radioactivity bound without any inhibiting antigen) as a function of the amount of inhibiting antigen added.
Heterologous standard curves using iodinated SRSIELF and anti-SRSIELF (1:1000 final dilution) with various antigens as inhibiting antigens, shown in per cent of $B_0$ as a function of the amount of inhibiting antigen added.

LH-system (Fig. 4) (performed by Dr. M. M. de Reviers, Nouzilly) LHα and SRS give a slope parallel to that of LH, but at a higher amount of added antigen.

3. Complement fixation assay (Fig. 5)

As can be seen in Fig. 5 a, anti-LH fixes the complement with either SRSITF or SRSIELF, and the similarity between the two systems is well demonstrated. Anti-SRSITF fixes the complement with LH or SRSITF (Fig. 5 b) but not with FSH (Fig. 5 c), and moreover, anti-FSH with SRSITF could not fix the complement (Fig. 5 d). An addition of SRSITF to anti-FSH before the test could not inhibit the ability of the FSH-anti-FSH-system to fix the complement. Anti-SRSIELF was not able to fix the complement with any antigen, nor was anti-LHβ. Anti-LHα was not tested because of the low titer of the serum.

4. Immunodiffusion (Fig. 6)

Anti-SRSITF gives precipitates with LH, SRSITF and SRSIELF (Fig. 6 a), and also with LHα and LHβ (Fig. 6 b) where it can be seen that the strongest
precipitates are formed with LHβ. Up to three different lines have been distinguished using SRSITF, and two lines when using LH in the anti-SRSITF system. The two lines of LH-anti-SRSITF are apparently the same as two of the three lines of SRSITF-anti-SRSITF. Furthermore as can be seen in Fig. 6 a, weak spurs occur showing partial identity between LH and SRSITF as well as between LH and SRSIELF. Weak spurs, indicating partial identity, are sometimes also formed using anti-SRSITF with LHα and SRSITF or with LHα and SRSIELF. However, in Fig. 6 b no spurs can be seen at all. Anti-LH gives very weak precipitates, but one precipitate seems to be identical between LH and SRSITF. Very weak precipitates are formed with the systems FSH-anti-SRSITF and SRSITF-anti-FSH, while the homologous systems give very dense precipitates. This coincides well with the results obtained using complement fixation assay, showing a difference between SRSITF and FSH. The weak precipitates of the systems with anti-SRSITF and anti-FSH seem

![Graph](image)

**Fig. 4.**
Standard curves using iodinated LH and anti-LH with various antigens as inhibiting antigens, shown in per cent of B₀ as a function of the amount of inhibiting antigen added.
however to be identical with both SRSITF and FSH as antigens. No precipitates are formed using anti-SRSELF with any antigen, nor using anti-LHβ. This is perhaps partly due to a species specificity, since anti-LHβ is raised against porcine LHβ.

DISCUSSION

Fig. 2 shows that iodinated SRSIELF reacts only with the antibodies against LH, LHα and FSH. LH and FSH both contain the α-subunit, and it can thus be expected that the antisera against these three proteins have antibodies against LHα. This similarity of SRS to the pituitary gonadotrophins is also shown in immunofluorescence (Fig. 1), as the gonadotrophic cells are fluorescent after an incubation with either of the two antisera against SRS.

Figs. 3a and b.

a. Complement fixation assays with the systems SRSITF-anti-LH and SRSIELF-anti-LH. Upper horizontal line shows absorbance when the antigen was omitted, and the lower horizontal line absorbance when the complement was omitted.

b. Complement fixation assays with the systems SRSITF-anti-SRSITF and LH-anti-SRSITF. Horizontal line shows absorbance when the complement was omitted.
c. Complement fixation assays with the systems SRSITF-anti-SRSITF and FSH-anti-SRSITF. Upper horizontal line shows absorbance when the antigen was omitted, and the lower horizontal line absorbance when the complement was omitted.

d. Complement fixation assays with the systems SRSITF-anti-FSH and FSH-anti-FSH. Upper horizontal line shows absorbance when the antigen was omitted, and the lower horizontal line absorbance when the complement was omitted.

Of the gonadotrophins, LH reacts similarly to SRS using many different immunological methods, while the reactions of FSH differ more or less from those of SRS. As can be seen from the immunodiffusion plates, LH shows identity or partial identity with SRSITF or SRSIELF (Fig. 6 a). Apparently two immunological systems are identical in LH and SRS. The significance of the reaction of partial identity is discussed below. Moreover in complement fixation assay LH reacts similarly to SRSITF and SRSIELF when using either anti-LH or anti-SRSITF, indicating homology between the two proteins. This similarity can also be seen from the curves of radioimmunoassay (Fig. 4), where the standard curves are parallel.

The reactions of the other pituitary gonadotrophin, FSH, show great differences from those of SRS, however. No fixation of complement of the system
Immunodiffusion. Anti-SRSITF has been added to the central basins (5 and 10 µl, respectively), and the antigens have been added to the surrounding basins as shown in the photos (µg antigen per basin).

FSH–anti-SRSITF (or the inverse) can be seen (Fig. 5 a and b), and SRSITF in high concentrations cannot inhibit the ability of complement fixation of the homologous FSH-system. Using immunodiffusion, only weak precipitates are formed with FSH and anti-SRSITF (or the inverse), while the homologous systems give very strong precipitates. The standard curves of radioimmunoassay also indicate a difference between SRS and FSH. In this case the type of reaction differs between the two different antisera against SRS with FSH as inhibiting antigen. It is, however, very difficult to give an explanation for these differences.

There are some paradoxes concerning the relations of SRS to the subunits of LH when comparing the results obtained by different methods. Using radioimmunoassay, there is a great similarity between the two preparations of SRS and L.Hα (and their antibodies). By studying the immunofluorescent cells, it can be stated that anti-SRSIELF does not have affinity for the same cells as anti-LHα, but for the same as anti-LHβ. These differing results could be due to the different denaturation steps used in the different methods. The radioiodination of the peptide for radioimmunoassay includes a denaturating, oxidizing step, and the tyrosine residues are blocked. The fixatives formol and sublimate, used when fixing the microscopical preparation, are strongly denaturating, acting especially on the sulphur-containing amino acids and on the free amino groups. Thus, the immunological determinants may be altered
in different ways when using the different methods. No denaturing step is used in immunodiffusion, where a similarity between SRS and both LHα and LHβ can be seen. This could be in parallel to the differences obtained when using anti-FSH. By radioimmunoassay, anti-FSH, reacting toward highly purified [125I]FSH, is not inhibited by LH, but by immunofluorescence on bovine or ovine pituitary, the same anti-FSH-antiserum is inhibited totality by amounts of LH, ten to twenty times smaller than the smallest amount of purified FSH required.

As SRS has some similarities with LHα, it could be expected that there would also be similarities between SRS and FSH. A masking or alternation of the immunological determinants of the α-subunit of FSH by the β-subunit, but the absence of such a masking in the native LH, could explain why FSH does not react like LH. From Fig. 4 it can be seen that SRSIELF and LHα react strongly in the LH radioimmunoassay. However, FSH does not cross-react more than 1–2 % in this system (Blanc, personal communication), probably due to a masking of determinants. However, as is indicated by the precipitation patterns of the immunodiffusion, it is possible that the SRS-molecule contains a “β-part” (but hardly as a subunit) with reactions specific to SRS. This “β-part” may also alter the immunological reactions of the “α-part” (these hypothetical “parts” of SRS must not be confused with the α- and β-subunits of LH and FSH). But if SRS is derived from the LH-molecule, the spurs of the immunoprecipitates could be the result of a unmasking of the determinants of LH during the preparation of SRS. However, attempts to prepare SRS from LH have been carried out, but no sperm-releasing activity could be detected after such preparation (Lakomaa 1974). The fact that both LHα and LHβ react with the antibodies against SRS could be due to the similar sequences of these two peptides (Ward et al. 1973), and to the fact that SRS also contains these sequences. But, on the other hand, however, the same sequences occur in the two subunits of FSH (Shome & Parlow 1974a,b) and in TSH (Pierce et al. 1971) (Fig. 7). Unfortunately, no subunits of FSH or TSH have been available during this study.

The differences between the reactions of SRSITF and SRSIELF and their antibodies show the need for the last purification step namely isoelectrical focusing. The less purified peptide, SRSITF (or the corresponding antibody) reacts more or less as LHα, also in immunofluorescence, and the fluorescence of anti-SRSITF can not be inhibited by large amounts of LHβ. Anti-SRSIELF has a pattern of fluorescence similar to that of anti-LHβ, and can be inhibited by LHα as well as by LHβ. However, the reactions of complement fixation are very similar when using either SRSITF or SRSIELF. This shows that the major component in SRS is the same in the two preparations, but that there are probably contaminants in SRSITF giving antibodies which react like anti-LHα in immunofluorescence.
Comparisons between the amino acid sequences in the subunits of the pituitary glycoprotein hormones. The amino acids which are similar in LHα and LHβ (not human) are underlined. The numbers refer to the number of amino acids, counted from the amino end of the peptide chain (numbers in brackets refer to FSHα). In hLHβ, hFSHβ and bTSHβ only the amino acids differing from bLHβ are noted. The Cys-Cys-bridges have been established only in oLHβ. Data from Pierce et al. (1971), Shome & Parlow (1973, 1974a,b) and Ward et al. (1973). (o = ovine, h = human, b = bovine).

It cannot be excluded, that SRSIELF also contains a contaminating part, and that this contaminating part is a part of LH. If the cross-reaction in the LH-system (Fig. 4) is due only to contamination of SRS with LH, it can be calculated that SRSIELF contains approximately 20% contaminating LH-reactive antigen. However, it can be presumed that there is only one immunologically reacting antigen.

The molecular composition of SRS still remains obscure. These results show a great similarity between SRS and the two subunits of LH. However, since no similarities between the other pituitary glycoproteins (FSH and TSH) have been found, it is unlikely that SRS is a part of LHα. SRS may be a peptide, single-chained, with some immunological determinants similar to those of LHα and LHβ, and with some specific determinants. Only an analysis of the sequence of amino acids in SRS can give the final solution to the problem of the molecular relations between SRS and the gonadotrophins.

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