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PROBLEMS WITH THE ASSAY OF LH-RH IN BIOLOGICAL FLUIDS

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SUMMARY

While sensitive assays of hypothalamic peptide regulatory hormones are readily established in buffer solution, their application to biological fluids has been associated with a number of problems.

The concentration in peripheral fluids would be expected to be very low. There is also evidence for pulsatile secretion with peaks that are rapidly cleared : this means that frequent sampling has to be used. Several tissues (for example : hypothalamus) contain peptidases which can destroy both the biological and immulogical activity. Finally it is necessary to authenticate the LH-RH assay by biological correlations, immunochemical and chromatographie properties.

Some results have been obtained from hypothalamus, isolated netve terminals and hypophysial portal blood, but there are some doubt concerning results in human and sheep peripheral blood.

I. — INTRODUCTION

The ability to measure the low levels of the hypothalamic peptide regulatory hormones in biological fluids would be of great value in increasing our understanding of the role of the central nervous system in the control of the pituitary gland in health and disease. Unfortunately, whilst sensitive assays are readily established in buffer solution, their application to biological fluids has been associated with a number of problems.

The first problem with regard to the decapeptide, luteinising hormone-releasing hormone (LH-RH) is one of nomenclature. A list (perhaps incomplete) is given in figure 1. Everyone appears to agree on the « R » but some sceptics still prefer « F » to « H » and four abbreviations have used in front of the « R ». It is worth noting that at a two-day meeting at the Royal Society of Medicine in London in February

1975, seven of these eight possible permutations was used. Internationally « gonadorelin » and « gonadoliberin » are being advocated which at least have the merit of recognising that there is a single gonadotrophin releasing hormone. In this paper I shall use the term « LH-RH ».

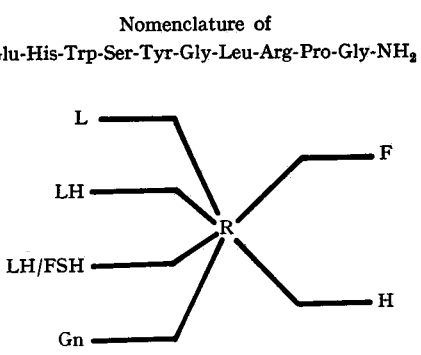

FIG. 1. — *Possible nomenclature of the hypothalamic decapeptide, gonadotrophin releasing hormone*

The presence of immunoreactive LH-RH-like material has been reported in many anatomical situations from the cerebral cortex to urine (JEFFCOATE *et al.*, 1975). Many of these reports require further validation before they can be accepted. In this paper I shall describe the problem associated with LH-RH assays, the criteria we use in an attempt to authenticate the LH-RH radioimmunoassays and discuss their application to the study of the immunoreactive peptides found in hypothalamic tissue, human urine, human peripheral blood and sheep jugular venous blood.

2. — PROBLEMS ASSOCIATED WITH ASSAY OF LH-RH IN BIOLOGICAL FLUIDS

A. — *Low levels*

Because LH-RH is secreted into the hypothalamo-hypophysial portal system, the resulting large dilution means that the concentration in peripheral fluids would be expected to be very low. For instance in human peripheral blood, levels are frequently undetectable (less than 0.25 pg/ml) and the mean level may be 0.8 pg/ml. (JEFFCOATE and HOLLAND, 1974 *a*). Thus, assays need to be highly sensitive.

B. — *Episodic secretion and rapid clearance*

For LH-RH there is evidence for pulsatile secretion with peaks that are rapidly cleared. There is evidence for this in sheep (CRIGHTON *et al.*, 1973 ; FOSTER *et al.*,

1974) and in humans (JEFFCOATE and HOLLAND, 1974 *a* ; SEYLER and REICHLIN, 1974 *a*). This means that frequent sampling has to be used to establish patterns of secretion.

C. — *Enzymic degradation*

Although LH-RH is stable in blood and urine, at least for periods up to 60 min at 37° (JEFFCOATE *et al.*, 1975) several tissues contain peptidases which can destroy both the biological and immunological activity of LH-RH. These are shown schema-

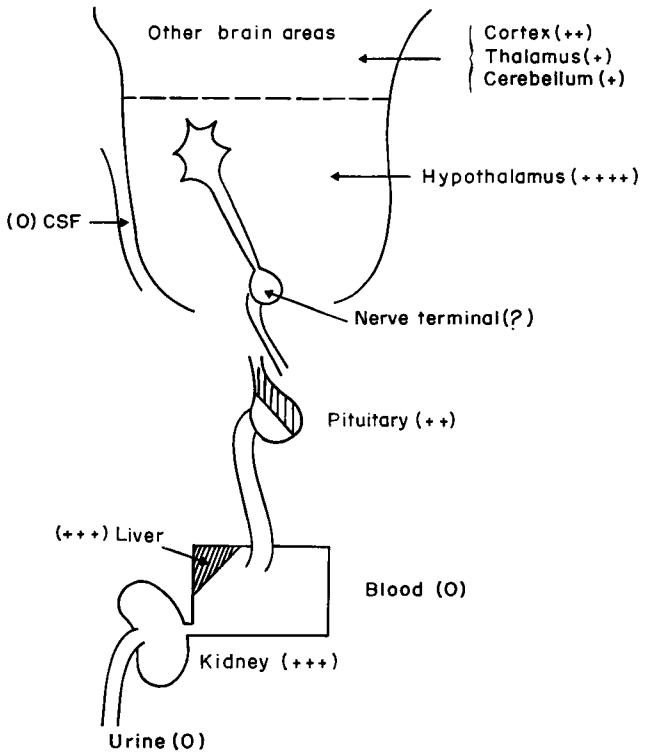

FIG. 2. — *Anatomical sites at which peptidase inactivation of LH-RH takes place.*
The degree of enzyme activity is indicated roughly by the scale (+) ⟶ (++++).
0 : no inactivation

tically in figure 2. The hypothalamic peptidases are of particular interest since their level of activity is related to the gonadal status of the animal and they may thus play a role in the feedback control of pituitary gonadotrophin secretion.

D. — *Doubts about the identity of immunoreactive material*

This applies to any immunoassay and we have attempted to apply a number of criteria in an effort to authenticate the LH-RH assay.

3. — AUTHENTIFICATION OF LH-RH RADIOIMMUNOASSAY

A. — *Biological correlations*

We have found two situations in which LH-releasing activity measured biologically was different in a tissue extract from its LH-RH immunoreactivity. These are : in chicken hypothalamus where there is about 50 times more biological than immunological activity ; and in sheep jugular venous blood where the peaks of immunoreactive material have no detectable biological activity.

B. — *Immunochemical properties*

A dose-response curve of the unknown immunoreactive material parallel to that of the standard is a *sine qua non* of radioimmunoassay validation. Unfortunately, in assays such as that of LH-RH, the sensitivity potential of the assay is being stretched and there is insufficient material to perform dilutions. Even when parallel curves are obtained the interpretation depends on a full knowledge of the immunochemical specificity of the antisera. All our antisera (JEFFCOATE, FRASER and HOLLAND, 1975) cross-react with LH-RH analogues and fragments provided residues tyr 5 to gly-amide 10 are intact. Thus possible metabolites such as the 2-10 nonapeptide and 3-10 octapeptide which might occur in biological fluids would not be distinguished from the decapeptide. With a highly specific antiserum (e.g. that of NETT *et al.*, 1973) the possibility of metabolites cross-reacting is small.

C. — *Chromatographic properties*

In order to establish identity between an unknown and a standard peptide, similar mobilities in as many chromatographic systems as is practicable should be demonstrated. For LH-RH, gel filtration, thin-layer adsorption chromatography, ion-exchange chromatography and electrophoresis have been valuable.

D. — *Enzymatic inactivation*

LH-RH is inactivated by peptidases present in many tissues as described above. Since the kinetics of inactivation with respect to time and enzyme concentration can be accurately quantitated, unknown peptides can be compared with the standard peptide for their ability to act as substrates for these enzymes. This approach is best illustrated by studies with TRH (JEFFCOATE and WHITE, 1975) but can be also applied to LH-RH.

4. — IMMUNOREACTIVE LH-RH IN THE HYPOTHALAMUS

Immunoreactive LH-RH has been extracted from hypothalami from a number of mammalian species and has the immunochemical and chromatographic characteristics of the decapeptide LH-RH (JEFFCOATE and HOLLAND, 1974). Extracts

of chicken hypothalami also contain small amounts of immunoreactive LH-RH but this is only about 2 p. 100 of the total LH-releasing activity of the extract.

Isolated nerve terminals (« synaptosomes ») from rat and sheep hypothalami can secrete LH-RH (and TRH) when incubated in *in vitro*. This technique has proved of value in the study of factors controlling LH-RH secretion (BENNETT *et al.*, 1975) including neuro-transmitters and steroids. This secreted material is also indistinguishable from the synthetic decapeptide using the criteria described above.

Rat hypophysial portal blood contains biologically and immunologically active LH-RH which is chromatographically similar to the decapeptide.

Above the level of the pituitary gland, then, the immunoreactive LH-RH in hypothalamic tissue, in the synapse and in portal blood appears to be identical with the decapeptide and the radioimmunoassay is valid in these situations. Below the pituitary however there is considerable doubt about the validity of the assay, not only because of the large dilution factor but also because the pituitary appears to metabolise the peptide.

5. — IMMUNOREACTIVE LH-RH IN HUMAN URINE

After intravenous injection of LH-RH in man a small amount is excreted as immunoreactive material within 4 hours. The relevant literature is summarised in table I. Although in our earliest study (JEFFCOATE *et al.*, 1973) we suggested that it was the decapeptide we now know that it is not. Following the injection of [^{3}H]-Glu_1 LH-RH, for instance (REDDING *et al.*, 1973), although a high proportion of the ^{3}H is excreted none is associated with unchanged decapeptide but with p-glutamic acid and the di-peptide, p-glutamyl-histidine. The immunoreactive material also has a different mobility compared with that of the synthetic decapeptide on TLC (BOLTON, 1974) and CM-cellulose (fig. 3) (JEFFCOATE and HOLLAND, 1975).

TABLE I

Urine excretion of LH-RH-like immunoreactivity after intravenous injection in man

Literature survey

Authors	Assay	Amounts	Comments
JEFFCOATE *et al.*, 1973 and 1974	RIA	1-2 %	? decapeptide
REDDING *et al.*, 1973	[^{3}H]-LH-RH	No unchanged	decapeptide
BOLTON, 1974	RIA	0.5 %	? 3-10 octapeptide
VIRKUNNEN *et al.*, 1974	Bioassay	« 1.5-2.4 ng/ml »	
JEFFCOATE and HOLLAND, 1975	RIA	0.2-1 %	not LH-RH not 3-10-octa ? 2-10-nona ? glu_1-LH-RH

Since it reacts with our antisera which are specific for the C-terminus, this end of the molecule must be intact and metabolism must have occurred at the N-terminus. The 3-10 octapeptide has been excluded because of its mobility on CM-cellulose (fig. 2) (JEFFCOATE and HOLLAND, 1975).

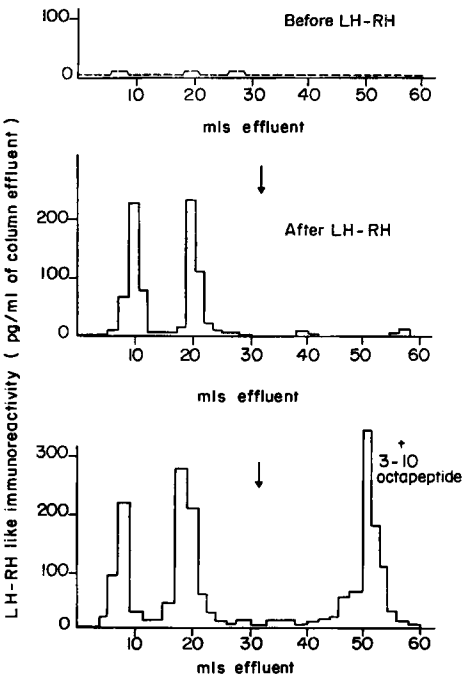

FIG. 3. — *CM-cellulose ion-exchange chromatography of Florisil extracts of urine before and after LH-RH in man*

Upper figure: baseline sample, before LH-RH. *Centre figure:* sample within 60 min of LH-RH injection. Two immunoreactive peaks are seen, neither of which is the decapeptide (position indicated by arrow). *Lower figure:* same sample plus 3-10 octapeptide fragment of LH-RH.

6. — IMMUNOREACTIVE LH-RH IN HUMAN PERIPHERAL BLOOD

Many attempts have been made to assay LH-RH in human peripheral blood, both by bioassay and by radioimmunoassay. The relevant literature is summarised in table 2. A series of papers from Reichlin's group using *in vivo* bioassay technique has shown : (*a*) increased concentrations at mid-cycle in women ; (*b*) levels in post-menopausal women, not correlated with LH levels ; (*c*) evidence for episodic secretion in men with increased values after orchidectomy. The levels reported are rather higher than those reported using the radioimmunoassay. ARIMURA *et al.* (1974) also suggested that there were mid-cycle peaks but their conclusions were based on scanty data. In our own studies (JEFFCOATE *et al.*, 1974 *a*, 1975) samples have been obtained from indwelling cannulae in the antecubital veins of normal human subjects at 10 min intervals for 5 hr. Values are frequently undetectable

(< 0.25 pg/ml) and the highest value found is 3.5 pg/ml. There is no apparent relation to LH secretion and in females no clear evidence for mid-cycle elevations in, or increase in the frequency of pulses.

TABLE 2

Assay of apparent LH-RH in human peripheral blood
Literature survey

Authors	Assay	Levels (pg/ml)	Comments
MALACARA *et al.*, 1972	Bioassay	[ΔLH]	Increase at mid cycle.
SEYLER and REICHLIN, 1973	Bioassay	[ΔLH]	Postmenopausal women negative correlation with LH
SEYLER and REICHLIN, 1974 *a*	Bioassay	N ♂ 12 (< 10-50) ♀ < 10-89) (estimated)	Episodic secretion, no correlation with LH. ♂ values up to 400 pg/ml
SEYLER and REICHLIN, 1974 *b*	Bioassay	[ΔLH]	♀ increased after orchiectoy
JEFFCOATE *et al.*, 1973	RIA	< 10	Non-detectable
KEYE *et al.*, 1973	RIA	30 (children) 70 (adults)	COOH-linked antiserum.
ARIMURA *et al.*, 1974	RIA	F.P. nd-1.8 Mid. 1.5-17.0 L.P. nd-6.0	Paradoxical binding Single samples
JEFFCOATE *et al.*, 1974 and 1975	RIA	N 0.25-3.5	Episodic secretion parallelism doubttul.

Clearly, a lot more information is required before the validity of these assays in human peripheral blood can be accepted.

7. — IMMUNOREACTIVE LH-RH IN JUGULAR BLOOD OF THE SHEEP

The relevant literature is summarised in Table 3. Using 15 min sampling (CRIGHTON *et al.*, 1973) and later 4 min sampling (FOSTER *et al.*, 1974) we found at the time of oestrus in the sheep, high peaks (up to 10 ng/ml) of LH-RH-like immunoreactivity at 90-120 min. intervals. This pattern was quite different from those observed either by KERDELHUÉ *et al.* (1973) or by NETT *et al.* (1974). In none of these studies was any correlation with LH levels observed. In our studies, peaks of immunoreactivity were not limited to the time of oestrus but were also observed at other times e.g. 48 hr. after oestrus and on day 10 of the cycle and even during anoestrus.

Chromatographically, on CM-cellulose, this LH-RH-like material is heterogenous and distinct from LH-RH (JEFFCOATE and HOLLAND, 1974 *b*). It is also biologically inactive.

TABLE 3

Assay of LH-RH-like immunoreactivity in jugular venous blood of the sheep
Literature survey

Authors	Findings	Comments
CRIGHTON *et al.*, 1973	Peaks of ng/ml (oestrus)	Episodic release Periodicity 60-120 min No correlation with LH
KERDELHUÉ *et al.*, 1973	Peaks of ng/ml (oestrus)	Few data
NETT *et al.*, 1973	Rams 71 pg/ml Castrate ♂ 128 pg/ml	
FOSTER *et al.*, 1974 and 1975	Peaks of up to ng/ml throughout oestrous cycle	No correlation with LH
NETT *et al.*, 1974	Ewes : 15-96 pg/ml (oestrus) : 120-370 pg/ml (castrate)	Periodicity 15 min No correlation with LH

Because our antisera cross-react with peptides related to LH-RH decapeptide that lack residues at the N-terminus it seems likely that the cross-reacting material, found in the sheep jugular venous blood are metabolites of this end of the molecule. Such metabolites would not be detected by more specific antisera. This explanation for the difference between our results and those of others is shown schematically in figure 4.

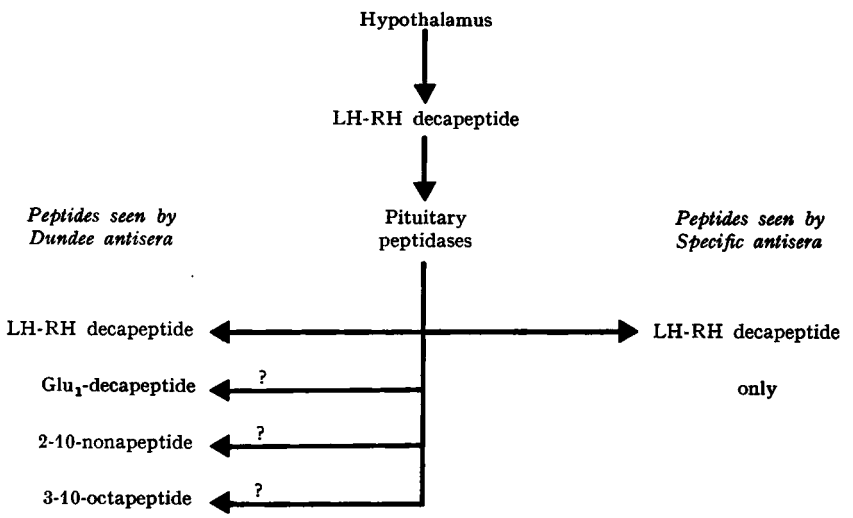

FIG. 4. — *Scheme showing how possible metabolites of LH-RH formed at the pituitary may be detected in peripheral fluids by non-specific antisera but not by specific antisera*

Since these metabolites are not present in the hypothalamic nerve terminal, nor in portal blood but are found in the jugular vein, the pituitary gland appears to be the likely site for their formation.

8. — CONCLUSION

The main conclusion to be drawn from our own work and that of other groups on the application of radioimmunological techniques to the assay of LH-RH or its metabolites in biological fluids is that such assays are associated with considerable problems, both technically and for validity, and until these are resolved such assays can reveal little about the secretion of LH-RH from hypothalamus in health and disease.

Symposium Gn-RH, Tours, 25 mars 1975.

RÉSUMÉ

PROBLÈMES LIÉS AU DOSAGE DE LH-RH DANS LES LIQUIDES BIOLOGIQUES

Tandis que les dosages des neuropeptides sont facilement effectués dans les solutions tampons, leur application aux liquides biologiques a été associée avec un certain nombre de problèmes.

Tout d'abord, on peut craindre que la concentration dans les liquides périphériques soit très basse. Il existe aussi de fortes chances que la sécrétion du LH-RH soit pulsatile avec disparition rapide des pics : ceci signifie qu'il faut de fréquentes prises d'échantillons. Plusieurs tissus, dont l'hypothalamus, contiennent des peptidases qui peuvent détruire à la fois les activités biologique et immunologique. Enfin, il est nécessaire d'authentifier le dosage de LH-RH par les corrélations biologiques et les propriétés immunochimiques et chromatographiques.

Quelques résultats ont été obtenus pour l'hypothalamus, les terminaisons nerveuses isolées et le sang du système porte hypophysaire. Toutefois, il subsiste quelques doutes concernant les résultats obtenus dans le sang périphérique chez l'Homme et le Mouton.

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