Definition and Measurement of Follicle Stimulating Hormone

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ABSTRACT

FSH has a key role in the development and function of the reproductive system and is widely used both diagnostically and therapeutically in developmental and reproductive medicine. The accurate measurement of FSH levels, in patients for diagnosis and monitoring and in therapeutic preparations for clinical use, is essential for safe and successful treatment. Historically, FSH was defined on the basis of classical *in vivo* endocrine activity, and early therapeutic preparations were calibrated using *in vivo* bioassays. There was early recognition that reference preparations were required for calibration if the results from different laboratories were to be comparable. In response to the perceived need, the World Health Organization established the first standard for such preparations in 1959. Subsequent developments in biotechnology have led to recognition that there is no single molecule that can be uniquely defined as FSH, and that FSH can induce a range of biological activities. Several highly purified standards for FSH are now available, but discontinuity and heterogeneity of estimates of FSH activity in terms of these standards made using *in vitro* assays and binding assays have been noted. It is thus essential that any measurement of FSH include specification both of the standard with which the measured FSH is compared and the assay method used for that comparison. (*Endocrine Reviews* **21:** 5–22, 2000)

I. Introduction

FSH is a member of the glycoprotein hormone family that has a central and essential role in reproduction. FSH determination is fundamental to elucidating reproductive physiology, regulating fertility, and diagnosing and treating disorders of reproduction. FSH exists in many different molecular forms, which may have different reactivities both in physiological systems and in different assay types. Thus FSH is not a single entity but is a heterogeneous population of different forms, which changes under different physiological and pathological situations. Moreover, FSH exerts a number of biological effects that have served as the basis for the different types of assay that have been developed for its estimation. Use of a particular assay depends upon the purpose for which the measurement is being made, the nature of the sample that is being analyzed, and the availability of different assay types. Thus, the "true" estimation of FSH, each form of which may react differently in the different assay systems used for estimation, is an ideal that will not be readily achieved.

This review considers how FSH is defined either in terms of its biological activity or in molecular terms, summarizes the different assay formats developed for its determination, and describes how the properties of FSH may influence its determination and hence the conclusions that are drawn from such determinations. Although many of the principles that apply to FSH apply to all members of the glycoprotein hormone family (*i.e.,* FSH, TSH, LH, and CG) and to other clinically important glycoproteins such as erythropoietin, there have been particular problems associated with setting up international standards for calibration of FSH assays.

II. Clinical Importance of FSH

A. Therapeutic uses

Preparations derived from human menopausal urine [menotrophin, human menopausal gonadotropin (hMG)]

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and containing FSH have been in clinical use since the 1960s (for historical review see Ref. 1). Advances in biotechnology have enabled industrial production of therapeutic grade urinary FSH (urofollitropin) and recombinant DNA (rDNA)-derived human FSH with high specific activity and minimal contamination by non-FSH materials. The properties of hMG and urofollitropin are defined by pharmacopeial monographs (*e.g.*, Ref. 2), as are the requirements of the (*in vivo*) bioassays used to test their potency.

Therapeutic preparations of FSH are widely used in the treatment of infertility (Table 1). The principles and practices for the gonadotrophic manipulation of the human ovary have been reviewed (3). Their use in assisted reproduction technology can be divided into three categories:

1. Induction of ovulation when a single healthy oocyte is required.

2. Induction of multiple ovulation or superovulation to maximize efficiency when assisted reproductive technologies are used that allow replacement of a fixed number of embryos.

3. Stimulation of spermatogenesis.

Treatment of female infertility is a situation in which patients are otherwise generally healthy and common disorders of reproduction such as anovulatory infertility can be treated in a safe and effective way (4). However, there is a narrow dose-range for use of FSH between a threshold level required to stimulate growth of a follicle(s) and the maximal dose (ceiling) above which overstimulation can occur (5). Thus there is a significant risk to health due to the iatrogenic induction of ovarian hyperstimulation syndrome or multiple pregnancies. Different physiological and clinical states can affect the levels of the threshold and ceiling for FSH treatment. Thus careful dose adjustment and monitoring of FSH levels and ovarian responses are required, particularly for patients with polycystic ovary syndrome (*e.g.,* Ref. 6). This cannot be achieved without accurate and reproducible calibration of therapeutic products. However, the end point used for patient response to therapeutic preparations should also be carefully considered.

B. Diagnostic use

The measurement of FSH in the circulation is widely employed in the diagnosis of disorders of reproduction and development (Table 2). In general, immunoassays are used for these measurements because of their practical advantages. A disadvantage is that immunoassays may not provide information about the biological activity of the FSH measured, although this may be less relevant in routine clinical management than in detailed studies.

The primary use of FSH measurements is for assessment of gonadal function. Through classical endocrine feedback pathways, an elevated level of FSH indicates reduced gonadal function or gonadal failure, whereas a normal serum concentration of FSH suggests normal gonadal function. A low serum FSH may indicate a problem at the level of the hypothalamus or pituitary.

A measurement of serum FSH, with measurement of LH and either estradiol or testosterone, may be helpful in children with suspected premature puberty or in cases of delayed puberty, particularly as the application of sensitive assay methodologies permits detection of hormonal changes before clinical changes of puberty are observed (7). FSH measurement is indicated in men with azoospermia or severe oligospermia to help determine the degree to which the problem is due to gonadal failure (8).

Ovarian reserve, or the total number of remaining oocytes within the ovary, declines with ovarian age, but this does not always equate with the age of the woman. A baseline measurement of serum FSH concentration, usually on day 3 of the menstrual cycle, is a fairly good predictor of ovarian reserve in women of reproductive years (9). A fluctuating baseline FSH level is indicative of compromised ovarian function. The picture is further enhanced if measurement of FSH is combined with serum estradiol and inhibin (reviewed in Ref. 10). In an irregular menstrual cycle it can be difficult to time collection of samples correctly, and therefore more than one sample may have to be taken, often in combination with an ultrasound scan of the ovaries, to help determine the stage in the cycle (11–14). Measurement of FSH is also helpful in determining the presence of common disorders of reproduction such as polycystic ovary syndrome, when classically the serum LH concentration is elevated, while FSH is usually normal (15). A single measurement of FSH is not predictive of the timing of menopause and is not usually recommended for this purpose, although it may be useful in developing a differential diagnosis to exclude other causes (endocarditis

TABLE 1. Common causes of infertility and treatments that require FSH

Cause	Treatment
Female infertility	
Ovulatory failure (oligo- or amenorrhea)	Ovulation induction
Primary ovarian failure	Superovulation followed by IVF using donated oocytes
Tubal/pelvic damage[a]	Superovulation and IVF
Endometriosis[a]	Superovulation and IVF, GIFT, IUI
Cervical mucus dysfunction or defects	Superovulation and IUI, GIFT, IVF, ZIFT
Antisperm antibodies	Superovulation and IVF, IUI
Idiopathic infertility	Superovulation and IVF, GIFT, IUI
Male infertility	
Sperm dysfunction	IVF, ICSI
Azoospermia	Stimulation of spermatogenesis with FSH if due to hypogonadotrophic hypogonadism or pituitary failure

Abbreviations: IVF, *In vitro* fertilization; GIFT, Gamete intra-fallopian tube transfer; IUI, Intra-uterine insemination; ICSI, Intra-cytoplasmic sperm injection; ZIFT, Zygote intra-fallopian tube transfer.
[a] May be treated surgically in appropriate cases.

TABLE 2. Summary of clinical situations where FSH determinations are useful or are commonly requested

Clinical condition	Comments
Anovulatory infertility (oligo/amenorrhea)	To help determine whether cause is pituitary or gonadal in origin and to aid diagnosis of conditions such as polycystic ovary syndrome
Suspected premature puberty	In addition to steroid hormone levels
Delayed puberty	In addition to steroid hormone levels
Azoospermia/severe oligospermia	To differentiate pituitary and gonadal causes
Ovarian reserve	As a biological marker for the number of releasable oocytes; may be enhanced by measurements of inhibin and ovarian ultrasound to accurately stage the timing of the sample
Menopausal status	A frequently requested test; FSH is not a good marker for timing of the menopause or of perimenopausal state

or pheochromocytoma for example) of symptoms such as hot flushes. Although various studies have been performed to characterize the perimenopausal status (16, 17), the practical use of FSH measurement is in the prediction of ovarian response to stimulation in the context of assisted reproduction.

Significant between-assay heterogeneity is observable for FSH assays. However, it is not clear whether this may have a significant effect on diagnoses based on results obtained from different assay systems. In the United Kingdom a large clinical chemistry laboratory may perform several thousand FSH assays per year. The majority will be for investigations of menopausal status, diagnoses of infertility/amenorrhea, and infertility in men (see above). Essentially, the clinician will wish to detect gross changes in FSH levels from the normal ranges, and it is it unlikely that variations between assays will mask gross changes in FSH concentration associated with primary gonadal failure and hypogonadotrophic hypogonadism. The change to more acidic forms of FSH at menopause may be an example of a qualitative assay providing diagnostically useful information since a change in isoform composition may precede the observed increase in levels of FSH associated with the menopause. The levels of FSH measured by immunoassay at this time could also be affected by a change in isoform profile relating to the selectivity of the assay system used. However, in some circumstances, minor variations between assays could have a more profound effect, *e.g.,* partial gonadal failure/resistant ovary syndrome. Intensive investigations of hormonal levels have revealed underlying gonadotropin disturbances and imbalances in polycystic ovary syndrome (18) and subtle changes relating to idiopathic infertility (19). The ratio of LH to FSH has been proposed as a good predictor of ovarian hyperstimulation syndrome (20). In such cases, particularly where a ratio of two measurements is made, it is important to maintain continuity of unitage between estimates derived from different assays over a period of time and thus from one standard preparation to the next.

III. Definition of FSH

The history of the elucidation of FSH has been briefly reviewed (21). The salient features are that gonadotropins, or gonadotrophic principles as they were originally described, were first identified and defined in terms of their biological activities and that the assays developed for gonadotropins were based on classical endocrine principles. These early assays had two main drawbacks (21). First, there was no assay specific for FSH, and second, quantification and hence between-laboratory comparisons were made difficult by a lack of standardization. Thus, the early assays measured a number of different biological responses to the different gonadotrophic principles and, furthermore, the extracts which defined gonadotrophic activity were composed of mixtures of the gonadotrophic principles.

After further scientific progress the structure of the glycoproteins for a variety of species in terms of amino acid sequence (22–29), carbohydrate composition (30–33), and gene sequences (*e.g.,* Refs. 34–36) have been determined and extensively reviewed (37–42). Human FSH can be defined in molecular terms as a heterodimeric glycoprotein hormone consisting of two noncovalently linked subunits designated α and β, which consist of 92 amino acids and 111 amino acids, respectively, and which are products of different genes. Each subunit has two N-linked glycosylation sites, which are on Asn 52 and Asn 78 for the α-subunit and Asn 7 and Asn 24 for the β-subunit and which are essential for expression of FSH bioactivity.

IV. Function of FSH

The biological actions of FSH can be summarized as follows; in the male, FSH, in combination with testosterone (which is under the control of LH), is required for the initiation and maintenance of qualitatively and quantitatively normal spermatogenesis (reviewed in Ref. 43). Although recent data in transgenic mice suggest that FSH appears to be not essential for male fertility (44), spermatogenesis is not completely normal in the absence of FSH and, furthermore, the requirement for FSH is more critical in primates than in rodents.

In the female FSH is necessary for the selection and growth of ovarian follicles and for the production of estrogens from androgen substrates. The gonadotrophic effects of FSH may be subserved by a number of intermediaries (reviewed in Ref. 45) that form part of the cellular and tissue (*e.g.,* Ref. 46) response to FSH stimulation culminating in ovulation. Such cellular responses illustrate the complex nature of FSH since they indicate that FSH activity has many components, *i.e.,* FSH is a growth factor or tropic hormone, a secretagogue, and a modulator of cellular development (*e.g.,* Ref. 47). It is generally thought that FSH exerts most of its intracellular actions via the cAMP-mediated signaling pathway, although

FSH may also utilize other signal transduction pathways such as Ca^{++} (48).

The biological activity of FSH is the sum of a complex combination of processes: release from the pituitary, survival in the circulation, transport to the site of action (*i.e.*, the gonad), binding to the receptor, and activation of signal transduction pathways. These processes may be modified by other factors that may affect release (pulse frequency or amplitude), clearance from the circulation, receptor binding and desensitization, cellular responsiveness, and modifications to the hormone during circulation such as neuraminidase action.

A. Structure/function relationships

1. Amino acid sequence. The α- and β-subunits of the gonadotropins are noncovalently linked but their individual conformations are determined by intramolecular disulfide bonds. Recently, glycoprotein hormones have been identified as members of the cysteine knot family of growth factors (49, 50). The disulfide bonds of the cysteine knot of the α-subunit have been found to be critical for heterodimer formation and/or secretion (51, 52), and conformational changes that occur in association with different hormone-specific β-subunits are assumed therefore to occur outside this essential core region of the α-subunit molecule.

The amino acid sequence ^{48}QKTCT^{52} of the β-subunit appears to be essential for formation of the FSH heterodimer (53), and the disulfide bonds 7–31 and 59–87 are important for heterodimer formation of LH but not of human (h)CG and hFSH (54). Further studies showed that the residues Phe^{33}, Arg^{35} and Arg^{42}-Ser^{43}-Lys^{44} are essential for receptor binding of hCG and hTSH but not for hFSH (55), whereas His^{90}-Lys^{91} of the α-subunit is essential for binding of FSH to its receptor but not for binding of hCG to the LH/hCG receptor (56, 57). Interestingly, this sequence is required for stimulation of cAMP by both hormones. Receptor binding sites have been identified within the 93–99 amino acid sequence of the C-terminal region of the FSH β-subunit (58). In the hCG α-subunit the sequence between amino acids 33–45 contains several residues that are essential for binding to the β-subunit and some of which are involved in receptor binding (59). The hormonal specificity of each subunit is thought to reside in a determinant loop formed between residues Cys 93 and Cys 100 of CG β-subunit and the equivalent sequences of the other β-subunits (60). This loop has been shown to form a seat belt around the α-subunit in the heterodimer (49, 50).

2. Oligosaccharides. The functions of the oligosaccharides have been investigated in two main ways. Earlier studies made use of chemical (61–63) or enzymatic alterations (64, 65) to the glycan moieties. The enzymatic approach allows investigation of the function of each sugar in the glycan by using sequential exoglycosidases to expose each sugar in turn (66), whereas chemical methods rapidly remove key sugar groups leaving a core structure intact. One possible drawback of chemical methods is that the treatment may alter the polypeptide chain in some way. More recently, molecular biology techniques have been used to produce molecules that were not glycosylated at individual sites by induction of

mutations in the polypeptide backbone, which prevent glycosylation (67). Site-directed mutagenesis has the advantage of permitting investigation of the role of individual glycosylation sites but also has the effect of removing the entire glycan structure and does not therefore allow investigation of the effect of sugar heterogeneity at any one site. A combination of site-directed mutagenesis and enzymatic or chemical modification may address this issue. The available evidence suggests that the protein structure was similar in intact and chemically treated preparations (62) and that the thermal stability of chemically deglycosylated hCG may be enhanced compared with the native hormone (68).

Hormones with deglycosylated α-subunit are antagonists of the action of intact hormones. Oligosaccharides on the α-subunit are required for signal transduction, in particular α52 (69), intracellular stability, and association with the β-subunit (70). Those on the β-subunit are required for β-subunit folding (71, 72) and for correct disulfide bond formation (73).

Although substantial work has been done, the complexities of the situation arising from variations of glycosyl content at each of the four possible glycosylation sites and the interactions of these structural features with biological systems are still incompletely understood.

B. Relationship of FSH structure to physiology

FSH is heterogeneous and the pattern of FSH forms changes with different physiological situations (reviewed in Refs. 74 and 75). Variability in the sugar chains is the major cause of heterogeneity in the gonadotropins, although microheterogeneity exists in the polypeptide chain.

An overall change in FSH isoform pattern resulting in a more basic composition has been observed in the middle of the menstrual cycle (76), in response to a GnRH challenge during puberty (77, 78), and in girls and young women compared with boys and men (79). In women, more acidic forms of FSH appear after the menopause (79, 80), the longer half-life of which may contribute to the increased serum FSH concentrations observed.

The profile of observed isoforms has also been shown to change with various hormonal treatments in humans and experimental animals and cultured rat pituitary cells (*e.g.*, Ref. 81). The changing patterns of isoforms observed under different conditions in humans (79–85) and other species (86–88) have been described. These data strongly implicate a role for sex steroids in controlling the isoform profile of FSH in women; in contrast, in men (82) and male rats (87) the evidence for androgens exerting the same effect is not as strong, and other factors such as inhibin are also implicated in controlling both the isoform profile and the amount of FSH secreted. The association of particular isoform patterns or molecular forms of FSH to particular actions or cellular and physiological responses is difficult to make because of the pleiotropic actions of FSH and the complexity of its molecular structure.

C. Biological clearance

Data from *in vivo* bioassays suggest that one of the major factors that controls FSH action is the relative rate of clear-

ance of different isoforms. The main mechanism of clearance of glycoproteins appears to be the hepatic route (89) or the renal route (90).

1. Hepatic clearance. Terminal sialic acid and sulfate groups are important for regulating biological half-life of glycoproteins (91). Subterminal galactose residues are recognized by the hepatic asialoglycoprotein receptor (92), and a sulfated glycoprotein receptor (93) is responsible for rapid clearance of sulfated glycoprotein hormones such as LH and TSH (94, 95). It is likely that molecules with a low sialic acid content but whose sugar structures are recognized by the asialoglycoprotein receptor may be cleared by this mechanism although hCG does not appear to be desialylated *in vivo* to render it a substrate for hepatic clearance (96).

Cellular uptake of asialoglycoproteins can be investigated *in vitro* by use of cultured liver cells or cell lines (97–99). There are, however, differences in the uptake of asialoglycoproteins by liver cells from different species (98) and between hormone molecules of natural or synthetic origin (95). These differences could contribute to differences in potency observed between natural and recombinant molecules (95) and in different situations (*e.g.*, pharmacopeial *in vivo* bioassay *vs.* clinical use of therapeutic preparations). With further development this model might be used to assess hepatic clearance when estimating FSH activity in serum or in therapeutic products *in vitro*.

2. Renal clearance. The renal route results in excretion of biologically active gonadotropins in the urine (100) or in degradation (90). Radiolabeled FSH and deglycosylated FSH (which is cleared from the circulation faster than intact FSH) appear to be cleared largely through the renal route rather than the hepatic route (101). Excretion may occur after filtration, which may be regulated by the surface charge and relative molecular size of the molecule (102). The loss of negative charge caused by removal of terminal sialic acids may therefore enhance renal filtration of desialylated molecules. Cellular uptake and degradation (90) may also be influenced by surface charge or by recognition by cell surface receptors or may require a cellular uptake mechanism that could be mediated by a mechanism similar to the transendothelial trafficking described previously (103). There is good evidence that the β-core of hCG is produced during elimination of the hCG molecule through the kidney (104). It would be difficult to model the renal route and to include models of both renal and hepatic routes of biological clearance into routine *in vitro* assays.

V. Metrological Considerations and the Need for Standards

The process of discovering that a property is "measurable" and setting up a procedure for measuring it depends entirely upon experimental inquiry, and is an important part of experimental science (105). More specifically, measurement of a quantity consists of ascertaining its ratio to another fixed quantity of the same kind, known as the "unit" of that quantity. Any unit is an abstract concept and cannot be used as a basis of measurement until it has been defined in one of two

ways: by reference to an arbitrary material standard, or by reference to a natural phenomenon. Thus, an essential requirement for any meaningful measurement is definition of the "quantity" being measured, of the "units" in which it is measured, and of the process by which the measurement is carried out. The need for metrological definition in the context of assays has been discussed both generally (106, 107) and in the context of specific types of assay (108, 109). Failure to define clearly what is meant by "measurement" of FSH (or of hormones more generally) can be the source of ambiguity and apparent inconsistencies.

The main assumption underlying a valid determination of any measurement is that the quantity to be measured and the standard with which it is compared are "of the same kind". This requirement and its implications for bioassays have been extensively discussed (110–113). Although FSH was initially defined in terms of its ability to produce specific biological effects, it is now known both that materials with different molecular structures can produce these effects, and that there is no unique molecule that can be defined as FSH (*Sections III* and *IV*). Thus, definition of the "quantity to be measured" and provision of a sample "of the same kind" are not simple. In certain cases, the ability to produce specific effects in a biological system may still serve as the basis for definition of FSH. Biological responses, while they may be used to provide a definition of FSH, are not capable of defining the "units" of FSH, or indeed of any other hormone, as was recognized early in the study of such materials (114). Nevertheless, biological responses may serve as the basis of a measuring system for "FSH" in the form of bioassays.

The structure of an assay involves a subject (*e.g.*, an animal or a cell culture in the case of a bioassay, or an antibody-coated well of a microtiter plate in the case of an immunoassay) that responds in some measurable way to an application of a defined amount of the hormone. The relationship between the response and the amount or dose of hormone (the dose-response relationship) will be subject to random variations arising from the variations between replicate subjects. The availability of a material standard of the hormone with a defined unitage permits the application of "known" amounts of the standard to an array of subjects, while defined amounts of the substance to be measured are applied to a similar array of subjects. Subject to certain assumptions about validity, the comparison of the dose-response relationships for the standard and the unknown to be measured then gives a value of the FSH unitage to the substance being measured (110, 113).

Measurements of FSH are required for a variety of reasons (*Section II*). The definition of "FSH," its measurable property (*e.g.*, biological activity), and the corresponding measurement process (*e.g.,* bioassay) must be related to the purpose for which the measurement is required.

One of the more important requirements for measurement of FSH is to determine the potency of therapeutic products. For this purpose, the quantity to be measured is the product, which may be defined by its production process and various physical or chemical specifications. The property to be measured is the capability of this quantity of material to produce a biological effect in patients. The units in terms of which this property is measured are International Units defined by ref-

erence to the International Standard, a material reference, and the measurement process is a pharmacopeially defined *in vivo* bioassay (*Section VI.A*).

"Measurements of FSH" are also made for samples of biological fluids from patients. The measuring system most commonly used is an immunoassay, in which case the property being measured is the binding of particular molecular forms. However, the molecular forms bound are seldom well defined (*Section VI.B*).

A. Standards for FSH

The First International Reference Preparation (IRP) of hMG was established in 1959, in response to a request by the International Federation of Gynaecology and Obstetrics, with no defined unitage, and was described as "ampoules containing 22 mg of dried active principle from urine of post-menopausal women" (115). This early preparation was found to be toxic in some assays, and a second preparation was evaluated by international collaborative study and became the second IRP (116). Subsequently, international standards for hMGs were established by the World Health Organization (WHO) and were calibrated by bioassay in terms of the second IRP (117). IRPs derived from pituitary extracts were also calibrated by bioassay in terms of the second IRP for menopausal gonadotropins. The unitage of biological activity assigned to the second IRP has served as the basis for definition of FSH activity of all subsequent WHO international standards and reference preparations (Table 3). The principles, use, and availability of standards for gonadotropins have been reviewed (118, 119) and have been recently updated (120).

These standards have been of service to the scientific and medical communities, although some problems have been noted. The change from use of relatively impure preparations to use of purified preparations as therapeutic products was followed by some studies indicating that on a unit-for-unit basis recombinant preparations appeared to be more potent clinically than their urinary counterparts (121), although such findings were not universal (*e.g.*, Ref. 122). Thus, international standards of the purified preparations have been prepared and calibrated (123), and at present therapeutic preparations of gonadotropins are calibrated in terms of the appropriate international standard (Table 3) using the pharmacopeial *in vivo* bioassay.

There is at present no single standard that may be considered appropriate for general use in *in vitro* bioassays and immunoassays. Although some of the international standards have been used for these assays and may help to decrease interassay variation (124), significant interassay heterogeneity of estimates has been observed in two international collaborative studies (123, 125, 126). Furthermore, different international standards have been found to have different molecular compositions (127, 128) demonstrating the difficulty of preparing candidate standard materials from natural sources with reproducible properties. The possibility of standardization of the assay systems has been considered (124, 129–132). However, the difficulties inherent in standardization of bioassays have long been recognized (111), and similar considerations apply to immunoassays.

B. Future prospects for FSH standards

Further developments arising from the application of biotechnology may lead to the generation of purified preparations of defined but differing isoform composition or new molecules with therapeutic potential (133–135), which may require new assays and new standard preparations for their calibration. High purity preparations can be analyzed using physicochemical techniques that were not applicable to hMG preparations. At present, however, therapeutic materials, including those derived from rDNA sources, are calibrated by *in vivo* bioassay (see *Section VI.A*).

If immunoassay or lectin-antibody systems can be developed that select for certain forms of FSH such as acidic forms, then it may be appropriate to prepare standards of differing

TABLE 3. World Health Organization International Reference Preparations (IRP) and International Standards (IS) for FSH

Preparation	Code	Source	Calibration	
			Potency (IU FSH/ampoule)[a]	Standard
1st IRP for menotrophins[b]	hMG 24	Dried active principle from urine of postmenopausal women	22 mg	N.A.
2nd IRP for menotrophins[b]		Pergonal 23	40 (by definition)	1st IRP for menotrophins
1st IS for menotrophins[b]	70/45	Pergonal batch P49 E229C	54	2nd IRP for menotrophins
2nd IS for menotrophins[b]	71/223	Pergonal batch P49 E229C	54	2nd IRP for menotrophins
3rd IS for menotrophins[b]	71/264	Pergonal batch P49 E229C	54	2nd IRP for menotrophins
1st IS for urofollitropin	92/512	Urofollitropin	121	2nd IS for menotrophins
1st IS for rDNA-derived hFSH	92/642	RDNA-derived hFSH	138	2nd IS for menotrophins
1st IRP for FSH and LH pituitary[b]	69/104	Pituitary extract FSH/LH LER 907	10	2nd IRP for menotrophins
2nd IRP for FSH and LH pituitary[b]	78/549	Pituitary FSH/LH LER 907	10	1st IRP for FSH and LH pituitary
Interim reference preparation	94/632	Pituitary FSH/LH LER 907	20	2nd IRP for FSH and LH pituitary
1st IS for FSH, human pituitary	83/575	Highly purified pituitary FSH	80	2nd IRP for FSH and LH pituitary
1st IS for FSH, rDNA for immunoassay	92/510	RDNA-derived hFSH	60	2nd IRP for FSH and LH pituitary

The table shows the origin and relationship of unitages for the different preparations. All preparations were calibrated by *in vivo* bioassay in terms of the stated standard except preparations coded 94/632 and 92/510 for use in immunoassays.
[a] Unless otherwise indicated.
[b] Preparations no longer available.

isoform composition for the calibration of these assays. Alternatively, standards might be given a unitage based on the molar content of FSH in the ampoule or in reconstituted solution. The heterogeneous nature of FSH would necessitate that such a standard should have a defined isoform composition and that it can be reproduced for the preparation of replacement standards. Even with such a standard, discrepancies would arise between immunoassay systems that have different selectivities based on their epitopic recognition. Paradoxically, with the advances in preparation and analysis of therapeutic products, such a standard may also have applications in calibration of new therapeutics.

VI. Assay Systems Used for Measurement of FSH

Assays for FSH can be classified into a variety of groups (136). One broad classification might be those that determine some response of a biological system to stimulation with FSH (bioassays, both *in vivo* and *in vitro*), those that estimate high affinity binding to molecules which exhibit specific properties of molecular recognition (immunoassays, receptor assays and lectin binding assays), and those that determine structural features or molecular properties by physicochemical means.

A. Bioassays

1. In vivo bioassays. FSH was originally discovered and therefore defined on the basis of its biological action. Early studies (reviewed in Ref. 21) were confusing and contradictory. The activities being measured in unfractionated biological fluids (urine from pregnant and nonpregnant women) had to be resolved first into activities of placental and pituitary origin and subsequently into follicle stimulating and luteinizing activity. A number of different *in vivo* bioassays, which were essentially based upon the effects of aqueous extracts of pituitary, placenta, or urine on the reproductive tracts of mice and prepubertal rats, were used in the early stages of discovery of FSH. The initial methodology of Ascheim and Zondek (137) was based on the biological effect of urine from pregnant women on the development of the reproductive tract in mice. Subsequently, increase in uterine weight and ovarian weight were used as biological endpoints (138). The assay developed in 1953 by Steelman and Pohley (139) based on the stimulation of ovarian weight in gonadotropin (LH)-treated immature rats, has proved to be a robust specific *in vivo* bioassay for FSH activity. This assay remains the basis of pharmacopeial monographs for the statutory determination of the FSH potency of therapeutic preparations (EP).

The major drawback of this assay is that the daily dosing regimen may preclude FSH forms that have a short half-life from exerting a biological effect. Evidence from studies on rDNA-derived FSH indicates that FSH isoforms with a pI value greater than about 5.1 exert no biological action in this assay (140). For most urinary derived preparations this may have little consequence since FSH produced in menopausal women is of an acidic nature, but it may be important in calibrating recombinant forms of FSH and in assigning specific activities related to protein content.

Acute or short-term *in vivo* bioassays that have been de-

scribed for LH are the ovarian ascorbic acid depletion test and the measurement of peripheral testosterone levels (141, 142). When preparations of different molecular composition are compared with each other in these two assays, different relative potencies are derived depending upon the assay type. No equivalent acute *in vivo* assays have been described for the determination of FSH. The *in vivo* effects of synthetic peptides corresponding to amino acids 34–37 of human FSH β-subunit have recently been evaluated using several parameters based on regulation of the estrous cycle (143). This illustrates other quantifiable FSH-dependent responses which might form the basis of a relevant bioassay for some preparations of potential therapeutic value that interfere with the action of FSH. Other endpoints in an *in vivo* bioassay could include the secretion of inhibins that have been related to subtle changes in FSH levels in women (144) and may allow an acute response to FSH to be determined.

In vivo biological activity is sometimes considered to be that which best defines FSH since it has two important components: that of biological action at the target tissue and that of biological clearance (145). However, use of genetic engineering techniques has enabled the modification of gonadotropins in terms of their biological activity by substitution of key amino acid sequences (146–148). The calibration of these materials as therapeutics will require careful thought. The current *in vivo* bioassay may be differently affected by forms of FSH with extended or shortened biological half-lives and may only detect some forms of FSH depending upon how the assay system is designed. Therefore, it might be necessary to redesign *in vivo* bioassays or to devise new assay systems to accommodate differences in activity of different molecular forms of FSH. Novel use of existing units, which may not reflect different aspects of the activity of some preparations, will need to be defined clearly to avoid confusion in their clinical usage. Clinicians using such preparations would need to distinguish between the expected patient responses to preparations with different properties calibrated in different ways. A unitage based on mass, although apparently providing a common basis, would not be appropriate unless combined with a range of physicochemical and/or biological assays since materials with different half lives exemplify the lack of relationship between mass and activity. Additionally, new unexpected activities can be generated (148) that will necessitate careful evaluation of the full biological potential of any chimeric molecules. If new materials produced through biotechnology are able to give significantly improved therapeutic treatments, then such problems would have to be overcome.

2. In vitro bioassays. A number of *in vitro* bioassays have been described (149–162), and this particular subject has been reviewed (163, 164). *In vitro* bioassays are largely based upon a quantifiable cellular response to stimulation with FSH. Such responses include stimulation of cAMP production (151), aromatase activity (154), or tissue plasminogen activator production (161) by Sertoli cells or granulosa cells in culture (*e.g.,* Refs. 155 and 157). More recent developments have included the use of cell lines transfected with the gene for expressing rat (165) or human (166, 167) FSH receptors and estimates of changes in cell shape or size (168) or cou-

pling of the gonadotropin receptor to a reporter gene (169). Recent advances in molecular biology may enable genetically engineered cell lines of various types to become common methods for estimating hormone activity (170). *In vitro* bio-assays can provide very sensitive methods for estimating the biological activity of FSH in biological fluids (160) or isolated FSH isoforms (171). The two major drawbacks are that no comparisons of biological half-life can be made in such systems and that usually only one biochemical endpoint is measured. Furthermore, the systematic designs adopted for many *in vitro* assays may lead to biased results. Nevertheless, *in vitro* bioassays have proved to be valuable tools in elucidating the physiology of FSH.

B. High-affinity binding assays

1. Immunoassays. Immunoassays are widely used for clinical determination of FSH for diagnosis and in physiological studies because they are rapid, readily available, relatively cheap, and sensitive. Immunoassays are generally considered to be more precise than *in vivo* bioassays although, with the exception of a few documented cases (*e.g.,* Ref. 172), they are assumed to give no information about biological activity.

Early immunoassays for FSH were based on the RIA format (173), which gave robust assays that are still in use (174). However, RIAs have been largely superseded by assays based on monoclonal antibody technology (175). Most commercially available assays are now based on sandwiches of monoclonal or monoclonal-polyclonal antibodies with a variety of detection modes and are generally more sensitive and precise than one-site assays. The latter assays are highly specific and may exclude some forms of the hormone of interest (*e.g.,* Ref. 176). Thus, a hormone-specific polyclonal antibody-based assay that is less selective for hormone isotypes may be preferred for some purposes.

Although assays of apparently high specificity are commercially available, there is still considerable variability between the results of different assays for gonadotropins (124, 129–132, 177). The reasons for such variations have been ascribed to differences in calibration of different assay kits (130), cross-reactivity between gonadotropin subunits and nonlinearity between kit standard and internationally available standards (132), differential recognition of different samples obtained for example at different stages of the menstrual cycle (131), and varying dose-response characteristics between different assay systems (178). Details of the methods of calibration and sources and composition of kit and in-house standards are frequently unknown to the user, which makes evaluation of the standardization of calibration and hence comparisons between different immunoassay kits difficult. By comparison, therapeutic products and in-house standards are calibrated by a common methodology (pharmacopeial bioassay), and regulatory authorities may test the potency of products released onto the market to ensure compliance with statutory requirements and consistency.

2. Epitopic structure and molecular recognition. In many cases it is either not known or not stated what particular epitopes on the molecules are recognized by different antibodies even though the overall areas of greatest antigenicity have been mapped on FSH (179–183) and hCG (184, 185). The methods used and results of epitopic mapping studies on gonadotropins have been succinctly reviewed (186). Epitopic maps of gonadotropins have several uses including the identification of antigenic regions, regions important for bioactivity (*e.g.,* for the purposes of designing vaccines), the functional importance of structural features such as the subunit contact sites (187) and receptor interaction sites (188–190), and the definition of topology (191).

The main antigenic epitopes of the subunits of FSH have been identified. Using a panel of 181 monoclonal antibodies, Berger *et al.* (186) identified nine antigenic sites; five on the α-subunit, two on the β-, and two dependent on the conformation of the αβ-dimer. Dias and co-workers (179, 187, 189–193) have extensively mapped FSH epitopes and the effects of conformation on these. Although the α-subunits of the glycoprotein hormones have the same amino acid sequences in each of the four hormones, some of the identified epitopes on the FSH α-subunit (*e.g.,* 3A epitope) are different between the different glycoprotein hormone heterodimers, suggesting some flexibility of this epitope, the conformation of which is affected by binding to the β-subunit (192).

A study of affinities for recognized epitopes has been done on commercially available immunoassay kits for LH (194) with some success in identifying the causes of between-assay variation. Furthermore, standardization of epitopic recognition perhaps directed at "rigid" epitopes may contribute to further minimizing between-assay variability. Assay of variable epitopes in a hormone-specific fashion may enable further observations on how structural features of hormones change with different physiological conditions. Antibody responses to epitopes in the region covered by amino acid residues 33–53 of the FSH β-subunit have been found to both enhance (195) and neutralize (196) FSH action. These observations raise the possibility of new methods with which to manipulate ovarian function or to produce contraceptive agents.

Structural features of FSH known to change with different circumstances are the carbohydrate side chains attached to the protein backbone. Studies are currently underway to investigate whether antibody binding is affected by glycan heterogeneity. The current thinking derived from epitopic studies is that oligosaccharides do not play a significant role in antigenic structure of gonadotropins but that removal of or alteration of the structure of oligosaccharides may alter the affinity of binding of monoclonal antibodies (197). There are, however, reports of antibodies that can distinguish between native and desialyated hCG (198–202), and there may be some difference in orientation of the α-subunit assessed immunologically following chemical deglycosylation (200). Further evidence for antigenic recognition of oligosaccharide structures was derived from studies of antibodies raised against rDNA-derived hCG that contains glycans high in mannose as a result of the glycosylation produced in the baculovirus system (201). Direct screening of glycosides released from glycoproteins shows that some antibodies at least can recognize specific carbohydrate sequences (202). It is likely that there are both antibodies whose binding is not affected by alterations in sugar structure and antibodies whose binding is affected. For the purposes of defining assay

specificity, it therefore appears necessary to determine experimentally whether particular antibodies are affected by glycan structure.

In addition to differences in epitopic specificity, differences in antibody affinity, and differences in molecular recognition, there are other factors that contribute to the heterogeneity observed between immunoassay types. These factors can include assay type such as RIA *vs.* immunoradiometric assay or enzyme-linked immunosorbent assay (ELISA), etc., kinetics of the assay [few commercial assays are taken to completion (steady state) for the sake of rapid production of results], orientation of antibodies in the assay, and comparisons between different standards. Comparisons between commercial assay kits would be made easier if epitopic recognition and other properties of the antibodies, such as affinity for epitopic sites as well as source and composition of the kit and in-house standards, were clearly described.

3. Lectin-binding assays. Although the extent to which glycan structures may influence binding of antibodies to FSH molecules cannot be generally ascertained, the replacement of a detector antibody in an ELISA format with a labeled lectin gives rise to assays that can detect changes in oligosaccharide structures (203). This methodology is not generally quantitative but has been used to probe qualitative differences between different preparations of rDNA-derived human FSH preparations. It has also been used to independently confirm enzymatic modifications of gonadotropin molecules but has yet to be developed for application to samples of serum or pituitary tissue. Lectin-affinity chromatography (204), in which samples of serum are applied to a lectin column and eluted in a stepwise fashion with increasing concentrations of a suitable sugar, results in the identification of FSH isoforms that are either not bound to the column, weakly bound, or strongly bound. Gross differences in sugar structures are thus inferred. In the lectin-ELISA format, ratios of signals originating from terminal sialic acid, free galactose, and core structures can be determined and related to ELISA data for estimating the relative levels of these molecular structures in the preparation. Either protocol may be a first step in development of a clinically useful assay that provides qualitative data on changes in glycan structures on FSH (and other glycoproteins).

4. Receptor assays. Receptor assays involve the estimation of the binding of unknown samples to preparations of specific receptors of either natural or rDNA-derived origin. Early receptor assays were based upon the solubilized FSH receptor from calf testis (205–207). This has proved to be a robust assay and remains in use with further developments leading to a receptor assay that can be used for quantifying FSH in unextracted serum (208). Variations of the assay utilize intact cells or cell lines transfected with recombinant receptors. The inclusion of receptor assays in international collaborative studies has given rise to results that are relatively consistent in comparison with *in vitro* assays. This may result from the different properties that are determined by these two assay types (209).

A combination of a (recombinant) receptor and an antibody preparation in an ELISA-type format could give rise to a useful and practical assay that estimates functional as well as immunologically reactive structural features (210).

5. Summary. Although high-affinity binding assays have provided much information about FSH, those that do not discriminate between different glycosylation forms of FSH may not be able to detect variant or antagonist forms of FSH (211). Analysis of binding kinetics can give results that are different for intact and deglycosylated FSH (212). These assays may also be affected by FSH-like molecules (213) or other inhibitory materials (214). The physiological basis of such molecules remains to be clarified.

C. Physicochemical assays

In impure preparations such as hMG, it is not possible to estimate FSH by physicochemical techniques, although in combination with immunoassays or *in vitro* bioassays, for example, the physicochemical properties of FSH molecules can be explored. The separation of different forms of pituitary or circulating FSH by physicochemical methods, such as zone electrophoresis with an immunoassay (215), or a bioassay as the detection method has been described (for a review see Ref. 216). Such studies show that FSH can change with different physiological or pathological situations but have not provided definitive structural information.

The availability of highly purified preparations derived from rDNA methods has now enabled a wider range of physicochemical techniques to be applied. The amino acid sequence and molecular mass (by mass spectrometry) of separated subunits can now be determined along with pI values for isolated bands on isoelectric focusing (IEF) gels. The structures of the glycan moieties and polypeptide chains can be determined by nuclear magnetic resonance (217, 218), and *in vivo* biological activity has been correlated with protein amount after separation of isoforms by IEF (140). However, despite all these methods for structural determination, no one method has been shown to predict biological activity directly.

Advanced techniques of mass spectrometry have been applied recently to the analysis of gonadotropins, particularly hCG (219–221). In these studies the heterogeneity derived from carbohydrate modifications resulted in limited ability to unambiguously identify hCG. After digestion by trypsin and separation of the various peptides before mass spectrometry, clearer signals were obtained, which enabled confirmation of hCG. This approach can be used to identify unambiguously a purified preparation of hCG for purposes such as drug enforcement (219) but would not enable quantitative determination of therapeutic products. It may, however, enable qualitative comparisons between different batches of therapeutic products (221) which could be used to assure batch-to-batch consistency.

Analysis by high-performance anion-exchange chromatography of glycosyl residues after enzymatic removal from the polypeptide backbone has been used to derive a hypothetical N-glycan charge value termed Z (222). The overall charge contributed by terminal sialic acids present on different glycosyl structures was calculated for a number of glycoproteins and was found to be a highly accurate and

reproducible measurable characteristic of glycoproteins. In light of the importance of sialic acid in regulating biological half-life and hence in *in vivo* activity, this could prove a useful step in estimating biological potency of therapeutic proteins by physicochemical means.

Although a number of advanced physicochemical techniques have been applied to characterize rDNA-derived hormones in detail (223–225), the determination of biological activity solely by physicochemical means requires further work.

VII. Interpretation of FSH Measurements

No assay system currently available clearly describes or estimates all features and actions of FSH. The relative amount of FSH determined by different assay types will depend upon the assay type chosen and the standard used. A major difficulty encountered in estimating FSH is the lack of clarity of definition of FSH. The concept of biological specificity associated with structural descriptions of FSH implies that it is a 'biological recognition' that defines FSH (and similar molecules) rather than a purely physicochemical definition. However, heterogeneity in the polypeptide backbone and in the glycosyl residues gives rise to a large number of isoforms that vary not only in molecular mass and overall charge but also in biological (and immunological) activity (potency and type of actions). It is this 2-fold heterogeneity of FSH that renders it difficult to define: chemically it is not possible to apply the concepts of purity and amount to an inherently heterogeneous substance; and biologically there is no single unique FSH bioactivity. Additionally, the matrix that contains FSH (*e.g.,* serum, plasma, follicular fluid) usually obviates any attempt at chemical or physicochemical analysis and may also interfere with biological assays.

A. Effect of different isoforms in different assay systems

1. In vivo bioassays. It would appear from experimentation that MCR is related to *in vivo* bioactivity (226). Those isoforms of FSH that are relatively low in sialic acid content, and are hence relatively basic in nature, have a relatively low potency in *in vivo* bioassays (140) due presumably to their rapid clearance from the circulation. However, there are conflicting reports that different isoforms of FSH do not exhibit differences in clearance (227). Furthermore, while differences in *in vitro* bioactivity were not found to be reflected by differences in clearance of *in vivo* bioactivity (228), *in vivo* biological activity of LH was found by others (42) to reflect *in vitro* biological activity rather than clearance. The reasons for these discrepancies in the literature are unclear but may be related to selection of endpoint in the assay (chronic effect *vs.* acute effect) and structural details of pituitary and circulating FSH in relationship to the mechanisms of clearance.

Studies carried out to correlate biological activity with isoform profile showed (140) that the protein amount, as determined by laser densitometry of IEF gels, correlated with bioactivity, as determined by ovarian weight augmentation, only for those forms of FSH that have a pI of less than 5.1. An increase in the proportion of protein with a pI of greater than 5.1 resulted in a decrease in *in vivo* biological activity.

It is not known whether the same would be true in an acute or short term *in vivo* bioassay. The response, if any, that these basic forms of FSH evoke may be transient and thus may not contribute to the endpoint determined in the Steelman-Pohley test. If rDNA-derived forms of FSH are selected for a relatively short half-life to enable fine tuning of the ovarian cycle (229), then the format of the *in vivo* bioassay will need to be redesigned for calibration of such materials.

2. In vitro bioassays. In vitro bioassays can offer insights by enabling the biological activity of short-lived species to be determined and specific cellular responses to be evaluated. However, variations in assay design and standards can lead to different interpretations of physiological events. Thus, the biological signal measured in one *in vitro* bioassay of FSH increases in the mid- to late luteal phase, when immunologically reactive FSH is apparently decreasing (167), and the biological activity of FSH appears to continue increasing even when estradiol levels are declining. Other reports have found an unchanging biological signal (159), an increase in the early follicular phase (230), and an increase in the mid to late luteal phase (167). There is strong evidence for specific effects of different FSH forms, which are produced at different stages of the cycle as proposed by Chappel *et al.* (231). Thus, FSH bioactivity rather than amount may be more important physiologically. Development of *in vitro* models that can distinguish between specific actions of FSH, such as recruitment and growth of follicles or the induction of hormonal components of follicular activity or which have multiple endpoints (75), would broaden our knowledge further.

3. Immunoassays. The effect of isoform composition on immunoassay determinations is not resolved. It is claimed that immunoassays are blind to the carbohydrate content of FSH and while the antigenic sites of FSH do not appear to involve oligosaccharides, it is likely that differences in overall charge resulting from variations in sugar structures could affect antibody binding (232). Whereas some immunoassay types may not provide an estimate of bioactive circulating FSH (232, 233), others have been reported to reflect biological activity well (172). Certainly there is significant variation in the estimates of activity of the same preparations of FSH by immunoassays (124) that has been observed in a recent WHO international collaborative study (123).

B. Effect of different forms of FSH on FSH determinations

1. Determination of FSH activity by in vivo bioassays and the relationship between in vivo bioactivity and clinical efficacy. The potency of therapeutic products is derived from *in vivo* bioassays in terms of in-house standards, which are calibrated in terms of WHO International Standards. Comparisons in international collaborative studies suggest that continuity and consistency of estimates may be obtained by *in vivo* bioassay for therapeutic products although the limits of activity defined by the pharmacopeia indicate the relative lack of precision when a limited number of assays are done. Moreover, although a common standard and a common assay method are intended to maintain consistent unitages for patients irrespective of the source of the material used, this cannot be guaranteed (*Sections VI* and *VII*).

TABLE 4. Summary of examples of assays for FSH and their main uses, advantages, and disadvantages

Assay type	Uses and Main advantages	Main disadvantages
Bioassays	Measure biological activity.	Do not necessarily imply any information on amount of substance; require reference material that is difficult to define
In vivo assays	Useful for calibrating therapeutic products; take account of biological clearance	Cumbersome, insensitive
In vitro assays	Sensitive, specific, high throughput; useful for measuring physiological samples	Do not take account of biological clearance; single endpoint often measured; difficult to standardize
Immunoassays	Measure immunoreactivity; useful diagnostically	Require reference material that is difficult to define; epitopes often poorly defined
RIA, ELISA, Lectin-ELISA	Sensitive and specific with high throughput; lectin may provide some structural information	May be too specific; do not necessarily imply any information about biological activity or amount
Physicochemical assays		
Electrophoresis Chromatography	Can be used quantitatively in absolute terms for some determinations (*e.g.*, protein concentration)	Require pure samples in large quantities
Mass spectrometry	Can be used to characterize structural details (*e.g.*, identify peptide map for drug testing)	Cannot (yet) infer information about biological activity; some systems, *e.g.*, mass spectrometry, very expensive
Chemical analysis NMR		

The biological action of FSH determined in the Steelman-Pohley assay is ovarian growth against a background of excess LH/hCG in immature rats. The desired clinical action in patients is the recruitment and stimulation of a single follicle or a cohort of follicles and the stimulation of estradiol production, possibly against a background of low endogenous LH levels.

The advent of therapeutic forms of FSH made from "pure FSH" derived from rDNA-sources (234) has led to the application of physicochemical techniques to FSH analysis (235, 236), to comparative analysis of *in vitro* and *in vivo* biological activity (237–240), and to a plethora of studies in which recombinant DNA-derived hFSH has been evaluated for toxicity (241), comparative pharmacokinetics (242, 243), and clinical pharmacology (122, 244–247). In many studies urinary and well-characterized recombinant materials have been compared for efficacy, quality, and safety (*e.g.*, Ref. 248). Most studies have found little difference between therapeutics derived from different sources. However, there is a significant body of data supporting the conclusion that recombinant preparations are more clinically efficacious than their urinary counterparts on a unit-for-unit basis (121, 249). Assuming that the materials under test in these studies were calibrated against WHO International Standards raises questions about the validity of the *in vivo* bioassay for calibration of therapeutic products from different sources and about the relationship of the biological assay of FSH to its clinical effectiveness and safety. Even when allowed variability between preparations defined by pharmacopeial monographs is taken into account (actual potency may be between 80–125% of the stated potency), it is difficult to explain why a recombinant DNA-derived material should be more effective in inducing follicular growth than a highly purified urinary material.

One explanation is that recombinant DNA-derived FSH, with a higher content of basic isoforms that do not contribute to the *in vivo* bioassay (140), may more closely resemble those FSH forms produced at midcycle than urinary FSH and may

therefore be more effective at producing the required physiological response (76). It has also been reported that relatively basic isoforms of rDNA-derived FSH are more effective in stimulating follicular growth *in vitro* (250).

2. Determination of FSH by immunoassay and clinical diagnosis. In the diagnosis of clinical conditions the level of biologically active FSH in the circulation is of importance. However, diagnostic tests are generally made by immunoassay that may recognize FSH molecules that are not biologically active. Conversely, some highly specific assays may discriminate against some forms of FSH, although this is a matter of controversy. Some anti-LH antibodies are reported not to recognize some forms of LH that arise by genetic variation (251, 252). There are reports of genetic variants of FSH that have been associated with delayed sexual development and infertility (253–255) but these are rare and result in drastic alterations to the FSH molecule rather than to an antigenic epitope as is the case with the more common LH polymorphism. Whether antibody selectivity could compromise diagnosis is not clear. Nevertheless the particular selectivity of any one assay system is seldom known, and it is therefore not possible to define FSH in terms of immunoreactivity. Since FSH (and LH) levels and isoform composition change drastically throughout life and through menstrual cycles, clinical determinations usually require some additional clinical data such as stage of cycle or repeat measurements to distinguish between different diagnostic possibilities. A combination of quantitative and qualitative assays would be a major advance in clinical utility of gonadotropin determinations.

Other complications of the gonadotropin system that have been implicated in disorders of reproduction and that may affect clinical diagnoses based on FSH determinations include mutations in the FSH receptor and circulating antibodies against FSH (256–258).

In combination with immunoassays, *in vitro* bioassays have been used to derive a biological activity- immunological activity (B:I) ratio. The rationale behind this concept has been

critically discussed (259). Unless either the biological activity or the immunoreactivity of different preparations can be shown to be constant for each set of assay systems in use, then the results of such studies will be misleading. Data derived from international collaborative studies indicate that the B:I ratio is not a stable parameter. Estimates of activity of different preparations obtained from both *in vitro* bioassays and immunoassays were variable, reflecting the different specificities of the different assay systems. Although B:I ratios may be misleading, a comparison of the results from receptor assays, immunoassays, and *in vitro* bioassays can provide an indication of changing isoform profile or of factors that interfere in FSH action in some circumstances (205, 206).

VIII. Conclusions

FSH is a heterogeneous hormone that can be defined in terms of both its molecular structure and its biological function. The molecular structure of FSH has been largely determined but variation in key structures leads to heterogeneity and renders impossible a definition of a single molecular entity as FSH. Biologically, FSH exerts multiple effects at its target tissues so that there is no single action that defines FSH.

Measurements of FSH are made using a variety of methods (summarized in Table 4), most of which are biological in nature. However, the interaction of molecular heterogeneity and biological actions and the difficulty in defining a standard preparation of the same type as the unknown sample that is to be measured can lead to uncertainties. This is particularly true if the characteristics of the standard and the assay system used are not clearly specified.

The World Health Organization has made available FSH standards derived from different sources that are representative of the main sources of bulk materials used in the preparation of therapeutic products. These international standards have similar biological actions to one another and to therapeutic products in the pharmacopeially defined *in vivo* bioassay. The use of appropriate standards for calibration of therapeutic products in the pharmacopeial assay has, to a large extent, achieved continuity of unitage of therapeutic products. However, these standards may be less suitable for general use in a range of assays where the interaction of molecular and biological heterogeneity of FSH may lead to inconsistent estimates with the "measured amount of FSH" depending on selection both of the standard and of the assay system.

As both the molecular structure of FSH and its biological actions are more clearly characterized, the need for appropriate standards for particular assays and purposes may become apparent. For some highly purified preparations, physicochemical techniques may become more useful. The availability of standards of defined isoforms of FSH might enable determination of the specificity of various immunoassays, and this might serve to clarify the meaning of some of these measurements. The definition of the particular isoforms that are of clinical relevance might permit the development of assays specific for them, as well as indicating the types of preparations that might be appropriate as standards.

FSH will continue to be used therapeutically and diagnostically in assessing and treating disorders of the reproductive system. Thus, the scientific community should be aware of the need to specify as far as possible what is meant by any particular measurement of FSH and, in particular, should be aware that such measurement is not meaningful without definition of both the standard and the measuring method used.

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